CASC15-S Is a Tumor Suppressor IncRNA at the 6p22 Neuroblastoma Susceptibility Locus

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Abstract

Chromosome 6p22 was identified recently as a neuroblastoma susceptibility locus, but its mechanistic contributions to tumorigenesis are as yet undefined. Here we report that the most highly significant single-nucleotide polymorphism (SNP) associations reside within CASC15, a long noncoding RNA that we define as a tumor suppressor at 6p22. Low-level expression of a short CASC15 isoform (CASC15-S) associated highly with advanced neuroblastoma and poor patient survival. In human neuroblastoma cells, attenuating CASC15-S increased cellular growth and migratory capacity. Gene expression analysis revealed downregulation of neuroblastoma-specific markers in cells with attenuated CASC15-S, with concomitant increases in cell adhesion and extracellular matrix transcripts. Altogether, our results point to CASC15-S as a mediator of neural growth and differentiation, which impacts neuroblastoma initiation and progression.

Introduction

Neuroblastoma, a cancer of the developing autonomic nervous system, is the most common malignancy diagnosed in the first year of life and accounts for approximately 10% of all pediatric cancer mortality (1–3). While the majority of low-risk neuroblastoma patients are cured with surgery alone, 50% of patients have the high-risk form of the disease, and only about half of these children survive despite highly intensive therapy (1). Neuroblastomas are thought to develop from cells derived from the neural crest committed to the sympathoadrenal lineage (peripheral autonomic nervous system; refs. 1–4). Because malignant transformation can occur at any point during sympathetic development, tumors may arise throughout the developing sympathetic nervous system (most commonly the adrenal gland), contributing to the hallmark heterogeneity observed in this disease (4). To address the etiology of sporadic neuroblastoma, we conducted the first pediatric cancer genome-wide association study (GWAS), leading to the identification of numerous validated susceptibility loci in several populations (5–12). Moreover, we showed that many of these susceptibility alleles are specifically associated with disease phenotype and patient outcomes. The majority of these SNPs act in cis to influence expression of protein coding genes at these loci, and several of these transcripts, such as LMO1, BARD1, and LIN28B, appear to play an oncogenic role in established tumors (5–12).

The first identified neuroblastoma susceptibility locus identified by GWAS, and the one that remains most significant, mapped to chromosome 6p22.3 and robustly replicated in three independent cohorts [rs6939340: P = 9.33 × 10−13, allelic OR 1.97, 95% confidence interval (CI), 1.58–2.45; ref. 5]. Like other subsequently identified loci, we observed a highly significant association with neuroblastoma susceptibility and clinically aggressive presentation. The minor allele (G) was over represented in neuroblastoma cases compared with controls, and presence of the G allele was further enriched in the high-risk subset of neuroblastoma (P = 0.007), tumors with MYCN amplification (P = 0.002) and stage IV disease (P = 0.025), implying the risk alleles were associated with a more malignant neuroblastoma phenotype. On the basis of HapMap data available at the time of this initial discovery, the associated SNPs tagged a 94.2 kb linkage disequilibrium (LD) block; this LD block overlapped two hypothetical genes (FLJ22536 and FLJ144180; ref. 5). However, both FLJ22536 and FLJ144180 lacked protein-coding potential, impeding further characterization of this region in neuroblastoma initiation.

Recent data obtained from whole-genome sequencing studies of neuroblastoma have illustrated far fewer recurrent mutations in protein-coding genes than previously predicted (13–16); however, it is now clear that as much as 70% of the genome is transcribed into products other than traditional protein-coding mRNAs (17, 18).
Although many of these transcriptionally active loci produce RNA species involved in translation (i.e., ribosomal and transfer RNAs), several other RNA classes have been functionally validated as bona fide regulatory molecules. The recently identified long noncoding RNAs (lncRNA), defined as RNA species >200 nt in length that lack a functional open reading frame, have been increasingly implicated in a wide variety of cellular functions (19). lncRNAs share several transcriptional features in common with mRNAs, they are often spliced, demonstrate RNA polymerase II occupancy, contain a 5' methylguanosine cap, and are commonly (though not always) polyadenylated (20, 21). Although lncRNA function is highly context dependent, they commonly play a prominent role in the spatiotemporal regulation of gene expression during developmental processes (22–24), and therefore exhibit a tendency to be located throughout the genome, in sites proximal to developmentally critical protein-coding genes (25). Indeed, several lncRNAs reside near protein-coding genes known to regulate lineage commitment in neural crest cells (26), serving as an attractive hypothesis to explain the etiology of embryonal cancers such as neuroblastoma.

As might be expected, lncRNAs have been increasingly implicated in a variety of oncogenic processes through association with epigenetic complexes and modification of chromatin accessibility, ultimately influencing gene expression (27–30). To date, there are few reports concerning the role of lncRNAs in the initiation and progression of solid pediatric neoplasms, despite the fact that many childhood cancers are fundamentally defects of normal human development (31). Here we describe the identification and characterization of a novel lncRNA, CASC15, which contributes to the GWAS association signal on 6p22.3 by functioning as a tumor suppressor in neuroblastoma.

**Materials and Methods**

**GWAS and imputation**

In an effort to refine the association signal and search for a causal variant at the 6p22 locus, we performed genotype imputation in a previously described European ancestry cohort of 2,101 neuroblastoma cases and 4,202 controls (10) using the 1000 Genomes Phase I Release 3 as a reference. Genotyping and quality control methods have been previously published (5). GWAS imputation and statistical tests are detailed in Supplementary Materials and Methods.

**Data sources**

The human February 2009 (GRCh37/hg19) genome assembly was used throughout the study. Transcript structures and annotations were obtained from GENCODE version 19. Details on the various data sources used are available in Supplementary Materials and Methods.

**Neuroblastoma data.** The neuroblastoma RNAseq, SNP profiling and HuEx databases are part of the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative, supported by NCI Grant U10 CA98543. The low-level sequence data have been deposited in the Sequence Read Archive at the National Center for Biotechnology Information, and are further accessible through the database of genotypes and phenotypes (dbGaP, accession number phs000218). The gene expression and copy number data, as well as clinical information on the NBL cases studied, is available via the TARGET Data Matrix (32).

5’/3’ Rapid amplification of cDNA ends

5’ and 3’ Rapid amplification (RACE) was performed via the First Choice RLM-RACE Kit (Ambion) using 10 μg of RNA obtained from fetal brain or NB69 neuroblastoma cells following the manufacturer’s protocol. Specificity for CASC1S-S was achieved by nested PCR using the following gene-specific primer (GSP) pairs:

- 5’ RACE: Outer GSP: 5’-CTAGCCCCATCGTCTCCCTCG-3’
- 5’ RACE: Inner GSP: 5’-TTCACCCCTGCTCTCAAGTCTG-3’
- 3’ RACE: Outer GSP: 5’-TGGTACCTGAGGTCGTCTCTT-3’
- 3’ RACE: Inner GSP: 5’-CTGACCGCCGTGCAACACAAC-3’

Gene products were cloned into a pCR4-TOPO vector for sequencing.

**RNA sequencing**

**Neuroblastoma.** PolyA selected RNA libraries obtained from 108 high-risk neuroblastoma patients as part of the NCI TARGET project were prepared using TruSeq v3 (Illumina) for RNA sequencing on Illumina HiSeq2000 sequencers. The 101 bp paired-end reads generated were aligned to the hg19 build of the human reference genome using TopHat v2.2.0, yielding a median of 115 million total aligned reads per patient sample (range: 48–250 million total aligned reads). The HTSeq package (v0.6.1) was used to map aligned reads to the VISTA-annotated enhancer region, h5335, as well as all transcripts annotated in RefSeq (v66) and/or the UCSC Genome Browser. Transcript expression values were normalized and quantified using the metric of reads per kilobase per million reads (RPKM). For isoform-specific quantitation of CASC15 and CASC1S-S, only exons and exon–exon junctions that were unique to each isoform were used in computing RPKM.

Other. Mapped RNA-seq reads from 16 primary tissues (Illumina Human Body Map) and 51 cell lines (Human ENCODE) were used to quantify gene- and transcript-level expression, based on GENCODE v19 annotations, using Cufflinks v2.2.0 with default parameters and “–min-frags-per-transfrag 0 –compatible-hits-norm –min-isofrom-fraction 0.0” Expression data heatmaps were generated using the heatmap.2 function of the gplots R package.

**RNA FISH**

We performed RNA FISH and counted RNA in single cells as described in ref. 33. Fluorescently labeled, nonoverlapping oligonucleotide probes (20 mer) were designed to tile RNA transcripts, including CASC14 (LOC729177), h5335, CASC15, and CASC1S-S. Probes were then divided into odd and even pools and hybridized to neuroblastoma cells. Details of image acquisition are available in Supplementary Materials and Methods.

**Quantification of CASC15-S in neuroblastoma cells**

Quantification of RNA transcripts was performed on a panel of neuroblastoma cancer lines using TaqMan RT-PCR. For quantification of CASC15-S, we developed a custom primer/probe set spanning exons 1–2 and consisting of the following primers:

- Forward: GCTGTCGACGAGAAGACGTGAT
- Reverse: GTCCAAGTCAGAAATGTCATCAGAAGA
- Reporter: CTTGTCAGGCCTAGCC

Primers/probe sets for CASC15 (Hs01371949_m1) and CASC14 (Hs04275511_s1) are commercially available (Life Technologies).
Quantification was normalized to the geometric mean of housekeeping genes TBP and GUSB.

**Cell growth and siRNA assays**

ON-TARGETplus SmartPool siRNAs containing four constructs per target (Thermo Scientific) were used for GAPDH and PLK-1 knockdown. ON-TARGETplus Non-targeting Control Pool (containing four constructs) was used as a control. Two constructs were used for each lncRNA, and were purchased as Silencer Select siRNA or custom siRNAs from Life Technologies.

siRNA constructs were transfected into neuroblastoma cells in triplicate, using 50 nmol/L of siRNA in 0.1%–0.2% DharmaFECT 1 (Thermo Scientific). Knockdown was assessed by TaqMan RT-PCR, comparing levels 72 hours after transfection to nontargeting control-transfected cells. For all siRNA experiments, the minimum knockdown achieved was 71.5% (average = 78.6, maximum = 92.0).

For growth assays, both tested, the siRNA with the best knockdown efficiency for each target is shown. Cell growth assays were conducted using the xCELLigence real-time growth (RT-CES, ACEA Biosciences) and/or Cell-TiterGlo (Promega) assays according to manufacturer protocols.

**CASC15-S add-back experiments**

For CASC15-S addback experiments, expression plasmids containing the cDNA for either CASC15-S or GFP were transfected in triplicate in a 96-well plate using 250 ng of DNA and 3 μL of Lipofectamine 2000 per well. These experiments were conducted using the growth assays described above, and the expression levels of the CASC15-S transcript were confirmed by TaqMan RT-PCR to be highly expressed in the addback condition. Transfection efficiency was estimated to be approximately 90% based on the GFP-transfected condition.

**shRNA expression and lentiviral constructs**

The shRNA construct for CASC15-S (targeting exon 1) was used to create a double-stranded shRNA construct and was subsequently cloned into the pLenti-GFP DEST lentiviral vector (Addgene). A total of 5 × 10^5 293T cells were transfected with 15 μg of the plenti transfer vector, 15 μg of pLP1, 6 μg of pLP2, and 3 μg of pNS-G vectors. Lentiviral particles were collected at 48 and 72 hours after transfection. Lentiviral transduction of neuroblastoma cells was carried out overnight at 37°C using 5 μg/mL of polybrene. For each neuroblastoma line used, serial dilutions of lentiviral particles were assessed at 48 hours after transduction for GFP expression. The minimum lentiviral concentration resulting in >95% GFP expression was chosen for expansion and knockdown was validated by TaqMan PCR. For all shRNA experiments, knockdown of at least 70.3% (average = 82.6, maximum = 91.8) was achieved. Each neuroblastoma line with confirmed knockdown was then frozen at low passage (<5) and thawed as needed for subsequent experiments.

**Luciferase reporter assay**

The respective risk (A_A) and non-risk (T_T) genotypes of Lan-5 and CHP-134 cells at rs9295534 were verified by PCR and Sanger sequencing. Subsequently, a 1,500 bp fragment was cloned from each cell line (750 bp on either side of the rs9295534) and was purified using the Qiaquick Gel Extraction Kit (Qiagen). The fragment was inserted into the pGL4.23 vector upstream of a minimal promoter used to drive firefly luciferase expression. Fragment-containing vectors were then transfected into HEK-293 cells, along with a Renilla luciferase vector (pGL4.75) to control for transfection efficiency. Seventy-two hours after transfection, luciferase activity was assayed by Dual-Glo reporter assay (Promega) using the GloMax Multi Detection System (Promega). Firefly luciferase was normalized to Renilla luciferase and a minimum of three replicates was used for each condition.

**In vitro transcription/translation assay**

CASC15-S was cloned into a T7-promoter containing plasmid (T7-CFE-Chis) and subsequently verified by Sanger sequencing. Coding potential of CASC15-S was assayed using the Tnt Quick Coupled Transcription/Translation System according to the manufacturer’s protocol. Incorporation of biotinylated lysine residues was visualized via Western blot analysis using a 1:10,000 anti-biotin HRP-labeled antibody (Cell Signaling Technology) and chemiluminescent detection (Thermo Scientific).

**Wound-healing assays**

Scratch assays were carried out on SK-N-BE2 and SK-N-SH cells stably depleted of CASC15-S plated at 85% confluence in 60 mm dishes, and were scratched using a sterile 200 μL pipette tip. Cells were photographed at regular intervals using a previously calibrated 5 × light microscope (Nikon). Assessment of cell migration was carried out by measuring scratch closure as a percentage of initial scratch size in ImageJ, and was compared with control cells using a linear regression function in GraphPad Prism 6.

**Cell culture**

Neuroblastoma cell lines were obtained from the neuroblastoma cell line bank maintained at the Children’s Hospital of Philadelphia, Philadelphia, PA. Cell line identity is routinely confirmed via AmpFLSTR Identifier (Applied Biosystems), last done in November 2013. Non-neuroblastoma cell lines were purchased from ATCC and all cell lines are routinely tested for mycoplasma. All cell lines are maintained in basal media (either RPMI1640 or DMEM) supplemented with 10% FBS and 1% gentamycin and cultured at 5% CO2.

**Statistical analysis**

All group comparisons were conducted using nonparametric testing (Mann–Whitney U) in GraphPad Prism.

For Kaplan–Meier analysis: optimal cutoff was determined by employing a scanning approach to the Kaplan–Meier method by iteratively splitting the ordered genes expression values across samples into two groups and calculating the P value by the Mantel–Haenszel log-rank test. The lowest P value corresponds to the optimal breakpoint. A Benjamini–Hochberg correction was applied to reflect the presence of multiple hypotheses testing.

For multivariate analyses, a Cox Proportional Hazard model was used to evaluate the effect of each gene expression on overall survival, adjusting for clinical factors such as age (as a continuous variable), MYCN amplification, and 1p/11q LOH status. Proportional hazard assumption was evaluated by the log–log plot and there was no evidence of violation of this assumption.

**Results**

Fine mapping of the 6p22.3 neuroblastoma susceptibility locus

The initial GWAS study illustrating 6p22.3 as a neuroblastoma susceptibility locus identified three common polymorphisms...
clustered on chromosome 6p22.3 that were highly associated with aggressive disease and a significantly increased risk of neuroblastoma development (5). These SNPs were shown to tag a 94.2 kb LD block overlapping two hypothetical genes and flanked upstream by SOX4. To map this region with finer detail, and identify putative causal/functional SNPs, we performed genotype imputation in our recently published discovery cohort of 2,101 cases and 4,202 controls (10). We first applied SHAPEIT (34) to infer haplotypes, and then utilized IMPUTE2 (35) with default parameters and Ne = 20,000, along with a multipopulation reference panel from the worldwide 1000 Genomes Project Phase 1 release to impute genotypes across the region. Genotyped and imputed variants were tested for association with neuroblastoma using the frequentist association test under the additive model imputed variants were tested for association with neuroblastoma development (5). These SNPs were shown to tag a 34.9-kb LD block demonstrated by the dashed red vertical line and rightmost black vertical dashed line.

Identification of CASC15 isoform expression

CASC15 is annotated as an lncRNA, prompting us to examine the difference in lncRNA transcript levels between 220 high-risk and 30 low-risk primary tumors, for which we possessed microarray expression data. This analysis revealed that only 3.5% of annotated lncRNAs (hg19: 8/229) appear significantly differentially regulated between high- and low-risk tumors (Table 1). Furthermore, high-risk neuroblastomas exhibit a 4.4-fold lower level of CASC15 expression as compared with low-risk disease, the most highly significant difference identified was with the high-risk tumors (CASC15 isoform expression).

Several computationally predicted lncRNAs map to the 6p22.3 locus, including multiple CASC15 isoforms and an antisense-strand IncRNA, CASC14 (formerly LOC729177) (Supplementary Fig. S1A).
nonoverlapping, transcript-specifically expressed in abundance in the brain, but at modest to low levels in other tissues (Fig. 2B). We next examined RNA expression data from a panel of 16 primary human tissues as part of the Illumina Human Body Map project, where we found that the short CASC15 isoform was expressed in abundance in the brain, but at modest to low levels in most other tissues (Fig. 2B). We subsequently generated RNA sequencing data from 108 primary neuroblastoma tumors using nonoverlapping, transcript-specific reads to investigate the expression of lncRNAs transcribed from this locus. We augmented these findings with our own strand-specific RNA sequencing data from two primary tumors, confirming nearly complete alignment to the plus strand (average = 92.6%, minimum = 86.9%, maximum = 98.6%). Together, these RNA sequencing data identify the short CASC15 isoform as the predominant transcript expressed from this locus in neuroblastoma, with expression values 20- to 40-fold higher than CASC14 and full-length CASC15, respectively (Fig. 2C). Finally, RNA sequencing data identified a third unspliced transcript, spanning part of exon 1 of the short CASC15 isoform through a downstream noncoding element (hs1335) with a validated enhancer function in the developing murine neural tube, further supporting the role of this locus in neural development (37).

To validate the isoforms identified from RNA sequencing data, we performed 5' and 3' RACE for CASC15, subsequently cloning and sequencing these transcripts from two neuroblastoma cell lines and fetal brain tissue. We verified the sequence of both the 12-exon 1.9 kb CASC15 transcript (NC_015410.1, Ensembl: CASC15-003) and the 4-exon short (1.2 kb) variant (Ensembl: CASC15-004), hereafter referred to as CASC15-S. CASC15-S resembles a known cDNA clone (GenBank: AK094718), containing a unique first exon, yet sharing its remaining sequence with the last three exons of CASC15 (Fig. 2A and Supplementary Fig. S3A). Despite several attempts, we were unable to isolate a 5'-capped product for the intron-less transcript (Ensembl: CASC15-006) predicted to overlap exon 1 of CASC15-S and extend to the noncoding enhancer element (hs1335). Indeed, although the presence of this isoform is indicated on the SK-N-SH RNAseq track (Fig. 2A), it is absent in RNA-PET data (Supplementary Fig. S1B), supporting this result. Finally, RNA-FISH experimentally confirmed the presence, relative abundance, and cellular localization of these transcripts in both NB-69 and NGP neuroblastoma cells; using nonoverlapping, strand-specific probes to label CASC15, CASC15-S, CASC14, and hs1335. These studies revealed exclusively nuclear CASC15-S and hs1335 transcripts (Fig. 2D) and virtually no CASC14 or CASC15 expression (Supplementary Fig. S3B and S3C). Taken together, these experimental results confirm our predictive bioinformatic data identifying CASC15-S as a bona fide lncRNA transcript in neuroblastoma.

Identification of candidate functional SNPs at 6p22.3

Because risk alleles at several other neuroblastoma susceptibility loci have been shown to function in cis to influence mRNA expression levels of nearby transcripts (6, 9, 10), we attempted to associate our previously published, highly significant polymorphism, rs6939340 (P = 1.67 × 10^{-14}; OR, 1.80; 95% CI, 1.53-2.10; ref. 5) with expression of the CASC14, CASC15, and CASC15-S gene products at this locus. However, we did not observe a significant correlation between risk alleles and transcript expression levels in either a set of 250 primary neuroblastomas (Supplementary Fig. S4A) or a representative panel of 20 neuroblastoma cell lines (Supplementary Fig. S4B). Furthermore, all three of our previously published polymorphisms lie in regions devoid of DNase hypersensitivity or other epigenetic marks indicative of transcriptional activity. In fact, eQTL analysis of all imputed genotypes with CASC15-S expression failed to reach significance after multiple comparison testing, leading us to postulate that polymorphisms contributing marginal effects may aggregate to impact function at this locus.

We therefore took advantage of our genome-wide imputation data to identify other highly significant polymorphisms lying within putative regulatory regions. As demonstrated in other post-GWAS follow-up studies (38), we employed the following workflow (Fig. 3A) to narrow the field of potentially functional polymorphisms: (i) we first chose polymorphisms with a GWAS P value < 1 × 10^{-10}, resulting in 32 candidates; (ii) we further refined this list by selecting only those SNPs within regions of DNase hypersensitivity (indicating open chromatin) and H3K27 acetylation marks (indicative of enhancer activity) leaving us with four candidates: rs1543310, rs6905441, rs9295534, and

<table>
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<th>Ratio</th>
<th>Disease state</th>
<th>P</th>
<th>Location</th>
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Table 1. Differential expression analysis of lncRNAs between high- and low-risk neuroblastomas

NOTE: Differential gene expression analysis (>2.0-fold; P < 0.05; FDR < 0.05) was conducted using 220 high- and 30 low-risk primary neuroblastoma tumors, focusing on differences in lncRNA expression. The top differentially regulated lncRNA was CASC15 (annotated here as LINC00340), which was significantly lower in high-risk disease (4.4-fold decrease; ANOVA, P = 3.60 × 10^{-14}).
Finally, we looked for SNPs with evolutionary conservation, resulting in a single candidate polymorphism, rs9295534 (\(P = 3.51 \times 10^{-12}\); OR, 1.63; 95% CI, 1.4–1.89) upstream of CASC15-S, and this variant localizes to an expanse of regulatory chromatin and dense transcription factor binding sites in several cell lines (Fig. 3B). Moreover, this region exhibits enhancer activity, evidenced by H3K27Ac CHIP-Seq data in several fetal tissues available from the NIH Roadmap Epigenomics Mapping Consortium (Fig. 3C).

We next verified rs9295534 genotypes in Chp134 (homozygous non-risk) and Ln5 (homozygous risk) neuroblastoma cells by Sanger sequencing of a 1.5 kb region encompassing this SNP, and subsequently cloned risk and non-risk fragments from these lines. To assess the impact of rs9295534 genotype on transcriptional activity, we inserted risk (A/A) and non-risk (T/T) fragments into luciferase reporter constructs upstream of a minimal promoter. Results from these experiments demonstrated significantly decreased transcriptional reporter activity following insertion of the risk genotype fragment (Fig. 3D), suggesting this region possesses an enhancer-like function that is disrupted following the inclusion of a neuroblastoma risk allele.

CASC15-S is differentially expressed in neuroblastoma and highly associated with disease outcome

Because the rs9295534 homozygous risk genotype, by virtue of its linkage with rs6939340, is associated with aggressive neuroblastoma, poor survival (5), and demonstrates decreased...
transcriptional activity in a minimal promoter assay, we postulated that patients with high-risk disease would have reduced CASC15-S expression. Indeed, we observed significantly lower CASC15-S expression in high-risk patient tumors (n = 230) compared with low-risk patient tumors (n = 30; Fig. 4A). This finding appeared independent of MYCN amplification, a known oncogenic driver in neuroblastoma (P = 0.29, Supplementary Fig. S5A). In addition, patients with tumors enriched in CASC15-S expression exhibited superior survival when compared with patients harboring neuroblastoma with low CASC15-S expression, both when low- and high-risk patients were included in the analysis (adj. P = 3.2 × 10^{-10}, Fig. 4B) but also when comparing expression from only high-risk patients (adj. P = 0.002, Supplementary Fig. S5B). Furthermore, this finding remained significant (P = 0.0084) after multivariate analysis adjusting for clinical factors such as age, MYCN amplification, and 1p/11q deletion status, all known prognostic variables in this disease (Supplementary Table S2). Although expressed at much lower levels than CASC15-S, the long isoform of CASC15 demonstrated a similar pattern in patient tumors (Supplementary Fig. SSC–SS5E). Taken together, these data indicate that low CASC15-S expression correlates with a more aggressive phenotype in neuroblastoma and decreased overall survival probability.

To understand the contribution of CASC15-S in the initiation and progression of neuroblastoma, we performed gene set enrichment analysis (GSEA) restricted to high-risk neuroblastoma samples (n = 220), where we utilized the same 1.9-fold difference in median CASC15-S expression as the Kaplan–Meier analysis to define 146 low- and 74 high-expressing CASC15-S samples (Fig. 4C). The top differential gene expression profile (normalized enrichment score = -2.69, nominal P value < 0.0001, FDR Q value < 0.0001) that emerged from these analyses consists of a 55 gene signature previously shown by Asgharzadeh and colleagues to be downregulated in neuroblastoma patients with poor outcomes (39). This result indicates that tumors with high CASC15-S expression are enriched in the expression of these genes, and again
Cancer Res; 75(15) August 1, 2015

Russell et al.

Figure 4.
Low CASC15-S expression correlates with poor clinical prognosis. A, microarray expression data from clinically annotated primary neuroblastoma tumors (n = 250) obtained at diagnosis reveals that high-risk (stage IV) neuroblastomas (n = 220) demonstrate significantly lower expression of CASC15-S than low-risk (stage I) tumors (n = 30). B, Kaplan–Meier analysis demonstrates significantly poorer overall survival for children with tumors expressing low levels of CASC15-S (n = 163 for group "low"; n = 87 for group "high"; adj. P = 3.2 x 10^{-6}). C, relevant metrics for selection of high-risk patient tumors used for differential gene expression analyses between patients with high (n = 74) and low (n = 146) CASC15-S levels. CASC15-S expression was increased 1.9-fold in the "high" group, and these patients exhibited a significant increase in overall survival. D, the most highly significantly regulated pathway using GSEA was identified to be a set of genes downregulated in poor outcome neuroblastoma (Asgharzadeh neuroblastoma poor survival down). Patients with high CASC15-S expression demonstrated enrichment of these genes, suggesting that CASC15-S acts in a protective manner (***, P < 0.001).

Figure 4.

CASC15 depletion in neuroblastoma cell lines enhances proliferation and invasive capabilities
Supporting a protective role for CASC15-S, resulting in less aggressive disease within even this subgroup of high-risk cases (Fig. 4D).

CASC15 depletion in neuroblastoma cell lines enhances proliferation and invasive capabilities
Having demonstrated a clinically relevant association with patient outcome for CASC15-S in neuroblastoma patients, we next sought functional validation for a role in tumorigenesis. We first assessed CASC15-S levels by qRT-PCR across a well-characterized panel of neuroblastoma cell lines (n = 21) where we observed differential expression similar to our primary tumor panel (Fig. 5A). To investigate the functional role of CASC15-S in neuroblastoma, we utilized an siRNA construct targeting exon 4 near the 3' end of the gene. Depletion of CASC15-S resulted in a highly reproducible increase in neuroblastoma proliferation as evidenced by real-time cell growth and viability assays (Fig. 5B), and we were able to recapitulate these results by subsequently targeting CASC15-S within its unique first exon in several neuroblastoma lines (Fig. 5C and Supplementary Fig. S6A and S6B). Depletion of full-length CASC15 or CASC14 had no observable impact on cell growth or viability, supporting our initial findings of CASC15-S as the functional isoform in neuroblastoma (Supplementary Figs. S6C and S6D and S7A–S7D). We next derived neuroblastoma cell lines stably depleted of CASC15-S (Supplementary Fig. S7E) and found that these cells also exhibited a substantial increase in cellular proliferation identical to what we observed in our transient siRNA-based CASC15-S depletion experiments (Fig. 4D and Supplementary Figs. S6E and S6F and S8). Furthermore, rescue experiments, conducted by ectopically expressing CASC15-S in these cells, were able to revert the accelerated growth (Fig. 5E). Microscopic examination revealed overt morphologic changes in shCASC15-S SK-N-BE2 cells, including striking changes in cell shape and size, with a resultant 3-fold increase in cell area (659.5 ± 50.2 μm² in control vs. 2024 ± 211.1 μm² in shCASC15 cells, P < 0.0001; Fig. 5F and G). Furthermore, both SK-N-BE2 (Fig. 6A and B) and SK-N-SH neuroblastoma cells (Fig. 6C) stably depleted of CASC15-S exhibited an increased migratory capacity and invasiveness as evidenced by wound-healing assays (SK-N-SH:...
P = 0.0006, SK-N-BE2; P < 0.0001). Taken together, these data suggest that CASC15-S loss promotes increased cellular growth and a more migratory phenotype in neuroblastoma.

CASC15-S regulates a subset of genes involved in neural crest development

To better characterize the phenotypic changes we observed following CASC15-S depletion, we surveyed gene expression signatures of neuroblastoma cells depleted of CASC15-S. For initial studies, SK-N-SH neuroblastoma cells were transfected in triplicate with either a nontargeting construct or siRNA specific for CASC15-S, and microarray gene expression signatures were examined at 48 hours following transfection. We observed substantial upregulation of several known cell adhesion genes, most notably entactin (NID1, P = 3.7 × 10⁻⁴) and activated leukocyte cell adhesion molecule (ALCAM, P = 1.05 × 10⁻⁷), after CASC15-S depletion. These gene expression data were used for Gene Ontology (GO) analysis to examine enrichment top level biologic processes. In support of the increased migratory phenotype we observed in wound-healing assays, both locomotion and cellular adhesion pathways were found among the top differentially regulated processes as a result of CASC15-S depletion (Fig. 6D).

We next assayed the result of persistent CASC15-S depletion on gene expression changes by comparing SK-N-BE2 neuroblastoma cells stably silenced for CASC15-S compared with control vector-transfected cells. We again examined gene expression signatures via microarray analysis, where we observed downregulation of...
several proneural gene family members with known roles in neurogenesis and differentiation such as neurogenin differentiation 1 (NEUROD1, $P = 1.1 \times 10^{-4}$), neural precursor cell-expressed, developmentally downregulated gene 9 (NEDD9, $P = 5.8 \times 10^{-4}$), and neurogenin 2 (NEUROG2, $P = 5.3 \times 10^{-4}$). In a manner identical to the SK-N-SH gene expression comparison, we utilized GO analysis of these differentially regulated genesets, with the top pathway, “cellular process” arising due to enrichment of the cell differentiation pathway node contained within this subset (Fig. 6E).

Finally, we submitted the differentially expressed gene lists from siCASC15-S SK-N-SH and shCASC15-S SK-N-BE2 cells to Ingenuity Pathway Analysis (IPA). Neuroblastoma cells depleted of CASC15-S demonstrated highly significant upregulation of pathways involved in cell migration, proliferation, and metastasis (Fig. 6F, top), and substantial decreases in proneural gene signatures (Fig. 6F, bottom). Taken together, these data suggest that loss of CASC15-S shifts the neuroblastoma gene expression away from a well-differentiated neural phenotype and promotes increased expression of cellular adhesion and migratory genes, a finding consistent with our phenotypic and morphologic observations.

### Discussion

High-risk neuroblastoma remains a major challenge due to a relative paucity of somatic mutations hindering the development of targeted therapies (1). The identification of mutations in ALK and PHOX2B has helped explain the origin of familial neuroblastoma; however, an understanding of the basis of sporadic disease has only recently begun to come into focus (40, 41). Here we identify and demonstrate CASC15-S as a neuroblastoma suppressor gene via a post-GWAS mechanistic evaluation of a complex region of the human genome. Despite this robust association, however, eQTL analyses correlating risk genotypes with transcript expression failed to reach statistical significance, a likely
CASC15 Is a Neuroblastoma Suppressor Gene

consequence of an underpowered patient data set and/or additional mechanisms capable of impacting expression (such as additional SNPs affecting expression, posttranslational modification/degradation, etc.). To address this shortcoming, we fine-mapped this locus, using orthogonal methodologies to identify the likely disease causal SNP, rs9295534, which localizes to the closest upstream enhancer of CASC15-S. Moreover, expression of the rs9295534 risk allele disrupts the enhancer function of this region, proving that genotype can indeed impact transcriptional ability at this locus. More importantly, we provide evidence for a potent effect of this IncRNA on neuroblastoma differentiation and migratory capacities, yielding mechanistic insights into why the GWAS signal at this locus is associated with metastatic disease and poor survival probability.

A growing body of work supports a defined role of IncRNAs as spatiotemporal-specific regulators of gene expression critical for ensuring proper differentiation during development. Thus, predominant expression of CASC15-S in brain (but not other tissues), the derivation of several cDNA clones from brain regions, and its proximity to a validated enhancer element (hs1335) strongly suggest that this IncRNA is uniquely involved in neural tube development. The role of IncRNA-mediated tumorigenesis in embryonal cancers provides a logical hypothesis to contribute to the understanding of the etiology of neuroblastoma tumors, which are typically devoid of activating somatic mutations (1).

A preliminary working model of how this transcript functions in neuroblastoma biology can be proposed from the functional and expression data we have demonstrated. CASC15-S expression strongly correlates with disease stage and overall survival, and patient tumors with high CASC15-S levels are enriched in genes typically lost in poor outcome neuroblastoma, demonstrating a protective role for CASC15-S. Furthermore, ablation of CASC15-S in neuroblastoma cell lines results in increased proliferative and migratory capacities, upregulation of adhesion and migration gene pathways, and a concomitant decrease in neural-specific transcripts. Therefore, reduced CASC15-S expression as the result of an inherited polymorphism would impact neural crest cellular lineage commitment and predispose these cells to undergo malignant transformation.

The phenotypic and gene signatures changes we observed signify that CASC15-S is responsible for maintaining a more differentiated and benign cell state, with CASC15-S loss leading to a poorly differentiated phenotype and expression of genes associated with transformed cells. It has been recently shown that CASC14 (renamed NBAT-1), although very lowly expressed, exerts a similar phenotype (42), suggesting that many IncRNAs in this region may cooperate. The precise mechanism by which CASC15-S (and potentially other transcripts near this locus) exerts its effect is currently under investigation, although it is likely that the observed transcriptional changes may be the result of modulation of cis elements. One possibility, given its proximity to a validated enhancer (hs1335) with neural tube expression and its position downstream of the developmental regulatory gene SOX4, is that CASC15-S functions as an enhancer RNA (43).

Future studies to identify direct interaction partners of CASC15-S will undoubtedly strengthen our understanding of IncRNA function and yield key insights into neuroblastoma tumorigenesis.

In summary, our findings support a recent and growing body of evidence that convincingly demonstrates involvement of the noncoding genome in the tumorigenesis of pediatric cancers in general, and neuroblastoma in particular (42, 44–47). While we certify CASC15-S as a neuroblastoma suppressor gene, we still have not elucidated all of the stochastic and/or epigenetic events that not elucidated all of the stochastic and/or epigenetic events that select for CASC15-S repression. Future studies focused on engineering CASC15-S depletion in vivo will further explore mechanisms for tumor initiation and progression as well as screen for synthetic lethal interactions to be leveraged therapeutically. Finally, this work provides the first highly significant GWAS-supported identification of IncRNAs in neuroblastoma, thus further stimulating exploration of this class of regulatory RNAs in human cancer etiology and clonal evolution.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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