Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

Cancer Therapy: Preclinical

Clinical Cancer Research

NNMT Silencing Activates Tumor Suppressor PP2A, Inactivates Oncogenic STKs, and Inhibits Tumor Forming Ability

Kamalakannan Palanichamy, Suman Kanji, Nicolaus Gordon, Krishnan Thirumoorthy, John R. Jacob, Kevin T. Litzenberg, Disha Patel, and Arnab Chakravarti

Abstract

Purpose: To identify potential molecular hubs that regulate oncogenic kinases and target them to improve treatment outcomes for glioblastoma patients.

Experimental Design: Data mining of The Cancer Genome Atlas datasets identified nicotinamide-N-methyl transferase (NNMT) as a prognostic marker for glioblastoma, an enzyme linked to the reorganization of the methylome. We tested our hypothesis that NNMT plays a crucial role by modulating protein methylation, leading to inactivation of tumor suppressors and activation of oncogenes. Further experiments were performed to understand the underlying biochemical mechanisms using glioblastoma patient samples, established, primary, and isogenic cells.

Results: We demonstrate that NNMT outcompetes leucine carboxyl methyl transferase 1 (LCMT1) for methyl transfer from principal methyl donor SAM in biological systems. Inhibiting NNMT increased the availability of methyl groups for LCMT1 to methylate PP2A, resulting in the inhibition of oncogenic serine/threonine kinases (STK). Further, NNMT inhibition retained the radiosensitizer nicotinamide and enhanced radiation sensitivity. We have provided the biochemical rationale of how NNMT plays a vital role in inhibiting tumor suppressor PP2A while concomitantly activating STKs.

Conclusions: We report the intricate novel mechanism in which NNMT inhibits tumor suppressor PP2A by reorganizing the methylome both at epigenome and proteome levels and concomitantly activating prosurvival STKs. In glioblastoma tumors with NNMT expression, activation of PP2A can be accomplished by FDA approved perphenazine (PPZ), which is currently used to treat mood disorders such as schizophrenia, bipolar disorder, etc. This study forms a foundation for further glioblastoma clinical trials using PPZ with standard of care treatment. *Clin Cancer Res; 23(9); 2325–34. ©2016 AACR.*

Introduction

The dysregulation of the cellular metabolism is a hallmark of cancer and plays a critical role in maintaining cancer cell viability. The cancer metabolism depends on alterations of key metabolic pathways, which has a profound effect on the expression of oncogenes and tumor suppressors. The protumorigenic effects of mutations in metabolic enzymes have been shown to be key regulators in the activation and inactivation of oncogenes and tumor suppressor genes, respectively (1). Although it has been shown that mutations in metabolic enzymes contribute to tumorigenesis, the role of hyperactive metabolic enzymes which are not mutated remains largely underexplored. We report that nicotinamide-N-methyltransferase (NNMT) is upregulated, however, rarely mutated in glioblastoma (Fig. 1A). The cytosolic enzyme NNMT catalyzes the transfer of the methyl group from S-adenosylmethionine (SAM; refs. 2–4), to nicotinamide (derivative of vitamin B3), producing S-adenosylhomocysteine (SAH) and methylnicotinamide (MNA), respectively (5, 6). NNMT is the only enzyme known to use nicotinamide as a methyl acceptor substrate and methylates nicotinamide as a marker for degradation and excretion. Therefore, NNMT plays an important role in controlling the intracellular concentration of nicotinamide, the precursor to NAD(+), an important cofactor linking cellular redox states with energy metabolism (7). Overexpression of NNMT has been linked to a variety of malignancies, including: lung, liver, kidney, thyroid, prostate, bladder, and colon cancers (8–17). In addition, the increased activity of NNMT has been linked to tumor aggressiveness and shown to promote the migration, invasion, proliferation, and survival of cancer cells (10, 11, 14, 18–20). Despite the correlation between NNMT and these malignancies, the mechanism by which NNMT enhances tumorigenesis is poorly understood. Here, we demonstrate that intact NNMT plays a key role in altering biochemical and cellular functions in glioblastoma by repressing the activity of the tumor suppressor enzyme PP2A. This decrease in the activation of PP2A enables the prolonged activation of key prosurvival kinases implicated in the enhanced tumorigenesis and aggressiveness of glioblastoma.

Materials and Methods

Study approval

This study was conducted in accordance with The Ohio State University Intuitional Review Boards for IRB (2009C0065 and 2014C0115), IACUC (2009A0127), and IBC (2009R0169).

Department of Radiation Oncology, James Cancer Hospital and Solove Research Institute, The Ohio State University College of Medicine and Comprehensive Cancer Center, Columbus, Ohio.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Kamalakannan Palanichamy, The Ohio State University College of Medicine and Comprehensive Cancer Center, 410 W. 12th Avenue, Room 385E, Wiseman Hall, Columbus OH 43210. Phone: 614-685-4245; Fax: 614-292-5435; E-mail: Kamalakannan.palanichamy@osumc.edu

doi: 10.1158/1078-0432.CCR-16-1323

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

Translational Relevance

Glioblastoma is a lethal brain tumor with a median patient survival of approximately 15 months and a 5-year survival rate of less than 10%. The activation of oncogenic kinases serves as a treatment escape mechanism in various targeted biological therapies, providing a barrier in improving treatment outcomes for glioblastoma patients. Therefore, identifying novel targets that modulate the activity of these kinases is imperative to improve treatment outcomes.

Cell culture

The three primary glioblastoma cell lines (VC3, MGH8, and OSU68) used in this study were isolated from glioblastoma patient tissues and authenticated by neuro-pathologist. The three commercially available cell lines (U87, LN18, and LN229) were obtained from ATCC. Glioblastoma cells were maintained in DMEM (Life Technologies), supplemented with 10% FBS (Sigma-Aldrich), and 1% antibiotic-antimycotic (Life Technologies). Cells were cultured at 37°C under a gas phase of 95% air and 5% CO_2. All studies were conducted within 10 passages.

Isogenic NNMT cell lines

Three NNMT MISSION shRNA lentiviral transduction particles were used to generate stable NNMT knockdown cell lines:

NM_006169.1-330s1c1 (Sequence: CCGGCCTCTCTGCTTGTGAATCCTTCTCGAGAAGGATTCACAAGCAGAGAGGTTTTTG), NM_006169.1-164s1c1 (Sequence: CCGGACCCTCGGGATTACCTAGAAACTCGAGTTTCTAGGTAATCCCGAGGGTTTTTTG), and NM_006169.1-448s1c1 (Sequence: CCGGGTGACCTATGTGTGTGATCTTCTCGAGAAGATCACACACATAGGTCACTTTTTG; Sigma-Aldrich).

Each construct was cloned into a pLK0.1-Puro vector. The U87-NNMT-OE cell line was generated using the true-ORF NNMT human cDNA clone in a pCMV6-Neo vector purchased from Origene.

MTT proliferation assay

The MTT Assay Kit was used according to the manufacturer's instructions (ATCC). Briefly, the proliferation rate was determined by measuring the amount of oxidoreductase enzymes. These enzymes reduce the yellow tetrazolium MTT to generate the insoluble purple product formazan. The samples were then measured at an absorbance of 570 nm to quantify the reducing activity and corresponding proliferation rate.

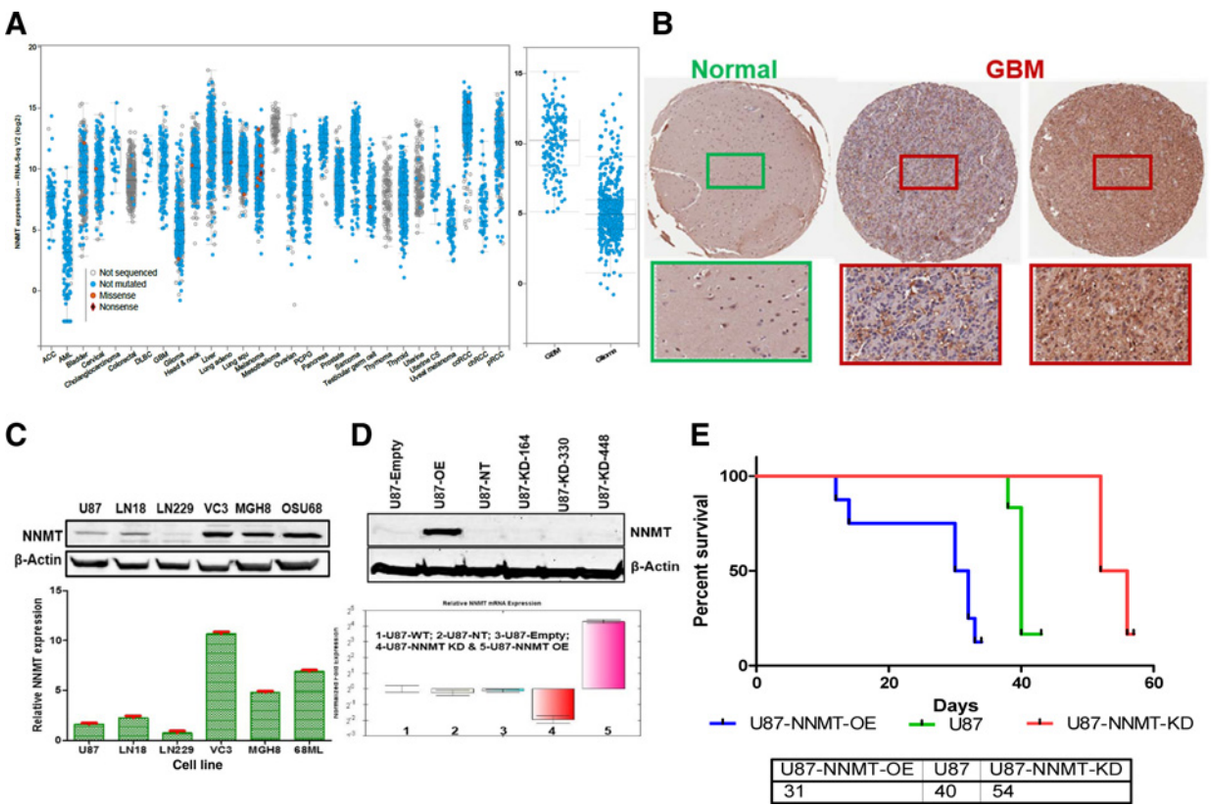


Figure 1.

NNMT is a prognostic marker for glioblastoma. **A,** The RNA-Seq data obtained from the cBioPortal shows the relative expression levels and mutational status of NNMT for a wide range of cancers including brain tumors. **B,** Representative tissue sections in TMA showing the NNMT staining in normal and glioblastoma tissues. **C–D,** Protein and mRNA expression levels of NNMT in (**C**) primary and established glioblastoma cell lines and (**D**) NNMT isogenic cell lines. **E,** Kaplan–Meier survival curves of NOD-SCID mice intracranially injected with NNMT isogenic cells.

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

Clonogenic survival assay

Cells were plated at 8,000, 4,000, 2,000, 1,000, 500, and 50 cells/well in petri dishes and radiated at 10, 8, 6, 4, 2, and 0 Gy, respectively. The plates were incubated in a humidified CO_2 incubator at 37°C, and grown for 10 to 14 days depending on colony size and cell type. After 10 to 14 days, the supernatant media was removed, and the surviving colonies were stained with methylene blue, dried, and counted. Colonies $\geq$50 cells were considered significant. The plating efficiency was calculated from the ratio of colonies formed over the number of cells plated.

In vitro PP2A-specific activity assay

The PP2A Assay Kit (R&D system) was used to detect PP2A activity according to the manufacturer's instructions. In brief, the active and inactive form of PP2A-C was bound by an immobilized capture antibody. The unbound material was washed away and a synthetic phosphopeptide substrate was added. Active PP2A dephosphorylated the substrate to generate free phosphate and unphosphorylated peptides. The free phosphate was then detected by a dye-binding assay using malachite green. The activity of PP2A in each well was determined by comparing the rate of phosphate release to that of the phosphate standard. Total PP2A concentration was measured using DuoSet IC sandwich ELISA Kit, according to the manufacturer's instructions. Relative PP2A activity was then determined by normalizing the PP2A activity to the total PP2A enzyme concentration in the cell lysate.

Western analysis

Cells were harvested and protein was extracted using RIPA buffer (Sigma) with 1% (v/v) protease inhibitor cocktail (Sigma) and 1% (v/v) phosphatase inhibitor cocktail (Sigma). Samples were run on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then incubated overnight with the following primary antibodies: P-p44/42 MAPK, p44/42 MAPK, P-Akt (Ser473), Akt, PP2A-C, NNMT, PARP, cleaved PARP, caspase-3, cleaved caspase-3, ASK1, p38, P-p38, LCMT1, β-Actin (Cell Signaling Technology), and Me-PP2A-C (Abcam). The membranes were then incubated for 1 hour with mouse or rabbit IgG–HRP conjugate secondary antibodies (Cell Signaling Technology) and developed.

Quantitative RT-PCR

The RNeasy Mini Kit and Superscript III First-Strand Synthesis Kit were used to isolate RNA from samples and generate cDNA according to the manufacturer's instructions. Using quantitative RT-PCR, the expression level *NNMT* (Hs00196287_m1; Thermo Fisher) was measured using the Taqman gene expression assay. *GAPDH* (Hs99999905_m1; Thermo Fisher) was used as the control. The gene expression profile data were generated using the C1000^{TM} Thermal Cycler-CFX96^{TM} Real-Time System and analyzed using the BIO-RAD CFX Manager.

Hematoxylin and Eosin staining and immunohistochemistry of coronal sections of mice brain tumors

Formalin-fixed, paraffin-embedded tissue sections were baked overnight at 60°C, deparaffinized, rehydrated using xylene and ethanol graded mixture, treated with epitope retrieval agent (Dako-TRS pH 6.1). Sections were stained with hematoxylin for 5 minutes, rinsed in running tap water, and counterstained in eosin for 15 seconds, and mounted after dehydration. Thorough washing with PBS (3×) was ensured after all incubation steps.

Metabolic profiling

The metabolic profiling protocol used has been outlined previously (21). Briefly, mass spectral profiling was done using Accurate Mass Q-TOF LC/MS instrument. The fragmentation pattern of the metabolites were analyzed using fixed collision energy. The product ions of precursor ion were confirmed with MS/MS data in Agilent METLIN Personal Metabolite database. The synthetic compound of each metabolite of interest was purchased and analyzed in targeted LC-MS/MS mode. The retention time and fragmentation pattern of the synthetic compounds were matched with the differentially regulated metabolites.

The metabolite validation and quantification was done using Triple Quad LC/MS through multiple reactions monitoring (MRM) method. The known concentration of the synthetic compound of targeted metabolites was used to quantify the differentially regulated metabolites. The Mass Hunter Optimizer (B.03.01) was used to find the optimum collision energy and fragmentor voltage for the targeted metabolite. Five microliters of each sample was injected to Triple Quad LC/MS to estimate the relative concentration of the targeted metabolites. The same column, mobile phase, gradient, and source parameters were followed as used in Accurate Mass Q-TOF LC/MS system. The Mass Hunter Quantitative Analysis (B.04.00) program was used to quantify the targeted metabolites. First, the standard calibration curve was generated by serial dilution with a known concentration of synthetic compound then the relative concentration of targeted ion in the sample was estimated.

Orthotopic injection of isogenic cell lines into NOD-SCID mice

The glioblastoma xenograft model used has been outlined previously (22). The sample size for intracranial injection was determined based on the power calculation from an *in vivo* experiment performed previously. It was determined that five mice would be sufficient to determine an effect size of 5 days with a power of 0.90 and significance level of 0.05. Differences in tumor growth between the different cell lines was determined using a *P*-value of 0.05.

Statistical analysis

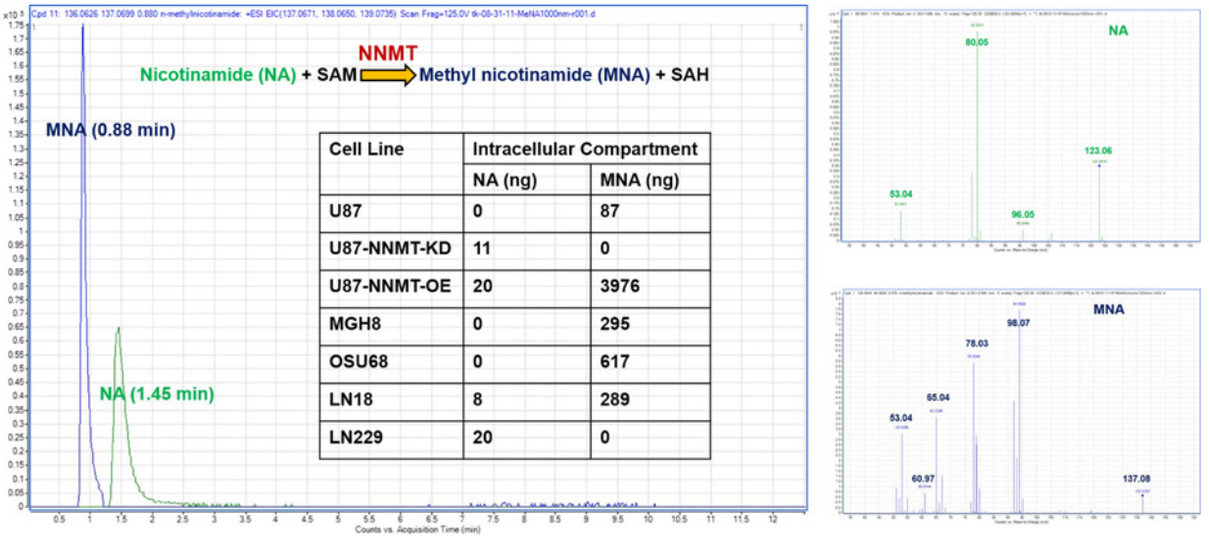
All results were confirmed in at least three independent experiments, and data from one representative experiment were shown. All quantitative data are presented as mean ± SD. The statistical analysis was performed using SAS 9.2 (SAS Institute) or Graph Pad Prism 5. Student *t* tests were used for comparisons of means of quantitative data between groups. Values of $P < 0.05$ were considered significant.

Results

Prognostic value of NNMT in glioblastoma tumors

We discovered from the The Cancer Genome Atlas (TCGA) data comprising more than 100 studies with different types of cancers showed that NNMT is mutated in only 3% of cancers (Fig. S1). We found that in brain tumors, the expression of NNMT in glioblastoma patients was significantly higher than that in other types of glioma by data mining publically available gene expression data sets (454 tumor biopsies; Fig. S2) and was validated using TCGA data that demonstrated that the expression of NNMT in

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323



Cell Line	Intracellular Compartment	
	NA (ng)	MNA (ng)
U87	0	87
U87-NNMT-KD	11	0
U87-NNMT-OE	20	3976
MGH8	0	295
OSU68	0	617
LN18	8	289
LN229	20	0

Figure 2.

LC-MS/MS was used to measure NNMT activity. The accurate mass LC-MS Q-TOF and LC-MS QQQ instruments were used in combination with the Mass Hunter qualitative analysis (B.03.01) software to identify the concentrations of NA and MNA. The smaller boxes adjacent to the ion chromatogram represent the target analysis MS/MS product ion spectrums of NA and MNA. The table within the ion chromatogram represents the intracellular concentration of NA and MNA in the patient-derived primary, commercially available, and NNMT isogenic cell lines.

glioblastoma was more than two-fold higher compared to low-grade glioma (Fig. 1A). Furthermore, we found that within glioblastoma tumors, that NNMT expression was inversely correlated with survival outcome (Fig. S3). Interestingly, this is one of the few enzymes which is not mutated but has a higher expression level in aggressive cancers. We further validated NNMT as a predictive and prognostic marker for glioblastoma using the gene expression profiles of 80 glioblastoma patient tissues following temozolomide and radiotherapy treatment (GSE7696; ref. 23). Using rigorous statistical conditions, we determined that there were 73 genes that were significantly differentially regulated in recurrent cases. Both NNMT probes were found among the differentially regulated genes thereby supporting our findings that NNMT is a predictive and prognostic marker (Fig. S4). The correlation between NNMT expression and recurrent glioma cases suggests that there may be an association between NNMT and treatment resistance as well as tumor recurrence.

NNMT expression levels are higher in glioblastoma compared to normal brain

Using a tissue microarray (TMA) consisting of normal brain and glioblastoma tissues, NNMT protein expression was analyzed by immunohistochemistry staining. The hematoxylin and eosin (H&E) and NNMT-stained TMA shows that in general glioblastoma specimens express higher amounts of NNMT when compared to normal brain specimens (Fig. 1B and Supplementary Fig. S5). Patient-derived primary and commercially available glioblastoma cell lines also expressed NNMT (Fig. 1C). Of note, the expression levels of NNMT were found to be higher in primary cells when compared to commercially available cells.

To determine the contribution of the increased expression of NNMT on the tumorigenicity of glioblastoma, we generated U87 NNMT knockdown (KD) and U87 NNMT overexpression (OE) isogenic cell lines (Fig. 1D). We decided to use the U87 cell lines due to the lack of availability of primary cell lines for other investigators, and also among U87, LN18, and LN229, U87 is the only commercially available cell line used which can form intracranial tumors. The U87 isogenic cell lines were implanted intracranially into NOD-SCID mice (Fig. 1E). The KD cell line formed tumors at a significantly slower rate than the U87 wild-type (WT) cell line (P-value = 3.93E−05). Conversely, the survival of the mice bearing the OE-derived tumors appear to have two outliers which prevented us from being able to determine any statistically significant changes in tumor growth without further experimentation. Nonetheless, the decrease in tumor growth resulting from NNMT silencing supports the role of NNMT in promoting tumor growth and aggressiveness.

NNMT silencing increases sensitivity of glioblastoma to radiation treatment

NNMT activity in the glioblastoma and isogenic cell lines was determined by measuring the concentration of nicotinamide (NA) and methyl nicotinamide (MNA) using LC-MS/MS in the multiple reactions monitoring (MRM) mode (Fig. 2). The NNMT expression levels in the cell lines tested corresponded with the concentration of MNA in each cell line. The proliferative potential of NNMT isogenic cell lines was determined using MTT assay. KD cells demonstrated a decreased proliferative potential compared to the WT and OE cell lines (Fig. 3A). After irradiating the isogenic cell lines, KD cells showed an increased growth inhibition and decreased colony forming ability compared to the other isogenic cell lines (Fig. 3B and C). Of note, adding temozolomide to radiation treatment did not significantly increase the growth inhibition of KD cells (Fig. 3D). Interestingly, Western blot analysis demonstrated that the KD cell line expresses a basal level of the apoptotic marker cleaved caspase 3 (Fig. 3E). Overall, this suggests that inhibition of NNMT alone may decrease

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

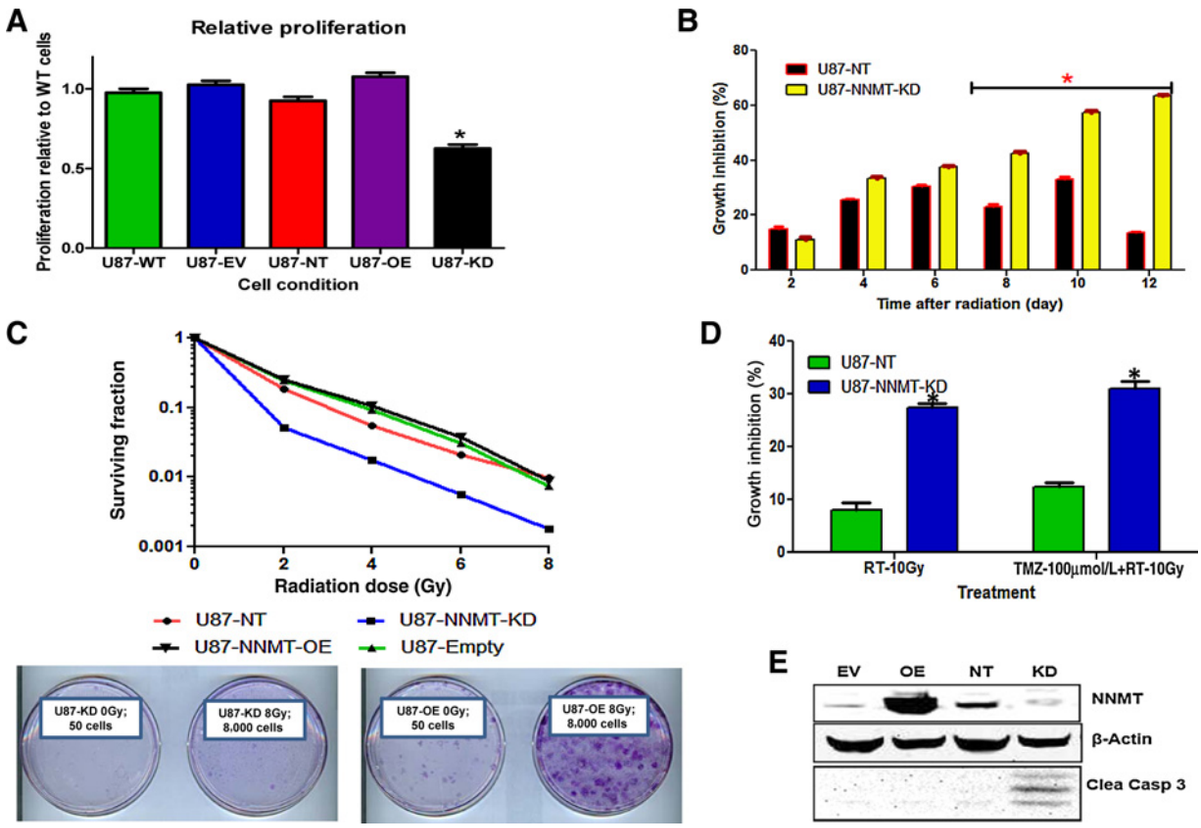


Figure 3.

NNMT silencing sensitizes glioblastoma cells to radiation treatment, decreases proliferation, and colony forming ability. **A,** The MTT assay was used to calculate the relative proliferation rate of the NNMT isogenic cell lines relative to the U87-WT cell line. **B,** Growth inhibition of the NT and KD cell lines following radiation and temozolomide treatment. **C,** The growth inhibition of KD and NT cells following 6 Gy radiation over a 12-day period. **D,** Clonogenic survival curves of the NNMT isogenic cell lines. Representative pictures of the clonal cells used to calculate the colonies and plot the survival curve have also been provided. **E,** Western blot analysis of the basal levels of NNMT and cleaved caspase 3.

glioblastoma viability, perhaps as a result of the deactivated prosurvival pathway.

NNMT silencing attenuates the activation of prosurvival kinases and induces apoptosis following radiation treatment

To determine the mechanisms behind the increased treatment sensitivity induced by NNMT silencing, we evaluated the expression levels of the prosurvival kinases Akt, along with several enzymes involved in the MAPK pathway. The KD cells showed significantly decreased activation of ASK1, SAPK/JNK, p38 MAPK, p44/42 MAPK, and Akt both with and without radiation treatment (Fig. 4A–C). In addition, NNMT expression inversely correlated with the concentration of cleaved PARP, a marker for apoptosis (Fig. 4D). The decreased activation of p44/42 MAPK and Akt following NNMT silencing shows a similar trend under *in vivo* conditions (Fig. 5A).

Downregulation of NNMT increases PP2A activity which inhibits oncogenic kinases

We next evaluated whether the dysregulation of the heterotrimeric serine/threonine phosphatase PP2A was responsible for the change in the activity of Akt and p44/42 MAPK. The regulatory subunit B of PP2A is recruited by a dimer composed of the catalytic subunit C (PP2A-C) and structural subunit A (24–26). Methylation of the C-terminal leucine residue (Leu-309) of PP2A-C allows for the assembly and activation of the PP2A trimer (27–30). PP2A-C methylation is regulated by the SAM-dependent trans-methylation reactions involving leucine carboxyl methyltransferase-1 (LCMT1) and protein phosphatase methylesterase-1 (PME1; refs. 31, 32). Western blot analysis revealed a higher expression level of methylated PP2A-C in KD cells compared to WT, nontarget (NT) knockdown control, and empty vector (EV) overexpression control (Fig. 5B). Conversely, OE cells showed a considerable reduction of methylated PP2A-C (Me-PP2A-C; Fig. 5C). Further *in vitro* studies confirmed the altered PP2A activity in the isogenic cell lines (Fig. 5D). The KD cell line exhibited a 1.5-fold decrease in cellular methylation potential compared to the OE cell line showing that NNMT expression correlates with the methylation potential (the ratio of SAM/SAH; Fig. 5E). Methylation potential is an important indicator of protein methylation status (posttranslational modification), DNA, RNA, and lipids. Cellular methylation is regulated by methyltransferases that use SAM as a methyl donor (2, 4). SAH is a product of methylation reactions and regulates the activity of these methyltransferases (33). In addition, the increased expression of Me-PP2A-C upon downregulation of NNMT was validated in U87-KD$_{330}$

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

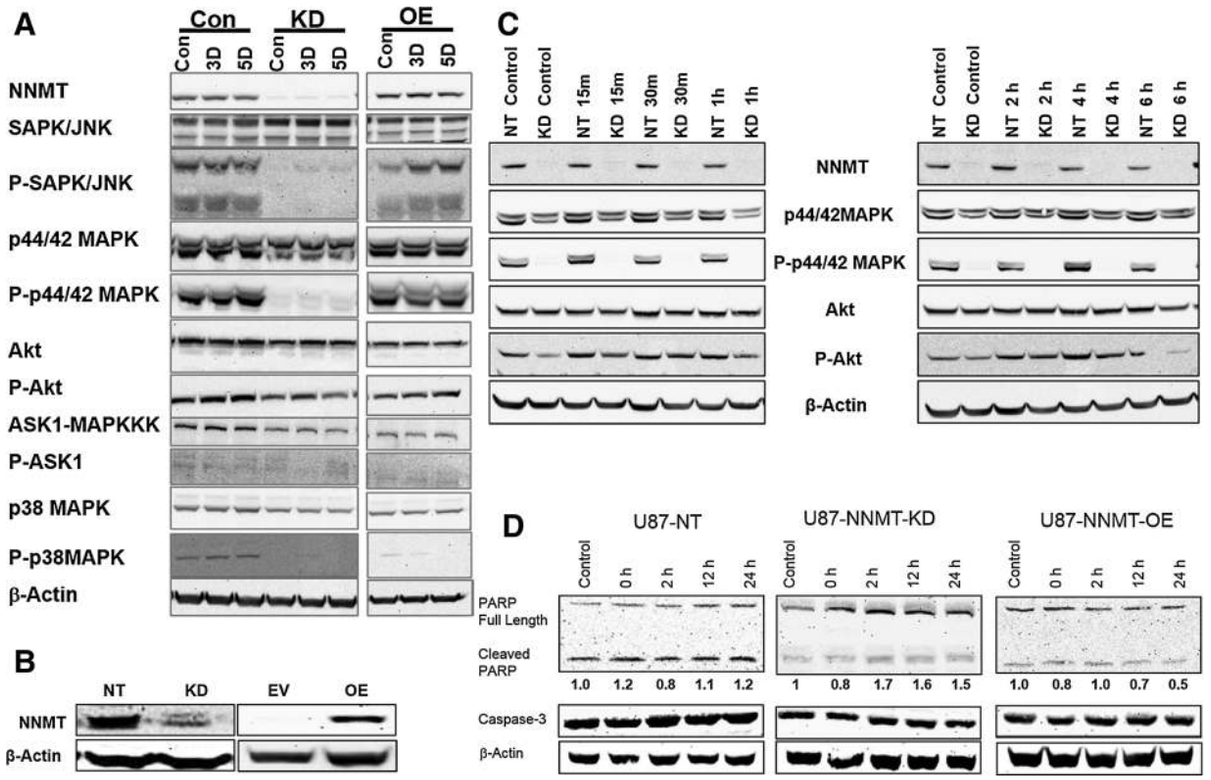


Figure 4.

NNMT silencing attenuates the activation of prosurvival kinases and induces apoptosis following radiation treatment. **A–D,** Western blot analysis of (**A**) the activation of the Akt and MAPK kinases in the WT, KD, and OE cell lines following 6 Gy radiation over a 5-day time period; (**B**) the basal NNMT expression levels in the isogenic U87 cell lines; (**C**) the activation of the Akt and MAPK kinases in the NT and KD cell lines following 6 Gy radiation over a 6-hour time period; (**D**) Caspase-3, PARP, and the apoptotic marker cleaved PARP in the NT, KD, and OE cell lines following 6 Gy radiation over a 24-hour period.

(a different Sh-RNA) and patient-derived MGH8-KD glioblastoma cell lines (Fig. 5F). The STK signaling cascades show a similar trend as that of U87-KD_{164}. Based on these results, we show that NNMT-PP2A-LCMT1 methylation plays a key role in activating oncogenic kinases.

Antagonist-agonist approach validates PP2A as a secondary target of NNMT

The basal expression level of Me-PP2A-C shows a significant down regulation in OE cells compared to KD cells. To determine whether the changes in the methylation of PP2A-C were dependent on NNMT, we exogenously added the PP2A inhibitor okadaic acid (OA) and PP2A activator perphenazine (PPZ) to the NNMT isogenic lines (Fig. 6A). The KD, OE, and WT cell lines were treated with increasing concentrations of OA with and without 25 μmol/L PPZ. There was an increase in the methylation of PP2A-C in the KD cells upon the addition of PPZ, across all concentrations of OA. Conversely, the methylation of PP2A-C was increased marginally in the WT (due to high basal expression level of NNMT) and OE cell lines when PPZ was added with the lowest concentration of OA. However, the WT and OE cells showed no significant increase of PP2A methylation when higher concentrations of OA were added. These data indicate that PP2A activation is modulated by NNMT expression. PP2A is one of several phosphatases that regulate the oncogenic activity of Akt and MAPK in cancer cells (24–26). To determine whether the methylation of PP2A-C was specifically dependent on NNMT expression and responsible for the observed changes in Akt and p44/42 MAPK activation, we treated the KD cells with the same treatment regimen of OA and PPZ. In all PPZ and OA treatments, we observed a decrease in Akt and p44/42 MAPK activation (Fig. 6B). These data clearly indicate that the methylation of PP2A-C is NNMT dependent, which in turn affects the phosphorylation of the prosurvival kinases.

Discussion

Previous reports have connected the increased expression levels of NNMT in cancer cells with chemotherapy and radiation resistance as well as increased tumor aggressiveness (14, 20, 34). The downregulation or silencing of NNMT expression has been shown to increase the sensitivity of carcinoma cells to radiation therapy and decrease tumorigenicity, providing key support for the role of NNMT in promoting treatment resistance and tumorigenesis in cancer cells (17, 35, 36). Existing literature reports the underlying mechanism for NNMT's role in resistance due to alterations at the epigenetic level, including histones. Here, we report that NNMT plays a novel role in modulating protein methylation at translational level and inhibiting tumor suppressive phosphatases to fuel oncogenic kinases.

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

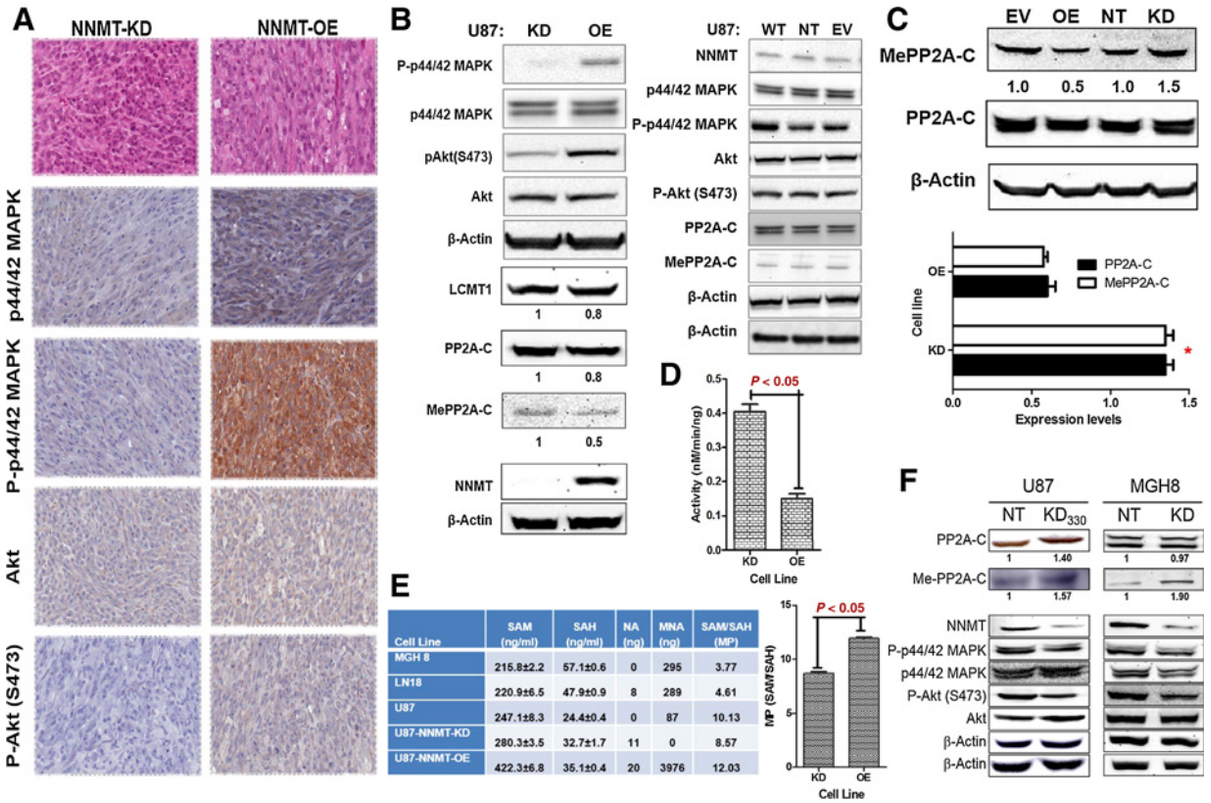


Figure 5.

NNMT silencing decreases STK activation by methylating PP2A-C. **A,** Immunohistochemical staining of tumors harvested from mice injected with KD and OE cells. The tissue sections were stained with H&E and Akt, P-Akt, p44/42 MAPK, and P-p44/42 MAPK antibodies for immunodetection. **B,** Western blot analysis of the change in PP2A activation, corresponding LCMT1 expression levels, and activation of STKs in the KD and OE cell lines (Ratio = expression level normalized to β-actin) [Ratio* = Expression level of (Me-PP2A-C:β-actin):(PP2A:β-actin)]. The control group of wild type, knockdown, and overexpression is shown on the right. **C,** Basal level of PP2A activation in the NNMT isogenic cell lines [Ratio = expression level of (Me-PP2A-C:β-actin):(PP2A:β-actin)]. **D,** The *in vitro* activity of PP2A in the KD and OE cell lines. **E,** LC-MS/MS was used to determine the concentrations of the substrates and products involved in the NNMT enzymatic reaction, along with the methylation potential (MP) for the glioblastoma cell lines tested. The graph to the right represents the relative MP of the KD and OE cell lines. **F,** Western blot analyses showing an increased methylation of PP2A-C and a consequent inhibition of P-Akt and P-p44/42 MAPK in U87-KD_{330} and MGH8-KD cells.

Nicotinamide has been identified as a radiation and/or chemotherapy sensitizing agent in carcinoma, sarcoma, and breast cancer (37, 38). In addition, nicotinamide has been the subject of several successful phase II and phase III clinical trials targeting carcinoma and non-melanoma skin cancer (39, 40). NNMT transfers the methyl group from SAM to nicotinamide to form MNA and is the only enzyme known to use nicotinamide as a methyl acceptor substrate. This N-methylation reaction marks nicotinamide for degradation and excretion. Therefore, NNMT plays an important role in controlling the intracellular concentration of nicotinamide, the precursor to NAD(+), which is involved in the regulation of the cellular energy supply, cellular resistance to stress or injury, and longevity. Nicotinamide induces increased radiation and chemotherapy sensitivity in cancer cells by inhibiting PARP, thus preventing efficient single-stranded DNA break repair (41). We show that when NNMT is inhibited in glioblastoma, increased intracellular nicotinamide inhibits PARP leading to radiosensitization. Apoptosis induced by radiation generated DNA fragmentation was measured by the increased cleaved PARP signal, a result of the increased activation of the apoptosis executioner protease cleaved caspase-3. This further reinforces the role of nicotinamide as a radiosensitizing agent and provides support for the use of nicotinamide in combination with radiation as a potential glioblastoma therapy.

A previous report showed the ability of NNMT to regulate the epigenetic landscape in the earliest steps of human embryonic stem cell development (42). In cancers, higher expression of NNMT has been reported to impair the cellular methylation potential by producing increased concentrations of MNA, resulting in an altered epigenetic state through hypomethylated histones (20). The kinetics of cellular methylation events are controlled by the rate constants K_m and K_i of methyltransferases for SAM and SAH, respectively. Here, we show that increased expression of NNMT ($K_m = 1.3$) selectively affects LCMT1 ($K_m = 1.8$), resulting in lower PP2A activation (5, 43). The IC_{50} for SAH is 3.0 mmol/L for LCMT1 and 40 to 100 mmol/L for NNMT (20), suggesting NNMT has a higher affinity for SAM as compared to LCMT1. This indicates that NNMT predominantly utilizes the methyl group from SAM to excrete or catabolize nicotinamide and under these circumstances LCMT1 lacks the ability to methylate

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

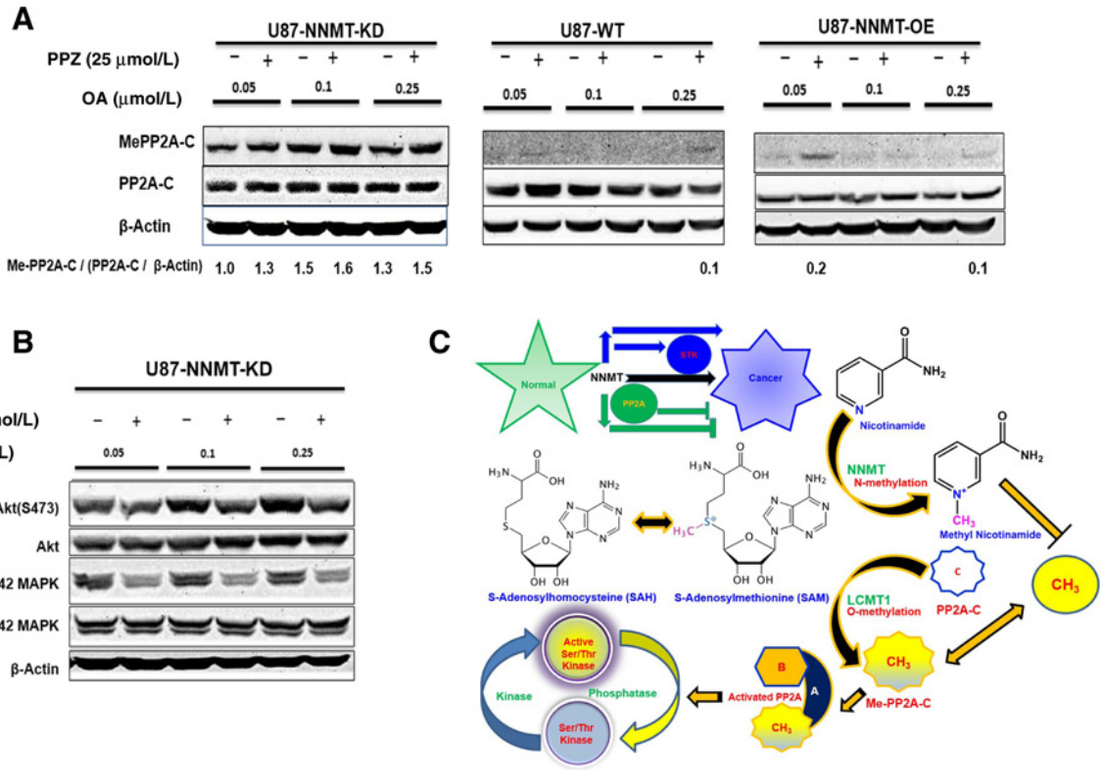


Figure 6.

NNMT silencing decreases the methylation potential and schematic illustration. **A,** Western blot analysis was used to determine the change in PP2A methylation/activation following different concentrations of PPZ and OA in the WT, KD, and OE cell lines. **B,** Western analysis of NNMT expression and Akt and p44/42 MAPK activation following the addition of OA with and without the addition of PPZ. **C,** A schematic illustrating the in cancer cells, the upregulation of NNMT utilizes an increased amount of SAM, directly modulating the expression of prosurvival kinases by influencing the activation of phosphatases.

PP2A. Even if there are methylated products in the system by LCMT1 which is a O-methylating enzyme could be potentially reversed to unmethylated product due to their reversible nature and availability of demethylases such as PME1. In contrast, N-methylating NNMT enzyme product MNA which is stable and acts as methylation sink (20) further favoring methyl transfer by NNMT compared to LCMT1.

A few cancer-relevant mechanisms of inhibiting PP2A tumor suppressor activity have been reported (44–48). However, cancer-associated mechanisms preventing PP2A-mediated inactivation of the MAPK pathway remain elusive. We have shown that silencing NNMT favors LCMT1-mediated PP2A activation followed by the inhibition of the oncogenic MAPK/Akt STKs. This is supported by a previous study that showed that decreased LCMT1 expression or SAH concentration decreases endogenous activated PP2A (49). Previous studies have also shown that PME1-mediated inhibition of PP2A promotes basal MAPK pathway activity and is required for efficient growth factor response (50). This suggests that the NNMT–PME1–LCMT1 signaling axis is one mechanism by which the activity of the MAPK/Akt pathways are maintained in cancer cells. However, in glioblastomas NNMT–PP2A–LCMT1 signaling axis regulates the activation of STKs and further suggests an important functional role of this signaling axis in the disease progression of gliomas. The schematic overview of NNMT altering SAM:SAH due to the formation of stable N-methylated product (MNA) by consuming the methyl groups which are otherwise utilized by O-methylating product by LCMT1. Blockage of MNA production by inhibiting NNMT rescued the LCMT1 mediated methylation of PP2A subunit C resulting in the activation of phosphatase PP2A and inhibition of STKs (Fig. 6C).

Through this study, we offer a biochemical mechanism to explain the role of NNMT in the epigenetic remodeling and modulation of phosphatases and oncogenic STKs in glioblastoma. In addition, we demonstrate the ability of increased intracellular nicotinamide to sensitize glioblastoma cells to radiation treatments and provide further support for the use of nicotinamide in combination with radiation treatment in the clinic.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

Authors' Contributions

Conception and design: K. Palanichamy, S. Kanji, A. Chakravarti

Development of methodology: K. Palanichamy, S. Kanji, A. Chakravarti

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Palanichamy, N. Gordon, K. Thirumoorthy, J.R. Jacob, K.T. Litzenberg, D. Patel

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Palanichamy, S. Kanji, K. Thirumoorthy, J.R. Jacob, A. Chakravarti

Writing, review, and/or revision of the manuscript: K. Palanichamy, S. Kanji, J.R. Jacob, K.T. Litzenberg, D. Patel, A. Chakravarti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Palanichamy, A. Chakravarti
Study supervision: K. Palanichamy, A. Chakravarti

Acknowledgments

We thank all the patients enrolled in our IRB for making this possible and OSU-CCC core facility services.

Grant Support

This work was supported by the NIH/NCI RC2CA148190 (AC, KP), RO1CA108633 (AC), RO1CA188228 (AC), U10CA180850 (AC), The Ohio State University Comprehensive Cancer Center (AC, KP) and RTOG (AC, KP).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 23, 2016; revised September 19, 2016; accepted October 12, 2016; published OnlineFirst November 3, 2016.

References

1. Wellen KE, Thompson CB. A two-way street: reciprocal regulation of metabolism and signalling. Nat Rev Mol Cell Biol 2012;13:270–6.
2. Lu SC.S-Adenosylmethionine. Int J Biochem Cell Biol 2000;32:391–5.
3. Ulrey CL, Liu L, Andrews LG, Tollefsbol TO. The impact of metabolism on DNA methylation. Hum Mol Genet 2005;14 Spec No1:R139–47.
4. Luo M.Current chemical biology approaches to interrogate protein methyltransferases. ACS Chem Biol 2012;7:443–63.
5. Aksoy S, Szumlanski CL, Weinshilboum RM. Human liver nicotinamide N-methyltransferase. cDNA cloning, expression, and biochemical characterization. J Biol Chem 1994;269:14835–40.
6. Riederer M, Erwa W, Zimmermann R, Frank S, Zechner R. Adipose tissue as a source of nicotinamide N-methyltransferase and homocysteine. Atherosclerosis 2009;204:412–7.
7. Houtkooper RH, Canto C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. Endocr Rev 2010;31:194–223.
8. Xu J, Moatamed F, Caldwell JS, Walker JR, Kraiem Z, Taki K, et al. Enhanced expression of nicotinamide N-methyltransferase in human papillary thyroid carcinoma cells. J Clin Endocrinol Metab 2003;88:4990–6.
9. Sartini D, Muzzonigro G, Milanese G, Pierella F, Rossi V, Emanuelli M. Identification of nicotinamide N-methyltransferase as a novel tumor marker for renal clear cell carcinoma. J Urol 2006;176:2248–54.
10. Roessler M, Rollinger W, Palme S, Hagmann ML, Berndt P, Engel AM, et al. Identification of nicotinamide N-methyltransferase as a novel serum tumor marker for colorectal cancer. Clin Cancer Res 2005;11:6550–7.
11. Tomida M, Mikami I, Takeuchi S, Nishimura H, Akiyama H. Serum levels of nicotinamide N-methyltransferase in patients with lung cancer. J Cancer Res Clin Oncol 2009;135:1223–9.
12. Emanuelli M, Santarelli A, Sartini D, Ciavarella D, Rossi V, Pozzi V, et al. Nicotinamide N-Methyltransferase upregulation correlates with tumour differentiation in oral squamous cell carcinoma. Histol Histopathol 2010; 25:15–20.
13. Lim BH, Cho BI, Kim YN, Kim JW, Park ST, Lee CW. Overexpression of nicotinamide N-methyltransferase in gastric cancer tissues and its potential post-translational modification. Exp Mol Med 2006;38:455–65.
14. Kim J, Hong SJ, Lim EK, Yu YS, Kim SW, Roh JH, et al. Expression of nicotinamide N-methyltransferase in hepatocellular carcinoma is associated with poor prognosis. J Exp Clin Cancer Res 2009;28:20.
15. Zhou W, Gui M, Zhu M, Long Z, Huang L, Zhou J, et al. Nicotinamide -methyltransferase is overexpressed in prostate cancer and correlates with prolonged progression-free and overall survival times. Oncol Lett 2014;8: 1175–80.
16. Sartini D, Muzzonigro G, Milanese G, Pozzi V, Vici A, Morganti S, et al. Upregulation of tissue and urinary nicotinamide N-methyltransferase in bladder cancer: potential for the development of a urine-based diagnostic test. Cell Biochem Biophys 2013;65:473–83.
17. Kassem H, Sangar V, Cowan R, Clarke N, Margison GP. A potential role of heat shock proteins and nicotinamide N-methyl transferase in predicting response to radiation in bladder cancer. Int J Cancer 2002;101:454–60.
18. Wu Y, Siadaty MS, Berens ME, Hampton GM, Theodorescu D. Overlapping gene expression profiles of cell migration and tumor invasion in human bladder cancer identify metallothionein 1E and nicotinamide N-methyltransferase as novel regulators of cell migration. Oncogene 2008;27: 6679–89.
19. Tang SW, Yang TC, Lin WC, Chang WH, Wang CC, Lai MK, et al. Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. Carcinogenesis 2011;32:138–45.
20. Ulanovskaya OA, Zuhl AM, Cravatt BF. NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. Nat Chem Biol 2013;9:300–6.
21. Palanichamy K, Thirumoorthy K, Kanji S, Gordon N, Singh R, Jacob JR, et al. Methionine and Kynurenine Activate Oncogenic Kinases in Glioblastoma, and Methionine Deprivation Compromises Proliferation. Clin Cancer Res 2016;22:3513–23.
22. Palanichamy K, Acus K, Jacob JR, Chakravarti A. Clinically relevant brain tumor model and device development for experimental therapeutics. J Analytical Oncol 2015;4:5–12.
23. Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. J Clin Oncol 2008;26:3015–24.
24. Sablina AA, Hector M, Colpaert N, Hahn WC. Identification of PP2A complexes and pathways involved in cell transformation. Cancer Res 2010;70:10474–84.
25. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, et al. MAP kinases. Chem Rev 2001;101:2449–76.
26. Ugi S, Imamura T, Ricketts W, Olefsky JM. Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling. Mol Cell Biol 2002;22:2375–87.
27. Shi Y.Serine/threonine phosphatases: mechanism through structure. Cell 2009;139:468–84.
28. Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. Mol Cell 2009;33:537–45.
29. Bryant JC, Westphal RS, Wadzinski BE. Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. Biochem J 1999;339 (Pt 2):241–6.
30. Ogris E, Gibson DM, Pallas DC. Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen. Oncogene 1997; 15:911–7.
31. Xie H, Clarke S. Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain. J Biol Chem 1994;269:1981–4.
32. Lee J, Chen Y, Tolstykh T, Stock J. A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain. Proc Natl Acad Sci U S A 1996;93:6043–7.
33. Cantoni GL.The role of S-adenosylhomocysteine in the biological utilization of S-adenosylmethionine. Prog Clin Biol Res 1985;198:47–65.
34. D'Andrea FP, Safwat A, Kassem M, Gautier L, Overgaard J, Horsman MR. Cancer stem cell overexpression of nicotinamide N-methyltransferase enhances cellular radiation resistance. Radiother Oncol 2011; 99:373–8.
35. Pozzi V, Sartini D, Morganti S, Giuliante R, Di Ruscio G, Santarelli A, et al. RNA-mediated gene silencing of nicotinamide N-methyltransferase is associated with decreased tumorigenicity in human oral carcinoma cells. PLoS One 2013;8:e71272.
36. Sartini D, Seta R, Pozzi V, Morganti S, Rubini C, Zizzi A, et al. Role of nicotinamide N-methyltransferase in non-small cell lung cancer: in vitro effect of shRNA-mediated gene silencing on tumourigenicity. Biol Chem 2015;396:225–34.

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

37. Horsman MR, Siemann DW, Chaplin DJ, Overgaard J. Nicotinamide as a radiosensitizer in tumours and normal tissues: the importance of drug dose and timing. Radiother Oncol 1997;45:167–74.
38. Dominguez-Gomez G, Diaz-Chavez J, Chavez-Blanco A, Gonzalez-Fierro A, Jimenez-Salazar JE, Damian-Matsumura P, et al. Nicotinamide sensitizes human breast cancer cells to the cytotoxic effects of radiation and cisplatin. Oncol Rep 2015;33:721–8.
39. Hoskin PJ, Rojas AM, Saunders MI, Bentzen SM, Motohashi KJ, investigators B. Carbogen and nicotinamide in locally advanced bladder cancer: early results of a phase-III randomized trial. Radiother Oncol 2009;91: 120–5.
40. Chen AC, Martin AJ, Choy B, Fernandez-Penas P, Dalziell RA, McKenzie CA, et al. A Phase 3 Randomized Trial of Nicotinamide for Skin-Cancer Chemoprevention. N Engl J Med 2015;373:1618–26.
41. Sauve AA.NAD+ and vitamin B3: from metabolism to therapies. J Pharmacol Exp Ther 2008;324:883–93.
42. Sperber H, Mathieu J, Wang Y, Ferreccio A, Hesson J, Xu Z, et al. The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol 2015; 17:1523–35.
43. De Baere I, Derua R, Janssens V, Van Hoof C, Waelkens E, Merlevede W, et al. Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. Biochemistry 1999;38:16539–47.
44. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med 2008;14:152–60.
45. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell 2007;130: 21–4.
46. Chen W, Arroyo JD, Timmons JC, Possemato R, Hahn WC. Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. Cancer Res 2005;65:8183–92.
47. Junttila MR, Puustinen P, Niemela M, Ahola R, Arnold H, Bottzauw T, et al. CIP2A inhibits PP2A in human malignancies. Cell 2007;130:51–62.
48. Sablina AA, Chen W, Arroyo JD, Corral L, Hector M, Bulmer SE, et al. The tumor suppressor PP2A Abeta regulates the RalA GTPase. Cell 2007; 129:969–82.
49. Sontag JM, Nunbhakdi-Craig V, Mitterhuber M, Ogris E, Sontag E. Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells. J Neurochem 2010;115:1455–65.
50. Puustinen P, Junttila MR, Vanhatupa S, Sablina AA, Hector ME, Teittinen K, et al. PME-1 protects extracellular signal-regulated kinase pathway activity from protein phosphatase 2A-mediated inactivation in human malignant glioma. Cancer Res 2009;69:2870–7.

Published OnlineFirst November 3, 2016; DOI: 10.1158/1078-0432.CCR-16-1323

Clinical Cancer Research

NNMT Silencing Activates Tumor Suppressor PP2A, Inactivates Oncogenic STKs, and Inhibits Tumor Forming Ability

Kamalakannan Palanichamy, Suman Kanji, Nicolaus Gordon, et al.

Clin Cancer Res 2017;23:2325-2334. Published OnlineFirst November 3, 2016.