Published in final edited form as:

Cancer Lett. 2016 February 28; 371(2): 214–224. doi:10.1016/j.canlet.2015.11.045.

MYCN controls an alternative RNA splicing program in high-risk metastatic neuroblastoma

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Data access

Sequence data used for this study are available at dbGaP under the accession number phs000868.v1.p1

Conflicts of interest: none

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Abstract

The molecular mechanisms underlying the aggressive behavior of MYCN driven neuroblastoma (NBL) is under intense investigation; however, little is known about the impact of this family of transcription factors on the splicing program. Here we used high-throughput RNA sequencing to systematically study the expression of RNA isoforms in stage 4 MYCN-amplified NBL, an aggressive subtype of metastatic NBL. We show that MYCN-amplified NBL tumors display a distinct gene splicing pattern affecting multiple cancer hallmark functions. Six splicing factors displayed unique differential expression patterns in MYCN-amplified tumors and cell lines, and the binding motifs for some of these splicing factors are significantly enriched in differentially-spliced genes. Direct binding of MYCN to promoter regions of the splicing factors *PTBP1* and *HNRNPA1* detected by ChIP-seq demonstrates MYCN controls the splicing pattern by direct regulation of the expression of these key splicing factors. Furthermore, high expression of *PTBP1* and *HNRNPA1* was significantly associated with poor overall survival of stage4 NBL patients ($p < 0.05$). Knocking down *PTBP1*, *HNRNPA1* and their downstream target PKM2, an isoform of pro-tumor-growth, result in repressed growth of NBL cells. Therefore, our study reveals a novel role of MYCN in controlling global splicing program through regulation of splicing factors in addition to its well-known role in the transcription program. These findings suggest a therapeutically potential to target the key splicing factors or gene isoforms in high-risk NBL with MYCN-amplification.

Keywords

neuroblastoma; splicing; MYCN-amplification; RNA-seq; RNA isoforms

Introduction

Pre-messenger RNA (pre-mRNA) splicing is the process of modifying primary messenger RNA into mRNA transcripts through intron removal and alternative exon usage. It is a universal phenomenon essential for regulating isoform expression in spliceosomes of all eukaryotic cells [24, 31, 44, 53], and is controlled by splicing factors[38]. The expression of different isoforms of a gene is determined by the transcription together with the splicing program [18]. Splicing factors regulate gene splicing in a tissue- and/or disease-specific manner, and some of them such as *FOX* and *hnRNP* family members are implicated in tumor initiation, progression and metastasis [6, 26, 33, 47, 52]. Using exon expression arrays and other high-throughput methods, several splicing profiles have been reported in human cancers including glioblastoma, colon, bladder, prostate, and lung cancers [6, 17, 49, 60]. With the advent of massively parallel sequencing technologies, it is now readily to

deconvolute the control mechanisms of RNA isoform expression through analyses of gene transcription and the splicing patterns of these genes in tumor samples.

Metastatic or stage 4 neuroblastoma (NBL) is characterized by heterogeneous clinical outcomes. Less than half of the patients older than 18 months with stage 4 disease survive, whereas those with *MYCN*-amplification (*MYCN*-A; 20–25% of all NBL) have an even worse outcome [11]. Stage 4S patients (2–5% of all NBL [1]), who are younger than 12 months of age, have a characteristic pattern of metastasis with rapid tumor growth followed by spontaneous regression and a good overall survival rate of >90% [27, 35]. Recent large-scale sequencing studies by our group and others [7, 29, 36, 40] have discovered only a few novel recurrent somatic mutations in NBL, with *MYCN*-A and *ALK* mutations being the most recurrent gene alteration events [14]. Despite *MYCN*'s well-studied roles in transcription, the effects of *MYCN* on RNA splicing have not been extensively studied. Previous expression studies by our group using exon microarrays demonstrated that tumors with and without *MYCN*-A have different splicing patterns [20]. However, the mechanisms underlying the *MYCN*-associated splicing signature have not been investigated.

In this study, we took advantage of massively parallel RNA sequencing to investigate the pattern of transcription and splicing of genes controlled by *MYCN*. We identified six splicing factors important in *MYCN*-A tumors and demonstrated that *MYCN* is responsible for a gene splicing pattern by directly controlling these splicing factors especially *PTBP1* and *HNRNPA1* in *MYCN*-A samples. Furthermore, we observed that high *PTBP1* and *HNRNPA1* expression is associated with poor survival in high-risk NBL patients ($p < 0.05$). Finally we demonstrated that knocking down of *PTBP1* and *HNRNPA1*, or their downstream target, the pro-tumor-growth isoform *PKM2*, resulted in suppressed proliferation in a *MYCN*-A neuroblastoma cell line.

Materials and Methods

RNA isolation and whole transcriptome library construction

Clinical features for the 29 pre-treated primary tumors are shown in the Supplemental Table S1. Total RNA was extracted, RNA-seq libraries were made and sequenced as previously described [55, 56]. The integrity of total RNA was evaluated using an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA), and only RNAs with RIN greater than 6.0 were used in this study.

RNA-seq reads alignment, differential expression and splicing analysis

First, 50 base nucleotide reads were filtered against a database of rRNA, tRNA, repetitive regions, and adapter sequences. The remaining reads were aligned to the reference human genome (hg 18) and splice junctions database using Bioscope v1.2 (ABI, Foster City, CA), with mapping mismatch penalty of -2.0 . Reads mapped to more than 10 different genomic locations were discarded.

We used Cufflinks 1.0.3 [50] for isoform abundance estimation based on Refseq hg18 for isoform sequences. Abundances in fragments per kilobase of exon per million fragments mapped (FPKM) were calculated for each annotated Refseq isoform. Isoforms expressed in

3 samples (n=29628) were included for principle component analysis using Partek software version 6.5 (Partek Inc., St. Louis, MO, USA). The cumulative proportion of the first 3 principle components was 34.6%. Proportion of variance: PC#1=13.3%, PC#2=12.3%, PC#3=9.02%.

Cuffdiff module of Cufflinks 1.0.3[50] was used to evaluate differential expression and differential splicing “TSS-genes”. In differential expression analysis, Cuffdiff compares the log ratio of “TSS-gene” expression in two conditions. Variance included the variability among biological replicates and uncertainty in the isoform expression estimation itself. “TSS-genes” with Benjamini-Hochberg FDR <0.25 and fold change >2 were selected as significantly differentially expressed. In differential splicing analysis, Jensen-Shannon divergence (JS; $0 \leq JS \leq 1$) was calculated for each cancer subtype by Cuffdiff, and a one-sided t-test was performed, with the null hypothesis that no alternative splicing causes relative abundances of isoform expression change. Differentially spliced “TSS-genes” were selected based on Benjamini-Hochberg FDR<0.05, and Jensen-Shannon divergence $\sqrt{JS}>0.2$.

MATS 3.0.8 [42] was used to determine differential splicing events in IMR5 cell line with PTBP1 and HNRNPA1 knockdown. Significant events were calculated using reads falling into Refseq annotated exon and splicing junctions. Differential splicing genes with FDR <0.05, and exon-inclusion difference >0.1 were used for downstream gene ontology analysis.

Gene ontology analysis

Gene ontology analysis was performed and visualized using Cytoscape [9] and ClueGO [2]. Gene symbols for DEG and DSG lists were inputted into the ClueGO. Enriched GO terms in biological process were calculated based on right-side hypergeometric test with Benjamini-Hochberg adjusted p-value<0.05. Enriched GO terms were grouped in networks based on their shared genes with GO term connection using ClueGO.

Splicing motif enrichment analysis

Exactly 200 nt upstream and downstream intron sequences of differentially spliced exons between 4S and MYCN-A subtypes were analyzed for enriched motifs. Oligo-analysis module of Regulatory Sequence Analysis Tools [48] (RSAT) was used for motif enrichment analysis. Second order Markov chain model was used for background model. P-value of over-representation of the motif occurrence and adjusted E-value were calculated as described by Helden *et al* [51].

Exon microarray hybridization and analysis

We used Affymetrix Human Exon 1.0 array to measure the exon expression level of tumor samples. Ribosomal RNA was removed from total RNA using a RiboMinus Transcriptome Isolation Kit (Human/Mouse) and processed to generate the labeled targets using an Affymetrix GeneChip WT Sense Target Labeling and Control Reagents. After hybridization of the labeled targets to the probes, the array was stained and scanned as per manufacturer’s instructions. The probe-level analysis was performed using R/Bioconductor packages:

aroma.affymetrix, biomaRt and Genomegraphs with the workflow described by Rodrigo-Domingo *et al* [39].

Luciferase reporters under PTBP1 or HNRNPA1 promoter

Luciferase reporter constructs under wild-type (pGL3-A1p) or mutant (pGL3A1pMu) HNRNPA1 promoter were obtained from Dr. Manley [13]. Human *PTBP1* promoter between genomic sequences GTGCTTTCTATCCTGCTGGC and GACAGTGCCCGAACAAC (chr11:797698+798124; hg19) was cloned into the pGL3-enhancer vector (Promega, Madison, WI) between the XhoI and HindIII restriction sites. Two-base inversion mutations were introduced to both E-box sites within the *PTBP1* promoter by mutagenesis to generate the mutant form of the reporter (GenScript, Piscataway, NJ). All *PTBP1* reporter vectors were verified by Sanger sequencing. Luciferase reporters were transfected into SK-N-AS cells with a β -galactosidase control plasmid using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). A MYCN expression vector under a CMV promoter was obtained from Origen (Rockville, MD). Luciferase activities were measured as described previously [57].

Cell culture, RNAi and western blot

Cell culture—NBL cell line *MYCN*-3 with a tetracycline-controlled MYCN expression construct was cultured and induced at 9 hours as described by Wei *et al* [57]. IMR32 were cultured in EMEM, IMR5 and SK-N-AS were cultured in RPMI-1640 (Quality Biological) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37°C in 5% CO₂. Cell lines were routinely checked for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, Walkersville, MD).

MYCN knockdown using siRNA

Non-silencing AllStars Negative Control siRNA (Qiagen, Valencia, CA) was used as negative control. siRNA was transfected into NBL cells by using Nucleofector (Amaxa Biosystems, Gaithersburg, MD) solution L following the manufacturer's protocol. IMR32 cells were washed and collected into cold PBS at 48 hours post-transfection, and the cell pellets were either used for protein or RNA extraction. Total protein lysates were prepared by re-suspending cells in RIPA buffer containing Halt Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) and EDTA. After extraction, suspensions were centrifuged for 10 min at 14,000 rcf at 4 °C and the protein supernatant was collected and quantified using a Bradford protein assay (Sigma-Aldrich, St. Louis, MO). We used two different MYCN siRNAs purchased from Qiagen (SI00076293, Hs_MYCN_2 and SI00076307, Hs_MYCN_4, Valencia, CA).

Knockdown of PTBP1 and HNRNPA1 in neuroblastoma cells

Reverse transfection was done using Lipofectamine RNAiMAX reagent (Life Technologies, Carlsbad, CA). In a clear bottom 6-well plate (Corning, Tewksbury, MA), 2 mL of Opti-MEM (Life Technologies, Carlsbad, CA) was dispensed into each well with 10–20 μ L of Lipofectamine RNAiMAX and a final concentration of 20 nM for each siRNA. All siRNAs

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were previously described [3, 19] and AllStars Hs Cell Death Control siRNA (Qiagen, Valencia, CA) was used as a positive control. All siRNAs were diluted in nuclease-free water (Life Technologies, Carlsbad, CA). The siRNA and Lipofectamine RNAiMAX was allowed to complex for 30–45 minutes in the well at room temperature. Then 2 mL of IMR5 or SK-N-AS cells in 20% FBS was seeded into each well so that the cells would reach 80% confluency after 48–72 hours. Confluence was monitored over time using the IncuCyte (Essen Bioscience, Ann Arbor, MI). Each data point is an average of confluence taken from 16 images. Confluence and cell number is assumed to be linear when cell morphology stays the same and full confluence is not achieved. SiRNA sequences targeting PTBP1 and HNRNPA1 were listed in Supplemental Table S4.

Western blotting

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After 48–72 hours siRNA transfection, cells were lysed in 50–100 μ L of RIPA buffer (Thermo Fisher Scientific, Waltham, MA) with 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA). Protein was quantified by BCA assay (Thermo Fisher Scientific, Waltham, MA) and reduced in 2 $\times$ SDS Protein Gel Loading Solution (Quality Biological, Gaithersburg, MD) containing 5% β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Samples were boiled at 95°C for 10 minutes and then 5–10 μ g of protein was loaded into 4–12% Tris-Glycine polyacrylamide gels (Life Technologies, Carlsbad, CA). The resolved protein was transferred to 0.2 μ m nitrocellulose membranes using the iBlot (Life Technologies, Carlsbad, CA). Membranes were blocked in room temperature with 5% nonfat dry milk in TBS (Corning, Tewksbury, MA) with 0.1% Tween-20 for one hour and subsequently probed overnight in 5% BSA (Sigma Aldrich, St. Louis, MO) in TBST at 4°C in the following antibodies: PTBP1 (1:1,000 Cell Signaling Technology #8776, Danvers, MA), hnRNPA1 (1:1,000 Cell Signaling Technology #8443, Danvers, MA), and β -actin (1:10,000 Sigma Aldrich #A1978, St. Louis, MO). Membranes were washed 3 times with TBST and then incubated in HRP-conjugated goat anti-mouse (1:10,000 Santa Cruz Biotechnology sc-2005, Dallas, TX) or goat anti-rabbit (1:10,000 Santa Cruz Biotechnology sc-2004, Dallas, TX) secondary antibodies in 1% nonfat dry milk in TBST for 1 hour at room temperature. Membranes were washed 3 times in TBST again and then bound antibodies were detected using SuperSignal (Thermo Fisher Scientific, Waltham, MA) and chemiluminescence was detected on CL-XPosure Film (Thermo Fisher Scientific, Waltham, MA).

RT-qPCR

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Quantitative RT-PCR was performed using Taqman assays (Life Technologies, Carlsbad, CA) on a Fluidigm system as previously described [46]. Briefly, cDNA was generated from 200 ng of RNA using reverse transcription. Then PCR was carried out on a 48 $\times$ 48 dynamic array using BioMark HD real-time PCR system (Fluidigm, San Francisco, CA). Triplicates were performed for each gene for every sample, and average threshold cycle (C_t) numbers were calculated. Gene expression levels were represented by normalized C_t ($-C_t$) against GAPDH. Primer catalog number and customized primer sequences used to amplify cDNAs are listed in Supplemental Table S5.

Chromatin Immunoprecipitation sequencing (ChIP-seq)

ChIP-seq experiment was performed on MYCN3 cell line either untreated (low MYCN) or treated with doxycyclin (high MYCN) as previously described [43]. Reads were mapped against human reference genome and stored using Genboree discovery platform [43]. The mapped ChIP-seq database was further visualized using UCSC genome browser (HG19) for MYCN binding at gene promoters or intergenic regions and E-box motifs at binding sites. Peaks were called using MACS [64] software, and followed by HOMER for genome feature enrichment analysis and GO term annotation.

Results

RNAseq of NBL samples

In order to investigate the role of MYCN on transcription and splicing control of transcripts, we performed massively parallel RNA sequencing (RNA-seq) on 29 stage 4 NBL tumors (10 *MYCN* amplified (*MYCN*-A), 10 *MYCN*-not-amplified (*MYCN*-NA), and 9 4S; Supplemental Table S1). The medium number of reads mapped to the genome was 103.7 million per sample (Supplemental Figure S1A), with 35.5% to the coding bases and UTRs, and 1.5% to the exon-exon junctions (Supplemental Figure S1B). We used random priming for construction of RNA-seq libraries for an even coverage of genes from their 5' to 3' ends to ensure unbiased detection of different transcripts of a gene (Supplemental Figure S1C).

NBL subgroups exhibit differential expression pattern at transcript level

The expression of each transcript isoform is the combined result of both transcription and splicing programs (Supplemental Figure S2). To distinguish the transcription versus splicing of isoform expression, we first grouped transcript isoforms transcribed from the same transcription starting site (TSS) as "TSS-genes", which are controlled by the same transcriptional machinery. Thus the sum of isoforms expressed from the same "TSS-gene" reflects transcription control. By contrast, differences in expression ratios of isoforms spliced from the same "TSS-gene" are the results of the splicing control [18]. There were 29628 transcripts detected in 3 samples representing 20486 TSS-genes. Of 5424 TSS-genes having more than 1 isoform, the average number of isoforms for each TSS-gene was 2.6 ± 1.1 (SD).

In order to examine if there is any specific isoform expression pattern among the stage 4 NBL subgroups, we next performed a principle component analysis (PCA) using all 29628 transcript isoforms. The complete separation of 4S and *MYCN*-A clusters demonstrated distinct expression patterns of gene expression between the two groups (Figure 1A). Interestingly, *MYCN*-NA samples fall between the 4S and *MYCN*-A clusters suggesting varied transcript expression for this subgroup probably due to their heterogeneous nature of these tumors [20, 29].

Differential transcription and splicing among NBL tumors

To dissect out the transcription and splicing controls of the transcript expression, we first examined the transcription control by differential expression analysis of "TSS-genes". The 4S and *MYCN*-A group comparison resulted in the highest number of differentially

expressed genes (860 DEGs), reflecting the diverse gene expression between 4S and *MYCN*-A tumors. In contrast, fewer “TSS-genes” were differentially expressed between *MYCN*-NA and 4S group (124 DEGs), or between *MYCN*-NA and *MYCN*-A group (93 DEGs, Figure 1B), possibly due to the heterogeneity among *MYCN*-NA samples.

We next examined the splicing program by differential splicing analysis using 5424 “TSS-genes” which have more than one annotated isoform detected in 3 samples. The largest difference was again seen in the 4S and *MYCN*-A groups comparison, with 460 differentially spliced “TSS-genes” (DSGs); whereas there were 308 and 332 DSGs in 4S vs. *MYCN*-NA, and *MYCN*-NA vs. *MYCN*-A comparisons respectively (Figure 1B). Notably, very few “TSS-genes” overlapped between DEGs and DSGs in their respective comparisons, indicating that only a small portion of “TSS-genes” were differentially regulated by both transcription and splicing programs.

In order to characterize tumors with the most extreme clinical outcomes, and to avoid reduced statistical power caused by large sample-to-sample variance among *MYCN*-NA tumors, we used 4S tumors as control to perform ontology (GO) enrichment analysis for the DEGs and DSGs in *MYCN*-A tumors. DEGs up-regulated in the *MYCN*-A tumors were enriched with genes involved in the cell cycle and DNA metabolism (Supplemental Figure S3A), whereas DSGs in *MYCN*-A tumors were enriched with genes of cell adhesion, cell cycle, immune system process, development, and programmed cell death (Supplemental Figure S3B). Thus, our data strongly suggest that transcription and splicing processes control different biological processes specific to their respective biology in NBL tumors.

Splicing factors that is differentially expressed in *MYCN*-A tumors

To identify the splicing factors responsible for the splicing signatures in these tumors, we searched for genes associated with RNA splicing (n=318; Gene Ontology: 0008380) and also differentially expressed in the *MYCN*-A tumors. Six splicing factors *RBFOX1*, *RBFOX3*, *CELF2*, *CELF6*, *PTBP1* and *HNRNPA1* showed significant differential expression in *MYCN*-A tumors (Figure 2A). Except for *RBFOX3* (the probes for which were not represented on the array), we were able to validate our findings in an independent cohort consisting of 151 stage 4 NBL tumors using exon array (http://target.nci.nih.gov/dataMatrix/TARGET_DataMatrix.html) (Supplemental Figure S4A–E). Thus, these splicing factors are likely responsible for the differential splicing pattern seen in the *MYCN*-A tumors.

In addition, we performed an *in-silico* analysis for the enrichment of the splicing factors’ binding motif sequences in the flanking introns of the differential spliced exons for DSGs. We found that the binding motif of the CELF family and PTBP1 family were significantly enriched in the adjacent intron in *MYCN*-A tumors (FDR<1.0E-3, Figure 2B). There are more than 100 different binding motifs reported for HNRNPA1 in the literature [34], as a result, no consensus sequence was meaningful for the HNRNPA1 binding motif analysis. The enrichment of splice binding sites upstream or downstream of alternatively spliced exons provide compelling evidence that splicing factors of the PTBP1, and CELF family have important roles in controlling the splicing program in NBLs.

MYCN directly regulates PTBP1 and HNRNPA1 in NBL

To experimentally examine if MYCN regulates the expression of these six splicing factors, we perturbed MYCN protein level and monitored the change of expression levels of these splicing factors using a MYCN inducible cell line (MYCN-3) [45, 57], and knockdown of MYCN in a MYCN-A NBL cell line (IMR-32) using 2 independent siRNAs. Among the six splicing factors, *PTBP1* and *HNRNPA1* showed positive correlation with MYCN expression (Figure 3A, B), whereas the other four splicing factors showed expression change only in one of the MYCN perturbation experiments (Supplemental Figure S5); indicating that *PTBP1* and *HNRNPA1* are likely the direct downstream targets of MYCN.

To test if MYCN directly controls these two splicing factors, we examined the binding of MYCN to the genomic loci of these splicing factors using chromatin immunoprecipitation sequencing (ChIP-seq) on a MYCN inducible cell line (MYCN-3) [43]. Of the 12754 peaks identified by ChIP-seq, 3584 (28.1%) were localized in the promoter regions which were significantly enriched from the genome (2.4% of human genome are of promoter, $p < 0.001$). Genes with peaks in promoter region were selected for biological process/pathway enrichment. Interestingly, RNA processing, spliceosome and mRNA splicing pathways were among the top enriched biological process/pathways using Gene Ontology, KEGG and REACTOME databases, suggesting the regulatory role of MYCN in mRNA splicing program in NBL (Supplemental Table S2; Splicing-related genes (n=142) showing MYCN binding at their promoter regions are listed in the Supplemental Table S6). At the loci of *PTBP1* and *HNRNPA1* genes near their promoter region, there are enriched MYCN binding signals, indicating the expression of these two splicing factors is directly controlled by MYCN (Figure 3C, D; left panels). This observation is consistent with a previous study reporting up-regulation of *PTBP1* and *HNRNPA1* by MYC [13]. However, we did not detect MYCN binding at the loci of *RBFOX3*, *RBFOX1*, *CELF2*, or *CELF6* (Supplemental Figure S6A–D), suggesting MYCN indirectly regulates the expression of these factors. Finally, in order to confirm that MYCN directly regulates the expression of splicing factor *PTBP1* and *HNRNPA1*, we performed the luciferase reporter assays in a MYCN-NA neuroblastoma cell line SK-N-AS using expression vectors under the control of *PTBP1* or *HNRNPA1* promoter. Luciferase assays clearly demonstrated that wild-type promoter reporters were responsive to the overexpression of ectopic MYCN in a dose-dependent manner; whereas promoters with mutant E-boxes significantly lost responsiveness to the ectopic expression of MYCN (Figure 3C and 3D; right panels). Therefore, we concluded that *PTBP1* or *HNRNPA1* are under the direct transcriptional control of MYCN.

Expression of splicing factors predict clinical outcomes in NBL patients

To evaluate the clinical relevance of the splicing signature in high-risk NBLs mediated by these splicing factors, we examined whether the expression of splicing factors predict patients' survival. Indeed, high expression of *PTBP1* and *HNRNPA1* predicted poor survival in a cohort of stage 4 NBLs (http://target.nci.nih.gov/dataMatrix/TARGET_DataMatrix.html, n=150, Figure 4A–B). In order to avoid the confounding effect of MYCN-amplification on survival, we further performed the survival analysis in patients with only MYCN-NA stage 4 tumors (n=103). Interestingly high expression of *PTBP1* still predicts poor outcomes for this group of patients ($p=0.03$, Figure 4C), suggesting its

important function of this splicing factor in neuroblastoma. PTBP1 has been reported to be regulated by MYC, a MYCN homolog, in NIH-3T3 cells [13], and high MYC pathway activity has been associated with poor outcome of MYCN-NA NBL [16]. Indeed, the expression of *PTBP1* mRNA was positively correlated with that of *MYC* (Figure 4D, $p=0.05$), but not with *MYCN* (Figure 4D, $p=0.4$) in these MYCN-NA tumors. Remarkably, the differentially expressed genes between PTBP1-high and PTBP1-low samples were significantly enriched with the MYC direct targets in a gene set enrichment analysis (Figure 4E, gene set: DANG_REGULATED_BY_MYC_UP [63], FDR<0.001; Figure 4F, gene set: PID_MYC_ACTIVPATHWAY [41], FDR<0.001), supporting the adverse effects of MYC in these poor outcome patients without MYCN amplification.

Knockdown PTBP1 and HNRNPA1 result in growth suppression of NBL cells

To test if splicing factor PTBP1 and HNRNPA1 are important for MYCN driven neuroblastoma cells, we knock them down using RNA interference in neuroblastoma cell lines. Combined knockdown of PTBP1 and HNRNPA1 suppressed proliferation of MYCN-A IMR5 cells (Figure 5A–B); whereas the same combined knockdown had a much milder suppression of the growth in MYCN-NA SK-N-AS cells (Supplemental Figure S7A–B). In addition, RNA-seq showed that 199 genes displayed differential splicing upon PTBP1 and HNRNPA1 knocking down in IMR5 cells (FDR<0.05, exon inclusion difference >0.1), with exon skipping event to be the most frequent event (Supplemental Figure S8). Interestingly the differentially splicing genes were enriched in neuron development and cell cycle process (Gene Ontology analysis, FDR<0.01, Figure 5C), indicating the splicing program may modulate these two important cell functions in this neuroblastoma cell.

PKM splicing is a major target of splicing of PTBP1 and HNRNPA1

The top most differentially spliced and highly expressed genes upon PTBP1 and HNRNPA1 knockdown was pyruvate kinase gene (PKM) (Figure 6A). Switching from PKM1 expressed commonly in differentiated tissues to its oncofetal counterpart PKM2 often occurs in actively growing human cancers [59], and splicing factors in the PTB and HNRNP families are reported to mediate the *PKM* isoform switching [10]. We found that *PKM* showed a significant switch from exon10 (PKM2) to exon9 (PKM1) usage (FDR<1E-13) followed by knockdown of the both splicing factors (Figure 6A–C, Supplemental Table S3). It is also one of the most differentially spliced DSGs in the stage 4 MYCN-A tumors. In addition, PKM exon10/exon9 ratio is correlated with the combined expression of PTBP1 and HNRNPA1 in NBL tumors (Supplemental Figure S9, $\text{cor}=0.42$, $p=9.0\text{E-}8$), as well as the ratio was significantly higher in stage 4 MYCN-A tumors compared to MYCN-NA tumors (Figure 6D, $p=0.0026$). Furthermore, the exon10/9 ratio of PKM also significantly associated with worse outcome in all neuroblastoma ($p=0.005$; Figure 6E) and in stage 4 MYCN-NA tumors ($p=0.001$; Figure 6F). This is in keeping with high expression of the tumorigenic isoform PKM2 which promotes tumor-favoring aerobic glycolysis in cancer including neuroblastoma [13, 20].

Suppression of PKM2 isoform leads to growth inhibition in MYCN-amplified cells

Since *MYCN*-amplification is predicted to increase expression of PKM2 and is associated with adverse outcome, we used two validated siRNAs [19] that target exon 10 of PKM to specifically knockdown PKM2 isoform in IMR5 and SK-N-AS cells. Western blot confirmed that, while the level of total PKM was intact, PKM2 levels were significantly reduced for both NBL cell lines using two independent siRNAs (Figure 6G, H).

Interestingly, knockdown of PKM2 expression suppressed proliferation of IMR5 cells (Figure 6G), whereas had no significant effect on *MYCN*-NA SK-N-AS cells (Figure 6H), suggesting *MYCN*-A cancer cells may be more sensitive to the suppression of PKM2.

Discussion

Differential isoform expression had been difficult to study prior to the advent of massively parallel sequencing technologies. Single nucleotide resolution of RNA-seq reads allow mapping to specific transcription start sites and splicing boundaries, making it feasible to deconvolute transcriptional control and splicing regulation for individual transcripts on a genome wide scale. In this study, we examined the transcriptome of stage 4 NBL tumors for the aberrations that would alter isoform expression. Our study is the first systematic attempt to examine the effects of splicing factors on transcript isoform expression in stage 4 NBL, which provides insight into how *MYCN* controls the expression of splicing factors, which in turn regulates a splicing signature at a genome-wide level in neuroblastoma tumors.

In agreement with a previous study on the NBL splicing program using exon arrays [20], we identify a splicing signature in *MYCN*-A tumors. Although the function of most of the spliced isoforms has not been elucidated, the differential spliced genes are enriched in biological processes such as programmed cell death and differentiation. Six splicing factors are found to be differentially expressed in *MYCN*-A tumors. In particular, we find by ChIP-seq, gene expression analysis and luciferase reporter assays that *MYCN* potentially directly regulates the expression of the splicing factors *PTBP1* and *HNRNPA1*. This finding is in keeping with the previous studies linking *MYC* to splicing factors *PTBP1* and *HNRNPA1* in HeLa cells and mouse embryonic fibroblast [13].

We found a significant association of high expression of *PTBP1* and *HNRNPA1* with poor survival in stage 4 NBL patients, whereas *PTBP1* expression showed the more significant difference in outcome. Interestingly we found that high *PTBP1* expression levels was associated with adverse outcome in stage 4 *MYCN*-NA NBL patients, and global gene expression analysis revealed that the difference between the two clinical subgroups was associated with a *MYC* signature. This indicates that even in stage 4 *MYCN*-NA NBL patients adverse outcome may be driven by high *MYC* activity.

We find that knockdown of the splicing factors *PTBP1* and *HNRNPA1* suppresses the proliferation of a *MYCN*-A IMR5 cell line, as well as a *MYCN*-NA SK-N-AS cell line albeit to a lesser extent, indicating that a further exploration is warranted to target these splicing factors as potential novel therapies for NBL patients. Differentially splicing genes upon *PTBP1* and *HNRNPA1* knockdown are enriched in neuron development and cell cycle processes, which is in line with known functions of these two splicing factors. Previous

studies have showed that repressing PTBP1 expression enhances neuron differentiation [37, 61]. Moreover, PTBP1 is involved in aberrant splicing in glioblastoma and breast cancer [15, 58]. Mutations in HNRNPA1 cause multisystem proteinopathy and ALS [25], and the function of this gene is linked to muscle differentiation and cell survival [22, 62]. Similar to PTBP1, HNRNPA1 is up-regulated in hepatocellular carcinoma and prostate cancer, which result in aberrant splicing in tumors [5, 30].

Furthermore, the most significantly differentially spliced and highly expressed genes upon PTBP1 and HNRNPA1 knockdown in IMR5 cells is pyruvate kinase (PKM). We observe a decreased expression of oncogenic exon10 (specific to the isoform PKM2), and an increased expression of exon9 (specific to the isoform PKM1) when repressing PTBP1 and HNRNPA1. PKM2 is a key regulator in cell metabolism by mediating glycolysis which favors cancer cell growth known as Warburg effect [8, 28]. It is known to be controlled by splicing factors PTBP1 and HNRNPA1 [4, 13], and is up-regulated in various cancer types [59]. Higher exon10/exon9 ratio is observed in *MYCN*-A tumors, and the high ratio of exon10/exon9 correlates with poor survival in stage 4 NBL patients. Knockdown of oncogenic isoform PKM2 suppressed growth of *MYCN*-A IMR5 cell line, but not *MYCN*-NA SK-N-AS cells up to 48 hours, suggesting *MYCN*-A neuroblastoma may be more vulnerable to inhibition of abnormal splicing program. One possibility is that hypoxia condition is required to influence cell proliferation by reduced PKM2-mediated glycolysis in fast growing SK-N-AS cells [21]. Another possibility is that there are alternative glycolysis metabolic pathways, such as the one activated by PKM1 in PKM2 null breast cancer model [23], as suggested in recent studies [12, 23]. Nevertheless, apoptosis is induced in multiple tumor cell lines and xenograft models using siRNAs targeting isoform-specific exon of PKM2 [19]. Additionally, multiple small molecule PKM2 activators, which induce PKM2 to obtain the similar enzymatic activity of PKM1, show antitumor properties *in vivo* and *in vitro* in preclinical studies [32, 54]. Taken together, targeting oncogenic isoform PKM2 is a potential therapeutic target in NBL patients.

In summary, our RNA-seq study reveals a unique gene splicing signature driven by *MYCN* or *MYC* through key splicing factors in neuroblastoma. Our findings highlight the important role of *MYCN* in regulation of a splicing program in addition to their well-known transcription regulation in cancer. Although the functions of many of the alternative spliced isoforms are not known, we show that *MYCN* is an upstream player of Warburg effect in the *MYCN*-PTBP1/ HNRNPA1-PKM2 pathway. The identification of the key splicing factors and their downstream oncogenic isoforms in neuroblastoma may lead to novel therapies in these aggressive *MYC* or *MYCN* driven cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Children's Oncology Group for the collection of samples for this study. This study utilized the high-performance computational capabilities of the Biowulf Linux cluster at the National Institutes of Health (<http://biowulf.nih.gov>).

Abbreviations

NBL	neuroblastoma
pre-mRNA	pre-messenger RNA
MYCN-A	MYCN-amplified
MYCN-NA	MYCN-not-amplified
RNA-seq	RNA sequencing
PCA	principle component analysis
DEG	differentially expressed genes
DSG	differentially spliced genes
ChIP-seq	Chromatin Immunoprecipitation sequencing

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Highlights

- A characteristic isoform expression pattern is detected in *MYCN*-amplified NBL tumors
- Several splicing factors control the splicing program in NBL tumors
- *MYCN* directly controls the expression of splicing factors *PTBP1* and *HNRNPA1*
- Splicing factors *PTBP1* and *HNRNPA1* and their downstream splicing target *PKM2* affect *MYCN*-amplified NBL cell proliferation

Figure 1. Transcriptional and splicing programs control isoform expression

A. PCA analysis of all transcripts shows separation of NBL subgroups. First three principle components were calculated using isoform expressions of 29 primary tumors. Red spheres represent MYCN-A samples; blue MYCN-NA samples; and green 4S samples. **B.** Differentially expressed genes (DEGs) and differentially spliced genes (DSGs) among stage 4 NBL subgroups. Numbers of DEGs and DSGs are plotted in pair-wise comparisons of three NBL tumor subtypes.

Figure 2. Differentially expressed splicing factors in MYCN-A tumors
A. Six splicing factors *RBFOX1*, *RBFOX3*, *CELF2*, *CELF6*, *PTBP1* and *HNRNPA1* are differentially expressed between tumor subtype *MYCN-A* to 4S and/or *MYCN-NA*. Box-plot diagrams show the minimum and maximum values with the quartiles' expression levels in samples of each NBL subgroup. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **B.** 200 nt upstream and downstream intronic regions of alternatively spliced exons in various *in vitro* and *in vivo* conditions were tested for splicing factors binding motif sequences enrichment. $-\log_{10}(\text{FDR})$ value was shown here and used for heatmap plot.

Figure 3. MYCN directly regulates splicing factors PTBP1 and HNRNPA1

A. MYCN perturbation alters the level of splicing factor expression. Venn diagram shows the positively-correlated splicing factor expression with MYCN induction or MYCN siRNAs knockdown. **B.** Real-time RT-PCR validation of the differentially expressed splicing factors PTBP1 and HNRNPA1 upon MYCN induction and MYCN siRNAs knockdown. Mean expression of genes is plotted. Error bars represent standard deviation. ChIP-seq (left panels) show MYCN binding to the promoter regions (red tracks) of *PTBP1* (**C**) and *HNRNPA1* (**D**). Actin antibody was used as a negative control (green tracks). Luciferase reporter assays (right panels) demonstrate that wild-type *PTBP1* (pGL3-PTBP1, **C**) and *HNRNPA1* (pGL3-A1p, **D**) promoters are directly controlled by MYCN; whereas mutant forms of these promoters (Mu) greatly attenuate the effect of MYCN.

Figure 4. High expression of PTBP1 and HNRNPA1 are correlated with worse overall survival in stage 4 NBL patients

High expression of **A. PTBP1** and **B. HNRNPA1** predicts overall survival in all 150 stage 4 NBL patients (log-rank test; $p=0.0095$ and $p=0.05$ respectively). **C. PTBP1** expression still predict outcome for 103 stage 4 MYCN-NA NBL patients (log-rank test, $p=0.03$). **D. MYC** is differentially expressed between PTBP1-high and PTBP1-low stage 4 MYCN-NA NBLs ($p=0.05$, mean $\pm$ SD). **E. and F.** Gene set enrichment analysis (GSEA) plots of ranked genes

of PTBP1-high samples compared to PTBP1-low samples show significant enrichment of *MYC* signatures (FDR<0.001).

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Figure 5. Knockdown of splicing factors PTBP1 and HNRNPA1 suppresses the growth of MYCN-A IMR5 cell line

A. IMR5 cell proliferation was monitored over 72 hours after double knockdown of splicing factors PTBP1 and HNRNPA1 using siRNAs (n=2, mean±SEM). **B.** Western blots show effective knockdown of PTBP1 and HNRNPA1 at protein level. **C.** Neural development and cell cycle process are the main functions of the differentially spliced genes after the knockdown of splicing factors. RNA-seq was used to profile gene expression after knockdown of the two splicing factors. Gene ontology networks show enriched gene ontology of differentially spliced genes detected upon PTBP1 and HNRNPA1 knockdown.

Figure 6. Knockdown of PKM2 suppresses cell growth of MYCN-A cell line

A. PKM is the highest expressed gene in IMR5 cells among the differentially spliced genes. RNA-seq was performed on IMR5 transcriptome and differentially spliced genes are ranked from highest to the lowest expression. **B.** Sashimi plot shows RNA-seq tracks upon PTBP1 and HNRNPA1 knockdown. The height of peaks indicates the expression level of exons and curved lines represent exon-exon boundaries. **C.** RNA expression ratio of Exon10/Exon9 was altered by knockdown of splicing factors indicating isoform switch from PKM2 to PKM1. Western blot of PKM2 and total PKM upon PTBP1 and HNRNPA1 knockdown

showed decreased PKM2 level. β -actin was used as a loading control. **D.** Higher PKM exon 10/exon 9 ratios were observed in stage 4 *MYCN*-A tumors (n=150). **E.** PKM exon10/exon9 ratio significantly predicts overall survival in all 150 stage 4 NBL patients, and **F.** in 103 stage 4 *MYCN*-NA NBL patients (log-rank test, $p=0.005$, $p=0.001$). **G.** IMR5 cell proliferation was suppressed by specific knockdown PKM2. Cell growth was monitored over 48 hours after PKM2 and control siRNA transfections. Western blot demonstrated decreased PKM2 protein level after 48 hours of PKM2 and control siRNA transfection in IMR5 cell line. β -actin was used as a loading control. **H.** SK-N-AS cell was not affected by PKM2 knockdown. The proliferation was monitored over 48 hours after PKM2 and control siRNA transfections. Western blot showed a decreased PKM2 protein level after 48 hours of PKM2 and control siRNA transfection on SK-N-AS cell line. β -actin was used as a loading control.