Microenvironment and Immunology

MYCN-Dependent Expression of Sulfatase-2 Regulates Neuroblastoma Cell Survival

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Abstract

Heparan sulfate proteoglycans (HSPG) play a critical role in the interaction of tumor cells and their microenvironment. HSPG activity is dictated by sulfation patterns controlled by sulfotransferases, which add sulfate groups, and sulfatases (Sulf), which remove 6-O-sulfates. Here, we report altered expression of these enzymes in human neuroblastoma cells with higher levels of Sulf-2 expression, a specific feature of MYCN-amplified cells (MYCN-A cells) that represent a particularly aggressive subclass. Sulf-2 overexpression in neuroblastoma cells lacking MYCN amplification (MYCN-NA cells) increased their in vitro survival. Mechanistic investigations revealed evidence of a link between Sulf-2 expression and MYCN pathogenicity in vitro and in vivo. Analysis of Sulf-2 protein expression in 65 human neuroblastoma tumors demonstrated a higher level of Sulf-2 expression in MYCN-A tumors than in MYCN-NA tumors. In two different patient cohorts, we confirmed the association in expression patterns of Sulf-2 and MYCN and determined that Sulf-2 overexpression predicted poor outcomes in a nonindependent manner with MYCN. Our findings define Sulf-2 as a novel positive regulator of neuroblastoma pathogenicity that contributes to MYCN oncogenicity. Cancer Res; 74(21); 5999–6009. ©2014 AACR.

Introduction

Heparan sulfate proteoglycans (HSPG) present at the surface and in the extracellular milieu of normal and malignant cells bind to a large variety of proteins, contributing to the mechanisms that control the communication between cells and their microenvironment (1, 2). In the extracellular milieu, proteoglycans serve as a reservoir of growth factors, cytokines and chemokines, regulating their bioavailability and binding to their receptors (3, 4). Proteoglycans play an important role in normal biologic processes, including organ development and tissue repair, as well as in pathologic conditions such as inflammation and cancer (3, 5). Their role in cancer is, however, complex and includes anti- as well as protumorigenic effects. This is in part due to the significant enzymatic modifications that these molecules undergo both intra- and extracellularly. Recently, attention has been focused on two extracellular sulfatases (Sulf-1 and Sulf-2) that selectively remove 6-O-sulfate groups from glucosamine residues, and thereby remodel HS chain sulfation (6). Sulfs have been implicated as inhibitors of tumor growth in some cancers (7), in which they have a bimodal effect (8–10), but are more commonly reported as being protumorigenic (11–14). The mechanism by which Sulfs contribute to tumorigenesis involves the modulation of ligand–receptor interactions and activation of downstream signaling pathways. By remodeling 6-O sulfation in HSPGs, Sulf-1 promotes Wnt signaling by increasing the interaction between Wnt and its receptor Frizzled (15), whereas Sulf-2 enhances the interaction between platelet-derived growth factor (PDGF) and its receptor PDGFRα (14).

Neuroblastoma is the most common extracranial solid tumor in children and a cancer that has a high potential to metastasize (16). In 40% of high-risk neuroblastoma cases, amplification of the MYCN (MYCN-A) oncogene has been identified as the oncogenic event responsible for aggressive progression and poor clinical outcome (17). The mechanisms by which MYCN promotes tumorigenesis are complex and linked primarily to its transcriptional activity, upregulating the expression of a large variety of genes involved in proliferation, survival, differentiation, DNA repair, drug resistance, and angiogenesis (18, 19). Owing to the important role that HSPGs play in the regulation of the interactions between tumor cells and the tumor microenvironment, their potential contribution in neuroblastoma progression was examined. Neuroblastoma cells have been shown to produce both cell surface and extracellular HSPGs (20, 21), but little is

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known of their role in neuroblastoma progression. Here, we report new findings regarding the role that Sulf-2 plays in neuroblastoma progression, indicating a unique function in MYCN-driven tumorigenicity.

Materials and Methods

Cell culture and reagents

Human neuroblastoma cell lines were obtained from the ATCC, and MYCN-A NB-19 cells from RIKEN (BioResource Centre). MYCN nonamplified (NA) CHLA-255, CHLA-90, SK-N-NA and SK-N-RA and MYCN-A CHLA-136, SMS-SAN and SK-N-BE(2) cell lines were obtained from Dr. C.P. Reynolds (Texas Tech University Health Sciences Center, Lubbock, TX) and grown as described previously (22). CHLA255-MYCN cells were obtained from Dr. L. Metelitsa (Baylor College of Medicine, Houston, TX), MYCN-NA SHEP and SHEP-21N cells were provided by Dr. M. Schwab (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Doxycycline was used at 10 ng/mL provided by Dr. M. Schwab (Deutsches Krebsforschungszentrum). MYCN nonamplified CHLA-255, CHLA-90, SK-N-RA and MYCN-A CHLA-136, SMS-SAN and SK-N-BE(2) cell lines were obtained from Dr. C.P. Reynolds (Texas Tech University Health Sciences Center, Lubbock, TX) and BE(2) cell lines were obtained from Dr. C.P. Reynolds (Texas Tech University Health Sciences Center, Lubbock, TX) and "Published OnlineFirst August 27, 2014; DOI: 10.1158/0008-5472.CAN-13-2513".

Cell transfection

Transfection of siRNA was in the presence of Lipofectamine RNAiMax (Life Technologies) according to the manufacturer’s instructions. siRNA oligonucleotides (sequences provided in Supplementary Table S1) were obtained from Life Technologies (Silencer Select Sulf-2, s31805, s31806; negative control AM4611). Two Sulf-2–overexpressing constructs, a full-length Sulf-2, and a catalytically inactive Sulf-2 (S2ΔC) form with a mutation (C88A) were used (Addgene; plasmid 13004 and 13006).

For shRNA, two previously published target regions of Sulf-2 [GGAGTGTGGGTGCATATAAA (1143) and GCTGAAGCTGCAGAAGAT (1413)] were used with AACAGTCGCGTTTGC [GGAGTGGTGGTGTCAATAA (1143) and GCTGAAGCTGCAGAAGAT (1413)] were used with AACAGTCGCGTTTGC-GACTGG as a control (scrambled, SCR; ref. 23). The pLVTHM backbone (Addgene; plasmid #11643) was used by transfection of siRNA. Plasmid deposition was verified by sequencing (GENEWIZ) using the human H1 promoter forward primer and/or SP6 forward primers.

Cell viability, proliferation, and apoptosis assays

CellTiter-Glo Luminescence cell viability assay (Promega #G7570) was used. For crystal violet staining, cells were fixed with 4% paraformaldehyde and subsequently stained with 1% crystal violet for 30 minutes. For apoptosis assays, cells were analyzed for caspase 3/7 activity (Apotix Live-Glo Multiplex Assay; Promega #G6410). Alternatively, cells were grown in 6-well plates (4 × 10⁴ cells/well) and Annexin V expression and propidium iodide (PI) staining were performed (FITC Annexin V Apoptosis Detection Kit; BD Pharmingen #556547). Cell-cycle analysis was performed as previously described (24) using an FITC BrdUrd Flow kit (BD Pharmingen; #552598). Annexin V and bromodeoxyuridine (BrdUrd) were analyzed by flow cytometry using CellQuestPro software.

Gene-expression analysis

Total RNA was extracted from cell lysates using the RNeasy Kit (Qiagen) and reverse transcribed into complementary DNA (cDNA) using SuperScript III Reverse Transcriptase (Invitrogen). RT-PCR was performed with cDNA corresponding to 50 ng total RNA using the LightCycler Roche 480 system (Roche) and the primers listed in Supplementary Table S1.

Western blot analysis

Western blots were performed using the following monoclonal antibodies (mAbs): a mouse mAb against human Sulf-2 (2B4; Novus Biologicals; #NB1-36727, 1:1,000 dilution), a mouse mAb against human Sulf-1 (Novus Biologicals; #H00023213-M01A, 1:500 dilution), a mouse mAb against human MYCN (Abcam; #16898, 1:500 dilution), an anti-poly ADP ribose polymerase (PARP) rabbit mAb (Cell Signaling Technology; #9542, 1:1,000 dilution), an anti-cleaved PARP (Asp214) rabbit mAb (Cell Signaling Technology; #9541, 1:1,000 dilution), an anti–caspase-3 rabbit mAb (Cell Signaling Technology; #9665, 1:1,000 dilution), an anti–caspase-3 rabbit mAb (Cell Signaling Technology; #9661, 1:1,000 dilution), an anti–β-actin rabbit mAb (Sigma-Aldrich; #A2066, 1:3,000 dilution) and an anti–β-tubulin mouse mAb (Sigma-Aldrich; #T4026, 1:1,000 dilution). Analyses of the membranes were performed with the Li-Cor Odyssey infrared imager using an anti-mouse IgG IRDye 680 (Li-Cor #926-32220) and an anti-rabbit IgG IRDye 800 (Li-Cor #926-32211) as secondary antibodies (1:5,000).

Animal experiments

In vivo experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Usage Committee of Children’s Hospital Los Angeles. SK-N-BE(2) cells stably transfected with pKRAB-SCR or KRAB-Sulf-2 were sorted for green fluorescent protein (GFP) expression by flow cytometry. Cells were injected s.c. in the left and right flank of 4-week-old NOD/SCID mice (n = 10 in each group). In each group, 5 mice were treated with doxycycline (2 mg/mL in the drinking water). Once tumors became palpable, tumor size was measured using a caliper and the tumor volume was calculated using the formula: tumor volume (mm³) = (width in mm)² × (length in mm) × π/6. When one of the two tumors reached the size of 1,500 mm³, a mouse was euthanized by O₂/CO₂.

Immunocytochemistry

Adherent cells grown on coverslips were fixed in 4% paraformaldehyde and examined in the presence of phage display anti-HS antibodies (HS387V and HS388V; gift from Professor A. van Kuppevelt, Nijmegen, the Netherlands). Secondary antibodies were anti-mouse IgG antibodies conjugated to AlexaFluor-488 or 594. Fluorescence and differential interference contrast images were captured using an LSM 710 confocal microscope with a 63x/1.4 Plan-APoCHROMAT oil-immersion objective lens (Carl Zeiss Microscopy, Thornwood, NY).
Gene-expression microarray data
The gene-expression studies were performed on neuroblastoma datasets published previously (27, 28). The GSE16716 dataset was obtained from samples of patients enrolled in clinical trials conducted by the German Consortium, Society of Pediatric Oncology and Hematology (GPOH). The GSE3446 dataset included 162 patients enrolled in clinical trials conducted by the COG. The transcript level data of array probes for each sample were obtained as log 2 expression levels and deposited in the Gene Expression Omnibus (GEO). The integrated density (ID) and area (A) of the pixels were quantified and optical density (OD) was calculated with the formula OD = log [IDmax (ID + IDmax − 255 × A)], where IDmax = 255 × 1388 × 1040, the maximum possible ID for an 8-bit image of 1388 × 1040 pixels. The samples were also classified for MYCN amplification and risk (high vs. low), according to the International Neuroblastoma Pathology Classification (26).

Statistical analysis
Differences between groups were compared using the Student t test or ANOVA as appropriate, with log_{10} transformation to normalize the data and stabilize variance when necessary. Comparisons of event-free survival between groups of patients were based on the univariate or stratified log-rank test as appropriate. Survival curves were based on the product-limit estimate (29). P values are based on two-sided tests unless otherwise specified. Statistical computations were performed using Stata 11 (Stata Statistical Software: Release 11; StataCorp LP). Statistical analysis for the gene-expression microarray data was performed with R Project Software (version 3.0.1) with one-way ANOVA followed by the Wald test for comparing differences between multiple groups and correcting for multiple testing when appropriate. Differences were considered significant at P < 0.05.

Results
Sulf-2 is specifically overexpressed in MYCN-A neuroblastoma cell lines
To explore the role of HS in neuroblastoma, we started with an unbiased analysis of the expression of key enzymes controlling their sulfation patterns, focusing on the biosynthetic sulfate transferases, and Sulfs that remove 6-O-sulfate from glucosamine residues. The analysis (Fig. 1) was performed by qRT-PCR, comparing eight neuroblastoma cell lines (four MYCN-A and four MYCN-NA) with six other malignant cell lines (A549 lung carcinoma, MG63 osteosarcoma, HT1080 fibrosarcoma, Hela cervical cancer, MDAMB231 breast cancer, and S8161 melanoma) and two nonmalignant cell lines (endothelial cells and fibroblasts; Supplementary Fig. S1). This analysis revealed a difference in the expression of Sulf-2, which was more abundantly expressed (ΔCt > 15) in four neuroblastoma cell lines [CHLA-136 and SK-N-BE(2), SMS-SAN and CHLA-259].

![Figure 1. Expression of sulfation-modifying enzymes in neuroblastoma cell lines measured by qRT-PCR. Data, negative mean ΔCt ± SEM (value of gene—control value of GAPDH) from three aliquots per sample from three separate experiments. Higher ΔCt values represent higher levels of expression. MYCN-NA, nonamplified; MYCN-A, amplified.](image-url)
NB-19] and also one melanoma cell line (C8161); no difference was observed in the expression of sulfate transferases and Sulf-1. Interestingly, the four neuroblastoma cell lines having the highest levels of Sulf-2 expression were all MYCN-A. The data were validated by Western blot analysis (Fig. 2A), confirming that Sulf-2 protein was more abundantly present in the four cell lines in which MYCN was highly expressed compared with the cell lines where the MYCN protein was low or absent. To determine whether overexpression of Sulf-2 affected sulfation pattern of HSPGs, immunocytofluorescence studies were conducted. Using HS3A8V that recognizes a common HS epitope, we observed the presence of similar levels of HSPG at the cell surface of both MYCN-A and MYCN-NA cell lines (Fig. 2B, i and ii). However, using HS3B7V that specifically recognizes highly sulfated HS epitopes, we demonstrated that the level of sulfation of HSPG was lower in MYCN-A cell lines, which expressed high levels of Sulf-2 (Fig. 2B, iii–vi). No staining was seen in the absence of primary antibody (Supplementary Fig. S2). Thus, among the enzymes controlling the pattern of sulfation of HSPGs, Sulf-2 was the only one that was differentially expressed in MYCN-A cell lines compared with all other cell lines. The data also demonstrated that higher levels of Sulf-2 were associated with a decrease in the level of sulfation of HSPG. These data led us to explore whether Sulf-2 could play an active role in MYCN-A–driven neuroblastoma.

**Sulf-2 increases neuroblastoma cell survival and its expression is linked to MYCN.**

To address this question, we first examined the effect of overexpression of Sulf-2 on the survival of two MYCN-NA cell lines (Fig. 3A and B). This analysis revealed that overexpression of Sulf-2 WT was associated with a corresponding increase in cell viability in cultures compared with untransfected parental cells, whereas overexpression of a catalytically inactive S2ACCC mutant had no effect. Importantly, the data also show that overexpression of Sulf-2 did not affect MYCN expression, indicating that MYCN is not downstream of Sulf-2. This observation, combined with the association between Sulf-2 expression and MYCN amplification reported in Figs. 1 and 2, raised the possibility of a link between MYCN and Sulf-2 expression. To address this question, we then examined Sulf-2 expression in a CHLA-255 cell line stably transduced with a MYCN cDNA (30); increased levels of Sulf-2 protein were observed compared with parental CHLA-255 cells (Fig. 3A, lanes 1 and 4). We then used SHEP-21N neuroblastoma cells, a subclone of SHEP cells transfected with a doxycycline-regulated (tet-off) MYCN expression vector (31). In the absence of doxycycline, these cells expressed MYCN and Sulf-2 (Fig. 3C) compared with SHEP parental cells that were negative for MYCN and Sulf-2 expression. Treatment of SHEP-21N cells with doxycycline resulted in a loss of MYCN protein expression that was followed by a progressive decrease in Sulf-2 protein at 12 and 18 hours. Removal of doxycycline resulted in a rapid reexpression of MYCN and a later reexpression of Sulf-2 (by 24 hours). The data further support a link between Sulf-2 and MYCN, and raised the possibility that Sulf-2 may actively contribute to its tumorigenic function.

**Knockdown of Sulf-2 expression in MYCN-A neuroblastoma cells results in loss of viability and an increase in apoptosis in vitro.**

To address this question, we examined the effects of downregulating Sulf-2 expression on the viability of MYCN-A cells. Two MYCN-A cell lines with strong expression of Sulf-2 [SK-N-BE(2) and NB-19] were transfected with either specific Sulf-2 siRNAs (Sulf-2 siRNA 1 or 2 and a combination of both), or with a SCR siRNA. Both siRNA-transfected cell lines showed an 85% to 90% decrease in the expression of Sulf-2 compared with the cells transfected with...
the SCR siRNA (Fig. 4A). The knockdown (KD) of Sulf-2 in these cells resulted in significant reductions in cell density (Fig. 4B), and in cell viability (Fig. 4C).

To determine whether this effect involved cell proliferation or apoptosis, the effect of Sulf-2 KD on cell-cycle and BrdUrd incorporation was examined in MYCN-A NB-19 cells. This analysis revealed a significant decrease in the percentage of BrdUrd-positive cells in S phase from 25.5% to 18.4% (Fig. 4D). An analysis of the cell-cycle distribution indicated a decrease in S and in G2 phases (from 9.5% to 4.4%) and an increase in the sub-G0 cell population (from 4% to 22%), suggesting an effect on both cell-cycle entry and apoptosis (Fig. 4E). This was confirmed by demonstrating an increase in caspase 3/7 activity upon Sulf-2 KD in SK-N-BE(2) and NB-19 cells (Fig. 5A), an increase in cleaved PARP and cleaved caspase 3 in Sulf-2 siRNA1- and 2-expressing cells (Fig. 5B), and a corresponding increase in Annexin V expression in NB-19 cells (Fig. 5C). The data indicated that suppression of Sulf-2 expression in MYCN-A neuroblastoma cells decreased proliferation and increased apoptosis, supporting the hypothesis that Sulf-2 expression is linked to MYCN, and is an active contributor to the protumorigenic function of MYCN.

**Downregulation of Sulf-2 expression in MYCN-A cells decreases tumor formation and tumor growth in xenotransplanted mice**

We then tested whether Sulf-2 would contribute to MYCN activity in vivo. SK-N-BE(2) cells were transfected with a doxycycline-regulated Sulf-2 shRNA lentiviral vector (pKRAB-Sulf-2) or control (pKRAB-SCR) and stably transfected GFP-positive cells were sorted by flow cytometry. Treatment of these cells with doxycycline (Dox) and examined for Sulf-2 and MYCN expression by Western blot analysis at indicated time points (0–18 hours). After 18 hours, doxycycline was removed from the medium, and cells were kept in culture for another 24 hours.

Figure 3. Sulf-2 increases neuroblastoma cell survival and its expression is linked to MYCN. A, left, Western blot analysis on Sulf-2 in CHLA-255 cells stably transduced with MYCN cDNA, a Sulf-2 WT cDNA, a mutant inactive Sulf-2 (S2ΔCC), or an empty vector control. β-Actin was used as a loading control. The data are representative of three independent experiments showing similar results. Right, CHLA-255 cells transfected as indicated in A were examined for viability over time. Data, mean (±SD) luminescence units (RLU) of triplicate samples; *; P < 0.05; **; P < 0.001. B, SHEP cells stably transduced with a Sulf-2 WT cDNA, a mutant inactive Sulf-2 (S2ΔCC), or an empty vector control were examined for Sulf-2 and MYCN expression (left) and viability (right) as shown in A. C, SHEP-21N cells were treated with doxycycline (Dox) and examined for Sulf-2 and MYCN expression by Western blot analysis at indicated time points (0–18 hours). After 18 hours, doxycycline was removed from the medium, and cells were kept in culture for another 24 hours.
In each group \((n = 9\) or \(10\) mice), 5 mice received doxycycline in the drinking water. In the 9 mice injected with SK-N-BE(2) pKRAB-SCR vector, 8 mice developed bilateral tumors and the administration of doxycycline had no effect on the rate of tumor growth (one mouse in the doxycycline-negative group did not develop tumors). By day 31 after tumor cell implantation, all mice had to be euthanized because one or both tumors had reached a volume greater than \(1,500\) \(\text{mm}^3\) (Fig. 6C).

In the group of mice injected with SK-N-BE(2) pKRAB-Sulf-2 vector, treatment with doxycycline resulted in a tumor formation rate of \(50\%\) only and in a significant delay in tumor growth with an average time for tumors to reach \(500\) \(\text{mm}^3\) of \(28 \pm 3.2\) days compared with a tumor formation rate of \(75\%\) and \(100\%\) and an average time for tumors to reach \(500\) \(\text{mm}^3\) of \(20 \pm 5\) and \(18 \pm 2.3\) days in the pKRAB-SCR control group (untreated and treated with doxycycline, respectively). Unexpectedly, the pKRAB-Sulf-2 group untreated with doxycycline, although having an expected tumor formation rate of \(100\%\), showed a delay in tumor growth with an average time to reach \(500\) \(\text{mm}^3\) of \(25 \pm 3.2\) days. This suggested that Sulf-2 expression may have been downregulated in this group due to the leakiness of the vector. This was confirmed by an analysis of Sulf-2 mRNA expression in tumor samples by qRT-PCR (Fig. 6E), which revealed a statistically significant decrease in Sulf-2 expression in the pKRAB-Sulf-2 subgroup (average \(\Delta C_t\) of \(12.7 \pm 0.8\) ) not treated with doxycycline \((P = 0.008)\). An analysis of the expression of Sulf-2 protein by immunohistochemistry (Fig. 6F) confirmed the downregulation of Sulf-2 in tumors derived from mice implanted with pKRAB-Sulf-2 cells and untreated with doxycycline. The data in this group of mice, thus, are explained by the leakiness of the pKRAB-Sulf-2 vector and provide further support to the demonstration that downregulation of Sulf-2 expression in MYCN-A cells inhibits tumorigenesis.
Sulf-2 is overexpressed in MYCN-A primary human NBL tumors and is associated with poorer outcome

To validate the observations made on established cell lines in human tumor samples, we examined 65 primary human neuroblastoma tumors for Sulf-2 expression by histology and immunohistochemistry. These samples included 22 tumors with MYCN-A and unfavorable histology (MYC-A-UH), 23 MYCN-NA tumors but unfavorable histology (NA-UH), and 20 MYCN-NA tumors with favorable histology (FH). The staining of Sulf-2 was predominantly present in the plasma membrane and in the pericellular space surrounding tumor cells (Fig. 7A). A quantitative analysis by color deconvolution (Fig. 7B) indicated much stronger expression of Sulf-2 in all MYCN-A tumors with levels 80-fold [95% confidence interval (CI), 28–233] and 330-fold (95% CI, 111–1,014) higher than in MYCN-NA-UH and MYCN-NA-FH tumors, respectively (both \( P < 0.0001 \)). There was an approximately 4-fold, and statistically significant, higher expression in the MYCN-NA-UH group compared with the MYCN-NA-FH group (\( P = 0.011 \)).

Additional confirmation of the link between Sulf-2 and MYCN expression in primary neuroblastoma tumors was obtained through analysis of gene-expression arrays in a cohort of 416 tumor samples obtained from the patients enrolled in GPOH clinical trials (33). This analysis revealed a statistically significant higher level of expression of Sulf-2 mRNA in tumors derived from patients with high-risk disease and MYCN amplification (HR-A) when compared with tumors of patients with high-risk disease, but lacking MYCN amplification (HR-NA), and of patients with low-risk disease (\( P < 0.0001 \); Fig. 6C). Similar results were obtained from a second cohort of 162 samples obtained from children.

Figure 5. Loss of Sulf-2 expression in MYCN-A neuroblastoma cells induces apoptosis. A, caspase 3/7 activity in Sulf-2 siRNA–transfected SK-N-BE(2) and NB-19 cells was measured using the ApoLive-Glo system. Data, mean (±SD) luminescence units (RLU) of triplicate sample from one experiment and are representative of three independent experiments showing similar results; **, \( P < 0.01 \); ***, \( P < 0.001 \). B, Western blot analysis of SK-N-BE(2) and NB-19 cells harvested 48 hours after transfection. C, apoptosis in NB-19 cells transfected as indicated in A was determined by flow cytometric analysis of Annexin V/PI staining; values, mean ± SEM of three independent experiments.
enrolled in the COG clinical trials ($P < 0.0005$). Event-free survival (EFS) analysis of the GPOH cohort of patients (Fig. 7D) revealed that higher levels of Sulf-2 expression were significantly associated with a lower EFS and predictor of poor clinical outcome (log-rank $P < 0.001$). However, owing to its strong association with MYCN expression, Sulf-2 expression was not found to be an independent prognosticator after controlling for risk amplification status and risk group (stratified log-rank $P = 0.96$ and $P = 0.12$ for GPOH and COG patients, respectively). Altogether these correlative data provide further support to the hypothesis that Sulf-2 contributes to the tumorigenic function of MYCN.

**Discussion**

We report two novel and relevant observations that shed light on the important role that HSPG sulfation patterns play in MYCN-driven neuroblastoma pathogenesis. First, among the enzymes controlling the sulfation of HSPGs, Sulf-2 was found a key contributor to the protumorigenic role of MYCN. Both Sulf-1 and Sulf-2 are involved in the pathogenesis of many cancers. They are highly expressed in hepatocellular carcinoma, head and neck, pancreatic cancer, lung adenocarcinoma, and squamous cell carcinoma, where their expression is typically associated with more aggressive behavior and poorer clinical outcomes (6). However, to our knowledge, this is the first report that Sulf-2 is specifically overexpressed in a subgroup of...
neuroblastoma tumors that are driven by MYCN. Our data suggest that Sulf-2 contributes to the oncogenic effect of MYCN by increasing cell proliferation and survival through a yet to be determined mechanism. Immunofluorescence studies demonstrated lower levels of sulfation in HSPGs in cells expressing Sulf-2. This raises the possibility that Sulf-2 may promote the release of growth factors and other ligands from undersulfated HSPGs, allowing them to interact with their respective receptors. In support of this concept is the observation that in malignant glioma loss of Sulf-2 expression decreases PDGF–PDGFRα interaction, resulting in growth inhibition in vivo in a subset of glioma cells that are more dependent on exogenous growth factors (14). Neuroblastoma cells express a variety of receptor tyrosine kinases (34, 35). It is conceivable that, as shown in glioma and breast cancer cells (36), Sulf-2 promotes the release of growth factors interacting with these receptors.

Figure 7. Sulf-2 is overexpressed in MYCN-A primary human neuroblastoma tumors and is an indicator of poor outcome. A, analysis of Sulf-2 expression in primary human neuroblastoma tumors. Representative sections of tumors stained with an anti–Sulf-2 antibody; i and ii, MYCN-A and UH; iii and vi, MYCN-NA and UH; and v and vi, MYCN-NA with FH; scale bar, 20 μm. B, data, mean (±SD) OD obtained from 10 fields (×20) for each tumor section from MYCN-A (n = 22), MYCN-NA, UH (n = 23), and MYCN-NA, FH (n = 20) tumors: ***; P < 0.0001 in the GPOH group and **; P < 0.0005 in the COG group based on ANOVA. C, expression of Sulf-2 RNA by gene array expression analysis generated from two cohorts of patients treated on clinical trials conducted by GPOH (n = 416) and COG (n = 162). The data were analyzed by comparing three groups according to their clinical risk stratification (HR-A, high risk with MYCN-A; HR-N, high risk with MYCN-NA; and LR, low risk): ****; P < 0.0001 in the GPOH group and **; P < 0.0005 in the COG group based on ANOVA. D, Kaplan–Meier regression analysis of EFS in the GPOH cohort according to the level of Sulf-2 mRNA expression.
One possible candidate is NTKR2 (TKB), which is abundantly expressed in MYCN-A tumors. Brain-derived neurotrophic factor (BDNF), the ligand for TKB, promotes tumor growth, drug resistance, and angiogenesis in neuroblastoma (37, 38). Like many other neurotrophic and growth factors, BDNF binds to oversulfated proteoglycans (39). Although there are currently no data supporting the concept that Sulf-2 could regulate the BDNF–TKB interaction, the observation that Sulf-1 and Sulf-2 positively regulate glial cell–derived factor–mediated signaling and esophageal innervation (40), suggests that Sulf-2 may regulate neurotrophic factor–mediated signaling.

The second important observation reported in this article is the close association between Sulf-2 and MYCN expression, which we demonstrated in cell lines and in human tumors in vitro and in vivo. These data demonstrate that there is a link between Sulf-2 and MYCN and that Sulf-2 is downstream of MYCN, but do not yet establish that the Sulf-2 gene is a transcriptional target of MYCN. A large number of transcriptional targets of MYCN have been reported, including genes involved in ribosome biogenesis and protein synthesis (41) and miRNA (42). Interestingly, Sulf-2 was recently identified among 36 genes downregulated in two MYCN-A neuroblastoma cell lines treated with the bromodomain and extraterminal domain inhibitor JQ1, that downregulates MYCN expression (43). The promoter regions of Sulf-2 have not yet been elucidated, but in preliminary studies we have identified an MYCN consensus sequence (CCCCACGTGGGC) located 1,675 nucleotides upstream of the Sulf-2 first exon on chromosome 20 that binds MYCN (data not shown). However, it is currently unclear whether this MYCN-binding sequence has a regulatory function for Sulf-2 expression.

Finally, our data raise the possibility that Sulf-2 could be a novel target for therapeutic intervention in MYCN-A neuroblastoma. A number of heparin- and HS-based drugs and mimetics based on sulfated oligosaccharides have been developed (44–49). The antitumorogenic, -metastatic and -angiogenic activity of several of these mimetics has been demonstrated and some, like PI-88 and M402, are undergoing clinical trials in patients with hepatocellular carcinoma and pancreatic cancer (46, 47). It is possible that such compounds could counteract the effect of Sulf-2 by sequestering growth factors released in the extracellular and pericellular space upon desulfation of HSPGs by Sulf-2. Furthermore, it is also possible that specific inhibitors of Sulf-2 could be developed (50). Considering the importance of targeting MYCN, this avenue should be actively explored.

In conclusion, our data point to Sulf-2 as a regulator of HS sulfation being downstream of MYCN and an important contributor to its oncogenic function. They also emphasize the critical role that HSPGs could play as regulators of survival and proliferation in neuroblastoma, a subject that has not been investigated so far.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: V. Solari, E.A. Yates, J.E. Turnbull, Y.A. DeClerck
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