Homozygous deletions of methylthioadenosine phosphorylase in human biliary tract cancers

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Abstract

The p16\(^{\text{INK4A}}\)/CDKN2A gene on chromosome 9p21 is a site of frequent allelic loss in human cancers, and in a subset of cases, homozygous deletions at this locus encompass the telomeric methylthioadenosine phosphorylase (MTAP) gene. The MTAP gene product is the principal enzyme involved in the salvage pathway for the synthesis of adenosine from L-alanosine. Inhibitors of the de novo purine synthesis pathway, L-alanosine, can be used to selectively blockade purine synthesis in cancer cells while causing minimal collateral damage to normal cells. In this study, we determine that 10 of 28 (35%) biliary tract cancers show complete lack of Mtap protein expression. In vitro analysis using a selective inhibitor of the de novo purine synthesis pathway, L-alanosine, shows robust growth inhibition in MTAP-negative biliary cancer cell lines CCK-1 and GBD-1 accompanied by striking depletion of intracellular ATP and failure to rescue this depletion via addition of exogenous methylthioadenosine, the principal substrate of the MTAP gene product; in contrast, no significant effects were observed in MTAP-expressing HuCCT1 and SNU308 cell lines. Colony formation studies confirmed that L-alanosine reduced both number and size of CCK-1 colonies in soft agar assays. Knockdown of Mtap protein by RNA interference in L-alanosine-resistant HuCCT1 cells conferred sensitivity to this agent, confirming that intracellular Mtap protein levels determine response to L-alanosine. Inhibitors of de novo purine synthesis can be a potential mechanism-based strategy for treatment of biliary tract cancers, one third of which show complete loss of MTAP function. [Mol Cancer Ther 2005;4(12):1860–6]

Introduction

Biliary tract cancers, which include cancers of the gallbladder and the intrahepatic and extrahepatic bile ducts, affect >7,500 individuals each year in the United States and many thousands more the world over (1). Biliary tract cancer is a lethal disease, and >50% of affected patients die from their cancer (2). Surgery is currently the best avenue for cure; however, the overwhelming majority of patients present with locally advanced or with distant metastatic disease, rendering the cancer inoperable. Conventional chemotherapy and radiation therapies have had limited success in improving survival. Clearly, there is an urgent need for identification and application of potent, “mechanism-based” therapeutic strategies for biliary tract cancer, particularly for the vast majority of patients in whom the tumors are deemed inoperable.

The revolution in our understanding of the genetics of cancer has brought with it the hope that novel therapies can be developed specifically exploiting the genetic deletions and resultant absolute biochemical deficiencies present in human biliary tract cancers. The p16\(^{\text{INK4A}}\)/CDKN2A gene is one of the most frequently targeted genes in periampullary neoplasms, including biliary tract cancers. The methylthioadenosine phosphorylase (MTAP) gene is located ~100 kb telomeric to the p16\(^{\text{INK4A}}\)/CDKN2A gene on chromosome 9p21. The MTAP gene is contained in the p16 homozygous deletion in 90% of malignant mesotheliomas, 75% to 100% of T-cell acute lymphoblastic leukemias, and 50% to 100% of pancreatic cancers (3–6). Importantly, immunolabeling for the Mtap protein closely mirrors gene status. For example, our group has shown recently that loss of Mtap protein expression in human pancreatic cancers is present only in cases with biallelic deletions of the MTAP gene, whereas cases with one or both retained alleles preserve MTAP labeling (7). Thus, immunohistochemical determination of Mtap protein expression in neoplastic cells can be a convenient assay for identifying cancers with biallelic MTAP gene inactivation.

Novel therapeutic approaches selectively targeting cells with loss of MTAP gene function have been developed recently. The product of the MTAP gene plays an important role in the salvage pathway for the synthesis of adenosine and adenine nucleotides. In the absence of functional Mtap protein, tumor cells are metabolically dependent on a de novo purine synthesis pathway, suggesting that molecules that selectively block the de novo pathway may be effective and selective inhibitors of cancers with MTAP gene deletions (6). This therapeutic approach would be

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effective against cancers with MTAP gene inactivation but not against adjacent normal cells with intact MTAP function, thereby providing a paradigm for “mechanism-based” cancer-specific therapy.

Here, we screen a series of human biliary tract cancers using an immunohistochemical assay to the Mtap protein and estimate the frequency of MTAP gene inactivation in these cancers. We then examine biliary tract cancer cell lines with and without MTAP gene deletions treated with a potent inhibitor of de novo AMP synthesis, l-alanosine (Salmedix, Inc., San Diego, CA). Our results confirm that growth inhibition by l-alanosine is restricted to cells with loss of MTAP function, whereas biliary tract cancer lines with functional Mtap protein show minimal effects. Thus, l-alanosine seems to be a potent targeted therapeutic strategy for that subset of biliary tract cancers with MTAP gene inactivation.

Materials and Methods

Human Biliary Tract Cancer Tissue Microarrays

The study was approved by the Johns Hopkins Institutional Review Board. Formalin-fixed, paraffin-embedded sections from 28 biliary tract cancers resected at the Johns Hopkins Hospital were retrieved from the surgical pathology archives. Tissue microarrays were constructed as described previously (8, 9). Briefly, four 1.4-mm cores were taken from each neoplasm and placed in the recipient block using a manual puncher (Beecher Instruments, Silver Springs, MD). We have shown previously that four 1.4-mm tissue cores are representative of the immunolabeling pattern in the parent tumor from which these are retrieved (8).

MTAP Immunolabeling

We used a newly developed immunohistochemical assay for the detection of absence of Mtap protein expression using a novel monoclonal anti-Mtap antibody developed by GeneTex, Inc. (San Antonio, TX) and Salmedix (7). Unstained 4-μm sections were cut from each tissue microarray block and deparaffinized by routine techniques. Antigen retrieval was done by incubating the tissue sections in Borg decloaker at 120°C (Biocare Medical, Walnut Creek, CA) for 3 minutes followed by trypsin incubation for 5 minutes at room temperature. The sections were incubated with 20 μg/mL of the monoclonal antihuman Mtap antibody (Clone 6.9, Salmedix). Incubation of labeled polymer (Envision Plus Detection kit, DAKO, Carpinteria, CA) was carried out for 30 minutes at room temperature. The peroxidase reaction was visualized by incubating with 3,3′-diaminobenzidine for 5 minutes. Immunolabeling was evaluated by a qualified gastrointestinal pathologist (A.M.), with complete loss of cytoplasmic labeling being considered “negative,” whereas any labeling was considered “positive.” Cases were considered “not evaluable” when the background nonneoplastic epithelial cells, stromal cells, or inflammatory cells failed to label.

Biliary Tract Cancer Cell Lines

Four biliary tract cancer cell lines (HuCCT1, GBD-1, SNU308, and CAK-1) were examined for MTAP gene deletions as described below. The sources and culture conditions of the first three biliary tract cancer cell lines have been described previously (10); CAK-1 was generated at Johns Hopkins University by two of the authors (C.A.K. and A.M.) by in vitro propagation of a first-passage extrahepatic bile duct adenocarcinoma xenograft as described previously (11). HuCCT1 cells were established from extrahepatic bile duct cancers, whereas GBD-1 and SNU308 cells are derived from gallbladder adenocarcinomas.

Genomic Real-time PCR Assay for MTAP Gene Deletion

To detect homozygous deletions of the MTAP gene in biliary tract cancer cell lines, we designed a real-time PCR assay using exonic primers directed against exon 8 of MTAP. We also designed a specific fluorophore-labeled probe (TaqMan probe, Applied Biosystems, Carpanteria, CA) and thereby used the 5′ exonuclease activity of Taq polymerase to determine fluorescent signal in real-time. Real-time assays were done on the Cepheid SmartCycler (Sunnyvale, CA). The specific primer and probe sequences are as follows:

- MTAP F3D 5′-CATGTGAATATCACTGCTCC-3′
- MTAP R3D 5′-CTCTTCTTCCTGCGGACG-3′
- MTAP pFamA FAM-TGGCCCAGTTTTCTGTTTTAT
- MTAP F3D 5′-CATGTGAATATCAGTTCC-3′
- MTAP R3D 5′-CTCTTCTTCCTGCGGACG-3′
- MTAP pFamA FAM-TGGCCCAGTTTTCTGTTTTAT

All assays were conducted using two controls: a pancreatic cancer cell line with a known MTAP homozygous gene deletion (Panc-1) and a pancreatic cancer cell line (Hs766T), which has retained both MTAP alleles.

Western Blot Analysis for Mtap Protein

The expression or absence of Mtap protein was ascertained by immunoblotting with anti-Mtap antibody 6.9 (lot 6.9.5) kindly provided by Salmedix. Briefly, protein lysates were made from cell pellets of the four above-described biliary tract cancer cells in addition to two controls: a pancreatic cancer cell line with a known MTAP homozygous gene deletion (Panc-1) and a pancreatic cancer cell line (Hs766T) with retained MTAP alleles (12). Radioimmunoprecipitation assay lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS], protease inhibitor cocktail (Sigma, St. Louis, MO), and phenylmethylsulfonyl fluoride were added before cell lysis. Cells were lysed on ice for 30 minutes with occasional gentle agitation of the tubes. Cell debris was separated by centrifugation at 13,200 rpm for 30 minutes at 4°C. Protein lysates were resolved by electrophoresis on 12% Tris-glycine gel (Invitrogen, Carlsbad, CA) and electrotransferred on nitrocellulose membrane (LC 2000, Invitrogen). Standard immunoblotting procedure was followed with slight modification: nitrocellulose membrane was blocked overnight at room temperature and incubated in primary antibody for 72 hours at 4°C. Anti-actin antibody (SC-1616-R, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control, and standard immunoblotting protocol was followed.

- 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenytltetrazo- lium Bromide Assay with l-Alanosine

Growth inhibition was measured using the CellTiter 96 Aqueous Cell Proliferation Assay (Promega, Madison, WI).
MTAP Deletions in Biliary Cancer

which relies on the conversion of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] to a colored formazan product by the action of living cells. Briefly, HuCCT1 and CAK-1 cells (1,500 per well) were plated in triplicate in 96 well-plate format in full serum (10% fetal bovine serum) condition. On day 1, full serum was replaced with low (0.5%) serum containing varying doses of L-alanosine (5–20 μmol/L) or vehicle [3% (w/v) NaHCO₃] only. The assay was terminated on day 4, and relative growth inhibition compared with vehicle-treated cells was measured using the CellTiter 96 reagent as described in the manufacturer’s protocol.

ATP Depletion Assay

The mechanism of action of L-alanosine is inhibition of AMP synthesis by the de novo pathway. In cells with loss of salvage pathway secondary to MTAP gene inactivation, blockade of de novo synthesis should therefore lead to depletion of intracellular ATP. The CellTiter-Glo Luminescent Cell Viability Assay (Promega), which generates a luminescent signal proportional to the amount of ATP present, was used to determine mechanistic specificity of L-alanosine in vitro. Briefly, HuCCT1 and CAK-1 cells (1,500 per well) were plated in triplicate in 96-well plate format in full serum (10% fetal bovine serum) condition. On day 1, full serum was replaced with low (0.5%) serum containing sub-IC₅₀ doses of L-alanosine (1–10 μmol/L) as determined for CAK-1 using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay above. The assay was terminated at 48 hours, and luminescence was measured as described in the manufacturer’s protocol.

Rescue Experiment with Methylthioadenosine

Methylthioadenosine is the direct substrate of the Mtap enzyme, which cleaves the former into adenine and 5-methylthioribose-1-phosphate (13). Adenine is then salvaged to form AMP by the action of the enzyme adenine phosphoribosyltransferase. In cells with intact MTAP function, the addition of exogenous methylthioadenosine will therefore permit salvage AMP generation even in the presence of Mtap expression in the adjacent nonneoplastic structures also confirmed that the salvage pathway is intact in these cells, whereas up to one third of biliary tract cancers can be potential candidates for treatment with specific inhibitors of the de novo purine biosynthesis like L-alanosine.

Subsequently, genomic quantitative PCR using MTAP exon 8 primers was done on the four biliary tract cancer cell lines and showed complete absence of signal in two cell lines (CAK-1 and GBD-1), whereas amplification of genomic product was seen in HuCCT1 and SNU308 (Fig. 2A); the normal control Hs766T cell line (retained MTAP) and deletion control Panc-1 (homozygously deleted MTAP) had expected signal intensities. Western analysis using monoclonal anti-MTAP antibody confirmed the genomic PCR data, with no expression seen in CAK-1 and GBD-1 and retained expression in the remaining two biliary tract cancer cell lines, HuCCT1 and SNU308 (Fig. 2B).

Further experiments were done using pairs of MTAP-positive and MTAP-negative cells to show mechanistic

Results

Complete loss of Mtap protein expression was seen in 10 of 28 (35%) archival biliary tract cancers. As shown in Fig. 1A and B, in all cases, appropriate internal controls (nonneoplastic biliary ductal epithelium or stromal elements) were available to confirm the adequacy of immunolabeling. The presence of Mtap expression in the adjacent nonneoplastic structures also confirmed that the salvage pathway is intact in these cells, whereas up to one third of biliary tract cancers can be potential candidates for treatment with specific inhibitors of the de novo purine biosynthesis like L-alanosine.
specificity of L-alanosine. The compound showed dose-dependent growth inhibition of CAK-1, with an IC₅₀ in the range of 10 μmol/L (Fig. 3A), whereas no apparent cytotoxicity was discernible in the MTAP-positive HuCCT1 line (up to 50 μmol/L L-alanosine dosage; data not shown). This experiment confirmed that activity of the drug was limited to cells with absent MTAP function. To further confirm the mechanism of action of L-alanosine, we assessed intracellular ATP levels at sub-IC₅₀ levels of the compound for CAK-1 (1–10 μmol/L) to minimize cytotoxicity. Striking and reproducible ATP depletion was seen in CAK-1 (up to ~80% reduction compared with control cells), whereas HuCCT1 showed only 20% to 30% reduction in cellular ATP levels (Fig. 3B). The attenuated ATP reduction in HuCCT1 was consistent with the salvage pathway ‘kicking in’ AMP synthesis once L-alanosine blocked de novo synthesis, which was untenable in the MTAP-negative CAK-1 cell line. To further accentuate the differential effects of L-alanosine on MTAP-negative and MTAP-positive cell lines, we cocultured the cells in the presence of the drug as well as the MTAP enzyme substrate, methylthioadenosine. The exogenous substrate would facilitate increased AMP (and subsequently ATP) production via the salvage pathway but only in cells with functional MTAP activity. Indeed, the addition of methylthioadenosine showed no significant effects on intracellular ATP levels in CAK-1 (Fig. 3C). The differential effects of L-alanosine were just as pronounced in the remaining pair of cell lines, with the MTAP-negative GBD-1 line showing profound growth inhibition in response to L-alanosine (IC₅₀, ~5 μmol/L), whereas MTAP-expressing SNU308 cells had minimal cytotoxicity from this compound (Fig. 3D). As with CAK-1, increasing dosages of L-alanosine were accompanied by progressive depletion of cellular ATP levels in GBD-1 (Fig. 3E).

We next assessed the ability of L-alanosine to inhibit colony formation in soft agar in CAK-1 cell line. As seen in Fig. 4A and B, CAK-1 showed a significant reduction in both colony count (40% relative decrease; P = 0.0026, ANOVA) and colony size in the presence of 10 μmol/L L-alanosine compared with vehicle [3% (w/v) NaHCO₃]–treated cells. Representative examples of control (left) and treated (right) soft agar colonies at 2 weeks are illustrated in Fig. 4C.

Although these experiments strongly suggested that loss of MTAP gene function was associated with sensitivity to L-alanosine, we wanted to conclusively establish this relationship by knockdown of MTAP in an otherwise MTAP-expressing cell line and compare differential susceptibilities to this drug versus the parental line. We selected HuCCT1, which as we have shown above has minimal cytotoxicity in the presence of L-alanosine. We confirmed that we were able to reproducibly knockdown Mtap protein levels in these cells using a siRNA pool directed against MTAP, whereas no effects were observed on transcript levels using a scrambled sequence (Fig. 5A). On exposure to varying concentrations...
of L-alanosine (Fig. 5B), MTAP siRNA-transfected HuCCT1 cells showed growth inhibition that was comparable with MTAP-negative cell lines (GBD-1 and CAK-1), whereas scrambled siRNA-transfected HuCCT1 lines essentially retained resistance to the compound.

Discussion
The MTAP gene product plays an important role in the synthesis of purines (13). AMP can be synthesized by one of two pathways in the cell. In the salvage pathway, the enzyme MTAP catalyzes the conversion of methylthioadenosine to adenine and 5-methylthioribose-1-phosphate. Adenine then serves as a substrate for AMP synthesis. In the de novo pathway for AMP synthesis, de novo purine biosynthesis leads to the production of IMP and IMP is then converted to AMP. L-Alanosine, the L-isomer of alanosine, is a potent inhibitor of the de novo pathway of AMP synthesis (4, 6, 14). The metabolite of L-alanosine inhibits the activity of the enzyme adenylosuccinate synthase that converts IMP to AMP. L-Alanosine specifically down-regulates AMP synthesis, in contrast to other purine synthesis inhibitors like methotrexate, which also affect the synthesis of GMP and thymidylate. The specificity of L-alanosine for the de novo AMP synthetic pathway ensures that cells with retained MTAP function (i.e., nonneoplastic cells) can be “rescued” by the exogenous administration of methylthioadenosine or other AMP salvage pathway intermediates, thus escaping cytotoxicity. In contrast, MTAP-deleted cancers will be unable to replenish their depleted ATP pools and perish, providing an “Achilles heel” for cancer-specific therapy.

Our in vitro studies have confirmed the hypothesis that the therapeutic effects of L-alanosine are restricted to MTAP-negative biliary tract cancer cell lines (e.g., CAK-1 and GBD-1), whereas cells with intact MTAP function (e.g., HuCCT1 and SNU308) show minimal cytotoxicity.
even at doses 5-fold higher than the IC₅₀ of susceptible cells. We have also confirmed that L-alanosine administration leads to profound reduction of intracellular ATP levels in MTAP-negative cell lines, which are not amenable to “rescue” by addition of exogenous methylthioadenosine, the principal substrate for MTAP enzyme. Importantly, we showed that knockdown of Mtap protein by RNA interference conferred sensitivity to L-alanosine in an otherwise resistant HuCCT1 cell line, confirming that levels of this enzyme modulate therapeutic response to purine synthesis blockade. The potential for rescue of normal (i.e., MTAP-retained) cells by methylthioadenosine is of potential clinical import, because pharmacokinetic or pharmacodynamic considerations in some biliary tract cancer cases may require higher doses of L-alanosine, and the concurrent administration of methylthioadenosine in such instances may provide a wider therapeutic window with minimal toxicity to nonneoplastic cells.

The challenge then becomes identifying those biliary tract cancer patients who would benefit from therapies targeting cancer cells with a MTAP deletion and sparing those with intact MTAP from unnecessary treatment. In the 1980s, clinical trials with L-alanosine in a variety of solid tumors and leukemias failed to show an effect as these were not screened for MTAP gene functional status (15–19). Currently, the availability of sensitive and specific
immunolabeling for the MTAP gene product suggests that patients can be identified who would benefit most from therapies targeting de novo purine biosynthesis (7). Resection specimens and, for inoperable cases, even excisional biopsy specimens can be immunolabeled for the MTAP gene product. Those patients whose cancers show a complete loss of MTAP expression can be offered treatment with specific inhibitors of the de novo purine synthesis pathway, whereas those patients whose cancers show intact MTAP expression can be spared an ineffective therapy. In fact, the New Approaches to Brain Tumor Therapy (http://www.nabtt.org) is currently conducting phase II trials in MTAP-negative glioma patients. In our study, we have found that approximately one third of patients with biliary tract cancers can be potential candidates with L-alanosine, as they show complete loss of MTAP protein labeling in the neoplastic cells. Importantly, in all cases, the adjacent nonneoplastic tissues retain robust MTAP expression, indicating that cytotoxicity is likely to be restricted to tumor cells.

In summary, we have presented an example of targeted therapy for human biliary tract cancers using a biochemical defect specific to the cancer cells. Our studies lay the foundation for initiating clinical trials with L-alanosine in subsets of biliary tract cancers that show loss of MTAP function, with the hope of alleviating the dismal prognosis associated with this devastating disease.

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Molecular Cancer Therapeutics

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