Loss of NAPRT1 Expression by Tumor-Specific Promoter Methylation Provides a Novel Predictive Biomarker for NAMPT Inhibitors

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Abstract

Purpose: We sought to identify predictive biomarkers for a novel nicotinamide phosphoribosyltransferase (NAMPT) inhibitor.

Experimental Design: We use a NAMPT inhibitor, GNE-617, to evaluate nicotinic acid rescue status in a panel of more than 400 cancer cell lines. Using correlative analysis and RNA interference (RNAi), we identify a specific biomarker for nicotinic acid rescue status. We next determine the mechanism of regulation of expression of the biomarker. Finally, we develop immunohistochemical (IHC) and DNA methylation assays and evaluate cancer tissue for prevalence of the biomarker across indications.

Results: Nicotinate phosphoribosyltransferase (NAPRT1) is necessary for nicotinic acid rescue and its expression is the major determinant of rescue status. We demonstrate that NAPRT1 promoter methylation accounts for NAPRT1 deficiency in cancer cells, and NAPRT1 methylation is predictive of rescue status in cancer cell lines. Bisulfite next-generation sequencing mapping of the NAPRT1 promoter identified tumor-specific sites of NAPRT1 DNA methylation and enabled the development of a quantitative methylation-specific PCR (QMSP) assay suitable for use on archival formalin-fixed paraffin-embedded tumor tissue.

Conclusions: Tumor-specific promoter hypermethylation of NAPRT1 inactivates one of two NAD salvage pathways, resulting in synthetic lethality with the coadministration of a NAMPT inhibitor. NAPRT1 expression is lost due to promoter hypermethylation in most cancer types evaluated at frequencies ranging from 5% to 65%. NAPRT1-specific immunohistochemical or DNA methylation assays can be used on archival formalin paraffin-embedded cancer tissue to identify patients likely to benefit from coadministration of a Nampt inhibitor and nicotinic acid. Clin Cancer Res; 19(24): 6912–23. ©2013 AACR.

Introduction

There has been a recent reawakening of interest in targeting the altered metabolic state of cancer cells to combat cancer. Although the observation that cancer cells utilize atypical metabolic pathways was originally made quite some time ago (1), it was unclear how to exploit this property for cancer therapeutic development. However, there are now a number of inhibitors of metabolic enzymes and a wealth of cancer-genome data that is opening new avenues to target cancer metabolism (2). One approach is to inhibit NAD biosynthesis (3). Cancer cells seem to require higher levels of NAD and NADH because they have high metabolic demands and rely heavily on glycolysis, a process that is far less efficient than oxidative phosphorylation for generating ATP. In addition, cancer cells may require more NAD due to increased activity of NAD-consuming enzymes such as the sirtuins and PARPS (4).

Nicotinamide phosphoribosyltransferase (NAMPT), also known as visfatin or pre-B-cell colony-enhancing factor, catalyzes the rate-limiting step in the primary salvage pathway used to generate NAD. It transfers a phosphoribosyl residue from 5-phosphoribosyl-1-pyrophosphate to nicotinamide to produce nicotinamide mononucleotide, which is subsequently converted into NAD+ by nicotinamide mononucleotide adenyl transferase (NMNAT; ref. 5–7). Three NAMPT inhibitors of two distinct structural classes have entered clinical trials for cancer, APO866 (formerly FK866; ref. 8), GMX1778 (formerly CHS828), and a
NAPRT1 Methylation Predicts Sensitivity to NAMPT Inhibition

Translational Relevance
Although two Nampt inhibitors have entered clinical trials, no attempt was made to select potentially responsive patients in these trials. Tumor-specific loss of nicotinate phosphoribosyltransferase (NAPRT1) is synthetically lethal with coadministration of a nicotinamide phosphoribosyltransferase (NAMPT) inhibitor and nicotinic acid. Therefore, identification of NAPRT1-deficient tumors can enable a diagnostically driven clinical strategy that entails selection of patients likely to benefit from coadministration of a NAMPT inhibitor and nicotinic acid. We demonstrate that the loss of NAPRT1 expression is due to DNA methylation, and that NAPRT1 expression is lost in a broader range of cancer indications than previously appreciated. However, the percentage of tumors that lack NAPRT1 suggests that prospective identification of patients will be required in most indications. To support this approach, we validate NAPRT1 immunohistochemical and DNA methylation assays to enable implementation of this novel diagnostic strategy in the clinic.

Materials and Methods
Cellular and biochemical assays
Cell lines were obtained from the American Type Culture Collection or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and stored in a central cell bank. Lines were authenticated by short tandem repeat and genotyped upon re-expansions. Cells were grown in RPMI-1640 medium supplemented with 10% FBS and 2 mmol/L glutamine (Invitrogen) and passaged not more than 20 times after thawing. To determine the IC50 values and nicotinic acid rescue status, cells were treated with nine point dose titrations of GNE-617 with or without 10 μmol/L nicotinic acid. At 96 hours post-drug addition, the GNE-617–treated cells were evaluated using CyQUANT Direct Cell Proliferation Assay (Invitrogen, Ltd.) followed by CellTiter-Glo Luminescence Cell Viability Assay (Promega Corporation) quantified with a Wallac EnVision 2104 Multi-label Reader. IC50 values were calculated using XLfit 5.1 (ID Business Solutions, Ltd). To examine the protein level, cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Cell Signaling Technology), run on SDS-PAGE (4%-12% Bis-Tris; Invitrogen), and evaluated by Western blotting using antibodies directed against NAPRT1 and β-actin (Sigma-Aldrich). For RNA interference (RNAi), A549 cells were plated at 1,500 cells per well in 96-well plates, allowed to adhere for 24 hours, and transfected with 25 nmol/L siRNA oligonucleotide using Dharmafect 4 (Thermo Scientific). NAPRT1-directed siRNA oligonucleotides were purchased from Ambion (s41083 and s41084) and from Thermo Scientific (J-016912-09, and J-016912-10). Nontargeting control siRNA was purchased from Thermo Scientific (D-001810-10-20). Transfected cells were treated with the indicated concentrations of GNE-617 for 72 hours and viability was evaluated with CellTiter-Glo (Promega). Lysates for detection of NAPRT1 protein were collected 72 hours after transfection of 1 million A549 cells in 10 cm dishes. For NAPRT1 re-expression, RERF-LC-MS cells were transfected with pCMV6-AC.NAPRT1 and empty vector pCMV6-AC (OriGene Technologies, Inc) using Amaza Nucleofector technology and selected with Geneticin (Life technologies).

LC/MS assay for NAD concentration
The concentration of NAD in cells was determined by a nonvalidated liquid chromatography/tandem mass spectrometry (LC/MS-MS) assay using [13C5]-NAD as an internal standard. Detailed methods are in the Supplementary Methods.

Immunohistochemistry
Immunohistochemistry for NAPRT1 was performed on a Ventana Discovery XT autostainer (Ventana Medical Systems). Formalin-fixed paraffin-embedded (FFPE) whole-tissue and tissue microarray sections were deparaffinized and pretreated with CC1 solution (Ventana Medical Systems) for 60 minutes followed by incubation with either 0.5 μg/mL NAPRT1 rabbit polyclonal antibody...
(Novus Biologicals) or naïve rabbit immunoglobulin G (Cell Signaling Technologies) for 60 minutes at 37°C. Detection was performed with OmniMap anti-rabbit HRP and DAB (Ventana Medical System) followed by counter staining with Hematoxylin II (Ventana Medical System).
Deparaffinization and DNA extraction of FFPE section for QMSP

FFPE sections were deparaffinized by soaking 3 times in Envirene (Hardy Diagnostics Cat No. CE-016) for 5 minutes, then 2 times in 100% ethanol for 5 minutes, then dried for 10 minutes at room temperature. Sections were scraped off the slides and Proteinase K digested in Tissue Lysis Buffer (from Roche High Pure FFPE RNA Micro Kit, Cat. No. 04823125001) overnight at 56°C. DNA was then purified using the QIAamp DNA FFPE Tissue Kit (Qiagen, Cat. No. 56404) starting at step 12.

RNA-seq and copy number analysis

RNA-seq reads were aligned to the human genome version GRCh37 using GSNAP (20). Gene expression was obtained by counting the number of reads aligning concordantly within a pair and uniquely to each gene locus as defined by consensus coding DNA sequence. The gene counts were then normalized using the DESeq Bioconductor software package (21). Illumina HumanOMni2.5_4v1 arrays were used to assay 906 cancer cell lines for genotype, DNA copy, and LOH at approximately 2.2 million single-nucleotide polymorphism (SNP) positions following methods published previously (PMID:23033341 and PMID:22895193). Copy number for NAPRT1 was calculated as the average absolute copy number for all SNPs within or directly adjacent to the bounds of the genomic region covered by any RefSeq transcript for that gene. Samples with a copy number less than 2 were counted as having a deletion.

DNA methylation analysis

DNA methylation was measured by Illumina Infinium 450 K BeadChip and preprocessed using Bioconductor lumi package (22) with default settings, as previously described (23). For next-generation sequencing, DNA samples containing equal amounts of 8 bisulfite PCR products (~500 ng DNA total) were treated with T4 DNA polymerase, Klenow large fragment, and T4 polynucleotide kinase to generate 5’-phosphorylated blunt ends. After concatemerization with T4 DNA ligase, the sample was sonicated to an average fragment length of 150 to 300 bp using a Misonix cuphorn sonicator 3000. Libraries were generated from these sonicated DNA samples using the standard Illumina protocol. The 24 samples were indexed with 7-bp barcodes (independent Illumina index read). Sequencing on Hi-Seq generated a total of 115 million 50-nt reads. Reads were aligned to the reference sequence using the bismark software (24). As reference sequence, either the PCR-amplified target region (in NAPRT1 gene promoter) or the entire chr8 (hg19) was used. Alignments were captured in SAM/BAM files, and percentage methylation at each CpG site was determined by running the appropriate bismark scripts. Read coverage at each CpG site was determined by generating a signal map at 1-bp resolution (using Active Motif software), and individual 50-nt reads with specific methylation status were counted using a combination of samtools (25) and standard UNIX commands. Gene schematic generated with FancyGene.

For quantitative methylation-specific PCR (QMSP) assays, sodium bisulfite converted DNA was amplified using previously described conditions (23).

Results

Evaluation of rescue identifies cancer types that may benefit from coadministration of NAMPT inhibitor and nicotinic acid

GNE-617 is a novel small-molecule inhibitor of NAMPT (Supplementary Fig. S1A) that inhibits the biochemical activity of NAMPT with an IC50 of 5 nmol/L and exhibits efficacy in xenograft models of cancer (26). As expected, this molecule causes rapid depletion of cellular NAD followed by a decrease in ATP (Supplementary Fig. S1B). We evaluated the activity of GNE-617 on a panel 53 non–small cell lung cancer (NSCLC) cell lines in the presence or absence of 10 μmol/L nicotinic acid. The majority of cell lines exhibit a steep dose response to GNE-617 when evaluated by decrease in ATP or total nucleic acid, and the cytotoxicity is completely rescued by simultaneous addition of nicotinic acid. However, some lines are not rescued by nicotinic acid (Fig. 1B), and we refer to these as “non-rescuable” cell lines. The majority of the cell lines tested have IC50 values below 100 nmol/L, with approximately half with IC50 values less than 10 nmol/L. We find a significant correlation between baseline expression of NAMPT and IC50—consistent with previous reports (16), and between NAPRT1 expression and IC50 (Supplementary Table S1). Eighteen cell lines were not rescued with nicotinic acid, and these non-rescuable cell lines tended to have lower IC50 values (P = 0.008, Fisher exact test, IC50 < 10 nmol/L vs. ≥10 nmol/L; Fig. 1C).

To explore the prevalence of the nicotinic acid rescue phenotype across indications, we evaluated a panel of cancer cell lines for response to 10 μmol/L GNE-617 in the presence or absence of 10 μmol/L nicotinic acid and found 16% (72/445) were not rescued with nicotinic acid (Supplementary Table S2). Cell lines derived from brain tumors or sarcomas exhibit the non-rescuable phenotype at a higher frequency than other types of cancer cell lines (45% and 67%, respectively), consistent with previous reports (16). In this study, we expand into a larger variety of cancer types and find at least one non-rescuable cell line from most indications tested. However, with the exception of sarcomas and brain tumors, the prevalence is less than 35% (Fig. 1D).

Rescue of NAMPT inhibitor toxicity by nicotinic acid is due to NAPRT1 deficiency

Previous studies have indicated that NAPRT1 is required for nicotinic acid rescue (16, 17). We wanted to test this observation in a larger panel of cell lines and identify additional determinants of nicotinic acid rescue across cancer cell lines from a diverse range of tissue types. We evaluated the cell line panel shown in Fig. 1D for correlation between gene expression and nicotinic acid rescue. The best correlate of the rescue phenotype is NAPRT1 (adjusted P = 6 × 10−30). The next best correlate gene is lymphotoxin β receptor (LTBR, TNFRSF15) with an adjusted P value of 10−20.
Figure 2. NAPRT1 level determines nicotinic rescue status in cancer cell lines. A, Western blot analyses of NAPRT1 in NSCLC lines; *, non-rescuable lines. B, NAPRT1 level evaluated by Western blot analysis versus rescue status, \( n = 38, P = 0.04 \) (two-tailed t-test), *, \( P < 0.05 \). C, left, GNE-617 dose response of A549 cells to GNE-617 with nontargeting siRNA (NTC, filled circles) or 4 independent NAPRT1 directed siRNA oligos from Thermo Scientific (D1, D2, filled symbols) or Ambion (A1, A2, open symbols), right, GNE-617 dose response of RERF-LC/MS cells stably transfected with NAPRT1 (squares) or empty vector (EV, triangles) in the presence (open symbols, dashed lines) or absence (filled symbols) of 10\( \mu \)mol/L nicotinic acid. D, fraction of cell lines for each cancer type with NAPRT mRNA \(<7.2\) RMA normalized from HGU-133P (Affymetrix), \( n = 663 \).
leading us to conclude that NAPRT1 level is the dominant determinant of nicotinic acid rescue status. We examined NAPRT1 protein levels in 38 NSCLC cell lines and found a good correlation between NAPRT1 protein and rescue (Fig. 2A and B). To demonstrate that NAPRT1 is necessary for nicotinic acid rescue, we reduced the level of NAPRT1 by siRNA in A549 cells. NAPRT1 reduction did not change the IC50 for GNE-617, but the cells were no longer rescued with nicotinic acid. To demonstrate that NAPRT1 is sufficient for rescue, we expressed NAPRT1 in the NAPRT1-deficient RERF-LC/MS cell line and observed nicotinic acid rescue in this line compared with the same line transfected with the empty vector control, which remains non-rescuable (Fig. 2C).

In the 32 cell lines for which we had NAPRT1 protein quantification and gene expression data, there was a strong correlation between mRNA and protein (Spearman $R = 0.88$, $P < 10^{-4}$; Supplementary Fig. S1C). In light of these data, we evaluated a panel of 551 cancer cell lines for NAPRT1 deficiency by gene expression to identify additional cancer types with and models that are likely to be non-rescuable (Fig. 2D and Supplementary Table S3). We set the threshold for NAPRT1 deficiency based on gene expression levels that allow nicotinic acid rescue in the NSCLC cell line panel. The threshold selected, 7.2, is close to the lower limit of detection on the Affymetrix array (Supplementary Fig. S1C), suggesting little or no gene expression. The most well represented indications, lung and breast cancer exhibited...
NAPRT1 deficiency at rates of 17% and 10%, respectively, similar to percentages of non-rescuable lines shown in Fig. 1D. There were no sarcoma cell lines in this panel, and the prevalence of NAPRT1 deficiency in gliomas, 9%, was lower than expected. Liver cancer cell lines had the highest prevalence of NAPRT1 deficiency (45%), slightly higher than the observation of 31% in the previous panel. This panel contains more lymphoma lines and these exhibited the second highest rate of NAPRT1 deficiency (38%), consistent with observations made on lymphoma tissue (19). In summary,

Figure 4. NAPRT1 is hypermethylated in a subset of cancer cell lines. A, heatmap of NAPRT1 DNA methylation in cell lines derived from lung cancer or nonmalignant lung epithelia (* measured by Infinium arrays. p values represent the fraction of molecules methylated at the CpG site. B, NAPRT1 methylation in breast cancer and (C) pancreatic cancer cell lines. D, there is a significant increase in NAPRT1 gene expression after exposure to 5-aza-2′-deoxycytidine in the in the non-rescuable cell lines (P = 0.02, t test, n = 9), but not the rescuable lines (P < 0.05. DMSO, dimethyl sulfoxide; NR, non-rescuable; R, rescuable.}

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lymphomas, sarcomas, and hepatocellular carcinomas tend to have higher rates of NAPRT1 deficiency.

**Immunohistochemistry can be used to identify NAPRT1-deficient tumors**

We next developed an immunohistochemical (IHC) assay for NAPRT1 using cell lines with known levels of NAPRT1 to validate the method. NAPRT1 staining on NSCLC tissue is cytoplasmic and has a large dynamic range. Benign cells stained with an intensity of immunohistochemistry 1+ whereas malignant cells stained across the full range of IHC scores (0–3+; Fig. 3A). To determine the level of NAPRT1 necessary for nicotinic acid rescue, we generated a cell pellet microarray from NSCLC cell lines with known rescue status and scored them for NAPRT1 on a scale of 0 to 3+. With only one exception, cell lines with IHC scores more than zero were rescued by nicotinic acid (Fig. 3B). We next stained tumor samples from a variety of tumor types and determined the fraction with NAPRT1 IHC scores of zero (Fig. 3C). Small cell lung cancer (SCLC) had a high prevalence of NAPRT1 deficiency (60%), but it should be noted that the number of samples was low (n = 10). In general, the prevalence of IHC 0 by tumor type was consistent with the prevalence of NAPRT1 deficiency and loss of nicotinic acid rescue in the cell lines. Thus, an IHC assay could be used to select patients for coadministration of NAMPT inhibitor and nicotinic acid. On the basis of these preclinical data, we propose that an IHC score of zero will be predictive of a tumor likely to respond to the combination of a NAMPT inhibitor and nicotinic acid.

**NAPRT1 is methylated in a subset of lung, pancreatic, and ovarian tumors**

The finding that loss of NAPRT1 expression has a strong negative association with nicotinic acid rescue suggested that NAPRT1 expression was completely absent in certain contexts. We examined copy number by SNP arrays in a panel of 906 cell lines and found that NAPRT1 underwent LOH in 18.6% of cell lines. In addition, we found four cell lines with single copy deletion at the NAPRT1 locus (BJAB, HCC1428, COLO-824, and NCI-H1882). We next examined NAPRT1 copy number in tumor tissue from The Cancer Genome Atlas (TCGA), and found the frequency of LOH to be 12.8% in ovarian cancer tissue (n = 405), and between 4.3% and 6.5% across 2066 breast, colon, glioblastoma, ovarian, and squamous lung cancer samples. There was single copy deletion of NAPRT1 in 1.5% of ovarian cancer samples, and 0.64% in the other cancer tissue samples that were evaluated for copy number. The observation that the NAPRT1 locus is subject to relatively frequent LOH and rare deletions suggests that NAPRT1 may have tumor suppressor activity in certain contexts. However, homozygous deletions were never observed, and thus cannot explain the absence of NAPRT1 mRNA and protein in some cancer cells.
We therefore considered epigenetic mechanisms as an explanation for the loss of NAPRT1 expression in cancer. Aberrant DNA methylation occurs frequently in cancer (27–31) and can be the major mechanism of loss of gene function at certain loci as in the example of RASSF1A (32). To determine whether NAPRT1 is silenced by promoter hypermethylation, we explored a previously published methylation dataset for lung cancer cell lines (23), as well as several new series for breast and pancreatic cancer. As shown in Fig. 4, hypermethylation of the NAPRT1 CpG island (indicated by high β values, colored red) is evident in a subset of NSCLC (14%), SCLC (18%), breast cancer (8%), and pancreatic cancer (18%) cell lines. In addition, partial methylation of the CpG island is evident in a subset of cell lines, indicated by the pink and light blue regions. Importantly, no methylation on the CpG island was detected in normal immortalized bronchial and small airway epithelial cells suggesting that the methylation we observed is tumor cell line specific. To demonstrate that hypermethylation of the NAPRT1 promoter causes transcriptional silencing, we treated 34 NSCLC cell lines with 5-aza-2′deoxycytidine (5-azaDC). NAPRT1 expression was detectable both before and after 5-azaDC treatment in cell lines that could be rescued by coadministration of nicotinic acid. In contrast, low or no expression of NAPRT1 could be detected in cell lines that could not be rescued by coadministration of nicotinic acid before 5-azaDC treatment, whereas after 5-azaDC treatment, NAPRT1 expression was comparable with rescuable cell lines (Fig. 4D).

We next evaluated the correlation of NAPRT1 methylation and gene expression as measured by RNA-seq (Fig. 5A). As expected, the strongest relationship between expression and methylation was at the extreme ends of the range of β values. Overall, there is strong correlation between expression and methylation in all three cell line panels, with a correlation coefficient of −0.69 (n = 179; Fig. 5A). We then evaluated the correlation between methylation and expression in publicly available data from TCGA for breast cancer and lung adenocarcinoma. We find a similar strong negative association between expression and methylation within the target region of the NAPRT1 promoter to predict nicotinic acid rescue. Using β values from the Infinium array, we calculated a positive predictive value of 1.0 (17/17), with a sensitivity of 0.94 (17/18) and specificity of 0.97 (34/35; Fig. 5D). Taken together, these data show that NAPRT1 expression is silenced by promoter hypermethylation in NSCLC and that promoter hypermethylation of NAPRT1 is inversely associated with nicotinic acid rescue in cancer cell lines.

**Bisulfite next-generation sequencing identifies tumor-specific methylation sites in NAPRT1**

On the basis of the strength of the association between NAPRT1 methylation, expression, and rescue, we considered development of a quantitative methylation assay for use in clinical samples. We used a next-generation bisulfite sequencing (NGBS) strategy to identify the most appropriate target region for QMSP assay development within the NAPRT1 CpG island. We sequenced the NAPRT1 CpG island in DNA derived from nine peripheral blood mononuclear cells (PBMC) preparations from healthy volunteers, 20 tumor cell lines with known NAPRT1 expression and nicotinic acid rescue status, as well as DNA derived from a variety of benign tissues (Fig. 6A). Consistent with the Infinium data, the NAPRT1 promoter was hypermethylated in seven cell lines, whereas no methylation was detected in eight cell lines. Five cell lines exhibited partial methylation. Methylation was not detected in benign breast, colon, or lung tissues. Immune cell infiltrates are frequently a source of contaminating DNA in preparations derived from biopsies. Our data suggest that at least a subset of individuals have detectable methylation over most of the NAPRT1 CpG island in their PBMCs. However, there was a core region in the CpG island (hg19, chr8:144660496-144660520) where there was no methylation in any of the benign samples (Fig. 6A, bottom).

These data suggest that a well-designed QMSP assay should be able to distinguish between tumors with complete promoter methylation and background signal in the tissue sample. We tested the QMSP assay in cell lines with known methylation and nicotinic acid rescue status. Consistent with bisulfite sequencing, QMSP distinguishes cell lines with methylation within the target region of the assay. Thus QMSP can be used to identify cell lines that lack NAPRT1 and are thus unable to be rescued by nicotinic acid (Fig. 6B). Most tumor samples obtained in clinical trials are archival diagnostic biopsies or surgical resections. These samples are almost always FFPE slides or blocks. Thus, to enable a diagnostic hypothesis in the clinic, a molecular assay needs to work well on these preserved tissue specimens. We tested the QMSP assay on tissue with known IHC status and we detected methylation only in the sample with IHC score of zero. Conversely, we did not detect any methylation in the tumor sample with an IHC score of 3+ (Fig. 6C).

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**Figure 6.** High-resolution mapping of NAPRT1 methylation enables design of a targeted methylation-specific PCR assay for detection of NAPRT1-low tumors.

A, targeted amplicon bisulfite deep sequencing quantifies DNA methylation status of NAPRT1 promoter-associated CpG island in nicotinic acid resuscuable and non-resuscuable cell lines. Columns represent percent methylation at individual CpG sites (average coverage 80,568 reads per site) across the NAPRT1 CpG island. Note that bisulfite sequencing of peripheral blood lymphocyte DNA from healthy donors and of normal breast, colon, and lung tissues indicate lowest methylation levels near the NAPRT1 transcription start site, an optimal region for QMSP primer design. B, comparison of NGBS and QMSP in the NSCLC cancer cells from part A. Left y-axis shows the percentage methylation based on NGBS (grey bars), 2^−ΔCt values (red dots, line) for the corresponding QMSP are shown on the right y-axis. C, representative QMSP results from DNA extracted from unstained sections of the tumors shown in the IHC slides above the graph. The positively stained area on the IHC D slide is benign tissue.
Discussion

Evaluation of NAPRT1 in cancer tissue provides the opportunity for a unique biomarker strategy for selection of patients likely to respond to NAMPT inhibitors. Selection of patients whose tumors do not express NAPRT1 allows simultaneous administration of nicotinic acid, which can mitigate the toxicity of NAMPT inhibitors in nonmalignant tissue that generally expresses NAPRT1. Although supplementing patients with nicotinic acid has the potential to improve the therapeutic index of NAMPT inhibitors, it carries with it an absolute requirement of identifying those patients whose tumors have lost the expression of NAPRT1, as nicotinic acid could also rescue tumors that express NAPRT1. Previous work has demonstrated that glioblastomas and neuroblastomas have a very high prevalence of NAPRT1 loss (16). We find that solid tissue carcinomas exhibited loss of NAPRT1 expression in approximately 15% of cell lines or samples evaluated. This greatly expands the potential responsive patient population, but requires a diagnostic test to identify patients eligible for coadministration of a NAMPT inhibitor and nicotinic acid.

By evaluating nicotinic acid rescue and gene expression in more than 400 cancer cell lines, we confirm that NAPRT1 level is the major determinant of nicotinic acid rescue status and define a threshold mRNA level below which nicotinic acid rescue was not observed. However, this will be challenging to translate into a clinical assay because even small amounts of contaminating tissue could result in an incorrect conclusion that NAPRT1 is expressed. Immunohistochemistry allows cell level resolution of staining and discrimination of staining in malignant tissue compared with adjacent normal tissue. NAPRT1 has been evaluated by immunohistochemistry in prior studies (16, 19), but there was no determination of the required threshold for rescue. In this study, we evaluated a panel of NSCLC cell lines for nicotinic acid rescue status in culture and prepared cell pellets for immunohistochemistry from the same cells. With this approach, we determined that an IHC score of zero correlated with lack of nicotinic acid rescue. Determination of the threshold is essential for the implementation of NAPRT1 immunohistochemistry as a diagnostic assay in solid tumor indications where there is a broad spectrum of NAPRT1 level.

We show that loss of function of the NAPRT1 gene is mediated primarily by hypermethylation of the CpG island that overlaps with the transcription start site of NAPRT1. Importantly, complete methylation of the CpG island is strongly associated with the absence of rescue in cell lines treated with GNE-617 and nicotinic acid. Reduction of DNA methylation by treatment of cells with 5-aza-dC was sufficient to induce NAPRT1 expression in cell lines in which the locus was hypermethylated. We mapped the sites of DNA methylation in non-locusable cell lines using NGBS and used this high-resolution map to develop a methylation-specific QMSP assay suitable for use on tissue preserved in FFPE slides. There are several advantages of using a QMSP assay as a molecular diagnostic to identify patients. Pre-amplification allows detection of NAPRT1 methylation from very small amounts of tissue which could enable the use of small biopsies or even fine needle aspirates, which are frequently the only tissue available for patients diagnosed with stage IV NSCLC, SCLC, or pancreatic cancer. As shown here, the QMSP assay works well on FFPE tissues, allowing easy analysis of archival tissue. The signal to noise ratio is excellent because the background of normal tissue is generally not methylated, allowing detection of a positive signal even if there is significant contamination from noncancerous tissue. Finally, because the assay detects a positive signal of DNA methylation rather than loss of protein expression, it is not prone to false positives (for loss of NAPRT1) that may occur with IHC assays due to poor tissue preservation. Our data strongly support the evaluation of NAPRT1 promoter methylation as an enrollment biomarker for clinical trials evaluating the safety and efficacy of NAMPT inhibitors.

Although the detection of DNA methylation has been explored extensively as a method for the early detection of cancer or as a prognostic indicator (33–36), there are very few examples in which DNA methylation of a specific gene predicts a responsive patient subset for a specific class of inhibitors. Two known examples are MGMT for dacarbazine (37) and BRCA1 for PARP inhibitors (38–40), and this study adds an additional example. Both MGMT and BRCA1 are tumor suppressor genes. The finding that NAPRT1 is subject to single copy deletion along with more frequent LOH and promoter hypermethylation suggests that NAPRT1 may act as a tumor suppressor gene in some contexts. It is not clear why loss of NAPRT1 expression would be advantageous to some cancers as the gene contributes to the NAD synthetic capacity of the cell. However, it is interesting to note that frequent hypermethylation of another metabolic enzyme, LDHB, has recently been reported in breast cancer (31), suggesting that alteration of metabolic pathways by DNA methylation may be a more general phenomenon in cancer.

Disclosure of Potential Conflicts of Interest

H. Koeppen and L.D. Belmont have ownership interest (including patents) in Roche stock. No potential conflicts of interest were disclosed by the other authors.

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