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

Valeria Solari¹²³, Lucia Borriello²³, Gianluca Turcati³, Hiroyuki Shimada⁴, Richard Sposto²⁵, G. Esteban Fernandez³, Shahab Asgharzadeh²⁴⁵, Edwin A. Yates¹, Jeremy E. Turnbull¹, and Yves A. DeClerck²³⁶

¹Centre for Glycobiology, Institute of Integrative Biology, University of Liverpool, Liverpool L69 7XB, United Kingdom; ²Division of Hematology-Oncology, Department of Pediatrics, University of Southern California, Los Angeles, USA; ³The Saban Research Institute of Children’s Hospital, Los Angeles, CA 90027, USA; ⁴Department of Pathology, ⁵Department of Preventive Medicine, ⁶Department of Biochemistry and Molecular Biology, University of Southern California, Los Angeles, USA.

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Address correspondence to: Yves A. DeClerck, MD, 4650 Sunset Boulevard, MS#54, Los Angeles, CA 90027. Tel: 323-361-2150; Fax: 323-361-4902; E-mail: declerck@usc.edu

or Jeremy E. Turnbull, PhD, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB. Tel: +44 (0)151 795 4427; E-mail: j.turnbull@liverpool.ac.uk

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Abstract

Heparan sulfate proteoglycans (HSPGs) 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 (Sulfs), 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 non-independent manner with MYCN. Our findings define Sulf-2 as a novel positive regulator of neuroblastoma pathogenicity that contributes to MYCN oncogenicity.

Precis

An enzyme that is responsible for removing sulfate groups from heparan sulfate proteoglycans appears to be an important mediator of the effects of the MYCN oncogene in a deadly pediatric tumor, with potential implications for understanding how MYCN contributes to malignancy by modifying interactions with the tumor microenvironment.
Introduction

Heparan sulfate proteoglycans (HSPGs) 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 biological processes including organ development and tissue repair, as well as in pathological conditions such as inflammation and cancer (3, 5). Their role in cancer is, however, complex and includes anti- as well as pro-tumorigenic 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), where they have a bimodal effect (8-10), but are more commonly reported as being pro-tumorigenic (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 HSPG, Sulf-1 promotes Wnt signaling by increasing the interaction between Wnt and its receptor Frizzled (15), while 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 TME, 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 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 American Type Culture Collection, and MYCN-A NB-19 cells from RIKEN (BioResource Centre). MYCN-non amplified (NA) CHLA-255, CHLA-90, SK-N-SN 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 and grown as described (22). CHLA255-MYCN cells were obtained from Dr. L. Metelitsa (Baylor College of Medicine, 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 to inhibit MYCN expression. Cell lines were authenticated by genotype in our laboratory using AmpFISTR Identification kit PCR reagents and Gene Mapper ID v3.2 (Applied Biosystems).

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 Table 1 in Supplementary Data) 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 (S2ΔCC) form with a mutation (C88A) were used (Addgene, plasmid 13004 and 13006).

For shRNA, two previously published target regions of Sulf-2 [GGAGTGGTGGTGTCAATAA (1143) and GCTGAAGCTGCATAAGTGC (1413)] were used with AACAGTCGCGTTTGCGACTGG as a control (scrambled) (23). The pLVTHM backbone (Addgene plasmid#12247) was used to insert the Sulf-2 oligonucleotides. Inserted sequences where subcloned into tet-inducible shRNA lentiviral vectors pLVCT-tTR-KRAB (Addgene plasmid #11643). 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 Luminescent 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 (ApoLive-Glo Multiplex Assay; Promega #G6410). Alternatively, cells were grown in 6-well plates (4x10^5 cells/well) and Annexin V expression and propidium iodide staining (PI) were performed (FITC Annexin V Apoptosis Detection kit, BD Pharmingen #556547). Cell cycle analysis was performed as previously described (24) using a FITC BrdU Flow kit (BD Pharmingen #552598). Annexin V and BrdU were analyzed by flow cytometry using CellQuestPro software.

Gene expression analysis

Total RNA was extracted from cell lysates using 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 Table 1 of Supplementary Data.

Western blot analysis

Western blots were performed using the following monoclonal antibodies (MAb) antibodies: a mouse MAb against human Sulf-2 (2B4; Novus Biologicals #NBP1-36727, 1:1000 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 #9542, 1:1000 dilution), an anti-cleaved PARP (Asp214) rabbit MAb (Cell Signaling #9542, 1:1000 dilution), an anti-caspase 3 rabbit MAb (Cell
Signaling #9665, 1:1000 dilution), an anti-cleaved caspase 3 rabbit MAb (Cell Signaling #9661, 1:1000,dilution), an anti-β-actin rabbit MAb (Sigma Aldrich #A2066, 1:3000 dilution) and an anti-β-tubulin mouse MAb (Sigma Aldrich #T4026, 1:1000 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 (1:5000).

**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 subcutaneously in the left and right flank of 4 week-old female 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 2 tumors reached the size of 1500 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 (HS3B7V and HS3A8V; 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 (DIC) 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).
Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) sections of 65 primary human neuroblastoma tumors were obtained from the Children’s Oncology Group (COG) Neuroblastoma Biology Study with the approval of the CHLA Institutional Review Board. Samples were obtained without patient identification. Tissue sections (5 μm) were subjected to heat-induced epitope retrieval using citrate buffer (pH 6) and incubated at 4°C overnight in the presence of a mouse monoclonal anti-human Sulf-2 (2B4) antibody (2 μg/ml). Following washing with PBS with 0.05% Tween-20 (PBST), the slides were incubated in the presence of a biotin conjugated anti-mouse IgG antibody and avidin/biotin complex (Vectastain ABC kit, Vector Laboratories). 3, 3'-diaminobenzidine (DAB; brown) was used to visualize antibody binding and Mayer’s hematoxylin to counterstain cell nuclei. DAB staining in the 20 x digital images was quantified using Fiji ImageJ software (25) in the following manner: Brown DAB and blue hematoxylin staining were separated into different images with the Color Deconvolution function using the H DAB matrix. The integrated density (ID) and area (A) of the pixels were quantified and optical density (OD) was calculated with the formula $\text{OD} = \log \left( \frac{\text{ID}_{\text{max}}}{\text{ID} + \text{ID}_{\text{max}} - 255 \times A} \right)$, where $\text{ID}_{\text{max}} = 255 \times 1388 \times 1040$, 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).

Gene expression microarray data

The gene expression studies were performed on neuroblastoma datasets published previously (27,28). The GSE16716 data set 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 averaged using the gene symbol annotations provided by the manufacturer.

**Statistical analysis**

Differences between groups were compared using Student’s $t$-test or Analysis of Variance (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 logrank 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. College Station, TX: 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 Wald’s 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, focussing on the biosynthetic STs, and Sulfs that remove 6-O-sulfate from glucosamine residues. The analysis (Figure 1) was performed by qRT-PCR, comparing 8 neuroblastoma cell lines (4 MYCN-A and 4 MYCN-NA) with 6 other malignant cell lines (A549 lung carcinoma, MG63 osteosarcoma, HT1080 fibrosarcoma, Hela cervical cancer, MDAMB231 breast cancer and C8161 melanoma) and 2 non-malignant cell lines (endothelial cells and fibroblasts) (Supplementary Data Figure S1). This analysis revealed a difference in the expression of Sulf-2, which was more abundantly expressed (\(\Delta CT >15\)) in 4 neuroblastoma cell lines (CHLA-136, SK-N-BE(2), SMS-SAN and NB-19) and also one melanoma cell line (C8161); no difference was observed in the expression of STs and Sulf-1. Interestingly, the 4 neuroblastoma cell lines having the highest levels of Sulf-2 expression were all MYCN-A. The data were validated by Western blot analysis (Figure 2A), confirming that Sulf-2 protein was more abundantly present in the 4 cell lines in which MYCN was highly expressed compared to 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 (Figure 2B, panel 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 (Figure 2B, panels iii to vi). No staining was seen in the absence of primary antibody (Supplementary Data Figure 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 to 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 2 MYCN-NA cell lines (Figure 3A and B). This analysis revealed that overexpression of Sulf-2 WT was associated with a corresponding increase in cell viability in cultures compared to untransfected parental cells, whereas overexpression of a catalytically inactive S2ΔCC 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 Figures 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 to parental CHLA-255 cells (Figure 3 A, lane 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 (Figure 3C) compared to 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 re-expression of MYCN and a later re-expression 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**

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To address this question we examined the effects of down-regulating 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 scrambled (SCR) siRNA. Both siRNA transfected cell lines showed an 85-90% decrease in the expression of Sulf-2 compared to the cells transfected with the SCR siRNA (Figure 4A). The knockdown (KD) of Sulf-2 in these cells resulted in significant reductions in cell density (Figure 4B), and in cell viability (Figure 4C).

To determine whether this effect involved cell proliferation or apoptosis, the effect of Sulf-2 KD on cell cycle and BrdU incorporation was examined in MYCN-A NB-19 cells. This analysis revealed a significant decrease in the percentage of BrdU positive cells in S phase from 25.5% to 18.4% (Figure 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 (Figure 3E). 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 (Figure 5A), an increase in cleaved PARP and cleaved caspase 3 in Sulf-2 siRNA1- and 2-expressing cells (Figure 5B) and a corresponding increase in Annexin-V expression in NB-19 cells (Figure 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 pro-tumorigenic 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 in vitro decreased Sulf-2 expression (Figure 6 A). We also noted that, in the absence of doxycycline, there was a partial decrease in Sulf-2 expression in pKRAB-Sulf-2 transfected cells compared to pKRAB-SCR cells suggesting “leaky” expression of the shRNA in the absence of doxycycline, as reported by others (32). Downregulation of Sulf-2 in these cells by doxycycline treatment resulted in decreased viability in vitro (Figure 6 B). These cells were then injected subcutaneously at 2 sites in NOD/SCID mice and monitored for growth (Figure 6 C and D). 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 mm$^3$ (Figure 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 mm$^3$ of 28±3.7 days compared to a tumor formation rate of 75% and 100% and an average time for tumors to reach 500 mm$^3$ of 20±5 and 18±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 mm$^3$ of 25±3.2 days. This suggested that Sulf-2 expression may have been down regulated 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 (Figure 6 E) which revealed a statistically significant decrease in Sulf-2 expression in the pKRAB-Sulf-2 subgroup (average ΔCT of 12.7 ± 0.8) not treated with doxycycline (p=0.008). An analysis of the expression of Sulf-2 protein by immunohistochemistry (Fig. 6 F) confirmed the down regulation 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 down-regulation 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 (Figure 7A). A quantitative analysis by color deconvolution (Figure 7B) indicated much stronger expression of Sulf-2 in all MYCN-A tumors with levels 80-fold (95% CI 28 to 233) and 330-fold (95% CI 111 to 1014) 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 to 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 to tumors of patients with high risk disease, but lacking MYCN amplification (HR-NA), and of patients with low risk (LR) disease (p < 0.0001; Fig. 6C). Similar results were obtained from a second cohort of 162 samples obtained from children 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 (logrank 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 logrank 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 (RTK) (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. One possible candidate is NTKR2 (TRKB), which is abundantly expressed in MYCN-A tumors. Brain-derived neurotrophic factor (BDNF), the ligand for TRKB 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-TRKB interaction, the observation that Sulf-1 and Sulf-2
positively regulate glial cell-derived factor (GDNF)-mediated signaling and esophageal
innervation (40), suggests that Sulf-2 may regulate neurotrophic factor-mediated signaling.

The second important observation reported in this manuscript 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 down-regulated in 2
MYCN-A neuroblastoma cell lines treated with the bromodomain and extra-terminal domain
inhibitor JQ1, that downregulates MYCN transcription (43). The promoter regions of Sulf-2 have
not yet been elucidated but in preliminary studies we have identified a 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 anti-
tumorigenic, -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 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.
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References


Legends to figures:
Figure 1. Expression of sulfation modifying enzymes in neuroblastoma cell lines measured by qRT-PCR. The data represent the negative mean ΔCT ± S.E.M. [(value of gene – control value of GAPDH)] from 3 aliquots per sample from 3 separate experiments. Higher - ΔCT values represent higher levels of expression. MYCN-NA = non amplified; MYCN-A = amplified.

Figure 2. Sulf-2 is specifically overexpressed in MYCN-A neuroblastoma cell lines. A, Western blot analysis of Sulf-2 in 8 neuroblastoma cell lines. Lanes 1-4 are MYCN-NA cell lines and lanes 5-8 are MYCN-A cell lines. B, The presence of Sulf-2 (red) and HSPGs (green) in neuroblastoma cells in culture was examined by immunofluorescence (left panels, MYCN-NA cells; right panels, MYCN-A cells). Panels i and ii were stained with an anti-HS antibody (HS3A8V). Panels iii to vi were double stained with an anti-HS phage display antibody (HS3B7V) that specifically recognizes highly 6-O-sulfated epitopes (green) and with an anti-Sulf-2 antibody (red). DAPI was used to stain cell nuclei (blue). Scale bars are 20 μm.

Figure 3. Sulf-2 increases neuroblastoma cell survival and its expression are linked to MYCN. A, Left panel: 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 is representative of three independent experiments showing similar results. Right panel: CHLA-255 cells transfected as indicated in A, were examined for viability over time. The data represent the 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 expression (left panel) and viability (right panel) as shown in A. C. SHEP-21N cells were treated with doxycycline (Dox) and examined for Sulf-2 and MYCN expression by Western blot.
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 4.** Knock down of Sulf-2 expression in MYCN-A neuroblastoma cells results in loss of viability. SK-N-BE(2) and NB-19 neuroblastoma cells were transfected with siRNA for Sulf-2 and examined for survival and proliferation. A, Western blot analysis of Sulf-2 expression on cell lysates obtained 48 hours after transfection. B, Representative crystal violet stains of cells in culture 48 hours after transfection (top: SK-N-BE(2), bottom: NB-19). C, Cell viability was measured at indicated time. The data represent the mean (±SD) fluorescence units (RLU) of quadruplicate samples and are representative of 3 separate experiments showing similar results. (*p < 0.05; **p < 0.01; ***p < 0.0001). D, BrdU and PI analysis of NB-19 cells by flow cytometry. The data are representative of 2 experiments showing similar results. Top panel: Cells transfected with SCR; bottom panel: cells transfected with siRNA 1 + 2. E, Bar diagram of the distribution of cells in phases of cell cycle according to PI content from the experiments shown in D.

**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. The data represent the mean (±SD) luminescence units (RLU) of triplicate sample from one experiment and are representative of 3 independent experiments showing similar results. (**p< 0.01 and ***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 are mean ± S.E.M. of three independent experiments.
**Figure 6:** Loss of Sulf-2 expression in MYCN tumor cells inhibits tumor formation and growth. MYCN-A SK-N-BE(2) cells were stably transfected with pKRABSULF2 and pKRABSCR and sorted for GFP expression. A, Cells were treated with doxycycline at the indicated concentrations and analyzed for Sulf-2 expression by Western blot after 4 day. Ratios of Sulf-2 to tubulin are shown below the panels.B. Cellular viability over 4 days in culture. Values represent mean ± SD. C, Cells were injected in the right and left flank of NOD/SCID mice and monitored for development and growth. The graph represents the tumor diameter (in mm$^3$) of each tumor measured at the indicated times in the four groups. n represents the number of mice. Because each mouse was injected at 2 sites, there are more growth curves than number of mice. D. The graph represents the percentage of tumors that developed in the 4 groups of mice. E. qRT-PCR analysis for Sulf-2 mRNA expression in tumors harvested at time of sacrifice. F. Analysis of Sulf-2 expression by immunohistochemistry in sections of tumors derived from the 4 groups of mice. Scale bar = 20 um

**Figure 7:** Sulf-2 is overexpressed in MYCN-A primary human neuroblastoma primary 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) are MYCN-NA with FH. Scale bar = 20 μm. B, The data represent the mean (+SD) OD obtained from 10 fields (20x) for each tumor section from MYCN-A (n=22), MYCN-NA, UH (n=23) and MYCN-NA, FH (n=20) tumors; ****p < 0.0001. 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 3 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 GPOH group and *** = p < 0.0005 in 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.
Figure 1
Figure 2
**Figure 3**

A. Western blot analysis showing expression levels of Sulf-2, MYCN, and β-actin in CHLA-255 cells treated with different conditions:
- CHLA-255
- CHLA-255 + Sulf-2
- CHLA-255 + S2ΔCC

B. Cell viability (RLU) over days for SHEP and SHEP-21N cells treated with:
- SHEP
- SHEP + Sulf-2
- SHEP + S2ΔCC

C. Western blot analysis for SHEP and SHEP-21N cells treated with Dox.
- SHEP
- SHEP-21N

D. Graphs showing cell viability over days for:
- CHLA-255
- CHLA-255 + Sulf-2
- CHLA-255 + S2ΔCC

E. Graphs showing cell viability over days for:
- SHEP
- SHEP + Sulf-2
- SHEP + S2ΔCC

**Note:**
- Sulf-2
- MYCN
- β-actin

**Legend:**
- *P < 0.05
- **P < 0.001
Figure 4

A

SK-N-BE(2)

NB-19

B

Sulf-2

β-actin

Sulf-2

β-actin

C

SK-N-BE(2)

NB-19

Cell Viability (RLU)

Days

Cell Viability (RLU)

Days

D

FITC-A

7AAD-A (x 1,000)

P4 25.5%

47%

9.5%

E

% of cells

subG1

G2M

G1

S

 SCR

siRNA 1

siRNA 2

siRNA 1+2

 SCR

siRNA 1

siRNA 2

siRNA 1+2

 ***

* ***

***

Figure 4

on May 28, 2017.
**Figure 5**

A) SK-N-BE(2) and NB-19

- **Caspase 3/7 activity (RLU)**
  - **Days:** 0, 1, 2, 3
  - **Graphs:**
    - SK-N-BE(2) and NB-19
    - Lines represent different treatments:
      - SCR
      - siRNA 1
      - siRNA 2
      - siRNA 1+2
    - Activity levels:
      - SK-N-BE(2) at 6×10^6 RLU
      - NB-19 at 2.4×10^6 RLU
      - SK-N-BE(2) at 4×10^6 RLU
      - NB-19 at 1.6×10^6 RLU
      - SK-N-BE(2) at 2×10^6 RLU
      - NB-19 at 8×10^5 RLU

B) SK-N-BE(2) and NB-19

- **Western Blots**
  - **Proteins:**
    - Sulf-2
    - PARP
    - Cleaved PARP
    - β-Tubulin
    - Caspase-3
    - Cleaved Caspase-3
  - **Treatments:**
    - siRNA 1
    - siRNA 2
    - siRNA 1+2
    - SCR

C) NB-19

- **Annexin V positive cells (%)**
  - **Graphs:**
    - SCR, siRNA 1, siRNA 2, siRNA 1+2
  - **Values:**
    - SCR: 20%
    - siRNA 1: 30%
    - siRNA 2: 25%
    - siRNA 1+2: 45%
**Figure 6**

**A**

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**B**

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**C**

*shSCR no Dox*

Avg. 500 mm³ = 20 ± 5 days

n=4 mice

*shSCR with Dox*

Avg. 500 mm³ = 18 ± 2.3 days

n=5 mice

*shSulf-2 no Dox*

Avg. 500 mm³ = 25 ± 3.2 days

n=5 mice

*shSulf-2 with Dox*

Avg. 500 mm³ = 28 ± 3.7 days

n=5 mice

**D**

**E**

**F**

shSCR no Dox

shSCR with Dox

shSulf-2 no Dox

shSulf-2 with Dox

**Days post- injection**
Figure 7
MYCN-dependent Expression of Sulfatase-2 Regulates Neuroblastoma Cell Survival

Valeria Solari, Lucia Borriello, Gianluca Turcatel, et al.

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