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Metabolism

Molecular Cancer Research

NAD Synthesis Pathway Interference Is a Viable Therapeutic Strategy for Chondrosarcoma

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Abstract

Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT) are rate-limiting enzymes in the NAD^+ synthesis pathway. Chondrosarcoma is a malignant cartilage forming bone tumor, in which mutations altering isocitrate dehydrogenase-1 and -2 (IDH1 and IDH2) activity have been identified as potential driver mutations. Vulnerability for NAD^+ depletion has been reported for *IDH1/2*-mutant cells. Here, the potency of NAMPT inhibitors as a treatment of chondrosarcoma was explored. Eleven chondrosarcoma cell lines were treated with NAMPT inhibitors, in which the effect on cell viability, colony formation, and 3D collagen invasion was assessed. The expression level of NAMPT and NAPRT transcripts in chondrosarcoma cells was determined by qRT-PCR. Methylation of the NAPRT promoter was evaluated using a previously published dataset of genome-wide methylation. In addition, a methylation dataset was used to determine methylation of the NAPRT promoter in 20 *IDH1/2*-mutated cartilage tumors. Chondrosarcoma cells showed a dose-dependent decrease in cell viability, 3D collagen invasion, and colony formation upon treatment with NAMPT inhibitors, in which nearly half of the cell lines demonstrated absolute IC_{50}s in the low nanomolar range. Increasing IC_{50}s correlated to increasing NAPRT expression levels and decreasing NAPRT promoter methylation. No correlation between *IDH1/2* mutation status and sensitivity for NAMPT inhibitors was observed. Strikingly, higher methylation of the NAPRT promoter was observed in high-grade versus low-grade chondrosarcomas. In conclusion, this study identified NAMPT as a potential target for treatment of chondrosarcoma.

Implications: Chondrosarcoma patients, especially those of high histologic grade with lower expression and hypermethylation of NAPRT, may benefit from inhibition of the NAD synthesis pathway. *Mol Cancer Res; 15(12); 1714–21. ©2017 AACR.*

Introduction

Chondrosarcoma represents a heterogeneous group of cartilage-forming tumors. It is the second most common primary bone malignancy in humans (1), with different outcomes depending on subtype and histologic grade. The far most prevalent subtype (72% of the cases) is conventional central chondrosarcoma, in which the tumor arises centrally in the medulla of the bone. This subtype can be histologically subdivided into atypical cartilaginous tumor (ACT), grade II and grade III chondrosarcomas. ACT (previously known as grade I) accounts for 61% of the cases. The first-line treatment is curettage with local adjuvant treatment, resulting in a 5-year survival rate of 83%. Grade II (36%) and grade III (3%) have a worse 5-year survival (combined 53%) due to the higher occurrence of metastases (1–3). These tumors are treated by en bloc resection. Two other subtypes with a worse prognosis are dedifferentiated chondrosarcoma, a highly malignant variant (4), and mesenchymal chondrosarcoma, a rare aggressive subtype in which distant metastasis can be identified even after 20 years (5–7). Chondrosarcoma patients with unresectable disease, due to tumor location, tumor size, or extensive metastatic disease, have a 5-year survival of only 2% as the overall efficacy of chemotherapy is limited (8, 9).

Gain-of-function mutations in the isocitrate dehydrogenase 1 and -2 (*IDH1* and -2) genes have been identified as potential driver mutations of chondrosarcoma because of their high prevalence (38%–70% depending on the subtype; refs. 10, 11). IDH1 and IDH2 are key enzymes in cell metabolism, as they convert isocitrate to α-ketoglutarate (α-KG) in respectively the cytoplasm and the mitochondria. The mutant enzyme acquires the activity to convert α-KG to D-2-hydroxyglutarate (D-2-HG). This leads to increased levels of this oncometabolite in cartilage tumors harboring an *IDH1/2* mutation (12), which competitively inhibits the α-KG–dependent enzymes by the high structural similarities (13). Although AGI-5198, a specific small-molecule inhibitor of the activity of mutant IDH1, was able to decrease D-2-HG levels in *IDH1*-mutant chondrosarcoma cell lines, this did not influence the tumorigenic properties of these cells (14, 15), which is in line with findings in *IDH2*-mutant leukemia (16, 17) and *IDH1*-mutant glioma models (15, 18). This suggests that although the *IDH1* or -2 mutations are an early event in tumorigenesis, at later stages,

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

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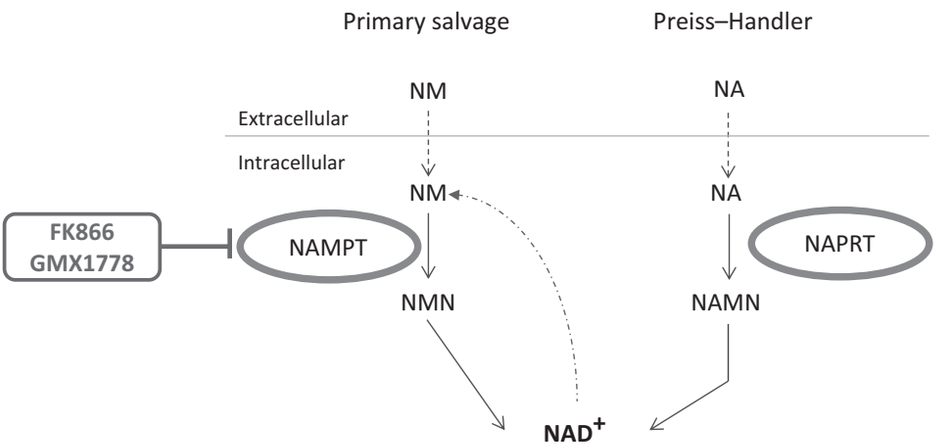

Figure 1.
Schematic representation of NAD^+ biosynthesis. NAMPT and NAPRT are the rate-limiting enzymes of the primary salvage pathway and the Preiss–Handler pathway, respectively. NAD^+ is recycled in the primary salvage pathway. NM, nicotinamide; NMN, nicotinamide mononucleotide; NA, nicotinic acid (vitamin B3); NAMN, nicotinic acid mononucleotide.

other processes involved in chondrosarcoma progression render these cells independent of the mutant IDH1/2 enzymes, which is in line with findings in other *IDH1/2*-mutated cancers (15, 16). To identify metabolic targets for *IDH1/2*-mutated glioma, Tateishi and colleagues performed a systematic metabolic profiling on glioma cells after short- and long-term mutant IDH1 inhibition (15). This study revealed a vulnerability to NAD^+ depletion in *IDH1/2*-mutant cells, demonstrated by an increased sensitivity for nicotinamide phosphoribosyltransferase (NAMPT) inhibitors. This increased sensitivity could be explained by decreased expression levels of nicotinic acid phosphoribosyltransferase (NAPRT) in *IDH1/2*-mutated tumors, potentially caused by an increased methylation of the NAPRT promoter (15). NAMPT and NAPRT are rate-limiting enzymes of respectively the primary salvage pathway and the Preiss–Handler pathway, which are involved in NAD^+ synthesis (Fig. 1). Tumor cells depend on these pathways for their rapid NAD turnover, as they lack expression of key enzymes in the *de novo* synthesis of NAD from tryptophan (19–21). Therefore, interfering with NAD^+ biosynthesis holds great promise as a therapeutic strategy for cancer, which is why we further explored this pathway in chondrosarcoma. We used our large chondrosarcoma cell line panel ($n = 11$) to determine sensitivity to NAMPT inhibitors. In contrary to glioma cell lines, chondrosarcoma cell lines harboring an endogenous *IDH1* or *IDH2* mutation can grow as a monolayer culture. We determined expression levels of NAMPT and NAPRT, and methylation of the NAPRT promoter in cell lines and in primary tumors. Our results indicate that NAMPT is a promising therapeutic target in chondrosarcoma.

Materials and Methods

Compounds

NAD^+ and the NAMPT inhibitors FK866 (also known as APO866) and GMX1778 (also known as CHS-828) were purchased from Sigma-Aldrich. FK866 and GMX1778 both likely function as substrate mimetics (19). FK866 and GMX1778 were dissolved in DMSO in a concentration of 20 and 36.16 mmol/L, respectively, and stored at −20°C. NAD^+ was dissolved in culture medium at a concentration of 10 mmol/L, stored at −20°C, and used in a concentration of 10, 50, and 100 nmol/L. AGI-5198 (Cayman Chemical) was dissolved in DMSO in a concentration of 10 mmol/L, stored at −20°C, and used in a concentration of 1 and 10 μmol/L.

Cell culture

The conventional chondrosarcoma cell lines JJ012 (22), SW1353 (ATCC), CH2879 (23), OUMS27 (24), L835 (25), and CH3573 (26), the dedifferentiated chondrosarcoma cell lines L3252B (25), NDCS-1 (27), and L2975 (25), as well as the chondrocyte cell line LBPVA (28) and the cell line HT1080 (ATCC) were cultured in RPMI1640 (Gibco, Invitrogen) supplemented with 10% (JJ012, SW1353, CH2879, NDCS-1, L2975, HT1080) or 20% (L835, L3252B, OUMS27, CH3573, LBPVA) heat-inactivated FBS (F7524, Sigma-Aldrich). HT1080 was originally reported to be derived from a fibrosarcoma of bone. As this is a diagnosis of exclusion, and this cell line is now known to harbor an *IDH1* mutation, this tumor cell line is most likely derived from a dedifferentiated chondrosarcoma (14). MCS170 (mesenchymal chondrosarcoma; ref. 29) was cultured in IMDM (Gibco, Invitrogen) with 15% FBS. The lowest passage number possible was thawed (passage number between 11 and 42), the authenticity confirmed by STR profiling with the GenePrint10 (Promega Benelux BV) and tested for mycoplasma using MycoAlert (Lonza) before the start of the experiments. The cells were grown at 37°C with 5% CO_2 in a humidified incubator. Cell lines were never cultured for more than 3 months and were tested for mycoplasma every 4 weeks (using RT-PCR).

qRT-PCR

RNA isolation and cDNA synthesis was performed as described previously (30). A standard qRT-PCR was performed (31) to determine the expression levels of NAMPT and NAPRT. Primers (Supplementary Table S1) were designed using primer3 software (http://bioinfo.ut.ee/primer3/). To correct for the amount of cDNA input, gene expression levels were normalized using the expression levels of CYPa, CPSF6, and GPR108 (32, 33). Data were normalized using the $\Delta\Delta C_q$ method using Bio-Rad CFX Manager (Bio-Rad).

Proliferation assay

The cell lines were plated in triplicate at a density of 3,000 to 20,000 cells per well depending on the growth rate. After the cells adhered overnight, the compounds were added in their corresponding concentrations. To determine the effect of AGI-5198 on NAMPT inhibitor sensitivity, cells were pretreated for 72 hours

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with 1 or 10 μmol/L AGI-5198, after which the cells were counted and the same number of cells were plated for the different pretreatment conditions. Treatment with AGI-5198 was continued when the NAMPT inhibitors were added. To validate that NAD^+ could reverse the effect of the NAMPT inhibitors, NAD^+ was added in a concentration of 10, 50, or 100 nmol/L at the same time as the NAMPT inhibitors. After 72 hours of incubation, cell viability was measured using the PrestoBlue Cell Viability Reagent (Promega Benelux BV) according to the manufacturer's instructions. Colorimetric values in the plates were subsequently measured using a Wallac 1420 VICTOR2 (PerkinElmer). Data were analyzed in GraphPad Prism 5.0 (www.graphpad.com). The results shown are the results of three independent experiments.

Absolute IC_{50}s for FK866 and GMX1778 were compared with NAPRT expression levels using Pearson correlation (IBM SPSS Statistics 20).

Cell counting

To confirm that a decrease in cell viability corresponds to an absolute decrease in cell number, the cell lines JJ012, SW1353, and CH2879 were plated in black 96-well μ-Clear Plates (Greiner) in a fully independent experiment. After the cells adhered overnight, the corresponding concentrations GMX1778 were added to the wells in duplicates. After 72 hours of incubation, the PrestoBlue Cell Viability Reagent was added as described above. After measuring the Colorimetric values, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 minutes, and stained with Hoechst 33342 (Thermo Fisher Scientific). The cells were counted using Cellomics (Thermo Fisher Scientific) according to the manufacturer's instructions. Results were normalized to mock-treated wells.

3D outgrowth/invasion assay

The invasion assay, based on invasion of cells in a 3D extracellular matrix scaffold, was performed as described previously (34). In short, trypsinized monolayer, cultured cells were suspended in PBS containing 2% polyvinylpyrrolidone (PVP; Sigma–Aldrich), after which they were printed into 70 μL solidified collagen gels in glass-bottom 96-well plates (Greiner) using injection robotics. Three droplets were injected per well, forming three collagen-embedded tumor spheroids per well. Subsequently, 130 μL medium containing compounds at indicated concentrations was added to each well. To assess outgrowth and invasion of the spheroids, images were taken 1 hour postinjection and 3 days postinjection using the Motic Motical 3 CMOS 3.0MP Color Digital Camera and the corresponding Motic Images Plus 2.0 ML Software.

Colony formation assay

The colony formation assay was performed according to the "plating before treatment" method as described by Franken and colleagues (35). After counting, 1,000 NDCS-1 cells and 1,500 JJ012 cells were seeded per well. The next day, FK866 and GMX1778 were added in the indicated concentrations. The 6-well plates were analyzed at day 10 after staining with 0.05% crystal violet, 6% glyceraldehyde. Colonies were quantified by manual counting, followed by normalization to the negative control. Quantification is done for at least three separate experiments.

Methylation analysis

Methylation of the NAPRT promoter in chondrosarcoma cell lines was determined using a previously described genome-wide methylation dataset (14). In addition, we used a previously conducted but unpublished methylation array of *IDH1/2* mt cartilage tumors, for which genomic DNA of four enchondromas and five ACTs, nine grade II chondrosarcomas, and four grade III chondrosarcomas was bisulfite-converted using the EZ DNA Methylation Gold Kit (Zymo Research) and used for microarray-based DNA methylation analysis, performed at ServiceXS using the HumanMethylation450 BeadChip array (Illumina). The bisulfite-converted DNA was processed and hybridized to the arrays according to the manufacturer's instructions. We performed data analysis in R version 3.2.3. "methylumi" (36) was used to load data from the raw data files and perform data quality checks. One grade II sample was excluded from the analysis based on an inflated average detection *P* value. Samples were normalized using the BMIQ procedure from the "wateRmelon" package (37). We selected probes around the NAPRT gene from base pair position 144658390 until 144668845 on chromosome 8. A heatmap from this region including all samples was generated from the β-values using Gene-E (Broad Institute). We compared the level of methylation of the promoter region of NAPRT (from 144659831 to 144660631; ref. 15) between low-grade (enchondroma and ACT) and high-grade (grade II and grade III) tumors. A *t* test was performed to compare the groups. In addition, we compared methylation of the CpG island of the NAPRT promoter region with NAPRT mRNA expression in chondrosarcoma cell lines and in a subset ($n = 13$) of these cartilage tumors using the Spearman correlation (IBM SPSS Statistics 20).

Results

NAD^+ depletion inhibits chondrosarcoma cell viability, invasion, and colony formation

To explore whether NAMPT could be a therapeutic target for chondrosarcoma, we treated 11 chondrosarcoma cell lines with FK866 and GMX1778. In contrast to the chondrocyte cell line LBPVA, all chondrosarcoma cell lines showed dose-dependent decreases in cell viability and 8 of 11 chondrosarcoma cell lines showed more than 75% reduction in cell viability upon treatment with 1 μmol/L NAMPT inhibitor (Fig. 2A). Sensitivities for FK866 and GMX1778 were highly comparable within cell lines (Fig. 2A). Comparing cell counts with cell viabilities confirmed that a decrease in cell viability was caused by an absolute decrease in cell number (Fig. 2B). Cell viability could be rescued by coincubation with NAD^+ in two cell lines tested, demonstrating that the treatment with FK866 and GMX1778 indeed caused on-target inhibition (Fig. 2C). Interestingly, high concentrations of NAD^+ also reduced cell viability in NDCS-1, suggesting that NAD^+ levels are tightly regulated in these cells. Absolute IC_{50} values varied between the different chondrosarcoma cell lines: six cell lines had absolute IC_{50} values below 10 nmol/L and three cell lines had absolute IC_{50} values above 100 nmol/L (Table 1). In addition to determining the effect of NAMPT inhibition on cell viability, we studied its effect on JJ012 spheroid outgrowth and invasion using a 3D collagen scaffold model (Fig. 2D). Upon treatment with FK866 or GMX1778, a clear decrease in invasive outgrowth was observed. In addition to cell viability and spheroid outgrowth and invasion, these compounds inhibited colony formation of JJ012 and NDCS-1 cells already at a concentration of 0.5 nmol/L (Fig. 2E and F). This demonstrates that chondrosarcoma cell lines depend on NAMPT for their NAD^+ generation and that NAD^+ is essential for their tumorigenic properties.

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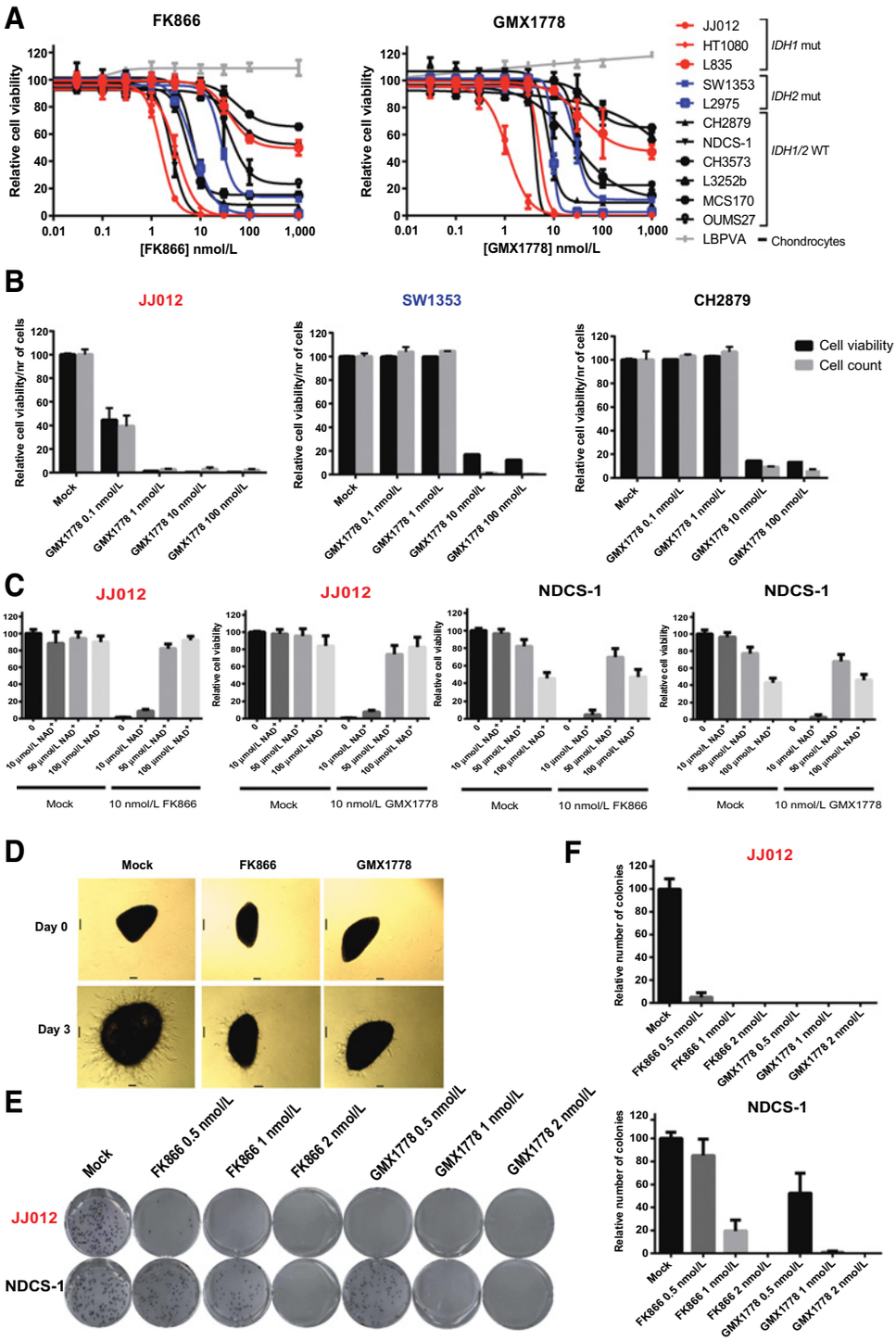

Figure 2.

Chondrosarcoma cell lines are sensitive for NAD^+ depletion. **A,** Eleven chondrosarcoma cell lines and a chondrocyte cell line were treated for 72 hours with two NAMPT inhibitors, FK866 and GMX1778, after which cell viability was assessed by a PrestoBlue assay. All cell lines showed a dose-dependent decrease in cell viability except the LBPVA control, and in 8 of 11 cell lines, this reduction was more than 75%. **B,** Cell count results (using Cellomics) were very similar to cell viability results (using PrestoBlue). **C,** JJ012 (*IDH1* mt) and NDCS-1 (*IDH* WT) were cotreated with NAMPT inhibitors and 10, 50, or 100 μmol/L NAD^+. NAD^+ abolished the effect of the NAMPT inhibitors, demonstrating that the effect of NAMPT inhibitors is caused by an NAD^+ depletion. **D,** 3D outgrowth and invasion of JJ012 cells in a collagen scaffold-embedded spheroid model. JJ012 spheroid outgrowth and invasion was inhibited by treating the cells with NAMPT inhibitors. A total of 130 μL medium containing 10 nmol/L compounds was added on top of the 70 μL collagen gel. **E,** Representative wells from JJ012 and NDCS-1 colony formation during treatment with FK866 and GMX1778. **F,** Quantification of at least three different colony formation assays.

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Table 1. Absolute IC_{50} values for the NAMPT inhibitors FK866 and GMX1778 of the chondrosarcoma cell lines

Cell line	IC_{50} FK866 (nmol/L)	IC_{50} GMX1778 (nmol/L)
JJ012	1.54	1.09
HT1080	3.04	5.02
L835	497.15	358.18
SW1353	28.08	29.11
L2975	7.17	9.44
CH2879	7.51	8.39
NDCS-1	2.49	4.09
CH3573	5.67	30.68
L3252b	>1,000	>1,000
MCS170	>1,000	>1,000
OUMS27	50.70	32.94

NAPRT methylation correlates with sensitivity for NAMPT inhibitors

We hypothesized that the variation in sensitivity to NAMPT inhibitors between cell lines could be attributed to expression of NAMPT or NAPRT, the rate limiting enzymes of pathways involved in NAD^+ synthesis. We performed qRT-PCR analyses in chondrosarcoma cell lines to determine NAMPT and NAPRT expression levels. Expression levels of NAMPT and NAPRT are highly variable between chondrosarcoma cell lines (Fig. 3A). NAPRT has the highest expression in CH2879, L3252b, L835, and OUMS27, of which three of four have IC_{50} values above 30 nmol/L for the NAMPT inhibitors. Plotting IC_{50s} for FK866 and GMX1778 versus NAPRT expression of 10 chondrosarcoma cell lines revealed a correlation between low IC_{50} values and low NAPRT expression levels ($P = 0.034$ and $P = 0.043$, respectively; Fig. 3B). As it has been suggested that low expression of NAPRT is mediated by methylation of the NAPRT promoter (15, 38), we assessed the methylation of the NAPRT promoter in 10 chondrosarcoma cell lines (Fig. 3C) using a previously published genome wide methylation array dataset (14). The analysis showed that high CpG island methylation was associated with low NAPRT expression levels (Fig. 3D; $P = 0.029$). JJ012, HT1080, SW1353, and NDCS-1 have high β-values, demonstrating high methylation of the NAPRT promoter. L835 and L2975 have medium β-values, whereas the *IDH* WT cell lines CH2879, CH3573, L3252b, and OUMS27 have low β-values. This suggested a potential correlation between *IDH1/2* mutation status and sensitivity for NAMPT inhibitors in chondrosarcoma cell lines. However, (pre)treating JJ012 with AGI-5198, a specific IDH1-mutant inhibitor, did not affect the sensitivity of JJ012 to NAMPT inhibitors (Fig. 2E). Furthermore, using previously published datasets and mRNAs of four chondrosarcoma cell lines treated for 10 and 20 passages with AGI-5198 and one *IDH* WT cell line for 10 passages with D-2-HG (14), we demonstrated that the mutant IDH1 enzyme did not influence the methylation of the NAPRT promoter nor NAMPT and NAPRT expression levels (Supplementary Fig. S1A and S1B).

The NAPRT promoter is hypermethylated in high-grade chondrosarcomas

To extend our study to primary tumors, we determined methylation of the NAPRT promoter in an available dataset of 20 *IDH1/2*-mutated cartilage tumors. Interestingly, we found significantly higher methylation of the CpG island of the NAPRT promoter in the high-grade (grade II and III) chondrosarcomas compared with the low-grade (ACT and enchondroma) cartilage tumors ($P = 0.002213$; Fig. 4A). To compare methylation with expression in this set of tumors, mRNA of 13 of these 20 tumors was collected, in which we could not identify a significant correlation between NAPRT CpG island promoter methylation and decreased NAPRT expression levels ($P = 0.271$; Fig. 4B). In addition, we determined NAMPT and NAPRT expression levels in an independent cohort of 32 cartilage tumors, in which 9 *IDH* WT tumors were also included (Fig. 4C and D). Similar to the cell lines, expression of NAMPT and NAPRT was variable in the primary tumors. Expression levels seemed slightly lower in the high-grade tumors as compared with low grade, although the difference was not statistically significant ($P = 0.255$, t test).

Discussion

The results of this study demonstrate that chondrosarcoma cell lines are vulnerable to NAD^+ depletion. Five of 11 chondrosarcoma cell lines have IC_{50} values below 10 nmol/L for the two tested NAMPT inhibitors, suggesting that these cell lines depend on the primary salvage pathway for NAD^+ synthesis. Strikingly, the chondrocyte cell line LBPVA was unaffected by NAMPT inhibition, further demonstrating the therapeutic potential of NAMPT inhibitors for chondrosarcoma patients.

To identify a possible biomarker for distinguishing sensitivity to NAMPT inhibition, we assessed methylation and expression levels of NAPRT, the rate-limiting enzyme of the Preiss–Handler pathway for NAD^+ synthesis. Comparing IC_{50} values with NAPRT expression levels demonstrated that low NAPRT expression is significantly correlated to increased sensitivity for NAMPT inhibitors. Therefore, NAPRT expression partly explains the difference in NAMPT inhibitor sensitivity between the different cell lines. Strikingly, we observed higher methylation of the CpG island of the NAPRT promoter in high-grade chondrosarcomas versus low-grade cartilage tumors, suggesting that NAMPT can be a promising target especially in these clinically challenging patients.

We did not observe a correlation between the presence of a mutant IDH1/2 enzyme and sensitivity for NAMPT inhibitors in chondrosarcoma. Furthermore, inhibition of the IDH1-mutant enzyme by AGI-5198 did not influence sensitivity for NAMPT inhibitors nor methylation of the NAPRT promoter. This is in line with previous studies from our group, where we demonstrated that *IDH1/2* mutations do not affect IHC levels of 5-hmC, 5mC, and trimethylation of H3K4, -9, and -27 (39), and prolonged inhibition of the IDH1-mutant enzyme does not affect global gene expression, CpG island methylation nor histone H3K4, -9, and -27 trimethylation in chondrosarcoma cell lines (14). Our observations are in contrast to the conclusion from Tateishi and colleagues, who suggest that mutant IDH1 downregulates NAPRT expression, making *IDH1*-mutant cell lines more sensitive to NAMPT inhibitors (15). However, only two chondrosarcoma cell lines were included in that study, which both harbor an *IDH1* or *IDH2* mutation and are sensitive to NAMPT inhibition. No cotreatment with a mutant IDH1 inhibitor and a NAMPT inhibitor was performed, and NAPRT expression was not assessed in primary tumors; the link between mutant *IDH1* and NAPRT expression was demonstrated by the introduction of an *IDH1* mutation in *IDH* WT glioma cells. Therefore, experimental differences and a different tumor type may explain the discrepancy between experimental results.

Phase I clinical trials to test the safety of NAMPT inhibition have been performed with FK866, GMX1778, and its prodrug

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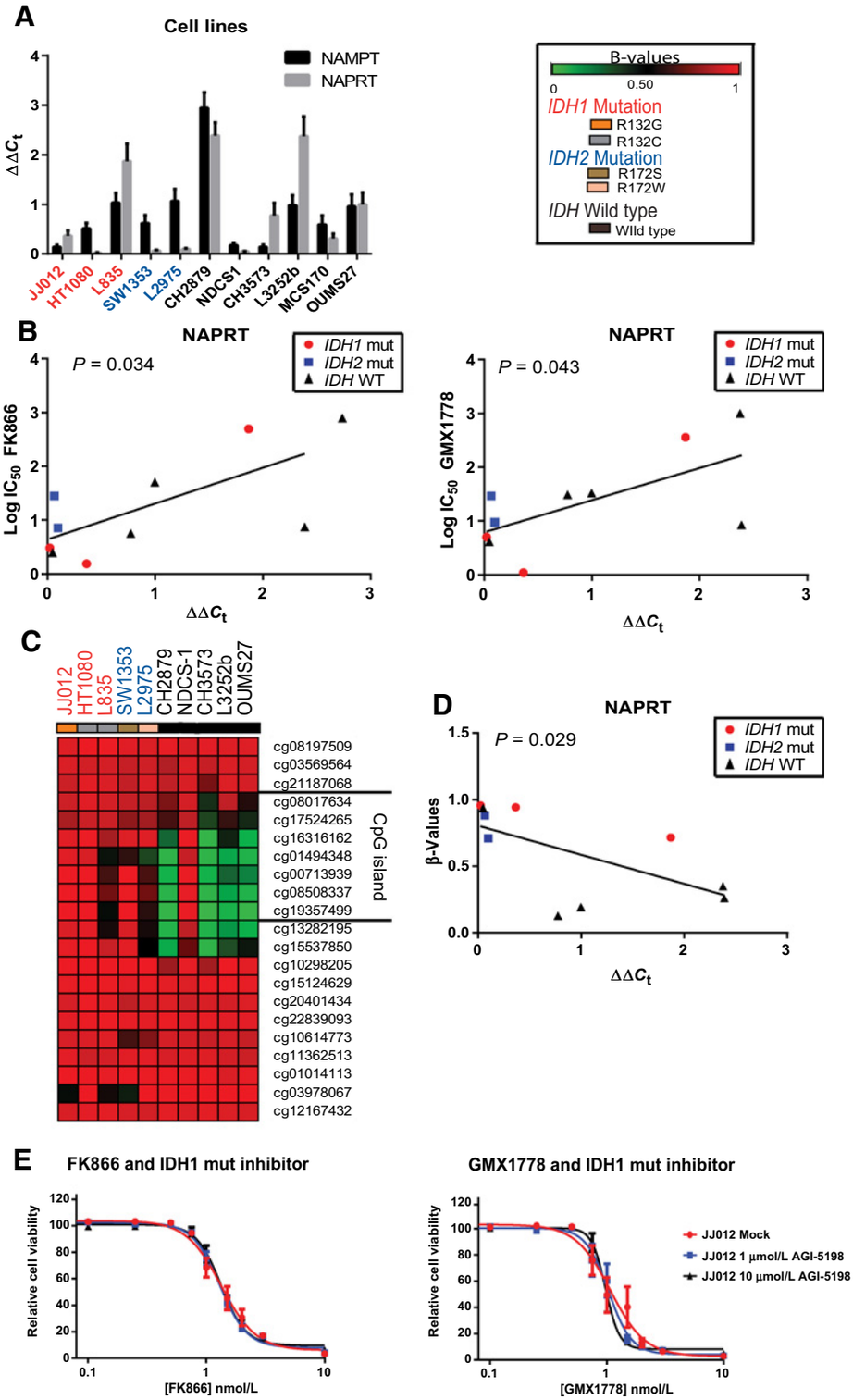

Figure 3.

NAPRT expression correlates to sensitivity for NAMPT inhibitors and to methylation of the CpG island of the NAPRT promoter. **A,** NAMPT and NAPRT are heterogeneously expressed in the different chondrosarcoma cell lines. Expression levels were determined using qRT-PCR analyses. **B,** Log IC_{50}s for the NAMPT inhibitors FK866 and GMX1778 significantly correlated to NAPRT expression levels. **C,** NAPRT methylation in a previously published methylation array of 10 chondrosarcoma cell lines. β-Values are visualized using Gene-E (Broad Institute), where red represents high methylation of the NAPRT promoter and green represents low methylation of the NAPRT promoter. **D,** NAPRT expression significantly correlates to methylation of the CpG island of the NAPRT promoter in chondrosarcoma cell lines. **E,** Pretreating JJ012 cells for 72 hours with AGI-5198, followed by a 72-hour combined treatment of AGI-5198 and the corresponding NAMPT inhibitor did not influence the dose-dependent decrease in cell viability. Cell viability was determined by a PrestoBlue assay.

GMX1777 (19). However, further evaluation was discontinued due to dose-limiting toxicities (19). To increase the therapeutic index of NAMPT inhibitors, coadministration with nicotinic acid (NA) has been proposed (19). NA can be used to synthesize NAD^+ in NAPRT-proficient cells, thereby decreasing the toxicity without interfering with its efficacy in the treatment of NAPRT-deficient tumors. Indeed, it was shown that the effect of NAMPT inhibitors on tumorigenic properties of HT1080 and SW1353

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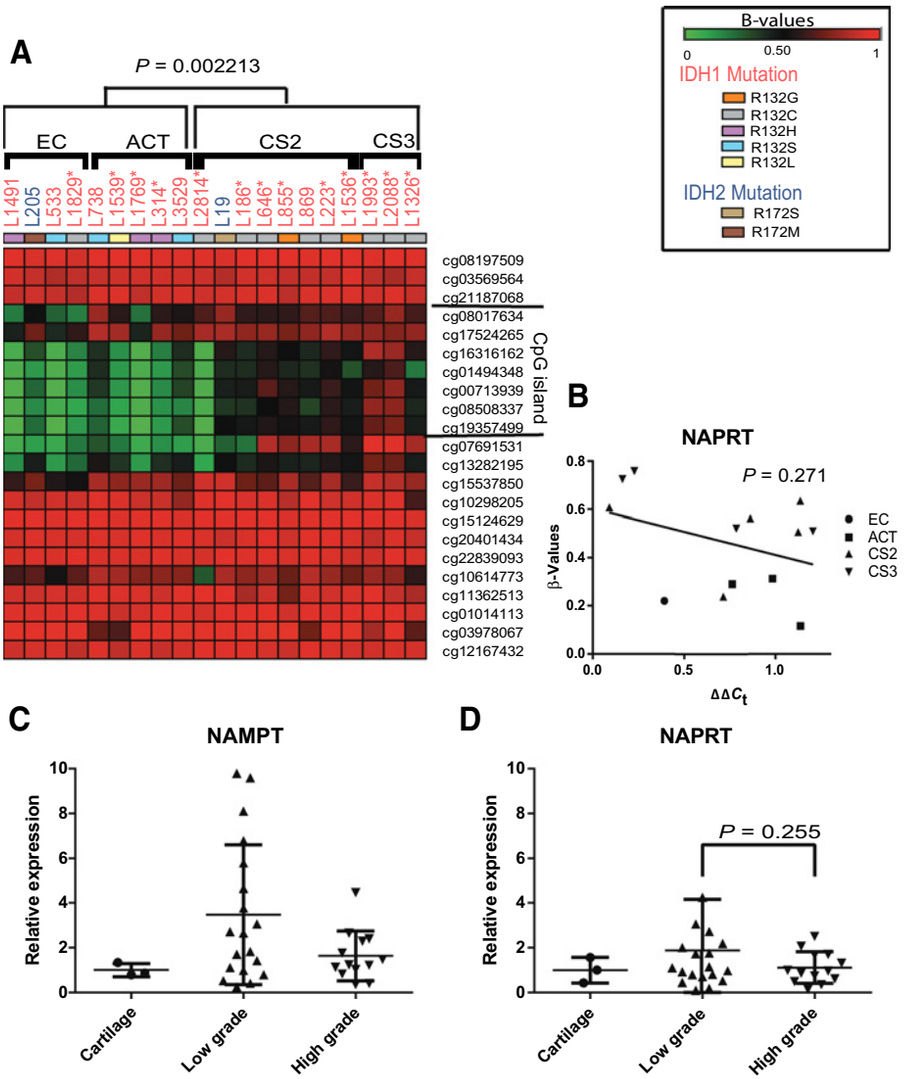

Figure 4.

A, The CpG island of the NAPRT promoter is hypermethylated in high-grade compared with low-grade *IDH1/2*-mutant cartilage tumors. β-Values are visualized using Gene-E (Broad Institute), where red represents high methylation of the NAPRT promoter and green represents low methylation of the NAPRT promoter. mRNA was available of cell lines marked with a *. **B,** Methylation of the CpG island of the NAPRT promoter weakly correlated to NAPRT expression in cartilage tumors *. **C,** qRT-PCR analyses of NAMPT expression in an independent cohort of 32 cartilage tumors. **D,** NAPRT expression in a cohort of 32 cartilage tumors shows a nonsignificant trend for higher expression in low-grade versus high-grade cartilage tumors. Expression was normalized to cartilage. EC, enchondroma; ACT, atypical cartilaginous tumor; CS2, chondrosarcoma grade II; CS3, chondrosarcoma grade III.

chondrosarcoma cell lines and HT1080 xenografts was not affected by coadministration of NA (40), suggesting that this could be a suitable approach to decrease dose-limiting toxicities in chondrosarcoma patients.

Collectively, this study demonstrates that NAMPT inhibitors hold potential therapeutic promise for chondrosarcoma patients, especially for those with high histologic grade as these, due to hypermethylation of the NAPRT promoter, are dependent on the primary salvage pathway for their NAD^+ synthesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E.F.P. Peterse, J.V.M.G. Bovée
Development of methodology: E.F.P. Peterse
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.F.P. Peterse, B.E.W.M. van den Akker, J. Suijker, Y. de Jong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.F.P. Peterse, B.E.W.M. van den Akker, B. Niessen, J. Oosting, J.V.M.G. Bovée
Writing, review, and/or revision of the manuscript: E.F.P. Peterse, B.E.W.M. van den Akker, J. Suijker, Y. de Jong, E.H.J. Danen, A.-M. Cleton-Jansen, J.V.M.G. Bovée
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.E.W.M. van den Akker, J. Suijker
Study supervision: A.-M. Cleton-Jansen, J.V.M.G. Bovée

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References

1. Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F. Conventional osteosarcoma. In: Rosenberg AE, Cleton-Jansen A-M, de Pinieux G, Deyrup AT, Hauben E, Squire J, editors. WHO classification of tumors of soft tissue and bone. Lyon, France: IARC; 2013. p 282–8.
2. Gelderblom H, Hogendoorn PC, Dijkstra SD, van Rijswijk CS, Krol AD, Taminiau AH, et al. The clinical approach towards chondrosarcoma. Oncologist 2008;13:320–9.
3. Bjornsson J, McLeod RA, Unni KK, Ilstrup DM, Pritchard DJ. Primary chondrosarcoma of long bones and limb girdles. Cancer 1998;83:2105–19.
4. Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F. Dedifferentiated chondrosarcoma. In: Inwards C, Hogendoorn PCW, editors. WHO classification of tumors of soft tissue and bone. Lyon, France: IARC; 2013. p 269–70.
5. Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F. Mesenchymal chondrosarcoma. In: Nakashima Y, de Pinieux G, Ladanyi M, editors. WHO classification of tumors of soft tissue and bone. Lyon, France: IARC; 2013. p 271–2.
6. Frezza AM, Cesari M, Baumhoer D, Biau D, Bielack S, Campanacci DA, et al. Mesenchymal chondrosarcoma: prognostic factors and outcome in 113 patients. A European Musculoskeletal Oncology Society study. Eur J Cancer 2015;51:374–81.
7. Xu J, Li D, Xie L, Tang S, Guo W. Mesenchymal chondrosarcoma of bone and soft tissue: a systematic review of 107 patients in the past 20 years. PLoS One 2015;10:e0122216.
8. van Maldegem AM, Gelderblom H, Palmerini E, Dijkstra SD, Gambarotti M, Ruggieri P, et al. Outcome of advanced, unresectable conventional central chondrosarcoma. Cancer 2014;120:3159–64.
9. Italiano A, Mir O, Cioffi A, Palmerini E, Piperno-Neumann S, Perrin C, et al. Advanced chondrosarcomas: role of chemotherapy and survival. Ann Oncol 2013;24:2916–22.
10. Amary MF, Bacsi K, Maggiani F, Damato S, Halai D, Berisha F, et al. IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumors. J Pathol 2011;224:334–43.
11. Meijer D, de Jong D, Pansuriya TC, van den Akker BE, Picci P, Szuhai K, et al. Genetic characterization of mesenchymal, clear cell, and dedifferentiated chondrosarcoma. Genes Chromosomes Cancer 2012;51:899–909.
12. Amary MF, Damato S, Halai D, Eskandarpour M, Berisha F, Bonar F, et al. Ollier disease and Maffucci syndrome are caused by somatic mosaic mutations of IDH1 and IDH2. Nat Genet 2011;43:1262–5.
13. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 2011;19:17–30.
14. Suijker J, Oosting J, Koornneef A, Struys EA, Salomons GS, Schaap FG, et al. Inhibition of mutant IDH1 decreases D-2-HG levels without affecting tumorigenic properties of chondrosarcoma cell lines. Oncotarget 2015;6:12505–19.
15. Tateishi K, Wakimoto H, Iafrate AJ, Tanaka S, Loebel F, Lelic N, et al. Extreme vulnerability of IDH1 mutant cancers to NAD+ depletion. Cancer Cell 2015;28:773–84.
16. Wang F, Travins J, DeLaBarre B, Penard-Lacronique V, Schalm S, Hansen E, et al. Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. Science 2013;340:622–6.
17. Chen C, Liu Y, Lu C, Cross JR, Morris JP, Shroff AS, et al. Cancer-associated IDH2 mutants drive an acute myeloid leukemia that is susceptible to Brd4 inhibition. Genes Dev 2013;27:1974–85.
18. Turcan S, Fabius AW, Borodovsky A, Pedraza A, Brennan C, Huse J, et al. Efficient induction of differentiation and growth inhibition in IDH1 mutant glioma cells by the DNMT Inhibitor Decitabine. Oncotarget 2013;4:1729–36.
19. Sampath D, Zabka TS, Misner DL, O'Brien T, Dragovich PS. Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic strategy in cancer. Pharmacol Ther 2015;151:16–31.
20. Xiao Y, Elkins K, Durieux JK, Lee L, Oeh J, Yang LX, et al. Dependence of tumor cell lines and patient-derived tumors on the NAD salvage pathway renders them sensitive to NAMPT inhibition with GNE-618. Neoplasia 2013;15:1151–60.
21. Heyes MP, Chen CY, Major EO, Saito K. Different kynurenine pathway enzymes limit quinolinic acid formation by various human cell types. Biochem J 1997;326(Pt 2):351–6.
22. Jagasia AA, Block JA, Qureshi A, Diaz MO, Nobori T, Gitelis S, et al. Chromosome 9 related aberrations and deletions of the CDKN2 and MTS2 putative tumor suppressor genes in human chondrosarcomas. Cancer Lett 1996;105:91–103.
23. Gil-Benso R, Lopez-Gines C, Lopez-Guerrero JA, Carda C, Callaghan RC, Navarro S, et al. Establishment and characterization of a continuous human chondrosarcoma cell line, ch-2879: comparative histologic and genetic studies with its tumor of origin. Lab Invest 2003;83:877–87.
24. Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H, et al. A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. Int J Cancer 1998;77:854–9.
25. van Oosterwijk JG, de Jong D, van Ruler MA, Hogendoorn PC, Dijkstra PD, van Rijswijk CS, et al. Three new chondrosarcoma cell lines: one grade III conventional central chondrosarcoma and two dedifferentiated chondrosarcomas of bone. BMC Cancer 2012;12:375.
26. Calabuig-Farinas S, Benso RG, Szuhai K, Machado I, Lopez-Guerrero JA, de Jong D, et al. Characterization of a new human cell line (CH-3573) derived from a grade II chondrosarcoma with matrix production. Pathol Oncol Res 2012;18:793–802.
27. Kudo N, Ogose A, Hotta T, Kawashima H, Gu W, Umezu H, et al. Establishment of novel human dedifferentiated chondrosarcoma cell line with osteoblastic differentiation. Virchows Archiv 2007;451:691–9.
28. Grigolo B, Roseti L, Neri S, Gobbi P, Jensen P, Major EO, et al. Human articular chondrocytes immortalized by HPV-16 E6 and E7 genes: Maintenance of differentiated phenotype under defined culture conditions. Osteoarthritis Cartilage 2002;10:879–89.
29. de Jong Y, van Maldegem AM, Marino-Enriquez A, de Jong D, Suijker J, Briaire-de Bruijn IH, et al. Inhibition of Bcl-2 family members sensitizes mesenchymal chondrosarcoma to conventional chemotherapy: report on a novel mesenchymal chondrosarcoma cell line. Lab Invest 2016;96:1128–37.
30. Cleton-Jansen AM, van Beerendonk HM, Baelde HJ, Bovee JV, Karperien M, Hogendoorn PC. Estrogen signaling is active in cartilaginous tumors: implications for antiestrogen therapy as treatment option of metastasized or irresectable chondrosarcoma. Clin Cancer Res 2005;11:8028–35.
31. Rozeman LB, Hameetman L, Cleton-Jansen AM, Taminiau AH, Hogendoorn PC, Bovee JV. Absence of IHH and retention of PTHrP signalling in enchondromas and central chondrosarcomas. J Pathol 2005;205:476–82.
32. Hameetman L, Rozeman LB, Lombaerts M, Oosting J, Taminiau AH, Cleton-Jansen AM, et al. Peripheral chondrosarcoma progression is accompanied by decreased Indian Hedgehog signalling. J Pathol 2006;209:501–11.
33. van Oosterwijk JG, Herpers B, Meijer D, Briaire-de Bruijn IH, Cleton-Jansen AM, Gelderblom H, et al. Restoration of chemosensitivity for doxorubicin and cisplatin in chondrosarcoma in vitro: BCL-2 family members cause chemoresistance. AnnOncol 2012;23:1617–26.
34. Truong HH, de Sonneville J, Ghotra VP, Xiong J, Price L, Hogendoorn PC, et al. Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens. Biomaterials 2012;33:181–8.
35. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc 2006;1:2315–9.
36. Triche TJ Jr, Weisenberger DJ, Van Den Berg D, Laird PW, Siegmund KD. Low-level processing of Illumina Infinium DNA Methylation BeadArrays. Nucleic Acids Res 2013;41:e90.
37. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 2013;29:189–96.
38. Shames DS, Elkins K, Walter K, Holcomb T, Du P, Mohl D, et al. Loss of NAPRT1 expression by tumor-specific promoter methylation provides a novel predictive biomarker for NAMPT inhibitors. Clin Cancer Res 2013;19:6912–23.
39. Cleven AHG, Suijker J, Agrogiannis G, Briaire-de Bruijn IH, Frizzell N, Hoekstra AS, et al. IDH1 or -2 mutations do not predict outcome and do not cause loss of 5-hydroxymethylcytosine or altered histone modifications in central chondrosarcomas. Clin Sarcoma Res 2017;7:8.
40. Watson M, Roulston A, Belec L, Billot X, Marcellus R, Bedard D, et al. The small molecule GMX1778 is a potent inhibitor of NAD+ biosynthesis: strategy for enhanced therapy in nicotinic acid phosphoribosyltransferase 1-deficient tumors. Mol Cell Biol 2009;29:5872–88.

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