EVALUATION OF METHYLENETETRAHYDROFOLATE REDUCTASE FOR TARGETED THERAPEUTICS IN CANCER

by

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A thesis to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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May, 1999

ABSTRACT

Folate derivatives are required for nucleotide/DNA synthesis and DNA methylation. Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the folate derivative required for homocysteine remethylation to methionine, the precursor of S-adenosylmethionine. Approximately 45%-50% of the general population is heterozygous for a common substitution (677C→T, A to V) in MTHFR. Due to loss of heterozygosity (LOH) in cancer cells, individuals who are heterozygous for MTHFR in their constitutional DNA may contain only one of the above alleles in their tumor DNA.

Loss of heterozygosity of MTHFR was observed in 40% of ovarian carcinoma tumor samples and in 16% of colon carcinoma samples suggesting that the chromosomal location to which the MTHFR gene maps (1p36.3) undergoes frequent LOH. Examination of cell viability of human fibroblasts and of human colon carcinoma cell lines in minimum essential media (MEM) lacking methionine found both cell types to be extremely sensitive to the methionine deficiency. Replacing methionine with homocysteine and vitamin B_{12} restored the growth of normal fibroblast lines to levels that approached those of replete MEM, but the transformed lines increased proliferation only slightly under these conditions. These results support earlier reports regarding the increased methionine dependence of transformed lines. Targeting specific MTHFR variants with the antisense oligonucleotide resulted in ~50% decreased survival of two carcinoma cell lines (V/V genotype), possibly due to MTHFR's involvement in methionine synthesis. Allele-specific targeting of MTHFR could therefore provide an effective approach for cancer therapy. Furthermore, cancer patients with the V/V genotype may require less aggressive anti-folate chemotherapy since V/V carcinoma lines were highly sensitive to drug treatment (IC_{50} < 25 nM) whereas the A/A lines were more variable in response.

"Obstacles are those frightful things you see when you take your eyes off your goal."

Henry Ford

"What lies before us and what lies behind us are small matters compared to what lies within us. And when we bring what is within out into the world, miracles happen."

Henry David Thoreau

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Les dérivés de l'acide folique sont nécessaires à la synthèse des acides nucléiques et aux réactions de méthylation de l'ADN. La méthylènetétrahydrofolate réductase (MTHFR) convertit le 5,10-méthylènetétrahydrofolate en 5-méthyltétrahydrofolate, le co-substrat requis pour la reaction de reméthylation de l'homocystéine en méthionine, le précurseur de la S-adénosylméthionine. Environ 45%-50% de la population est hétérozygote pour un polymorphisme fréquent du gène *MTHFR* (C677→T, A→V). Suite à la perte d'une allèle dans certaines cellules cancéreuses, il est possible que le génôme de celles-ci, bien qu'elles originent de cellules normalement hétérozygotes, ne présentent qu'une seule des deux allèles.

La perte d'une allèle de *MTHFR* a été observée dans 40% des échantillons de cancer des ovaires et dans 16% des cas de cancer du colon, suggérant que le locus *MTHFR* (1p36.3) est fréquemment délété. La viabilité de fibroblastes humains et de cellules provenant de cancers du colon, testée en modifiant la composition du milieu de culture, a démontré que les deux types cellulaires sont très sensibles à l'absence de méthionine dans un milieu minimal (MEM). La substitution de la méthionine par de l'homocystéine et la vitamine B12 rétablit la croissance des lignées de fibroblastes à des niveaux élevés, mais cet effet est minime pour les cellules transformées. Le ciblage de l'allèle variante de *MTHFR* avec un oligonucléotide antisens spécifique provoque la mort de 50% des cellules pour des lignées de carcinomes (génotype V/V), possiblement à cause de l'implication de *MTHFR* lors de la synthèse de la méthionine. Le ciblage spécifique d'une allèle de *MTHFR* pourrait offrir une approche thérapeutique efficace pour certains cas de cancer du colon. D'autre part, nos résultats suggèrent que les patients possédant le génotype V/V pourraient bénéficier d'une chimiothérapie anti-folate moins aggressive puisque les lignées V/V sont très sensibles à la drogue utilisée (IC_{50} < 25 nM) alors que les lignées A/A présentent une réponse plus variable.

ACKNOWLEDGEMENTS

Two years have flown by and my Master's thesis has reached its conclusion. Believe it or not, I'm still standing! I would like to thank several souls who helped keep me on my feet. Dr. Rima Rozen for the opportunity to pursue a fascinating project, and for her support and confidence in me. Andrea Schievella who always spared her time to provide me with valued experimental advice. Gail Dunbar for teaching me how to keep my cell cultures happy and healthy. Drs. Mukherjee and Rosenblatt for their feedback and suggestions that kept me thinking. To my lab-mates of past and present, for making lab life fun and for tolerating my constant teasing. Pam for all of her help, encouragement and support as well as for the airport rides, the girl talks, the tears we shared, and the laughs that sometimes brought us to tears. Aaron for being there when I needed him and for understanding my neat-freak ways. Sahar (kiddo) for laughing at my jokes, for movie-outings and for knowing how to 'relieve tension'. Eric for the car rides home and for his computer expertise. Mark for introducing me to Labre House and for being the unpaid handyman. Patricia for the good times in and out of the lab (especially the trip to New York City). Manuel for buying roller blades, for making spicy noodle dishes and for plasmid preps. Zhoutao for dropping everything to help me out, especially with the CO_2 tank. Ilan for never trashing my Canadian rock bands (yeah right!) and for his words that made me smile, laugh or just go 'huh?'. Irfan for his patience when I was just a clueless undergrad. Thida for the smile that could always lift my spirits and Daniel for chatting 'hockey' with me. Nelly for answering my stupid questions and for all her help in the lab. Francois and Qing for making sure my orders came in and Xin and Tak for Northern suggestions. Wei for helping me take over tissue culture. Phil and Maria for choosing CHOM-FM . Daniel for translating my Abstract. Mei and Li Yuan for their company. I would also like to thank Heather for the ice-storm memories, for not abandoning me in Montreal and most of all, for believing in

me. My pals across the miles who taught me that distance cannot break genuine friendships. Sonia and Candice, two of my closest friends in the world, for experiencing the best and worst of times with me throughout the years. Mom, Dad, Jude, Nana and Corine for their constant love and support, both financially and emotionally. And finally, nothing would be possible without the help and love of God.

ABBREVIATIONS

bp	Base pair
DEPC	Diethylpyrocarbonate
DHF(R)	Dihydrofolate (reductase)
dTMP	Deoxythymidinemonophosphate
5-FU	5-Fluorouracil
F-	Folate-deficient media
(F)dUMP	(Fluoro) deoxyuridinemonophosphate
FPGS	Folylpolyglutamate synthetase
LOH	Loss of heterozygosity
M-	Methionine-deficient media
MEM	Minimum essential media
MI	Myocardial infarction
MTHFR	Methylenetetrahydrofolate reductase
MTX	Methotrexate
NTD	Neural tube defect
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RPA	DNA Replication protein A
SAM	S-adenosylmethionine
TBE	Tris-borate-EDTA
TCA	Trichloroacetic acid
THF	Tetrahydrofolate
TS	Thymidylate synthase

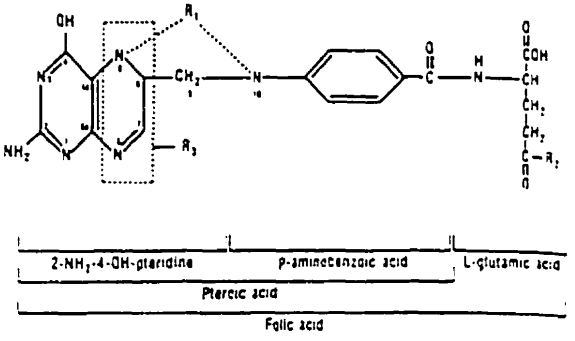
1. INTRODUCTION

1.1 Metabolism of Folates

Folates are pteridine compounds that act as coenzymes for several critical one-carbon transfer reactions, including those necessary for purine, thymidylate and methionine biosynthesis. As shown in Figure 1, there exists at least three stages of reduction of the pteridine group, at least six one-carbon groups substituted at the N^5 and/or N^{10} position, and varying lengths of gamma glutamyl-peptide chains (Rosenblatt 1995). The major metabolic pathways of the folates are shown in Figure 2. 5-Methyltetrahydrofolate is the predominant circulatory form of folate and is a co-substrate for homocysteine remethylation to methionine, a vitamin B_{12}-dependent reaction catalyzed by methionine synthase (Figure 2). Methionine is needed for protein synthesis and also serves as a precursor for S-adenosylmethionine (SAM), a methyl donor critical in many methylation reactions.

Altered homocysteine metabolism leading to varying levels of elevation may arise from genetic defects or nutritional factors such as vitamin B_6, vitamin B_{12} and folic acid deficiency. Many lines of evidence have supported the notion of hyperhomocysteinemia as a risk factor for vascular disease. High levels of homocysteine could have synergistic effects with other risk factors including smoking and hypertension (reviewed in Boers 1997). Elevation in homocysteine may be toxic to the cellular components of the arterial wall; however, reduction of blood homocysteine levels can be achieved by supplementation with B-vitamins and folic acid in particular (reviewed in Refsum 1998).

Figure 1. The structure of folic acid and its derivatives. From The Metabolic and Molecular Bases of Inherited Disease (eds. Scriver C.R., Beaudet A.L., Sly W.S. and Valle D., 1995).



R_1

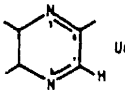
$-CH_3$ at N^5, $-H$ at N^{10}:	5-methyl
$-CHO$ at N^5, $-H$ at N^{10}:	5-formyl (citrovorum factor, folinic acid, leucovorin)
$-CHNH$ at N^5, $-H$ at N^{10}	5-formimino
$=CH-$ at N^5, N^{10}	5,10-methenyl
$-CH_2-$ at N^5, N^{10}	5,10-methylene
$-H$ at N^5, CHO at N^{10}	10-formyl

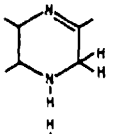
R_2

Poly(γ-glutamyl)$_n$ glutamic acids

$-OH$

R_3


Unreduced pteridine group


7,8 Dihydro (H_2Folate)

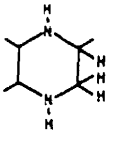
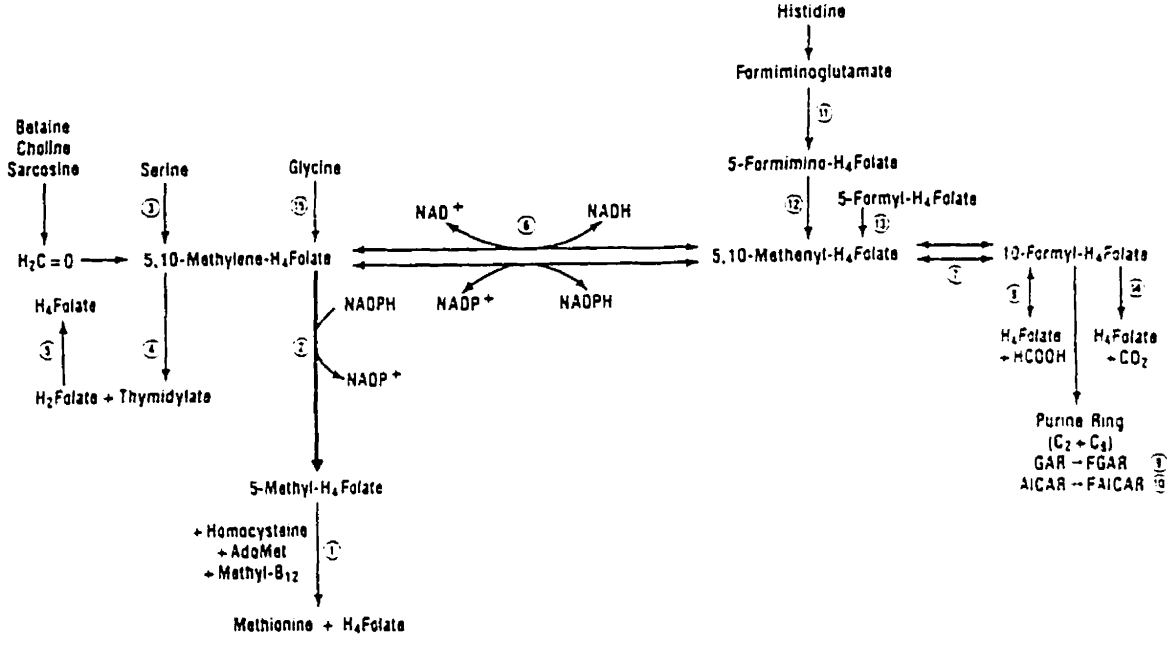

5,6,7,8 Tetrahydro (H_4Folate)

Figure 2. The major metabolic pathways of the folates. From The Metabolic and Molecular Bases of Inherited Disease (eds. Scriver C.R., Beaudet A.L., Sly W.S. and Valle D., 1995). 1. methionine synthase; 2. methylenetetrahydrofolate reductase; 3. serine hydroxymethyltransferase; 4. thymidylate synthase; 5. dihydrofolate reductase; 6. methylenetetrahydrofolate dehydrogenase; 7. methenyltetrahydrofolate cyclohydrolase; 8. 10-formyltetrahydrofolate synthase; 9. GAR transformylase; 10. AICAR transformylase; 11. glutamate formiminotransferase; 12. formiminotetrahydrofolate cyclodeaminase; 13. 5,10-methenyltetrahydrofolate synthetase; 14. 10-formyltetrahydrofolate dehydrogenase; 15. glycine cleavage pathway.


Histidine
Formiminoglutamate
Betaine
Choline
Sarcosine
Serine
Glycine
5-Formimino-H4Folate
5-Formyl-H4Folate
NAD+
NADH
H2C=O
5,10-Methylene-H4Folate
5,10-Methenyl-H4Folate
10-Formyl-H4Folate
H4Folate
NADPH
NADP+
NADPH
H4Folate + HCOOH
H4Folate + CO2
H2Folate + Thymidylate
NADP+
Purine Ring
(C2 + C3)
GAR → FGAR
AICAR → FAICAR
5-Methyl-H4Folate
+ Homocysteine
+ AdoMet
+ Methyl-B12
Methionine + H4Folate

The relationship between folate and homocysteine is also of relevance for the etiology and prevention of neural tube defects (NTDs). Maternal folate and vitamin B_{12} deficiency are independent risk factors for NTDs, whereas folate administration can prevent such malformations (reviewed in Fowler 1997). In addition, abnormal homocysteine metabolism has been observed in mothers of children with NTDs that may be related to problems in homocysteine remethylation. Deficiency of folate and vitamin B_{12} could provide an explanation for high homocysteine levels since the activity of methionine synthase is dependent on both vitamin B_{12} and folate (Mills 1995). Prevention of NTDs by folic acid supplementation may be achieved by overcoming the abnormality in metabolism.

1.2 Folate and Cancer

Folates play an essential role in DNA synthesis and methylation reactions. Aberrant DNA methylation may initiate carcinogenesis by altering gene expression. Several epidemiological studies have examined the relationship between folate and cancer, including cancers affecting the cervix, colorectum, lung, esophagus and brain, and propose an inverse association between folate status and early neoplasia (reviewed in Glynn 1994). Levels of methionine, an amino acid important for methylation reactions, and alcohol, an antagonist of folate and methyl-group metabolism, may also contribute to the development of colon cancer by interfering with the synthesis and methylation of DNA (reviewed in Ma 1997). High dietary intakes of folate and methionine reduce the risk of colon cancer while alcohol intake elevated the risk of this adenoma (Giovannucci 1993,1995). In fact, the combination of high alcohol with

low methionine and folate intake greatly increased the likelihood of colon cancer (Giovannucci 1995). Diets rich in fruits and vegetables, which are high in folate, show some protective properties (reviewed in Glynn 1994), suggesting that a methyl-deficient diet may be implicated in the early stages of carcinogenesis such as that observed in the colorectum (Giovannucci 1993,1995). The decrease in methyl availability can lead to hypomethylation that may affect gene regulation. As well, folate deficiency can raise uracil levels resulting in improper DNA synthesis and initiating neoplastic transformation (reviewed in Glynn 1994).

Once carcinogenesis has occurred, however, the functional importance of folates continues through the maintenance of such cancer cells. The high-affinity folate binding protein (FBP), involved in uptake of 5-methyltetrahydrofolate, is found over-expressed in many neoplastic tissues.

1.2.1 Loss of Heterozygosity in Cancer

Genetic variation or polymorphisms allow individuals to display heterozygosity for several genes in their constitutional DNA. Loss of heterozygosity (LOH) is an early event in the development of cancers that leads to the removal of large chromosomal regions, particularly those encoding tumor suppressor functions (reviewed in Basilion 1999). Due to LOH, genes situated close to tumor suppressor genes may also be lost leaving many in a hemizygous state in cancer cells. Eliminating heterozygosity as a result of LOH creates genetic differences between tumor and normal cells that can be exploited for cancer therapy. A strategy of

developing inhibitors targeting polymorphic genes that are essential for cell survival and which undergo frequent LOH has been described (Basilion 1999).

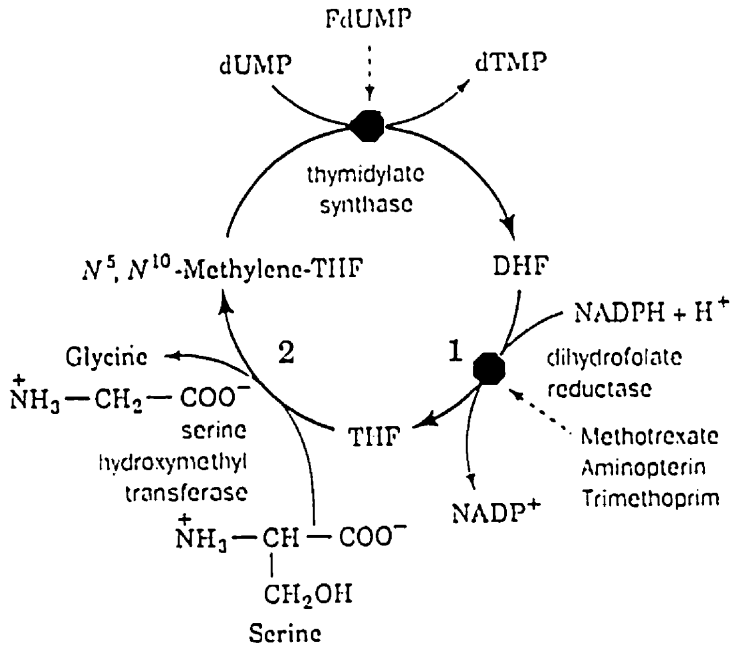
Examination of a key enzyme of folate metabolism, methylenetetrahydrofolate reductase (MTHFR), in ovarian tumors identified frequent allelic deletions or loss of heterozygosity (LOH) at this locus causing shortages of intracellular concentrations of 5-methyltetrahydrofolate (Viel 1997). This biochemical defect in folate metabolism, which can also alter methionine synthesis, may explain the physiological upregulation of FBP expression in neoplastic cells and suggests an increased demand for folates and methionine by such cancer cells (Viel 1997).

1.3 Effects of Chemotherapeutic Drugs on Folate Metabolism

1.3.1 5-Fluorouracil

5-Fluorouracil (5-FU) has chemotherapeutic uses in colorectal cancer, head and neck cancers as well as breast cancer. The conversion of 5-FU to its nucleotide form, 5-fluorodeoxyuridylate (FdUMP), leads to irreversible inhibition of thymidylate synthase (TS), the final enzyme in the de novo pathway that converts dUMP to thymidylate (dTMP) using 5,10-methylenetetrahydroflate as a co-substrate (Figure 3). Rapidly proliferating cancer cells require a constant supply of nucleotides such as dTMP for survival and are killed following treatment with the 5-FU anti-tumor agent (Voet 1995) possibly due to the induction of apoptotic cell death (Huschtscha 1996, Ueda 1997). Although 5-FU can be activated through different pathways that lead to at least two other cytotoxic compounds, fluorouridine triphosphate (FUTP) for RNA

Figure 3. Inhibitors of folate metabolism. From Biochemistry (eds. Voet D. and Voet J.G., 1995).


FdUMP
dUMP
dTMP
thymidylate synthase
N^5, N^{10}-Methylene-THF
DHF
NADPH + H^+
dihydrofolate reductase
Methotrexate
Aminopterin
Trimethoprim
$NADP^+$
THF
1
2
Glycine
$^+NH_3-CH_2-COO^-$
serine hydroxymethyl transferase
$^+NH_3-CH(CH_2OH)-COO^-$
Serine

incorporation and fluorodeoxyuridine triphosphate (FdUTP) for DNA incorporation, thymidylate synthase seems to be the preferred target when 5-FU is given by continuous infusion (reviewed in Cheradame 1997a). Optimal inhibition of TS by 5-fluorouracil requires the ternary complex formed between TS, FdUMP and 5,10-methylenetetrahydrofolate, with the reduced folate stabilizing the ternary complex when present at higher levels (reviewed in Cheradame 1997b).

1.3.2 Methotrexate

The antifolate methotrexate (MTX) is clinically ineffective against several human solid tumors but has been used as treatment for neoplasms such as acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, breast cancer and squamous cell carcinoma of the head and neck. MTX can enter cells by an active transport system utilized by 5-methyltetrahydrofolate and 5-formyltetrahydrofolate or possibly by diffusion when present at high concentrations (>20 μM) (reviewed in Jolivet 1983). Upon entry, methotrexate inactivates dihydrofolate reductase (DHFR), the enzyme that reduces dihydrofolate (DHF) to tetrahydrofolate (THF) using NADPH (Figure 3) (Voet 1995). As a DHF analog, MTX competitively binds DHFR preventing not only dTMP synthesis, but also other THF-dependent reactions such as purine, histidine and methionine synthesis.

The mode of cell death was examined in an acute leukemic T-lymphocyte cell line (CCRF-CEM.f2) after exposure to a broad concentration of cytotoxic drugs, including MTX. The first changes seen after MTX treatment were cell cycle perturbations, including the accumulation in G_1 phase, detected by flow cytometry,

and morphological alterations characterized by condensed chromatin segregating to the nuclear membrane. These changes preceded the appearance of internucleosomal DNA cleavage monitored by gel electrophoresis (Huschtscha 1996). Cytotoxic drug treatment may thus disrupt cell cycle progression, initiating drug-specific apoptotic sequences of dying cells.

1.4 Methionine Dependence

Cancer biologists have aimed to identify biochemical differences between normal and transformed cells that can aid in the treatment of human cancers. Altered methionine auxotrophy of transformed cells is one such biochemical change. In vitro, many tumor-derived cell lines are sensitive to a limited methionine supply and arrest in late-S/G_2 stage of the cell cycle termed MDCCB (methionine-dependent cell cycle block) (Guo 1993a, Hoffman 1980, Guo 1993b). This methionine dependence may be due to the high methionine requirement for transmethylation reactions resulting in low free-methionine levels and low S-adenosylmethionine/S-adenosylhomocysteine ratios that block cell division under conditions of low methionine provision (reviewed in Guo 1993a). An early study had shown that unlike normal cells, two SV40-transformed human cell lines and a rat carcinoma cell line (Walker-256) were unable to survive in media in which methionine was replaced with homocysteine and vitamin B_{12} (i.e. conditions of de novo methionine synthesis). The cessation in growth of the malignant and transformed lines occurred in spite of endogenous synthesis of methionine that was incorporated into high-molecular weight substances. In the presence of limiting amounts of exogenous methionine, homocysteine greatly

stimulated the growth of these cells (Hoffman 1976). These findings provide evidence for the necessity of preformed methionine to complement the high levels of de novo synthesis in order to fulfill functions for the rapid growth of transformed cells. Consistent with these results, another investigation demonstrated the ability of normal adult mammalian cells to utilize homocysteine in place of methionine to maintain their growth but the inability of three malignant cell types (rat breast carcinoma, W-256; mouse leukemia, L1210; human monocytic leukemia, J111) to do so (Halpern 1974).

The induction of tumor-specific cell cycle block has been attempted and achieved in vivo by depriving Yoshida sarcoma-bearing nude mice of dietary methionine. This also resulted in tumor regression and extended survival of the nude mice (Guo 1993a).

In light of such in vitro and in vivo studies, exploiting the methionine dependence of cancer cells has therefore been suggested for tumor therapy.

1.5 Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate reductase (MTHFR), an FAD-specific flavoprotein, catalyzes the NADPH-linked reduction of 5,10-methylenetetrahydrofolate (also used in thymidylate synthesis) to 5-methyltetrahydrofolate (reaction 2, Figure 2). Porcine liver MTHFR has been purified as a homodimer of 77 kDa subunits with an N-terminal catalytic domain of 40 kDa and a C-terminal regulatory domain of 37 kDa (Daubner 1982, Matthews 1984). A 1.3 kb human MTHFR cDNA, mapped to chromosome 1p36.3, was isolated using degenerate oligonucleotides based on the sequence of a porcine peptide, and assigned to the 5' or N-terminal domain (Goyette

1994). Using this original MTHFR cDNA, 900 bp of additional sequences at the 3' end (C-terminal) were obtained upon screening a human colon carcinoma cDNA library (Frosst 1995). The C-terminal regulatory domain of mammalian MTHFR contains the binding site for SAM, an allosteric inhibitor. Western analysis of several human tissues and of porcine liver reveals a 77 kDa polypeptide; however, another isoform of 70 kDa was observed in human fetal liver and porcine liver. In vitro expression of the 2.2 kb cDNA in *E.coli* yielded the smaller 70 kDa protein which was catalytically active (Frosst 1995). The remaining sequences that encode the larger isoform have yet to be isolated but are suspected to represent N-terminal sequences that may account for the homologous sequences missing from the N-terminal peptide of the porcine liver enzyme.

1.5.1 Severe MTHFR Deficiency

Hereditary MTHFR deficiency, an autosomal recessive disorder, is the most common inborn error of folate metabolism resulting in hyperhomocysteinemia, homocystinuria and low to normal plasma methionine levels. Patients with severe MTHFR deficiency (0-20% residual enzyme activity in cultured fibroblasts), diagnosed between infancy and adolescence, display variable phenotypes including developmental delay, motor and gait dysfunction, seizures and psychiatric disturbances (Rosenblatt 1995). Over 20 rare mutations that include nonsense mutations, amino acid substitutions, deletions and 5' splice-site mutations have been identified in the MTHFR gene (Goyette 1994,1995,1996, Kluijtmans 1998, S.Sibani

personal communication) and good correlation has been observed between genotype, enzyme activity and the clinical phenotype (Goyette 1995).

1.5.2 Thermolabile Variant of MTHFR

Mild MTHFR deficiency with ~50% residual activity and enzyme thermolability has been reported and suggested as an inherited risk factor for coronary heart disease (Kang 1988,1991). Enzyme activity was found to be ~50% of normal levels and following heating of lymphocyte extracts for 5 minutes at 46°C, consistent activity differences were seen between controls and patients suggesting decreased thermostability in MTHFR (Kang 1988, Kang 1991). This thermolabile form of the enzyme was first implicated with the development of coronary artery disease by measuring lymphocyte MTHFR in 212 patients with the disease and 202 controls. Thermolability (i.e. < 20% residual activity) was observed in 17% of cardiac patients compared to 5% of controls. These findings suggest that moderate deficiency of MTHFR, due to the thermolabile variant, can lead to higher plasma homocysteine concentrations that may be corrected by oral folic acid supplementation, and is positively correlated with the development of vascular disorders (Kang 1988, Kang 1991).

By SSCP analysis and direct sequencing of PCR fragments, a 677C→T mutation was identified in MTHFR that resulted in an alanine to valine (A→V) missense mutation which accounted for enzyme thermolability (Frosst 1995). The substitution is common in the general population with a 35%-40% allele frequency in French Canadians and North Americans. Analysis of lymphocyte extracts from

individuals with homozygous normal (A/A), heterozygous (A/V) and homozygous mutant (V/V) genotypes showed that V/V individuals had a significant decrease in MTHFR specific activity, increased enzyme thermolability and increased plasma homocysteine compared to the A/A or A/V counterparts (Frosst 1995). Activity and thermolability of heterozygotes were intermediate to A/A and V/V individuals. The mutation predisposes to high levels of homocysteine primarily in the presence of low plasma folate concentration, suggesting a genetic-environment interaction that influences the development of mild hyperhomocysteinaemia (Jacques 1996).

1.6 MTHFR Polymorphism and Multifactorial Disorders

1.6.1 Vascular Disease

As described earlier, hyperhomocysteinemia has been recognized as a risk factor for cardiovascular disease. A small study screened 60 Dutch cardiovascular patients and 111 controls to determine whether the A→V amino acid substitution in MTHFR serves as a risk factor for premature cardiovascular disease. The incidence of the homozygous transition (V/V), observed in 15% of the cases, was significantly higher than the 5.4% prevalence in controls, indicating that the homozygous form of the mutation may be an important risk factor for this disease (Kluijtmans 1996). A larger study conducted on participants in the Physicians Health Study included 293 American male physicians who had suffered from myocardial infarction (MI) and 290 controls. In this investigation, genotype alone did not influence the risk of MI but it

did seem to be a determinant of plasma homocysteine levels, particularly among men with low plasma folate status as well as younger men (< 60 years) (Ma 1996).

Combining the findings of several case-control studies has led to the general conclusion that thermolabile MTHFR is associated with elevated plasma homocysteine levels which, in itself, is an independent risk factor for vascular disease (Fletcher 1998). Furthermore, the effects of the thermolabile variant on homocysteine concentrations can be more dramatic when plasma folate levels are low, suggesting a critical role for folate in maintaining homocysteine homeostasis among V/V individuals (Fletcher 1998, Kluijtmans 1996, Ma 1996).

1.6.2 Neural Tube Defects

Periconceptional folic acid supplementation has been shown to reduce the risk of neural tube defects (NTDs) with the assumption that a large fraction of NTDs may arise as a result of abnormalities in folate metabolism. An investigation examining Dutch families with spina bifida included 55 patients and their parents (70 mothers, 60 fathers). Homozygosity for 677C→T was present in 16% of mothers, 10% of fathers and 13% of spina bifida patients compared to 5% of 207 controls. As seen previously, presence of the mutation was related to MTHFR activity and thermolability, with V/V individuals displaying decreased activity resulting in increased plasma homocysteine and decreased plasma folate compared to A/A and A/V individuals (van der Put 1995). This was the first evidence of a genetic explanation for NTDs. An Irish study examined the relationship between the alanine to valine polymorphism in MTHFR and NTDs in 82 cases and 99 controls. Homozygosity for the valine variant was observed

in 18.3% of individuals with NTDs which was significantly higher than the frequency (6.1%) in the control population resulting in an odds ratio of 3.47 (Whitehead 1995).

Reduced enzyme activity due to the A→V mutation in MTHFR can lower plasma folate levels, since 5-methyltetrahydrofolate is the primary circulatory form of folate. This folate derivative is needed for methionine synthesis from homocysteine. In turn, S-adenosylmethionine (SAM) may also decrease, possibly affecting critical methylation reactions such as those necessary for closure of the neural tube in early nervous system development (Whitehead 1995). Genotype alone may not be as critical as environmental influences in determining the ultimate phenotype with respect to NTDs. Folic acid administration may overcome the effects of decreased MTHFR activity, thus providing protection against the development of NTDs in individuals homozygous for the A→V mutation.

1.6.3 Colorectal Cancer and V/V Protection

The 677C→T polymorphism has been implicated in a third multifactorial disorder, colorectal cancer; however, in this case, the homozygous mutant form (V/V) provides a protective effect depending on folate status and alcohol intake (Chen 1996, Ma 1997). Males homozygous for the mutation, present in 15% of controls, showed one-half the risk of developing colon cancer compared to those with the homozygous normal or heterozygous genotypes (Ma 1997). Among men displaying adequate folate levels (> 3 ng/ml), those homozygous for the mutation showed a 3-fold decreased risk when compared with the A/A or A/V genotypes. However, the protective effect of the valine mutation is absent in men with folate deficiency (< 3 ng/ml). Alcohol intake

also plays a role in estimating the risk of colon carcinoma development since it can alter folate metabolism by decreasing its absorption and increasing its excretion. Using A/A men who drank little or no alcohol as the reference, V/V males with low alcohol intake exhibited an 8-fold decreased risk while moderate drinkers showed a 2-fold decrease in risk (Ma 1997). These findings suggest that the A→V mutation decreases colon cancer risk perhaps by increasing 5,10-methylenetetrahydrofolate levels for the thymidylate synthase reaction and proper DNA synthesis, but that its protective effect is negated if folate intake is low or if alcohol consumption is high.

5,10-Methylenetetrahydrofolate acts as the folate substrate for thymidylate (dTMP) synthesis from uridylate (dUMP) by the enzyme thymidylate synthase. Low cytosolic levels of this form of folate lead to a decrease in dTMP synthesis, a higher dUMP:dTMP ratio and ultimately an increase in uracil misincorporation into DNA (Blount 1997,Goulian 1980,Reidy 1988). Repair of uracil misincorporation is accomplished by uracil-DNA glycosylase and apyrimidine endonuclease through the generation of transient single-stranded breaks, or nicks (Dianov 1991). Two opposing nicks can cause double-stranded or chromosome breaks (Dianov 1991) that could increase the likelihood of cancer. The increase in DNA uracil levels and micronucleus frequency (a measure of chromosome breaks) can be lowered with folate supplementation (Blount 1997). Folate status is therefore a determinant of DNA strand breaks causing genetic instability and increasing cancer risk.

Individuals homozygous for the A→V mutation may have increased amounts of 5,10-methylenetetrahydrofolate at the expense of 5-methyltetrahydrofolate. 5,10-Methylenetetrahydrofolate is required for purine and thymidylate synthesis. Increasing

dTMP formation may decrease DNA uracil levels that could be responsible for strand breaks (Ma 1997).

2. RESEARCH PROPOSAL

Approximately 45%-50% of the general population is heterozygous for a common substitution (677C→T, ala to val) identified in MTHFR (Frosst 1995). The current research project aims to evaluate this polymorphism with respect to its cancer association in search of possible forms of cancer therapy. Four types of studies will be conducted for this investigation:

(1) Loss of heterozygosity (LOH), the loss of one variant form of a gene, occurs early in oncogenesis and frequently in cancer cells. MTHFR maps to chromosome 1p36 (Goyette 1994), a region often deleted in neuroblastomas, melanomas, and breast cancers (Caron 1993, White 1995, Dracopoli 1989, Genuardi 1989). As a result of LOH, individuals who are normally heterozygous for MTHFR in their constitutional DNA might contain only one of the polymorphic alleles in their tumor DNA. If the MTHFR gene product is essential for tumor cell viability, specifically targeting the remaining allele in such cells, by antisense oligonucleotides for example, could alter cancer cell survival without substantially affecting that of normal heterozygous cells (Variagenics Inc. 1996).

This project will examine two series of colon carcinomas to determine the frequency of LOH of MTHFR. Since an earlier report observed frequent loss of MTHFR in ovarian carcinomas (Viel 1997), a series of ovarian carcinomas will also be included for confirmation in our population and for comparison with colorectal carcinoma.

(2) In order to determine whether MTHFR is essential for cell viability, survival studies of human diploid fibroblasts and human colon carcinoma cell lines (A/A or V/V genotype) will be performed in folate and/or methionine deficient media since MTHFR provides the folate derivative required for methionine synthesis. Many cancer cells show methionine dependence that may be related to a higher methionine requirement as a result of the enhanced methylating capacity of tumor cells (Hoffman 1984). Exploitation of methionine dependence has therefore been suggested as a possible tumor therapy. 5,10-Methylenetetrahydrofolate reductase activity will be assayed in colon carcinoma cell extracts grown in deficient media in order to determine if a correlation exists between MTHFR activity and cell viability. To allow for survival in media lacking in folate and/or methionine, the carcinoma lines may show an upregulation in MTHFR activity. Results from viability studies and activity measurements could provide insight into the metabolic priorities of cancer cells.

(3) If MTHFR is important in cell viability, cell death resulting from the specific knockout of MTHFR will be attempted by utilizing antisense oligonucleotides directed against specific alleles of the MTHFR gene. Antisense work will be performed in collaboration with Variagenics Inc., a company that uses this approach of allele-specific targeting to develop novel drugs that are selectively cytotoxic to cancer cells. This same technique has been used to inhibit variants of the DNA Replication Protein A (RPA).

(4) Current methods of chemotherapy have included use of cytotoxic drugs such as methotrexate and 5-fluorouracil that inhibit folate metabolism and DNA synthesis. Individuals with MTHFR mutations might have different responses to the drugs. To

determine whether the MTHFR genotype (A or V) could influence responsiveness of carcinoma cells to chemotherapy treatment, carcinoma lines will be genotyped and treated with these drugs to examine effects on cell viability.

3. MATERIALS AND METHODS

3.1 DNA samples

Lymphocyte and tumor DNA samples from individuals with ovarian carcinoma or colorectal carcinoma were obtained in collaboration with Dr. W. Foulkes at the Montreal General Hospital. The study considered 43 United Kingdom (UK) women with ovarian carcinoma, 85 UK colorectal cancer patients and 63 colorectal cancer patients from Montreal.

3.2 MTHFR Genotype

Genomic DNA from lymphocytes was examined for the MTHFR 677C→T polymorphism by PCR amplification (Saiki 1988) and restriction digestion. The reaction was done using 10x PCR Buffer (Gibco-BRL, Burlington, ON), 1.5 mM $MgCl_2$ (Gibco-BRL), 0.2 mM dNTPs, 1.25 units Taq Polymerase (Gibco-BRL), 0.25 μg of sense and antisense primers, 0.5-1.0 μg DNA and distilled water to reach a final volume of 50 μl. Amplification was performed with an exonic primer, 1034 (5'-TGAAGGAGAAGGTGTCTGCGGGA-3') and an intronic primer, 1213 (5'-AGGACGGTGCGGTGAGAGTG-3'), to obtain a 198 bp PCR product following 36 cycles of 1 minute at 94°C, 1 minute at 68°C and 2 minutes at 72°C. Alternatively, PCR was used as a labeling reaction for the ovarian carcinoma study where fragments were internally labeled by direct incorporation of a radioactive nucleotide, $\propto^{35}$S-dATP (ICN Biomedical, Costa Mesa CA), during the amplification reaction. Temperatures and lengths of time for denaturation, annealing and elongation remained the same but

only 25 cycles were performed. Since the mutation creates a HinfI site, the 198 bp fragment was digested with HinfI (New England Biolabs, Mississauga ON) for 2 hours at 37°C; presence of the restriction site resulted in 175 bp and 23 bp digested fragments.

PCR products and digests were run on 9% acrylamide gels and visualized by ethidium bromide staining.

3.3 Loss of Heterozygosity Analysis

Individuals who were heterozygous for the 677C→T substitution in their lymphocyte DNA were evaluated for LOH in tumor DNA after PCR amplification and HinfI digestion. The banding patterns and intensities of the bands after restriction enzyme digestion of tumor DNA were compared to those of lymphocyte DNA following staining with ethidium bromide or X-ray film development of ^{35}S-labeled digests. Loss of or significant reduction in intensity of one allele in the tumor DNA was interpreted as complete or partial loss of heterozygosity.

3.4 Cell Lines

Cell culture experiments were conducted on 3 fibroblast lines and 8 colon carcinoma lines. Cultured fibroblasts were obtained from the Repository for Mutant Human Cell Strains at the Montreal Children's Hospital. MCH 51 and MCH 75 fibroblasts served as controls while WG 1554 is homozygous for a nonsense mutation in MTHFR. The human colon carcinoma cell lines CaCo-2, Colo 320DM, SW 620, SW 948, LoVo, DLD-1 and HCT-15 were obtained from American Type Culture

Collection (Rockville MD); human colon carcinoma cell line SW 1222 was a gift from Dr. N. Beauchemin (McGill Cancer Centre, Montreal QC). SW 1222, CaCo-2, SW 948, LoVo, DLD-1 and HCT-15 were genotyped and shown to be homozygous wild type (A/A) for the A→V polymorphism; Colo 320 and SW 620 are homozygous mutant (V/V). All cell lines were maintained in tissue culture treated 75 cm^2 flasks containing minimum essential media (MEM; Gibco-BRL) in a 37°C incubator, supplied with 5% CO_2. Cells were fed every 4-6 days. To release cells from the surface of the flask, 4 ml of 0.05% Trypsin-0.53 mM EDTA-4Na (Gibco-BRL) were added and cells were incubated at 37°C for 5-10 minutes. Cells were then harvested by centrifugation at 2000 rpm for 7 minutes.

3.5 Culture Media

Minimum essential media (MEM) and MEM without folate and without methionine (F-M-) were obtained from Gibco-BRL. (F-M- media was supplemented with 100 mM sodium pyruvate). To prepare media deficient for folate alone (F-), 0.1mM methionine (Sigma-Aldrich, Oakville ON) was added to the F-M- media. For media deficient for methionine alone (M-), 2.3 μM folate (Sigma-Aldrich) was added to the F-M- media. For all media, 1% penicillin/streptomycin (Gibco-BRL), 5% fetal bovine serum (Intergen, Purchase NY) and 5% iron enriched calf serum (Intergen) were added. For media supplemented with homocysteine and vitamin B_{12} (F-+; M-+), 0.44 mM homocysteine (Sigma-Aldrich) and 1.5 μM vitamin B_{12} (Sigma-Aldrich) were added to either the folate-deficient or methionine-deficient media. Dialyzed serum was used for all deficient media unless otherwise stated (see section 3.6).

3.6 Serum Dialysis

Spectra/Por molecular porous membrane tubing (MWCO 12,000-14,000) was used for dialysis (Fisher Scientific Ltd., Nepean ON).

Preparation of dialysis tubing: Tubing was boiled for 20 minutes in 5% sodium carbonate and 10^{-2} M EDTA, rinsed with distilled water and boiled for 15 minutes in distilled water.

Dialysis: Tubing was filled with serum and dialyzed against ~10x volume of 0.9% NaCl solution at 4°C with spinning for 24 hours with 3 changes of saline solution. Serum was then filtered (.45 μm).

3.7 Cell Survival Studies

Cell viability studies were performed in 6-well tissue culture plates starting with 30,000-50,000 cells per well and 3 replicates for each condition. Cell survival in MEM was used as a control for proliferation in deficient media (F-M-, F-, M-, F-+, M-+). To demonstrate the vitamin B_{12}-dependent de novo synthesis of methionine, cell survival studies were pursued in which methionine was replaced with 0.44 mM homocysteine and the growth media was supplemented with 1.5 μM vitamin B_{12} (designated M-+).

3.8 Cell Counting

The initial number of cells, for plating purposes, was estimated by use of a hemacytometer. Cells grown in 6-well plates for the survival or toxicity studies were counted using the FluoroReporter Colorimetric Cell Protein Assay Kit (Molecular

Probes, Eugene OR). A standard curve was constructed for each cell line using serial dilutions of the cell suspension of known cell titre. Cells were resuspended in serum-free media and added to the wells at densities in the range of 1000-100,000 cells in 1 ml serum-free media. To fix cells, 250 µl 80% (w/v) trichloroacetic acid (TCA) was gently added to each sample and plates were stored at 4°C for 1 hour. Wells were washed 3 times with tap water and plates were allowed to dry (approximately 2 hours, 37°C). Cells were stained with 750 µl sulforhodamine B (SRB) staining solution, containing 0.4% (w/v) sulforhodamine B dye (Sigma) in 1% acetic acid, and stored in the dark for 30 minutes at room temperature. Cells were washed 3 times with 1% acetic acid and the plates were dried (stored in the dark). 1 ml solubilization reagent (10 mM unbuffered Tris base; Gibco BRL) was added and mixed with each sample. Absorbance (565 nm) of each sample (~700 µl) was measured and a standard curve was plotted.

Following viability or toxicity studies, final cell numbers were determined. Cells grown in the 6-well plates were washed with serum-free media and 1 ml fresh serum-free media was added. The procedure was conducted as above, beginning with TCA-fixation. Absorbance readings at 565 nm for each sample were compared to the appropriate standard curve to obtain final cell numbers.

3.9 Induced Cytotoxicity using MTX or 5-FU

Similar to the viability studies, experiments were conducted on the colon carcinoma cell lines in which varying concentrations of the chemotherapeutic drugs, methotrexate (Sigma-Aldrich) or 5-fluorouracil (Sigma-Aldrich), were introduced

with the culture media (MEM). For storage at -20°C, MTX and 5-FU were dissolved in distilled water at concentrations of 1.1 mM and 50 mM, respectively. Before addition to MEM, both drugs were diluted in phosphate-buffered saline (PBS) pH 7.4 (Gibco-BRL). Cells were subjected to increasing concentrations of either MTX or 5-FU and counted after 3 days.

3.10 Protein Extraction

Protein was extracted from frozen cell pellets (i.e. harvested carcinoma cells stored at -70°C) and used to measure MTHFR activity. Pellets were frozen and thawed 3 times and 100-150 µl of 0.1 M potassium phosphate pH 6.3 was added for resuspension. The resuspended pellet was centrifuged at 14,000 rpm for 10 minutes at 4°C. Protein concentration of the supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules CA).

3.11 MTHFR Assay

Using a modification of a previous method (Rosenblatt 1977), enzyme activity was assessed from crude protein extracts of 4 colon carcinoma cell lines, grown in various media. Approximately 50-100 µg of the protein extract was incubated for 1 hour at 37°C in a reaction mixture containing 180 µM potassium phosphate pH 6.3, 3.6 mM menadione bisulfite, 1.43 mM EDTA pH 6.3, 7.17 mM ascorbic acid, 29 µM FAD and 400 µM [5-^{14}C]-methyltetrahydrofolate (753 cpm/nmol) as the barium salt in 33 mM 2-mercaptoethanol with a final volume of 280 µl. Following incubation, the reaction was terminated by the addition of 250 µl 0.6 M sodium acetate pH 4.5, 150 µl

0.4 M dimedone and 100 µl 0.1 M formaldehyde. Mixtures were boiled for 12 minutes and cooled on ice. 2.5 ml toluene was added to each sample, and tubes were then vortexed twice for 15 seconds. After 10 minutes centrifugation at 2000 rpm, 1 ml of the upper organic phase was sent for scintillation counting to quantify the [^{14}C]-5,10-methylenetetrahydrofolate product that had dissociated to form a [^{14}C]-radiolabeled formaldehyde-dimedone adduct. Enzyme activity was expressed as nmol formaldehyde/mg protein/hour.

3.12 Transfection with Oligonucleotides

Oligonucleotides (20 bp in length) with phosphorothioate backbones, used for transfections, were made by Synthetic Genetics (San Diego CA). The oligonucleotides were as follows:

- Control (13706) – 5' TGC TCG GCT GTA AGT GTG AG 3'
- 12790 – 5' TGG TCT GCA GTT AGG GTC AG 3'
- MTHFR 677C – 5' GAT GAA ATC GGC TCC CGC AG 3'
- MTHFR 677T – 5' GAT GAA ATC GAC TCC CGC AG 3'

The control (13706) oligonucleotide is 6 bp mismatched to the specific antisense (12790) oligonucleotide directed against the 3' UTR of Replication Protein A (RPA). Targeting RPA was used as a positive control to confirm the methodology and efficiency of transfections. MTHFR 677C targets the wild type MTHFR transcript while MTHFR 677T targets the mutant gene with respect to the 677C→T (A→V) polymorphism.

Colon carcinoma cells were plated in MEM at variable densities in 6-well plates or 10 cm tissue culture dishes and incubated (37°C, 5% CO_2) overnight. Each well was washed with OPTI-MEM I Reduced Serum Media (Gibco-BRL). OPTI-MEM media (1-3 ml) was added with 12 μg/ml Lipofectin reagent (Gibco-BRL) and 400 nM of the particular oligonucleotide. Media was replaced with MEM (2-6 ml) after incubation for 5 hours.

3.13 RNA isolation

RNA was isolated from carcinoma cells 1 day after transfection with oligonucleotides. Cells were washed twice with PBS and 500 μl of Solution 1 (2% SDS, 200 mM Tris-Cl pH 7.5, 1 mM EDTA) was added. After transferring to 1.5 ml eppendorf tubes, cells were vortexed for 15 seconds. 150 μl of Solution 2 (100 ml of 42.9 g potassium actetate, 11.2 ml acetic acid and water) were added to each tube and chilled on ice for 2 minutes. Tubes were centrifuged at room temperature (14,000 rpm) for 5 minutes and the supernatant was recovered. The first extraction was conducted with 300 μl phenol:chloroform:isoamyl alcohol. The upper-phase was recovered and extracted with 300 μl chloroform:isoamyl alcohol. Cold isopropanol (650 μl) was mixed with the recovered upper-phase and cooled on ice for 15 minutes. Tubes were centrifuged for 15 minutes at 14,000 rpm, 4°C and pellets were washed with 70% ethanol. The RNA pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated water and the concentration was calculated after measuring the absorbance at 260 nm.

3.14 Killing Assay

Transfection with oligonucleotides was conducted as described in section 3.12 for 2 to 3 consecutive days followed by a 1 to 6 day period of cell regrowth in MEM. Cells were counted using the FluoroReporter Colorimetric Cell Protein Assay Kit.

3.15 Gel Electrophoresis of RNA

RNA (5-20 μg) was resuspended in 10-20 μl of loading solution containing 105 μl 10x MOPS buffer (400 nM MOPS, 10 mM EDTA, 100 mM sodium acetate), 183 μl formaldehyde (Fisher Scientific), 525 μl formamide (Gibco-BRL), 237 μl DEPC-treated water, 2 μl ethidium bromide (Gibco-BRL) and 2 μl TBE dye. Denatured RNA was loaded onto an agarose gel (1.2 g agarose, 87 ml dH_2O, 10 ml 10x MOPS buffer, 3 ml formaldehyde) and electrophoresed at 70-80 volts for approximately 2.5 hours in 1x MOPS buffer.

3.16 Transfer of Denatured RNA to Nylon Membrane

RNA was transferred immediately after electrophoresis from agarose gel to Zetabind membrane (Cuno Inc.; Meriden CT) by capillary elution after washing the agarose gel in 10x SSC (Sambrook 1989) for 45 minutes. Filter paper on a plexiglass support served as a bridge with 10x SSC as the transfer buffer. The gel was placed on top of the support in an inverted position with the nylon membrane, cut to the gel's size and rinsed with DEPC-treated water, laid on top. Four pieces of filter paper, of gel size, were wet in 10x SSC and placed on top of the wet membrane. The gel was then surrounded with Saran Wrap and ~10 cm of paper towels stacked on top. A glass

weight was applied to the top of the paper towels to complete the transfer system. Transfer of the RNA proceeded for at least 16 hours after which the membrane was rinsed in 2x SSC and vacuum dried at 80°C for 30 minutes. The membrane was rinsed briefly in DEPC-treated water before Northern hybridization was conducted.

3.17 Probes for Northern Analysis

An 810 bp RPA cDNA probe corresponding to base pairs 1520 to 2330 of the RPA gene was a gift from Variagenics Inc. (Cambridge MA).

A pTrc99A plasmid (Pharmacia; Baie d'Urfe QC) containing the 2.2 kb MTHFR cDNA (pTrc99A MR) was digested with EcoRI and MscI (Gibco-BRL). Following electrophoresis on 1% agarose gel, the 1.26 kb band, representing the 3' end of MTHFR cDNA beginning from bp 943, was purified using Gene Clean (Bio 101; Vista CA).

RPA and MTHFR cDNA probes were radiolabeled with $\propto^{32}$P-dCTP (Mandel; Guelph ON) using Multiprime DNA labeling systems (Amersham; Arlington IL) to an average specific activity of 10^8 cpm/μg DNA.

3.18 Northern Hybridization

Northern analysis was conducted following the protocol outlined with QuikHyb Hybrdization Solution (Stratagene; LaJolla CA). After RNA transfer, the membrane was prehybridized in QuikHyb solution in a shaking 68°C waterbath for 1-2 hours. Fresh hybridization solution with 3 μg sonicated salmon sperm DNA and 12.5 x 10^6-25 x 10^6 cpm probe were used for hybridization at 68°C in a shaking

waterbath for 2 hours. The Northern blot was washed twice in 250 ml of 2x SSC/0.1% SDS for 15 minutes (room temperature). The final wash was with 500 ml of 0.1x SSC/0.1% SDS at 60°C for 30-45 minutes. The membrane was exposed to Kodak Biomax MS film (Rochester NY) (70°C; 1-2 days) and developed.

4. RESULTS

4.1 Loss of Heterozygosity of MTHFR

Allelic losses of the MTHFR gene were identified using PCR amplification and HinfI digestion. For the ovarian carcinoma cases, 37% (16/43) showed the heterozygous (A/V) genotype. Tumor DNA samples from 15 of the A/V individuals were assessed for LOH (one tumor DNA sample was unavailable). Ethidium bromide staining showed loss of heterozygosity of the MTHFR locus occurring in 6 of 15 tumor samples (40% frequency of LOH) (Figure 4A). Radioactive PCR and visual inspection of ^{35}S-labeled digests verified the methodology and confirmed the above results (Figure 4B). Three of the LOH samples lost the alanine allele, while 3 showed partial or complete loss of the valine allele (Table 1). Since both non-radioactive and radioactive methods gave similar results, only the non-radioactive method was used to evaluate the colon carcinoma samples.

Heterozygosity in the British colorectal carcinoma population was displayed at a frequency of 36.5% (31/85) with 6 of the tumor samples (19%) undergoing complete or partial loss of either the alanine (2 cases) or valine (4 cases) allele. In the Montreal population, 62% (39/63) of the colon cancer patients were A/V in genotype. Only 5 of the tumor samples from these 39 individuals (13%) exhibited LOH; 1 tumor lost the alanine allele and 4 lost the valine allele (Figure 4C). Table 1 presents a summary of the results (Pereira 1999).

Figure 4. Representative examples of LOH analyses of the alanine to valine polymorphism at bp 677 of MTHFR. The upper band represents the alanine allele and the lower band represents the valine allele. Arrows indicate the loss of one allele in the tumor DNA. Mainly partial LOH in the carcinoma samples was observed due to the possible incorporation of normal tissue during the preparation of the tumor samples. A) LOH analyses using ethidium bromide staining in ovarian carcinoma. B) LOH analyses using digests of ^{35}S-labeled PCR products for the same ovarian DNA samples shown in A. C) LOH analyses using ethidium bromide staining in colorectal carcinoma.
(N=Normal; T=Tumor)

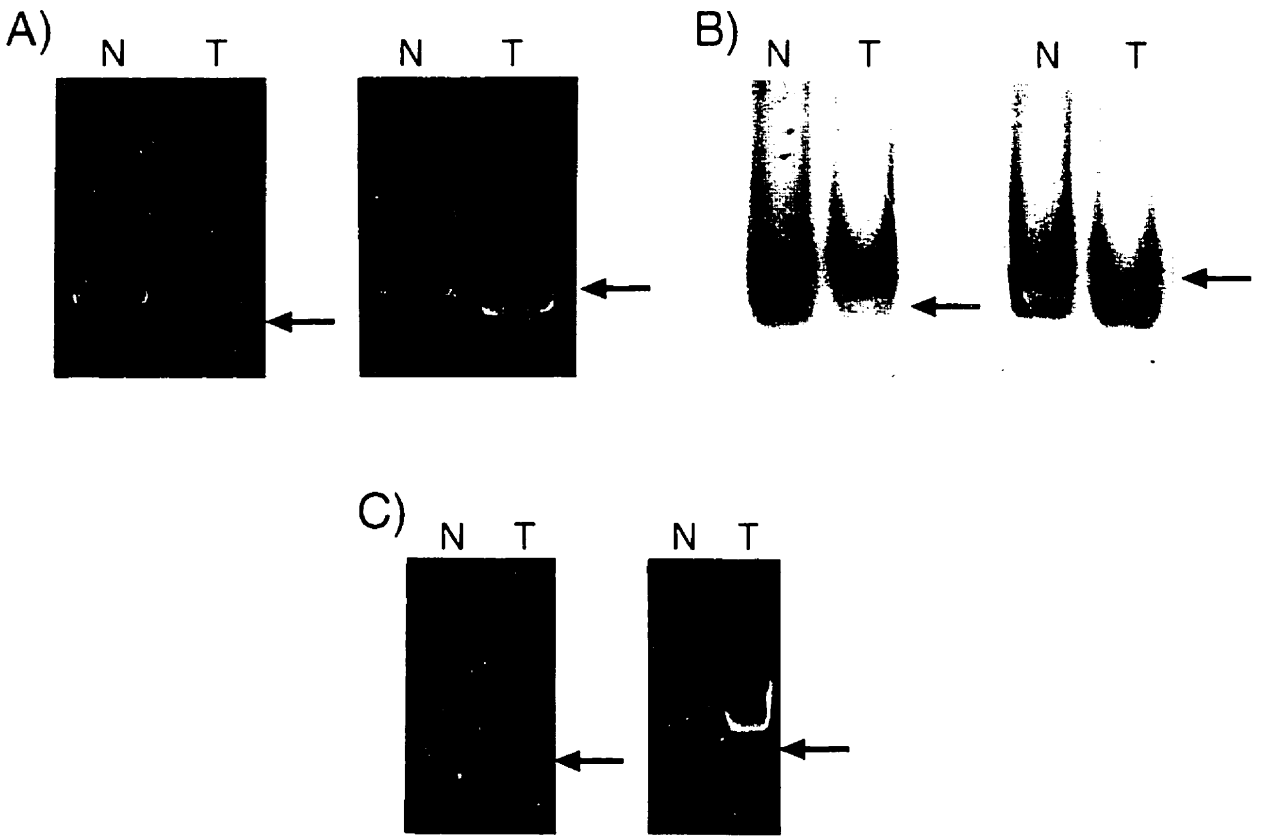

A)
N
T
N
T
B)
N
T
N
T
C)
N
T
N
T

Table 1. Loss of heterozygosity analysis of MTHFR in ovarian and colorectal carcinomas.

Carcinoma Type	% A/V Genotype	% LOH	Allelic Loss
Ovarian (UK)	37% (16/43)	40% (6/15)	3 alanine: 3 valine
Colorectal (UK)	36.5% (31/85)	19% (6/31)	2 alanine; 4 valine
Colorectal (Montreal)	62% (39/63)	13% (5/39)	1 alanine; 4 valine

4.2 Cell Survival Studies

The cell survival studies in MEM and deficient media showed that both the fibroblast and transformed lines were very sensitive to media lacking methionine, M-, (in the presence or absence of folate) such that growth was minimal and their viability was compromised compared to proliferation in MEM (Figures 5,6). During the time course of this experiment, fibroblast lines were unaffected by folate-deficient media (F-). Colon carcinoma cell lines showed variable growth in folate-deficient media possibly due to differences between cell lines in levels of intracellular folate, which is important for both DNA and methionine synthesis (Figure 2).

Replacing methionine with homocysteine and supplementing the growth media with vitamin B_{12} (M-+) in order to restore de novo methionine synthesis restored growth of control fibroblasts to levels that approached those obtained in replete MEM (Figure 7). The mutant fibroblast line (WG 1554), with zero MTHFR activity, was unable to restore growth due to its inability to synthesize 5-methyltetrahydrofolate, the folate derivative for methionine synthesis (Figure 7). In M-+, the carcinoma cell lines increased their proliferation only slightly through endogenous methionine synthesis (Figure 8), but their survival was just a small percentage of levels obtained in MEM. This supports the notion of methionine dependence of transformed lines and the necessity for exogenous methionine to meet demands for rapid cell proliferation. Numerical data for fibroblast and colon carcinoma cell survival studies are presented in Tables 2 and 3, respectively.

Figure 5. Growth response of fibroblast cell lines in deficient media. MEM (minimum essential media), F- (folate-deficient media), M- (methionine-deficient media). All 3 lines showed an inability to grow when methionine is lacking from the growth media. Data points represent the mean of 3 replicates.

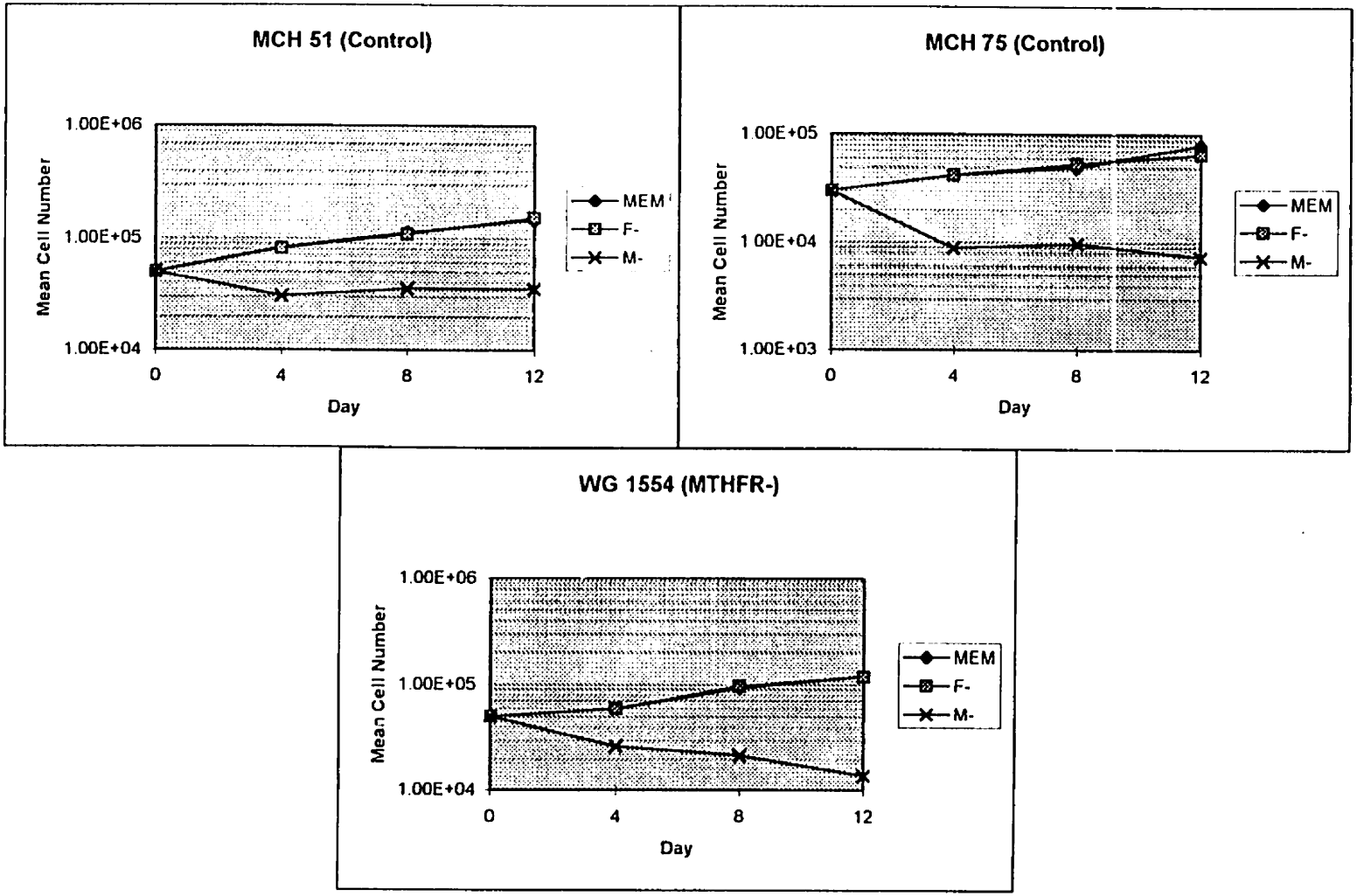

MCH 51 (Control)
1.00E+06
1.00E+05
1.00E+04
Mean Cell Number
0
4
8
12
Day
MEM
F-
M-
MCH 75 (Control)
1.00E+05
1.00E+04
1.00E+03
Mean Cell Number
0
4
8
12
Day
MEM
F-
M-
WG 1554 (MTHFR-)
1.00E+06
1.00E+05
1.00E+04
Mean Cell Number
0
4
8
12
Day
MEM
F-
M-

Figure 6. Growth response of colon carcinoma cell lines in deficient media. MEM (minimum essential media), F- (folate-deficient media), M- (methionine-deficient media). All 4 carcinoma lines (regardless of genotype) showed extreme sensitivity to methionine-deficient media. Data points represent the mean of 3 replicates.

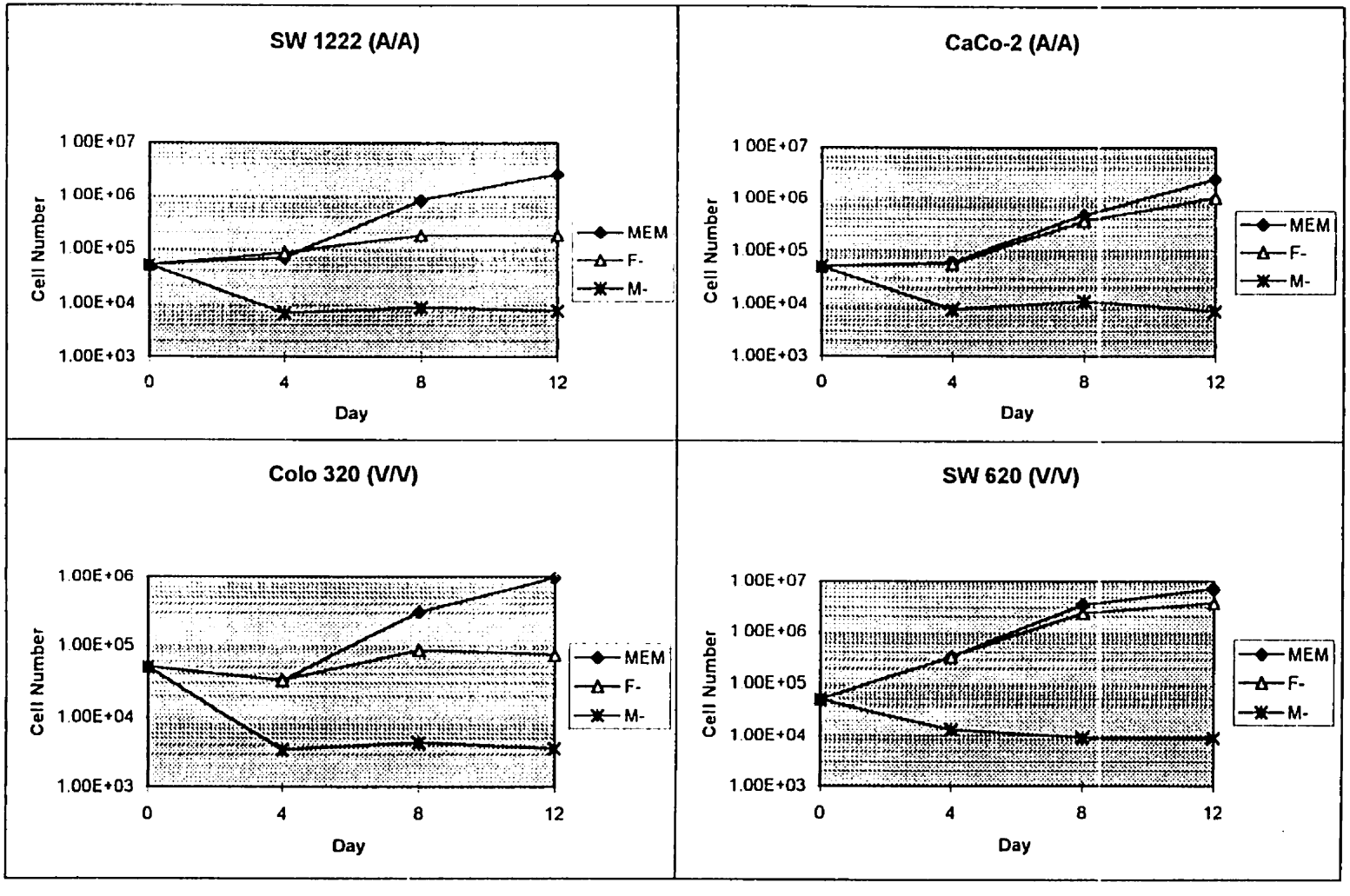

SW 1222 (A/A)
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Cell Number
0
4
8
12
Day
MEM
F-
M-
CaCo-2 (A/A)
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Cell Number
0
4
8
12
Day
MEM
F-
M-
Colo 320 (V/V)
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Cell Number
0
4
8
12
Day
MEM
F-
M-
SW 620 (V/V)
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Cell Number
0
4
8
12
Day
MEM
F-
M-

Figure 7. Supplementation of methionine-deficient media with homocysteine and vitamin B_{12} in fibroblast cell lines. The control fibroblast lines were able to restore growth in supplemented media (M-+) whereas the MTHFR null mutant was unable to do so. Data points represent the mean of 3 replicates.

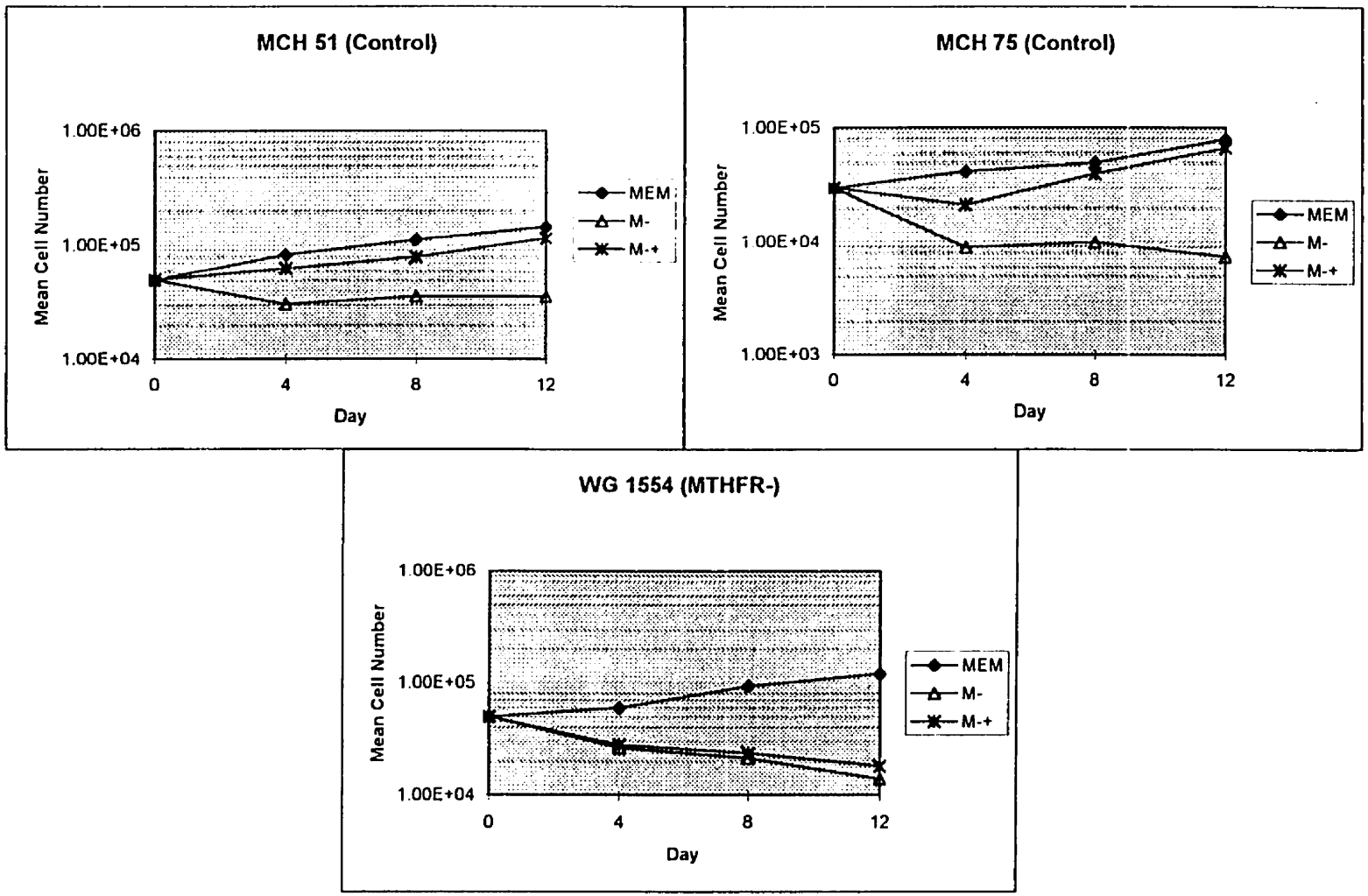

MCH 51 (Control)
Mean Cell Number
1.00E+06
1.00E+05
1.00E+04
0
4
8
12
Day
MEM
M-
M-+
MCH 75 (Control)
Mean Cell Number
1.00E+05
1.00E+04
1.00E+03
0
4
8
12
Day
MEM
M-
M-+
WG 1554 (MTHFR-)
Mean Cell Number
1.00E+06
1.00E+05
1.00E+04
0
4
8
12
Day
MEM
M-
M-+

Figure 8. Supplementation of methionine-deficient media with homocysteine and vitamin B_{12} in colon carcinoma cell lines. The carcinoma cells only slightly increased proliferation in the supplemented media (M-+). Data points represent the mean of 3 replicates.

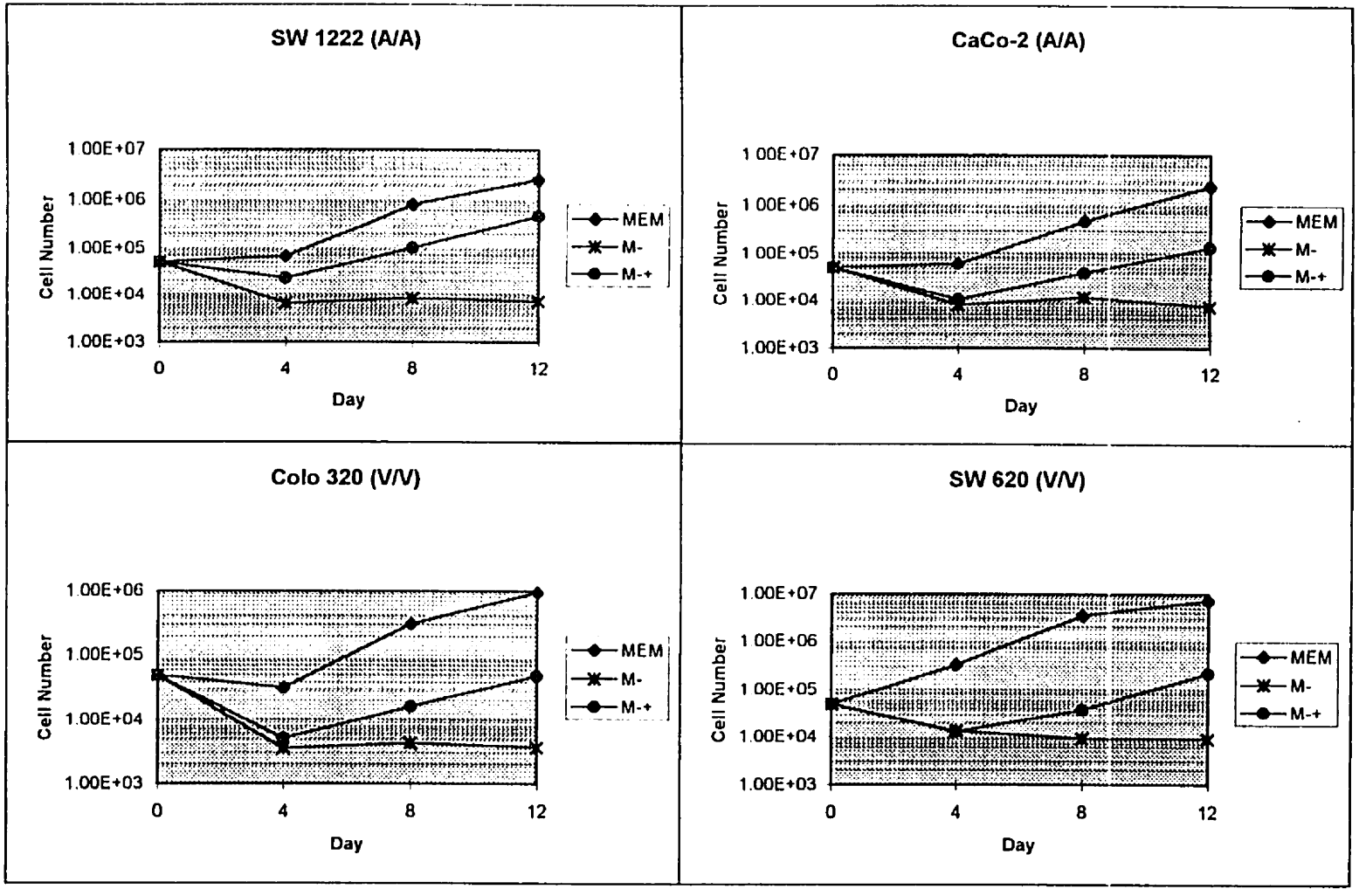

SW 1222 (A/A)
Cell Number
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
0
4
8
12
Day
MEM
M-
M-+
CaCo-2 (A/A)
Cell Number
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
0
4
8
12
Day
MEM
M-
M-+
Colo 320 (V/V)
Cell Number
1.00E+06
1.00E+05
1.00E+04
1.00E+03
0
4
8
12
Day
MEM
M-
M-+
SW 620 (V/V)
Cell Number
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
0
4
8
12
Day
MEM
M-
M-+

Table 2. Numerical results for fibroblast cell survival study. Standard deviation of 3 replicates presented in parentheses.

Cell Line	Media	Day	Mean Cell Count ($x 10^4$)
MCH 51	MEM	0	5.00
		4	8.28 (0.36)
		8	11.15 (0.67)
		12	14.59 (2.57)
	F-	0	5.00
		4	8.08 (0.53)
		8	10.82 (0.86)
		12	15.29 (3.35)
	M-	0	5.00
		4	3.05 (0.07)
		8	3.58 (0.47)
		12	3.57 (0.54)
	M-+	0	5.00
		4	6.30 (0.78)
		8	7.89 (0.44)
		12	11.61 (0.17)
MCH 75	MEM	0	3.00
		4	4.17 (0.27)
		8	4.95 (0.14)
		12	7.94 (0.38)
	F-	0	3.00
		4	4.23 (0.42)
		8	5.33 (0.48)
		12	6.49 (0.38)
	M-	0	3.00
		4	0.89 (0.04)
		8	0.98 (0.14)
		12	0.73 (0.17)
	M-+	0	3.00
		4	2.12 (0.35)
		8	4.00 (0.69)
		12	6.67 (0.91)

Cell Line	Media	Day	Mean Cell Count ($x 10^4$)
WG 1554	MEM	0	5.00
		4	5.94 (0.11)
		8	9.28 (0.12)
		12	11.99 (0.91)
	F-	0	5.00
		4	5.95 (0.52)
		8	9.59 (0.92)
		12	11.78 (0.84)
	M-	0	5.00
		4	2.63 (0.14)
		8	2.14 (0.17)
		12	1.38 (0.07)
	M-+	0	5.00
		4	2.78 (0.04)
		8	2.37 (0.13)
		12	1.80 (0.04)

Table 3. Numerical results for colon carcinoma cell survival study. Standard deviation of 3 replicates presented in parentheses.

Cell Line	Media	Day	Mean Cell Count ($x 10^4$)
SW 1222 (A/A)	MEM	0	5.00
		4	6.76 (0.23)
		8	81.00 (10.64)
		12	254.33 (41.40)
	F-	0	5.00
		4	8.40 (2.00)
		8	17.90 (0.13)
		12	17.68 (3.00)
	M-	0	5.00
		4	0.66 (0.10)
		8	0.83 (0.20)
		12	0.73 (0.17)
	M-+	0	5.00
		4	2.25 (0.36)
		8	10.25 (0.87)
		12	47.50 (0.64)
CaCo-2 (A/A)	MEM	0	5.00
		4	6.09 (0.69)
		8	48.17 (7.52)
		12	237.33 (29.69)
	F-	0	5.00
		4	5.60 (2.35)
		8	36.82 (10.47)
		12	103.33 (8.31)
	M-	0	5.00
		4	0.78 (0.16)
		8	1.13 (0.61)
		12	0.73 (0.13)
	M-+	0	5.00
		4	1.02 (0.19)
		8	3.83 (1.17)
		12	13.38 (1.73)

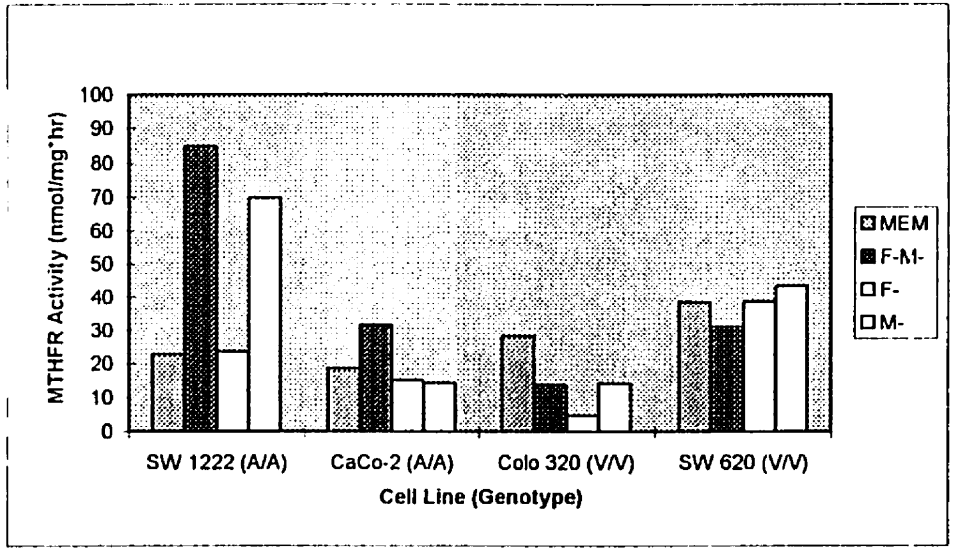
Cell Line	Media	Day	Mean Cell Count ($x 10^4$)
Colo 320 (V/V)	MEM	0	5.00
		4	3.20 (0.07)
		8	30.36 (9.21)
		12	96.22 (22.77)
	F-	0	5.00
		4	3.29 (0.28)
		8	8.54 (0.52)
		12	7.37 (0.24)
	M-	0	5.00
		4	0.35 (0.12)
		8	0.43 (0.09)
		12	0.36 (0.04)
	M-+	0	5.00
		4	0.50 (0.13)
		8	1.61 (0.50)
		12	4.90 (0.72)
SW 620 (V/V)	MEM	0	5.00
		4	32.60 (9.67)
		8	345.33 (56.16)
		12	720.33 (89.59)
	F-	0	5.00
		4	32.17 (6.50)
		8	234.33 (48.84)
		12	369.67 (19.73)
	M-	0	5.00
		4	1.30 (0.60)
		8	0.91 (0.23)
		12	0.90 (0.22)
	M-+	0	5.00
		4	1.26 (0.22)
		8	3.78 (1.62)
		12	22.21 (2.82)

4.3 MTHFR Activity

MTHFR activity was assayed in 4 transformed colon lines after 14 days of growth in MEM or deficient media containing non-dialyzed serum and after 5 days growth in MEM or deficient media, all containing dialyzed serum (Figure 9A, B). After 14 days in deficient media with non-dialyzed serum, only the two A/A carcinoma lines (SW 1222, CaCo-2) responded with an upregulation of MTHFR activity when both folate and methionine were lacking from the media (Figure 9A). The MTHFR activity of the V/V lines (Colo 320, SW 620) did not appear to be regulated under these conditions. These observations may be due to the presence of folate and/or methionine in the added serum. Therefore, the experiment was repeated using dialyzed serum in the four types of media. After 5 days, all 4 carcinoma lines displayed similar patterns in response by increasing reductase activity relative to levels in MEM when deprived of methionine, in the presence or absence of folate (Figure 9B). Differences in enzyme activity levels seen between the colon cancer lines may be explained by their degree of transformation and variations in intracellular folate and methionine stores. The increase in MTHFR activity for the 4 lines in M- media showed borderline significance by t-test ($p=0.05$-0.10). Interestingly, SW 620 also upregulated enzyme activity when grown without folate for 5 days. To examine whether the other lines respond to folate-depleted media in the same manner, enzyme activity was measured following 12 days growth in F- or M- as well as deficient media supplemented with 0.44 mM homocysteine and 1.5 μM vitamin B_{12} (Figure 10). Homocysteine and vitamin B_{12} utilization leads to production of methionine and SAM, an inhibitor of MTHFR. Over this extended period, all 4 lines increased activity in F-

Figure 9. MTHFR activity in colon carcinoma lines after 14 days growth in deficient media containing non-dialyzed serum (A) or 5 days growth in deficient media containing dialyzed serum (B). All 4 colon lines showed an increase in reductase activity when deprived of methionine in media utilizing dialyzed serum (B). Only the A/A colon carcinoma lines appeared to regulate MTHFR activity in media deficient in both folate and methionine (F-M-) with non-dialyzed serum (A).

A)


MTHFR Activity (nmol/mg*hr)
100
90
80
70
60
50
40
30
20
10
0
SW 1222 (A/A)
CaCo-2 (A/A)
Colo 320 (V/V)
SW 620 (V/V)
Cell Line (Genotype)
MEM
F-M-
F-
M-

B)

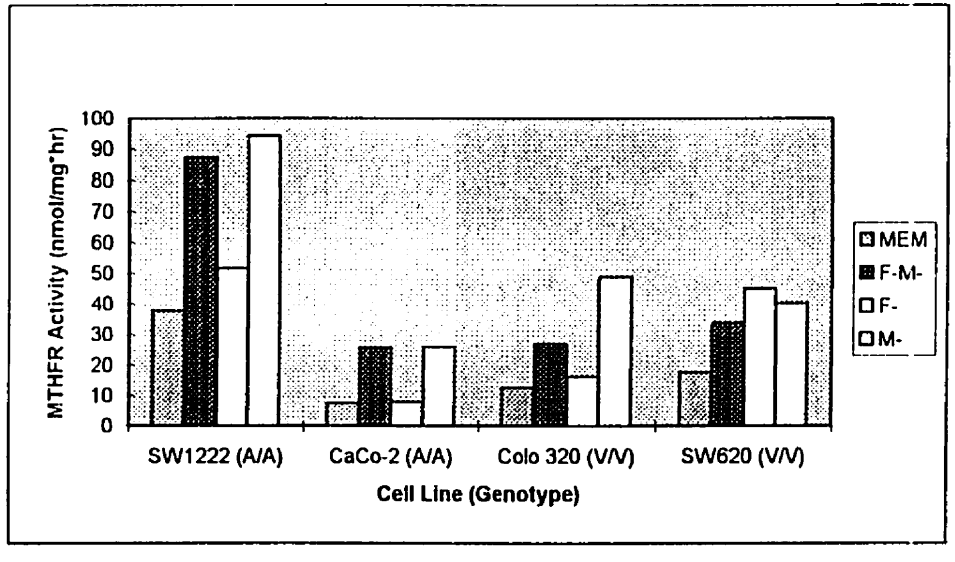
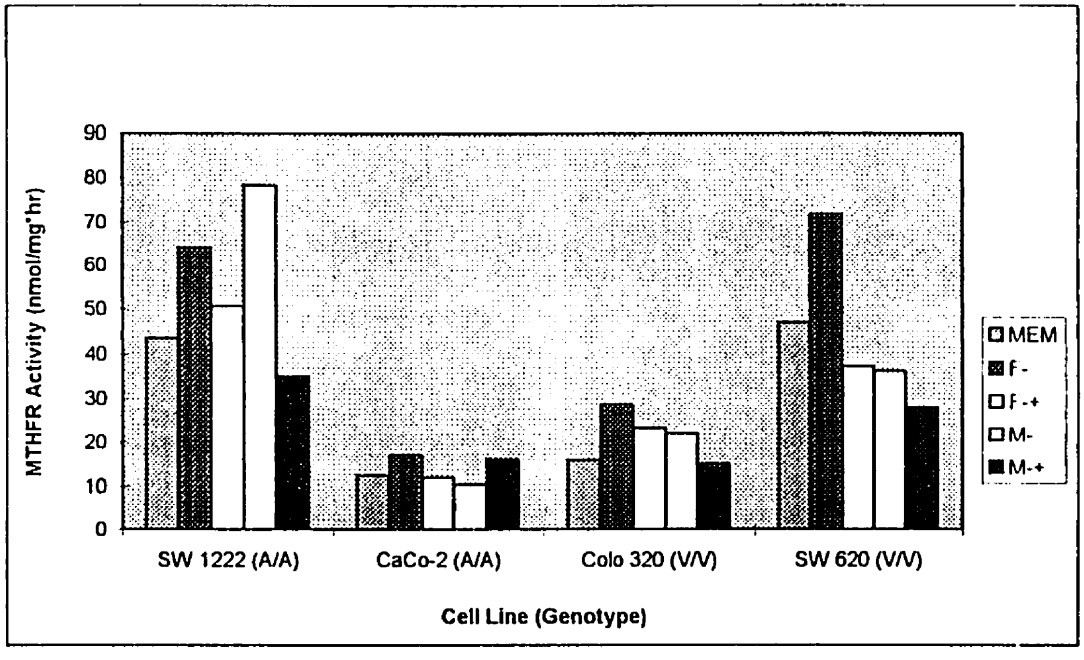

MTHFR Activity (nmol/mg*hr)
100
90
80
70
60
50
40
30
20
10
0
SW1222 (A/A)
CaCo-2 (A/A)
Colo 320 (V/V)
SW620 (V/V)
Cell Line (Genotype)
MEM
F-M-
F-
M-

Figure 10. MTHFR activity in colon carcinoma cell lines after 12 days growth in deficient media plus homocysteine and vitamin B_{12} (+). After 12 days, all 4 lines upregulated MTHFR activity in folate-deficient media.


MTHFR Activity (nmol/mg*hr)
90
80
70
60
50
40
30
20
10
0
SW 1222 (A/A)
CaCo-2 (A/A)
Colo 320 (V/V)
SW 620 (V/V)
Cell Line (Genotype)
MEM
f -
F -+
M-
M-+

media possibly in an effort to meet methionine requirements. As expected, the supplemented media (F-+, M-+) led to a reduction of the otherwise increased MTHFR activity observed without homocysteine or vitamin B_{12} addition (F-, M-) in 3 of the lines. When methionine is lacking, 3 of the 4 lines no longer showed the increased activity seen at 5 days. This may be explained by the high degree of cell death after 12 days (as discussed in section 4.2).

4.4 Allele-Specific Targeting

4.4.1 Targeting Replication Protein A

Transfection of SW 620 was conducted with the antisense oligonucleotide directed against Replication Protein A (RPA), used as a positive control in order to optimize transfection conditions. Variagenics Inc. has been able to decrease RPA mRNA levels considerably leading to cytotoxicity in culture. Reproducing antisense results of RPA was attempted before beginning antisense experiments against the MTHFR message. For transfection, the control oligonucleotide (13706) contained 6 mismatched base pairs compared with the specific antisense oligonucleotide (12790) that targets the 3' UTR of RPA. The antisense oligonucleotide decreased mRNA levels as seen by Northern analysis, but the amount of reduction was dependent on the initial cell density used for transfection. Transfection with 12790 at lower densities, in the range of 90,000 cells per 3.5 cm well, showed considerable decrease in the RPA transcript levels compared with 13706 control (Figure 11A). Reduction of mRNA levels, at higher cell densities was not as significant (data not shown).

Figure 11. Northern analysis following transfection with control or antisense oligonucleotides targeting RPA or MTHFR. Use of antisense oligonucleotides resulted in mRNA knockdown compared with the control transfections. A) 90,000 SW 620 cells per 3.5 cm well were transfected with control (13706) or antisense (12790) oligonucleotides directed against RPA. 4 μg of RNA were loaded in each lane as shown in the ethidium bromide stained gel (lower panel). B) 600,000 SW 620 cells per 10 cm dish were transfected with control, mismatched (MTHFR 677C) or antisense (MTHFR 677T) oligonucleotides directed against MTHFR. Approximately 10 μg of RNA were loaded in each lane as shown in the ethidium bromide stained gel (lower panel). Arrow indicates MTHFR full-length transcript; asterisk indicates an alternate product.

A)
13706
12790
12790
28S -
18S -
13706
12790
12790
B)
Control
MTHFR 677C
MTHFR 677T
*
28S -
18S -
Control
MTHFR 677C
MTHFR 677T

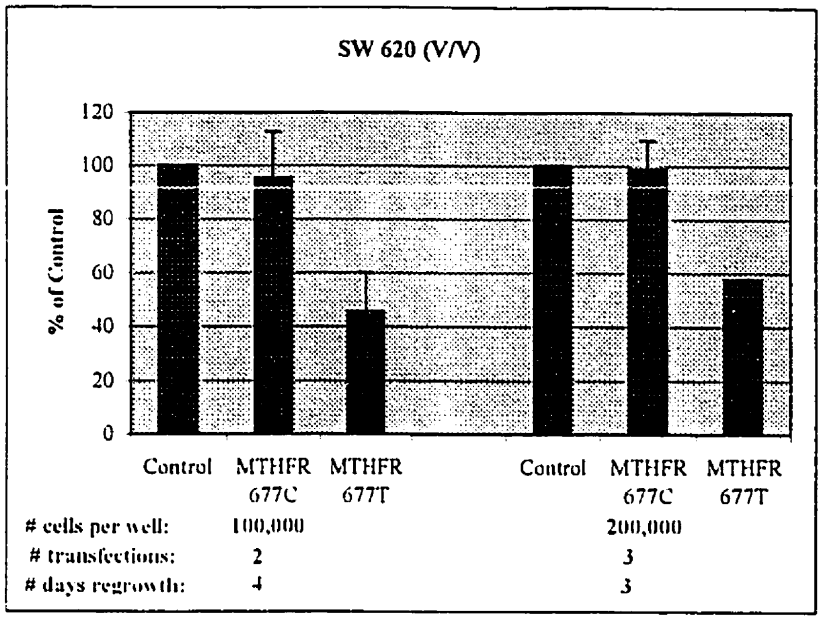
A killing assay using an initial cell density similar to that seen in Figure 11A showed a 3-fold decrease in survival of cells transfected with 12790 compared with the control (similar to the results obtained by Variagenics Inc.).

4.4.2 Targeting MTHFR

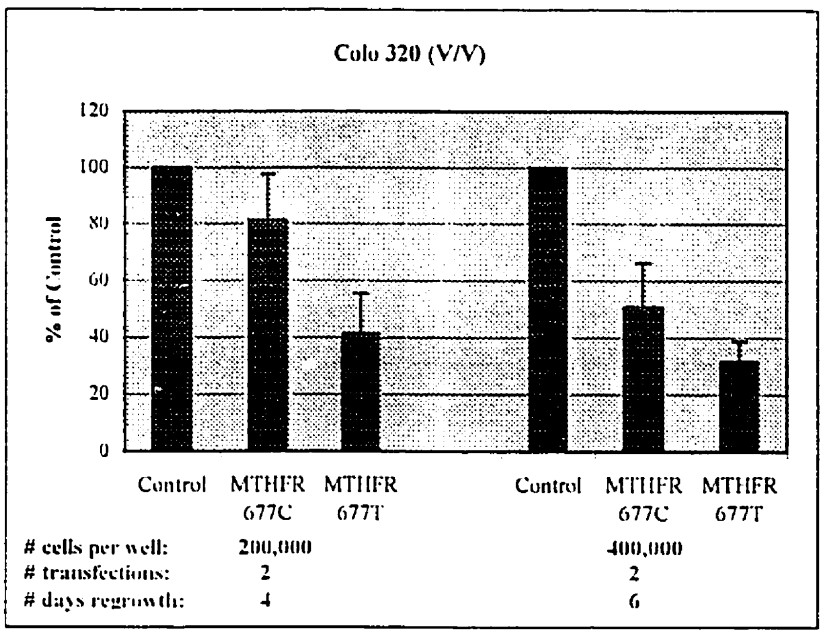
Following transfection of SW 620 cells (600,000 cells per 10 cm dish) with the antisense oligonucleotide (MTHFR 677T), reduction of levels of full-length MTHFR transcript was observed, compared with levels seen after transfection with the control oligonucleotide or mismatched (MTHFR 677C) oligonucleotide targeting the wild type allele that is not present in this cell line (SW 620 is homozygous mutant; V/V or T/T) (arrow, Figure 11B). However, an alternate-sized message seemed to arise after MTHFR allele targeting (asterisk, Figure 11B). The decrease in the full-length MTHFR mRNA may explain the ~50% reduced cell viability of SW 620 cells treated with the antisense oligonucleotide as observed from killing assays (Figure 12A). Use of the mismatched oligonucleotide showed no significant effect on cell survival relative to the control and confirms the specific nature of this allele targeting technique. Transfection of another homozygous mutant colon carcinoma cell line, Colo 320, with the 3 oligonucleotides under different conditions resulted in an approximate 3-fold decreased cell survival using the antisense oligonucleotide compared with control transfection (Figure 12B). The decreased viability with MTHFR 677T was ~2-fold greater than that observed with the mismatched MTHFR 677C, again providing evidence for allele-specific targeting.

Figure 12. Outcome of killing assays following multiple transfections with oligonucleotides targeting MTHFR mRNA. A) Transfection of SW 620. B) Transfection of Colo 320. Control, mismatched (MTHFR 677C), or antisense (MTHFR 677T) oligonucleotides were used for multiple rounds of transfection. Reduction in cell viability was greater with the specific antisense oligonucleotide in both cell lines. Bars represent the mean of 3 replicates.

A)


SW 620 (V/V)
% of Control
120
100
80
60
40
20
0
Control
MTHFR 677C
MTHFR 677T
Control
MTHFR 677C
MTHFR 677T
cells per well:
100,000
200,000
transfections:
2
3
days regrowth:
4
3

B)


Colo 320 (V/V)
% of Control
120
100
80
60
40
20
0
Control
MTHFR 677C
MTHFR 677T
Control
MTHFR 677C
MTHFR 677T
cells per well:
200,000
400,000
transfections:
2
2
days regrowth:
4
6

4.5 Induced Cytotoxicity

Survival of colon carcinoma cell lines was examined following 3 days growth in MEM containing increasing concentrations of either methotrexate (MTX) or 5-fluorouracil (5-FU). IC_{50} represents the observed concentration of drug that inhibits cell survival by 50% compared to survival without the drug.

4.5.1 IC_{50} with Methotrexate

Responses of the A/A carcinoma lines (SW 1222; CaCo-2) and the V/V carcinoma lines (Colo 320; SW 620) to MTX treatment are shown graphically in Figure 13. Both of the V/V lines, with $IC_{50} < 25$ nM, showed greater sensitivity to MTX than the 2 wild type cell lines (Figure 13). Differences between genotypes may be due to variability in intracellular folate and methionine levels. To explore the possibility that the MTHFR genotype may be predictive of tumor cell responsiveness to MTX treatment, 4 additional A/A colon carcinoma lines were tested. Unfortunately, no other V/V lines were available. With increasing MTX concentrations, only one of the 4 new lines, SW 948, displayed a high IC_{50} value (200 nM) compared with the V/V lines (Figure 14). The IC_{50} values of LoVo, DLD-1 and HCT-15 were similar to those of the V/V carcinoma lines (Figure 14). The high MTX sensitivity of these 3 A/A lines may be due to rapid proliferation that could deplete intracellular levels of folate and methionine required for cell survival. Cellular proliferation of the V/V carcinoma cell line Colo 320, was much less than SW 620 (Figure 6). Therefore, regardless of growth rate, V/V genotype showed MTX

Figure 13. Induced cytotoxicity by methotrexate. After 3 days in the presence of increasing concentrations of methotrexate (MTX), carcinoma cell lines with the homozygous mutant genotype (V/V) showed a greater sensitivity to the drug (IC_{50} < 25 nM) compared to the A/A counterparts. Data points represent the mean of 3 replicates.

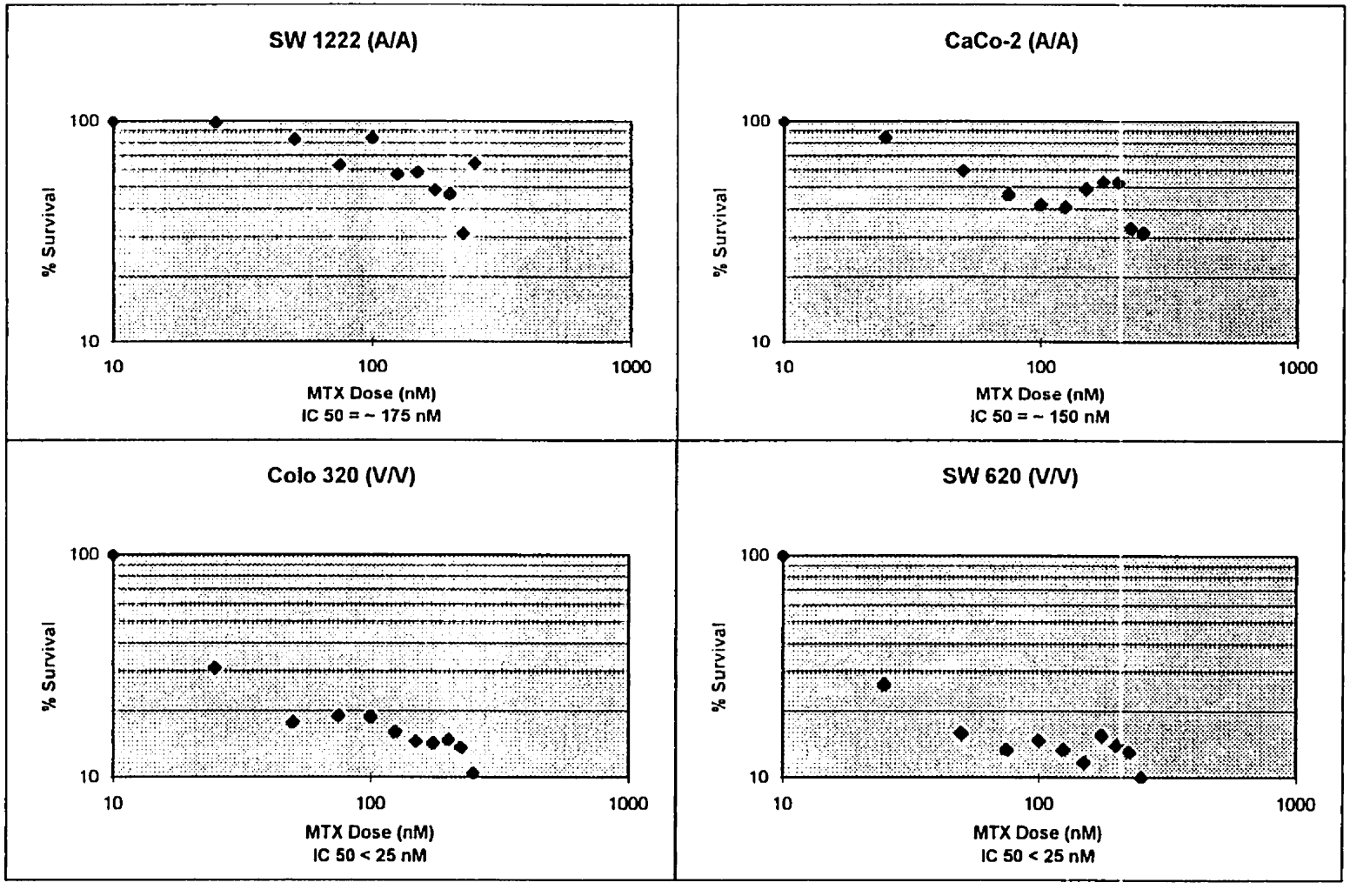
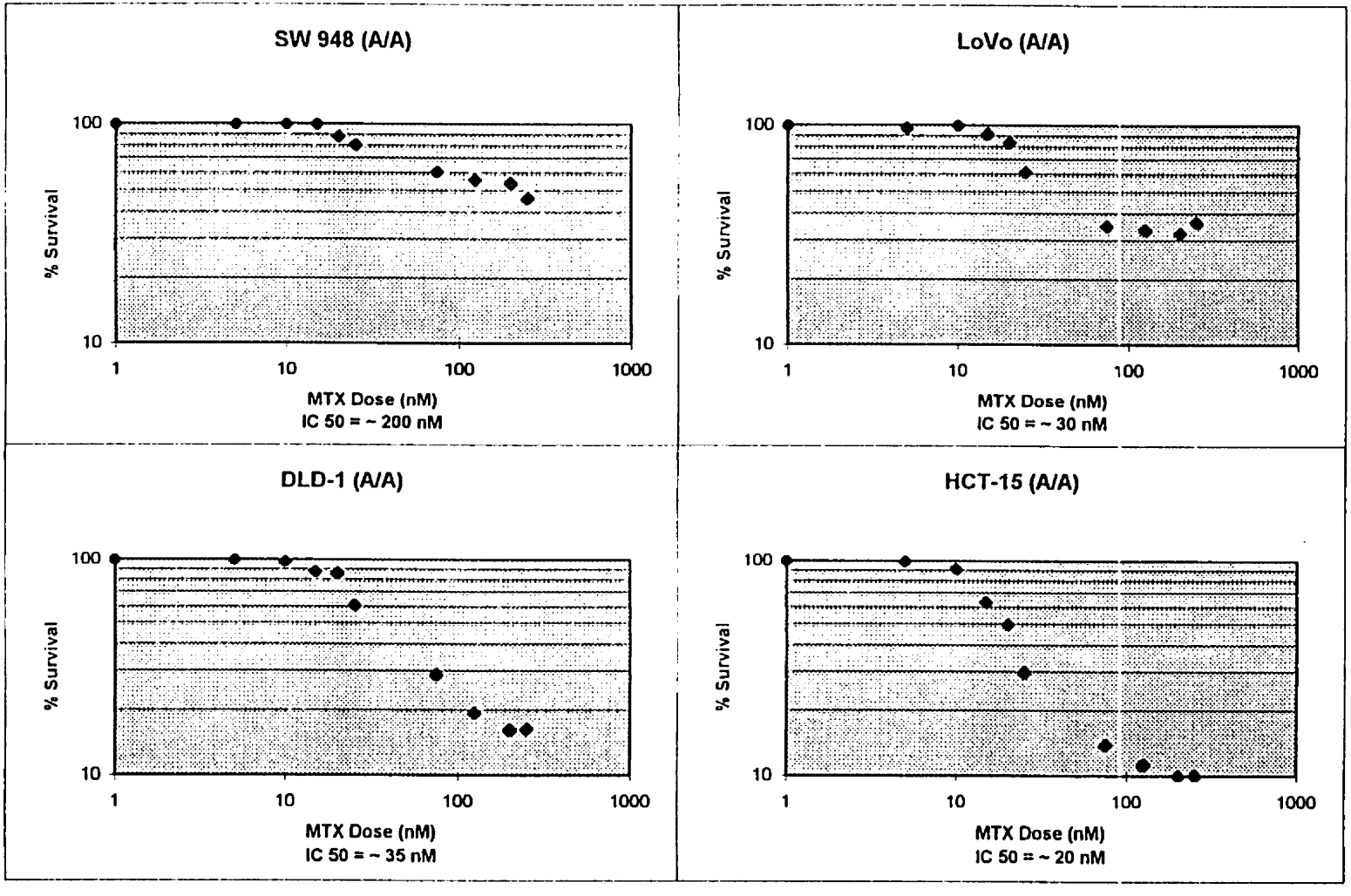

SW 1222 (A/A)
100
10
% Survival
10
100
1000
MTX Dose (nM)
IC 50 = ~ 175 nM
CaCo-2 (A/A)
100
10
% Survival
10
100
1000
MTX Dose (nM)
IC 50 = ~ 150 nM
Colo 320 (V/V)
100
10
% Survival
10
100
1000
MTX Dose (nM)
IC 50 < 25 nM
SW 620 (V/V)
100
10
% Survival
10
100
1000
MTX Dose (nM)
IC 50 < 25 nM

Figure 14. Induced cytotoxicity of additional A/A colon carcinoma cell lines by methotrexate. After 3 days in the presence of increasing concentrations of methotrexate (MTX), only SW 948 showed resistance to the drug. LoVo, DLD-1 and HCT-15 displayed IC_{50} values similar to those observed with the V/V lines. Data points represent the mean of 3 replicates.


SW 948 (A/A)
100
10
% Survival
1
10
100
1000
MTX Dose (nM)
IC 50 = ~ 200 nM
LoVo (A/A)
100
10
% Survival
1
10
100
1000
MTX Dose (nM)
IC 50 = ~ 30 nM
DLD-1 (A/A)
100
10
% Survival
1
10
100
1000
MTX Dose (nM)
IC 50 = ~ 35 nM
HCT-15 (A/A)
100
10
% Survival
1
10
100
1000
MTX Dose (nM)
IC 50 = ~ 20 nM

sensitivity. The A/A lines with high IC_{50} values (> 150 nM) exhibited rates of proliferation similar to or less than the V/V lines as determined by time-course experiments for 2 of the 3 resistant wild type lines (Figure 6). Values for the cell lines' responses to MTX treatment are presented in Table 4.

4.5.2 IC_{50} with 5-Fluorouracil

Induced cytotoxicity experiments with increasing concentrations of 5-FU showed only one of the V/V lines (Colo 320) being highly sensitive to the drug (IC_{50} < 5 μM). The observed IC_{50} of SW 620 (V/V genotype) was similar to that of the two A/A cell lines (Figure 15). Although there were no noticeable genotypic differences in 5-FU IC_{50} values, the percentage of surviving cells at the highest drug concentration was less in both V/V lines (20%-30%) compared with the A/A lines (~40%) (Figure 15; Table 5).

Table 4. Numerical results for induced cytotoxicity of colon carcinoma cell lines by methotrexate (MTX). Standard deviation of 3 replicates presented in parentheses.

Cell Line	MTX (nM)	% of MEM
SW 1222 (A/A)	0	100
	25	98.8 (0.8)
	50	82.5 (10.2)
	75	63.2 (10.8)
	100	83.4 (27.3)
	125	57.2 (5.4)
	150	58.5 (4.7)
	175	48.6 (2.4)
	200	46.5 (18.6)
	225	31.1 (18.5)
	250	64.6 (25.4)
CaCo-2 (A/A)	0	100
	25	84.0 (16.9)
	50	59.6 (12.0)
	75	46.1 (5.0)
	100	41.7 (2.0)
	125	40.6 (7.4)
	150	49.1 (15.0)
	175	52.7 (5.3)
	200	52.5 (12.4)
	225	32.8 (11.4)
	250	31.4 (10.3)

Cell Line	MTX (nM)	% of MEM
Colo 320 (V/V)	0	100
	25	30.9 (2.7)
	50	17.8 (2.6)
	75	19.0 (5.0)
	100	18.8 (2.5)
	125	16.1 (0.7)
	150	14.6 (6.1)
	175	14.3 (3.2)
	200	14.8 (3.5)
	225	13.7 (2.7)
	250	10.4 (0.6)
SW 620 (V/V)	0	100
	25	26.3 (12.3)
	50	15.9 (1.1)
	75	13.4 (1.7)
	100	14.8 (0.3)
	125	13.4 (1.5)
	150	11.7 (2.6)
	175	15.6 (1.6)
	200	14.0 (2.8)
	225	13.1 (1.6)
	250	9.2 (2.1)

Cell Line	MTX (nM)	% of MEM
SW 948 (A/A)	0	100
	5	>100
	10	>100
	15	>100
	20	88.0 (11.5)
	25	80.3 (12.5)
	75	60.9 (2.0)
	125	56.0 (6.4)
	200	53.9 (5.1)
	250	46.1 (11.2)
LoVo (A/A)	0	100
	5	97.3 (12.0)
	10	>100
	15	91.0 (5.3)
	20	83.1 (14.6)
	25	61.3 (9.9)
	75	34.8 (7.7)
	125	33.4 (9.7)
	200	32.2 (4.4)
	250	36.2 (5.5)

Cell Line	MTX (nM)	% of MEM
DLD-1 (A/A)	0	100
	5	>100
	10	97.9 (3.6)
	15	87.2 (7.1)
	20	85.7 (2.8)
	25	60.7 (6.2)
	75	28.7 (1.0)
	125	19.3 (2.3)
	200	15.9 (2.0)
	250	16.2 (1.7)
HCT-15 (A/A)	0	100
	5	99.3 (10.1)
	10	90.9 (6.5)
	15	63.5 (6.0)
	20	49.7 (9.0)
	25	29.7 (1.5)
	75	13.8 (1.8)
	125	11.2 (0.6)
	200	9.4 (1.1)
	250	9.9 (1.3)

Figure 15. Induced cytotoxicity by 5-fluorouracil. After 3 days in the presence of increasing concentrations of 5-fluorouracil (5-FU), only one V/V line (Colo 320) showed a low IC_{50} value. Both V/V lines had lower percentages of surviving cells at the highest concentration of 5-FU used in this study (20% and 30% for V/V compared to 40% survival for A/A). Data points represent the mean of 3 replicates.

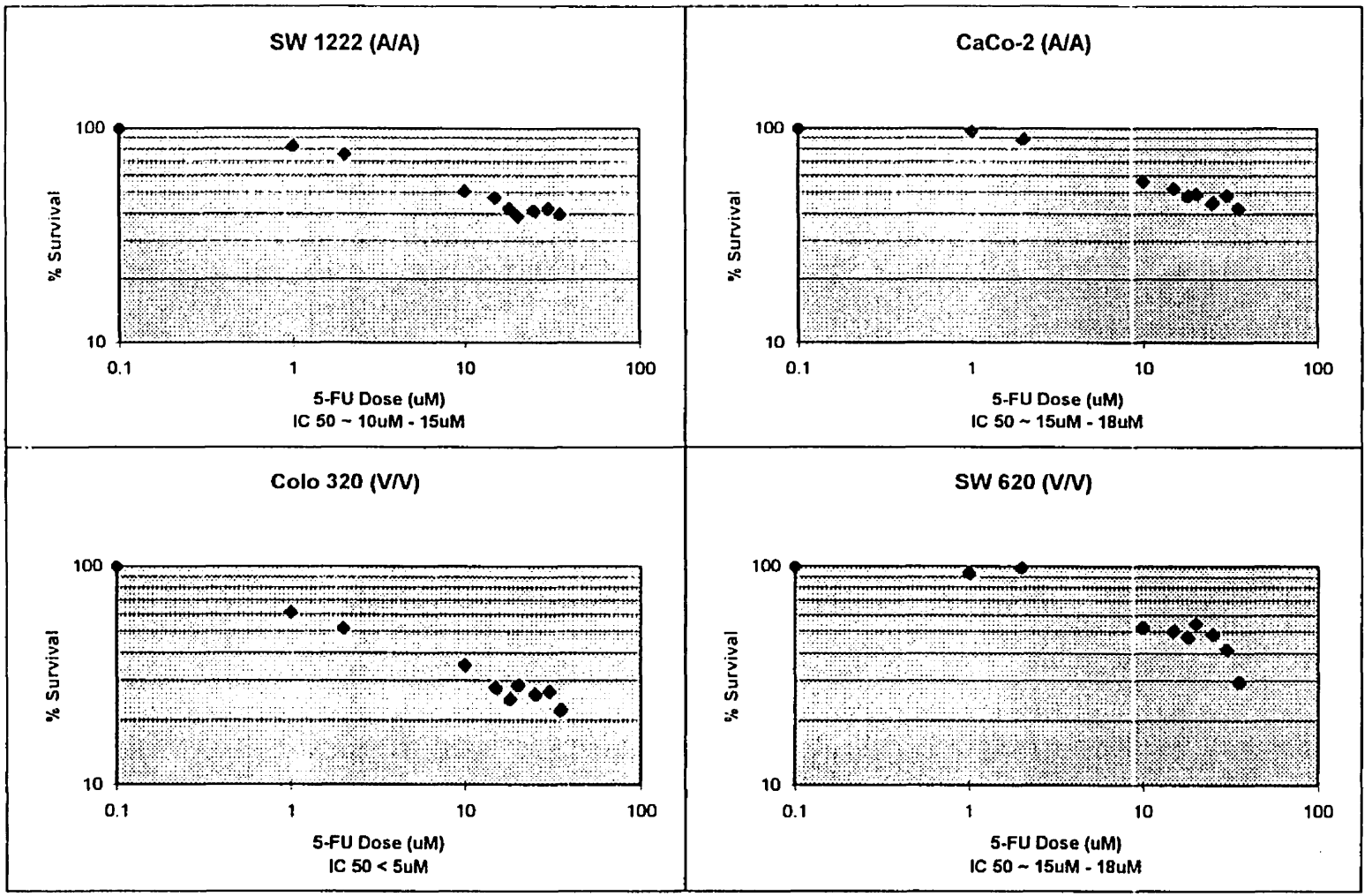

SW 1222 (A/A)
100
10
% Survival
0.1
1
10
100
5-FU Dose (uM)
IC 50 ~ 10uM - 15uM
CaCo-2 (A/A)
100
10
% Survival
0.1
1
10
100
5-FU Dose (uM)
IC 50 ~ 15uM - 18uM
Colo 320 (V/V)
100
10
% Survival
0.1
1
10
100
5-FU Dose (uM)
IC 50 < 5uM
SW 620 (V/V)
100
10
% Survival
0.1
1
10
100
5-FU Dose (uM)
IC 50 ~ 15uM - 18uM

Table 5. Numerical results for induced cytotoxicity of colon carcinoma cell lines by 5-fluorouracil (5-FU). Standard deviation of 3 replicates presented in parentheses.

Cell Line	5-FU (μM)	% of MEM
SW 1222 (A/A)	0	100
	1	82.5 (8.4)
	2	75.7 (10.6)
	10	50.6 (5.6)
	15	47.5 (5.7)
	18	42.3 (5.7)
	20	38.9 (3.9)
	25	41.2 (1.2)
	30	42.1 (7.3)
	35	39.6 (3.6)
CaCo-2 (A/A)	0	100
	1	96.9 (13.9)
	2	88.3 (7.6)
	10	56.6 (2.8)
	15	52.3 (6.3)
	18	48.2 (3.3)
	20	49.1 (7.3)
	25	44.8 (5.0)
	30	48.4 (10.2)
	35	42.3 (5.3)

Cell Line	5-FU (μM)	% of MEM
Colo 320 (V/V)	0	100
	1	61.5 (5.6)
	2	51.9 (0.7)
	10	35.2 (0.3)
	15	27.7 (3.1)
	18	24.7 (3.8)
	20	28.4 (7.3)
	25	25.9 (5.3)
	30	26.6 (5.3)
	35	22.1 (2.4)
SW 620 (V/V)	0	100
	1	93.4 (6.8)
	2	98.9 (12.5)
	10	52.1 (8.2)
	15	50.4 (4.5)
	18	47.1 (5.3)
	20	54.3 (7.1)
	25	48.6 (7.9)
	30	41.3 (1.7)
	35	29.4 (1.9)

5. DISCUSSION

In the general population, the A to V polymorphism in MTHFR is quite prevalent in the heterozygous state, at a frequency of 45%-50%. Due to loss of heterozygosity, cancer patients who are normally A/V in MTHFR genotype may retain only one of the variants in their tumor cells. If essential for viability, targeting the remaining MTHFR allele to eliminate its expression may prove to be cytotoxic to these cancer cells, without significantly affecting other tissues, and could therefore be utilized as a method of tumor therapy.

5.1 Allelic Losses of MTHFR

Analyses of two cancer types, ovarian and colorectal carcinomas, showed frequent loss of heterozygosity of MTHFR (Table 1). Comparison of the genotypes from tumor DNA with that from normal tissues of ovarian carcinoma patients identified LOH in approximately 40% of tumors. This high occurrence is consistent with data obtained from an earlier report (Viel 1997), confirming the sensitivity of our methodology. In Montreal and UK colorectal carcinoma patients, a much lower frequency of LOH was observed in tumor tissue (average of 16%). The 2-3-fold increased incidence of LOH of MTHFR in ovarian carcinoma compared to colorectal carcinoma suggests that a gene within this same chromosomal region (1p36.3) may be implicated in the oncogenic process of ovarian carcinomas, but has less influence on colon cancer progression (Pereira 1999). The frequent loss of distal chromosome 1p has been identified in several other tumor types (Caron 1993, White 1995, Dracopoli 1989, Genuardi 1989). These observations may be related to the p73 gene that maps

to the 1p36.33 region and that shares homology with the tumor suppressor p53 gene (Kaghad 1997, Jost 1997). p73 has the potential to activate p53-responsive genes, inhibiting cell growth by inducing apoptosis. Therefore, alteration of p73 gene expression, as a result of loss at chromosome 1p36, may contribute to tumorigenesis in a variety of cancers (Kaghad 1997, Jost 1997).

In combination with adequate folate levels and low alcohol consumption, the A to V mutation in MTHFR has been associated with reduced risk of colorectal carcinoma (Chen 1996, Ma 1997). Although the sample size in our study was small, colorectal tumors that displayed LOH more commonly lost the valine allele than the normal alanine allele. The more frequent loss of the mutant valine allele (8/11 tumors) is consistent with its protective effect observed in clinical studies. The mutation does not appear to influence ovarian carcinoma risk since no differential loss was observed in the ovarian carcinoma samples, nor was it seen in the earlier study (Viel 1997). Due to the importance of folates in DNA and methionine synthesis, additional studies are required to determine if the preferential allelic loss of MTHFR in colorectal carcinoma influences the oncogenic process of this cancer type.

5.2 Cell Viability in Deficient Media

Cell survival studies of fibroblast and colon carcinoma cell lines in media deficient of folate or methionine, with and without homocysteine and vitamin B_{12} supplementation (Figures 5-8), support earlier reports of the methionine dependence of transformed lines. Although normal fibroblast lines showed the ability to restore their growth by restoring endogenous methionine synthesis (Figure 7), the carcinoma cell

lines only exhibited a slight increase in survival under these conditions (Figure 8). The high methionine requirement of tumor-derived cell lines may be needed for the enhanced transmethylation capacity of such transformed cells (Hoffman 1984, Guo 1993a). Presence of exogenous methionine could complement endogenous levels that together meet the demands for rapid growth of carcinoma cells (Hoffman 1976). The upregulation of MTHFR activity (Figures 9B, 10) in M- or F- media, further supports the high methionine requirement of tumor cells. To compensate for a limited methionine supply, carcinoma cells may increase MTHFR activity to form more 5-methyltetrahydrofolate that can then be used for de novo methionine synthesis.

In addition, the importance of MTHFR for cell survival was evident from the growth response of fibroblast cell line, WG 1554, a homozygous nonsense mutant of MTHFR. With zero MTHFR activity, this cell line does not form the folate derivative for remethylation of homocysteine to methionine. As a result, WG 1554 was unable to restore growth in the homocysteine plus vitamin B12 supplemented (M-+) media (Figure 7). This observation suggests that MTHFR is essential for cell viability likely due to its role in the formation of methionine.

5.3 Allele-Specific Targeting

Antisense methods targeting specific MTHFR alleles were used in an attempt to slow or cease growth of tumor lines. This approach has been used by Variagenics Inc. to inhibit variants of the DNA Replication Protein A, resulting in lowered mRNA levels (Figure 11A) and cytotoxicity in cell culture. Targeting the mutant MTHFR allele in SW 620 (V/V) with the antisense oligonucleotide led to reduction of the full-

length transcript compared with mRNA levels observed following transfection with a control or a 1 bp mismatched oligonucleotide (Figure 11B). The decrease in full-length MTHFR mRNA due to allele targeting led to the formation of a smaller-sized message (asterisk, Figure 11B). It is possible that inhibiting the desired MTHFR product induces the cell to undergo a switch leading to the expression of an alternate product as a compensatory measure. Alternatively, this smaller transcript may be a degradation product resulting from the action of the nuclease, RNase H, postulated to be responsible for many of the effects of antisense oligonucleotides (Bennett 1995). During hybridization with the complementary mRNA sequence, deoxynucleotides may activate RNase H and then dissociate following hydrolysis at the double-stranded (DNA-RNA hybrid) area (Zamecnik 1995). This could form a stable mRNA fragment of reduced length that is detected, at an early time-point (i.e. 1-day post transfection), by Northern analysis. At a later time, this alternate product may disappear due to further cleavage by nucleases. The ~50% cell death of SW 620 following antisense treatment (Figure 12A) indicates, however, that the full-length transcript levels correlate with cell survival and that the smaller alternate-sized transcript may be missing those functions of the MTHFR gene that help maintain cell viability. Based on cleavage following oligonucleotide binding, levels of the smaller-sized transcript were expected to be greater following transfection with the 677T antisense oligonucleotide compared to the 677C mismatched oligonucleotide (Figure 11B). It remains unclear why this was not observed but it may be due to non-specific targeting and nuclease activity at later time points. Transfection of the homozygous mutant,

Colo 320, with the antisense oligonucleotide resulted in approximately a 3-fold decrease in cell survival compared with control transfection (Figure 12B).

The allele-specificity of this method of gene targeting is evident in SW 620, the survival of which was minimally affected following transfection with the mismatched oligonucleotide, and in Colo 320, which showed a 2-fold greater decrease in viability as a result of targeting with the antisense oligonucleotide compared with that of the mismatched oligonucleotide (Figures 12A,B).

The methionine dependence of cancer cells may therefore be exploited for allele-specific targeting of MTHFR. Due to its frequent allelic loss and involvement with methionine synthesis, MTHFR serves as a reasonable candidate for this approach of anti-tumor therapy.

5.4 Induced Cytotoxicity

Preliminary results of cytotoxicity experiments with methotrexate showed V/V colon carcinoma lines with at least a 5-fold increased sensitivity to treatment compared with two A/A lines (IC_{50} < 25 nM in V/V; IC_{50} ~ 150 nM in A/A) (Figure 13). These findings suggested that the A to V genotype may be predictive of tumor cell responsiveness to MTX treatment. To further explore this hypothesis, 4 additional A/A colon carcinoma lines were studied (no other V/V lines were available). In this case, only one A/A line, SW 948, showed a high IC_{50} value (~ 200 nM) while the other 3 lines displayed lower values (< 35 nM) (Figure 14) similar to those observed with the V/V lines (Colo 320 and SW 620). The correlation between tumor proliferative activity and response to chemotherapy has been documented in many

studies. For example, increased sensitivity to methotrexate was observed following growth factor-induced cell proliferation of normal and transformed T-lymphocytes and of a leukemia cell line (Ciaiolo 1988). As well, the response of breast cancer patients to chemotherapy that included MTX treatment positively correlated with a high rate of tumor cell proliferation (Collecchi 1998). The response of A/A carcinoma cells to MTX treatment may thus depend on their rate of growth, with increased proliferation leading to higher drug sensitivity perhaps due to intracellular folate and methionine depletion. Of the 2 V/V lines used in these studies, the rate of Colo 320 cell proliferation was considerably slower than that of SW 620, as observed from time-course experiments (Figure 6). Therefore, regardless of growth rate, the V/V genotype seems to be quite sensitive to MTX treatment while the A/A lines are more variable in their response. Although further studies using more V/V carcinoma lines are required, these results suggest that cancer patients who are V/V in genotype may benefit from a less aggressive form of anti-folate chemotherapy.

With respect to 5-FU IC_{50} values, there does not seem to be a clear difference between genotypes. However, V/V carcinoma lines showed decreased survival at high concentrations (> 35 μM) compared with the A/A lines suggesting that 5-FU treatment may be more effective in decreasing cell survival and slowing growth of mutant carcinoma cells. The V/V lines were expected to be more sensitive to 5-FU by having higher levels of 5,10-methylenetetrahydrofolate to stabilize the inactive complex formed between thymidylate synthase and fluorodeoxyuridylate, thus inhibiting TS activity (Cheradame 1997a). Reduced folates are polyglutamated by folylpolyglutamate synthetase (FPGS) inside cells; these polyglutamated forms result

in increased cellular retention and higher affinity for TS (Cheradame 1997a). FPGS activity may be affecting responsiveness of these carcinoma lines through its action on reduced folates, 5,10-methylenetetrahydrofolate in this case, and measuring FPGS levels in the 4 lines studied should provide an explanation for the inconclusive findings.

5.5 Future Experiments

The preferential loss of the valine allele observed in our colorectal carcinoma samples is consistent with its protective effect (Chen 1996, Ma 1997). Genotypic frequencies and loss of heterozygosity of MTHFR should be studied in additional populations while comparing different cancer types to determine the significance of our findings.

The specific elimination of MTHFR variants by allele-targeting must be attempted and proven effective using several other carcinoma cell lines of both the A/A or V/V genotypes before similar methods are used for anti-tumor therapy. Once established, these in vitro techniques need to ultimately be transferred to in vivo methods and the development of novel drugs directed against tumor cells that have undergone LOH at the MTHFR locus. These chemotherapeutic agents may take the nucleic acid-based approach targeting the specific MTHFR base changes with the antisense oligonucleotide. Due to the sensitivity to degradation by phosphodiesterases, the stability of the oligonucleotides needs to be improved by chemically modifying the phosphate backbone. This may take the form of a phosphorothioate linkage such as that used for transfection of cell cultures (Bennett

1995). The use of antibodies targeting the wildtype or mutant protein may also be an effective method to diminish MTHFR expression. Furthermore, in developing therapeutic drugs for this purpose, these agents must be internalized predominantly by cancer cells, show target specificity and low toxicity of normal tissues (Brysch 1996).

Although V/V carcinoma cell lines showed sensitivity to methotrexate regardless of their growth rate, the A/A lines were more variable in response. Measuring the proliferative activity of A/A carcinoma cells by [^{3}H]-thymidine labeling index or by flow cytometry may explain the differences seen between the wildtype carcinoma cell lines and could potentially serve as a prognostic factor for anti-folate chemotherapy. Similar to V/V cancer patients, individuals with the A/A genotype having high proliferative tumors may also benefit from a less aggressive form of chemotherapy. This study could be taken to the clinical level by comparing the response of cancer patients with A/A and V/V genotypes who have been given MTX chemotherapy. Genotype, MTX dose and the rate of tumor proliferation may predict the outcome and effectiveness of carcinoma treatment with this drug. In vitro, transfection of the mutant carcinoma cell lines with wild type MTHFR should increase resistance to the anti-folate drug and lend proof to the hypothesis that the V/V genotype is responsible for the sensitivity to methotrexate.

To improve the effectiveness of 5-fluorouracil on decreasing growth of carcinoma cells, inhibition of thymidylate synthase is necessary. This may be accomplished by increasing polyglutamation of 5,10-methylenetetrahydrofolate through the stimulation of folylpolyglutamate synthetase or by increasing the folate

substrate itself perhaps by reducing MTHFR activity and lowering its conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.

5.6 Summary

This research study has revealed that methylenetetrahydrofolate reductase, due to its frequent allelic loss and involvement with methionine synthesis and folate metabolism, serves as a reasonable target for tumor therapy. Specific reduction of MTHFR alleles in methionine-dependent tumor cells that have undergone LOH at this locus may be selectively cytotoxic to cancer cells without substantially affecting the viability of normal heterozygous cells. In addition, the A to V genotype in MTHFR and tumor proliferative activity in cancer patients may aid in determining the aggressiveness of anti-folate chemotherapy.

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