High Expression of CAI2, a 9p21-Embedded Long Noncoding RNA, Contributes to Advanced-Stage Neuroblastoma

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Abstract

Neuroblastoma is a pediatric cancer with significant genomic and biologic heterogeneity. p16 and ARF, two important tumor-suppressor genes on chromosome 9p21, are inactivated commonly in most cancers, but paradoxically overexpressed in neuroblastoma. Here, we report that exon γ in p16 is also part of an undescribed long noncoding RNA (lncRNA) that we have termed CAI2 (CDKN2A/ARF intron 2 lncRNA). CAI2 is a single-exon gene with a poly A signal located in but independent of the p16/ARF exon 3. CAI2 is expressed at very low levels in normal tissue, but is highly expressed in most tumor cell lines with an intact 9p21 locus. Concordant expression of CAI2 with p16 and ARF in normal tissue along with the ability of CAI2 to induce p16 expression suggested that CAI2 may regulate p16 and/or ARF. In neuroblastoma cells transformed by serial passage in vitro, leading to more rapid proliferation, CAI2, p16, and ARF expression all increased dramatically. A similar relationship was also observed in primary neuroblastomas where CAI2 expression was significantly higher in advanced-stage neuroblastoma, independently of MYCN amplification. Consistent with its association with high-risk disease, CAI2 expression was also significantly associated with poor clinical outcomes, although this effect was reduced when adjusted for MYCN amplification. Taken together, our findings suggested that CAI2 contributes to the paradoxical overexpression of p16 in neuroblastoma, where CAI2 may offer a useful biomarker of high-risk disease. Cancer Res; 74(14); 3753–63. ©2014 AACR.

Introduction

It is becoming increasingly apparent that long noncoding RNAs (lncRNA), which until recently were thought to have little functional significance, harbor diverse roles in regulating cell identity, differentiation, and development. They have been implicated in cellular and molecular regulation, with chromatin remodeling and gene regulation emerging as common functions (1). In addition to normal cellular regulatory roles, lncRNAs are often deregulated in a wide variety of diseases (1–4). For example, the lncRNA CRNDE, originally identified through its association with colorectal cancer, is elevated in multiple cancer types and is induced during neuronal differentiation (5). One of the most well-characterized lncRNAs, the HOX gene antisense lncRNA HOTAIR, has been shown to serve as a tumor biomarker in various types of cancers, including breast, esophageal, and pancreatic, and has also been correlated with tumor progression and prognosis (6–9).

Initiating from discrete first exons separated by approximately 20 Kb on 9p21, the transcripts of p16 (p16\textsuperscript{locus}\textsuperscript{+}) and ARF (p14\textsuperscript{ARF}) are spliced onto a common exon 2 in separate reading frames and code for two different growth regulatory proteins. In addition, a homologous third tumor-suppressor gene, p15 (p15\textsuperscript{locus}\textsuperscript{−}), lies on 9p21 at approximately 10 Kb telomeric of ARF. Given the central role these proteins play in cell-cycle regulation, it is not surprising to find that the inactivation of all three has been associated with the pathogenesis of many human cancers (10, 11). In addition, genome-wide association studies also implicate this locus with coronary disease, intracranial aneurysm, and diabetes (for review see ref. 12), demonstrating that this chromosomal region is a major player in a wide range of diseases. 9p21 is also the home of two lncRNAs, ANRIL and p15\textsuperscript{AS} (CDKN2AS; refs. 13, 14). Functionally, both act as regulators of growth. ANRIL has been shown to regulate its neighbor tumor suppressors p16 and ARF and thereby regulate cell proliferation and senescence (15–17). Expression of exogenous p15\textsuperscript{AS} caused p15 silencing and increased growth through heterochromatin formation and DNA methylation (14).

Neuroblastoma is the third most common malignancy of childhood. Diagnosed in stages based on clinical and biologic features, patients with a favorable (low-risk disease) diagnosis...
can often be cured. However, approximately 50% of patients have high-risk disease and have dismal outcomes despite multiagent and multimodality therapy, myeloablative therapy followed by autologous bone marrow transplantation, with more than half of the patients nevertheless relapsing and succumbing to the tumor. Recently, we have reported on the significant improvement in survival targeting the GD2 tumor-associated antigen uniformly expressed by neuroblastoma (18). Nevertheless, approximately one third of the patients continue to fail, highlighting the need to identify biomarkers to better predict outcome and improve efficacy.

While investigating the role of p16 in neuroblastoma, we observed that, in contrast to most tumors, p16 was not inactivated but paradoxically was overexpressed (19). Notably, p16 overexpression was significantly associated with advanced-stage disease and a poor outcome (20). Upon continued investigation in neuroblastoma cell lines, we identified a unique transcriptional variant, p16\textit{\textgamma}, which is formed by the splicing of the cryptic exon \textit{\gamma}, located in \textit{p16} intron 2, onto \textit{p16} exons 2 and 3 (21). It was our investigations into \textit{p16\textgamma} that led to the discovery we report here that \textit{\gamma} is also part of an lncRNA we call \textit{CAI2}, a single-exon, intron-oxidized, polyadenylated gene. \textit{CAI2} is expressed at high levels in many tumor cell lines, and its expression is highly correlated with the expression of \textit{p16} and \textit{ARF}. In neuroblastoma, \textit{CAI2} expression was significantly associated with advanced-stage disease and a poor outcome, suggesting that \textit{CAI2} may be a novel biomarker of neuroblastoma patient risk.

Materials and Methods

DNA and RNA preparation, RACE, and amplification

DNA was isolated using the Gentra DNA isolation Kit and total RNA from cell lines extracted using Trizol reagent (Life Technologies). The 5’ end of \textit{CAI2} gene was obtained using both the GeneRacer Kit (Life Technologies) and the SMART RACE cDNA amplification Kit (Clontech) with DNAse-treated total RNA from HeLa cells. RACE products were cloned into pCR4-TOPO vector (Life Technologies) and sequenced. For RT-PCR, DNAse-treated RNA was reverse-transcribed using SuperScript III (SSIII) First-Strand Synthesis System with oligo-dT primers (Life Technologies). qRT-PCR amplifications were performed in a 24 to 72 hours, after which cells were observed, counted, assayed, and/or harvested as appropriate. Primary sample and cell line RNA was isolated using Trizol (Invitrogen). All RNA samples were subjected to DNAse treatment and repurified.

Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>BS-ACT</td>
<td>5’-GAAACTACCTCTAACCATTCACT-3’</td>
</tr>
<tr>
<td>BR-ACT</td>
<td>5’-GTAGAAGCTTCTGGAGATGAGGGG-CC-3’</td>
</tr>
<tr>
<td>p16S</td>
<td>5’-CTGGGATCAGTCTGACCTGGACTTGGACAC-3’</td>
</tr>
<tr>
<td>c359R</td>
<td>5’-CTGGAATTCAGCATGGAGCCGGCGGCGGGAG-3’</td>
</tr>
<tr>
<td>c257F</td>
<td>5’-TGTCTGCTGATCTACTGAGG-3’</td>
</tr>
<tr>
<td>c257F</td>
<td>5’-TGTCTGCTGATCTACTGAGG-3’</td>
</tr>
<tr>
<td>refSYR</td>
<td>5’-CCATCATCATGACCTGGATCTTCTT-3’</td>
</tr>
<tr>
<td>TH-766F</td>
<td>5’-CAATTAGACCCCGCTCACAGG-3’</td>
</tr>
<tr>
<td>3p16</td>
<td>5’-CTACGAAGCCGGGTGTGTGTTGTT-3’</td>
</tr>
<tr>
<td>1888F-Bam</td>
<td>5’-ATCGGGATCCACCCCTCTTCAAGCACA-AT-3’</td>
</tr>
<tr>
<td>2252R-Xho</td>
<td>5’-ATCGGAGACAAGGGGAATAGCTTGAGGAGGAG-3’</td>
</tr>
<tr>
<td>TH-666F</td>
<td>5’-CTGACTCTGAGAGGGCAAGTAGCTTGAGGAGGAG-3’</td>
</tr>
<tr>
<td>Th-763R</td>
<td>5’-GTCGCCGTGCCCTGTACTG-3’</td>
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**Expression constructs**

Full-length \textit{p16} (pc\textit{p16}, ref. 21) and \textit{CAI2} (pc\textit{CAI2}) expression constructs were performed in pcDNA3.1 (Invitrogen); \textit{CAI2} (ps\textit{CAI2}) was also created in pSilencer4.1-CMV vector (Ambion). \textit{CAI2} was cloned from an ampiclon generated with primers H1698 and 3p16. All constructs were sequence and expression validated, with \textit{p16} further validated by Western blot.

For reticulocyte assay, we used full-length \textit{p16} or \textit{CAI2}, and \textit{ORF2} (amplicon from primer set 1888 F and 2252R) cloned into pCR2.1-TOPO (Invitrogen) in both the sense (\textit{CAI2} and \textit{ORF2}) and antisense (\textit{CAI2}) directions. All constructs were sequence validated and protein production assessed with the Promega TNT Coupled Reticulocyte Lysate System using [\textit{\textalpha}S]-Methionine and [\textit{\textbeta}S]-Cysteine.

**Tissue, cell lines, cell culture, cell growth, and transfections**

Normal human tissue RNA was from Clontech. FS15 are low-passage cultures of foreskin fibroblasts generously provided by Dr. Bruce Barshop (University of California, San Diego, La Jolla, CA). The source of the cell lines and culture conditions has been documented previously (19, 22). Cells were transfected at 70% to 90% confluence using Lipofectamine 2000 reagent (Invitrogen) or with the NEON electroporation apparatus (Invitrogen). After transfection, incubations proceeded for 24 to 72 hours, after which cells were observed, counted, assayed, and/or harvested as appropriate. Primary sample and cell line RNA was isolated using Trizol (Invitrogen). All RNA samples were subjected to DNAse treatment and repurification using the Zymo Research RNA Clean & Concentrator Kit.

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Primary tumor samples
A total of 62 samples of primary neuroblastoma tumors with a tumor cell content of ≥80% were obtained from the Children’s Oncology Group. The patients had an average age of diagnosis of 1.6 years (range, 0–12.2 years), with an average follow-up of those who had no event (relapse or death) of 3.8 years (range, 0.8–9.5 years). Samples were distributed as follows: 10 stage 4S, 11 stage 1, 12 stage 2, 9 stage 3, and 20 stage 4. The clinical stages were classified as favorable stage/low risk (stages 1, 2, and 4S), unfavorable stage/moderate risk (stage 3), or unfavorable stage/high risk (stage 4) based on Children’s Oncology Group staging criteria. Tumor samples were collected after informed consent and used under an University of California, San Diego-approved Institutional Review Board.

NMB7 cells
For the serial passage experiments, a fresh thaw of low-passage NMB7 was plated in complete media and, at the passage indicated, cells were released from the plate with trypsin, and a portion of the cells lysed in TRIzol for RNA extraction, whereas a separate portion was reseeded for continued growth. The serial passage experiments were done twice, whereas RNA extractions from low- and high-passage cultures were done multiple times. For treatment comparisons, NMB7 cells at passage 5 or passage 16 were plated in 6-well plates at 125,000 cells in 10% FBS-supplemented RPMI with antibiotics. After an attachment period, the media were removed and replaced with fresh media either without serum, with serum plus 0.5% DMSO, or with serum plus 5 μmol/L retinoic acid (RA; Sigma R2625 with 0.5% DMSO final concentration), and cells allowed to grow for an additional 24 or 72 hours, after which they were evaluated and, in some experiments, lysed in TRIzol. Cells were photographed in independent experiments either directly or after hematotoxin and eosin staining at 40× under phase-contrast. For growth experiments, 50,000 low-or high-passage cells were plated, allowed 24 hours to adhere, then observed and counted at 24-hour intervals up to 96 hours. Doubling time (DT) was calculated as DT = h * ln(2)/ln(c2/c1)), where h is time in hours, and c1 and c2 are the beginning and ending number of cells present.

Statistical analysis
A Spearman rank correlation (SRC) was used to assess correlations between expression data and a continuous outcome, a Kruskal–Wallis test was used to test the association between CAI2 expression and a categorical variable such as stage, and a Wilcoxon rank sum test used to test the association between CAI2 expression and a binary variable such as MYCN status. A log-rank test was used to assess the associations between a survival outcome and a binary predictor, and a Cox model was used to assess these associations adjusted for other covariates such as age at diagnosis. ANOVA was used to compare expression across multiple parameter or time points, with individual analyses compared by t test. P values less than 0.05 were considered statistically significant.

Results
Discovery of CAI2 (CDKN2A/ARF/intron 2 noncoding RNA) by RACE
While exploring p16γ expression, we observed that the γ exon was present in many more samples than those in which p16γ could be detected. Speculating that the γ exon may be part of another alternatively spliced transcript of p16, we used RACE (Rapid Amplification of cDNA Ends) to explore potential novel 5' ends using DNase-treated total RNA from HeLa cells. The 5' RACE amplification product using the Invitrogen GeneRacer Kit resulted in a sharp band and a uniform smear of increasing molecular weight (Fig. 1A). Sequence analysis of the extracted, purified, and cloned RACE products revealed the sharp RACE band encompassed not only exon γ, but extended upstream to 1,256 bases from the 3' end of p16 exon 2 (Fig. 1B). Sequence analysis of the smear yielded an identical sequence, except it contained a concatemer of the RNA oligonucleotide adapter on the 5’ end. A second RACE analysis using the DNA-oligo adaptor method (Clontech) also yielded a single ampollic that, as revealed by subcloning and sequence characterization, harbored a 5’ end that was within 30 bases of the transcript characterized using the GeneRacer Kit and a 3’ end that terminated 226 bases after the p16 stop codon (Fig. 1B). Amplifications with sense primers to exon 2, exon 1α, or exon 1β yielded amplicons with p16 3’-UTR (untranslated region) primers, but not with RACE-region antisense primers. On the other hand, RACE-region sense primers easily amplified with the 3’-UTR primers (data not shown). Furthermore, transcript amplification could only be demonstrated from RNA subjected to the RT reaction (Fig. 1E). Taken together, these data demonstrate that this intronless transcript is not DNA contamination and is independent of p16 and ARF.

CAI2 is a nonconserved noncoding RNA
A bioinformatic analysis of this transcript revealed five open reading frames (ORF) with a length of 40 amino acids or greater (Fig. 1C). The largest, ORF2, predicts a putative protein of 107 residues. A cross-species comparative analysis revealed only weak homologies (≤57%) that were limited to upper primates, consistent with the presence of an Alu element in this region. A comprehensive comparative analysis of the entire CAI2 nucleotide sequence also revealed no significant homologies with the exception of the expected p16 exon 3 and the Alu region of ORF2. We nevertheless assessed possible translation using the TNT Reticulocyte Lysate System (Promega Corp). However, no evidence of a protein was observed from the transcript oriented in either direction or ORF2 in the sense direction (Fig. 1D). These experiments revealed that the γ exon is part of an uninterrupted 1643 base noncoding transcript we call CAI2 (CDKN2A/ARF Intron 2 IncRNA) that is distinct from p16, ARF, and p16γ (Fig. 1B). The complete sequence is presented in Supplementary Table S1.

Expression of CAI2 in relation to the p16 and ARF
We compared the expression profile of CAI2 in 46 tumor cell lines and five nontumor samples, with representative amplifications shown in Fig. 1F and summarized in Supplementary Table S2. The expression profiles of p16, ARF, and CAI2 were
similar in most cell lines, where 20 of 26 cell lines with an intact 9p21 locus expressed all three genes robustly. Of the remaining six, all expressed ARF high and p16 low, whereas expression of CAI2 was mixed. In contrast to tumor cell lines, none of the five nontumor samples appreciably expressed CAI2. Considering whether this was a general characteristic of nonneoplastic cells, we undertook a quantitative assessment of CAI2 expression in 25 normal samples. When each gene was normalized and graphed individually against expression in fibroblasts, most tissues exhibited similar levels of expression (≥0.5- to <5.0-fold), with expression much lower (<0.5 of control) in just six and very high in two (spinal cord and adrenal). In contrast, p16 and ARF expression was much lower than fibroblasts in most tissues (Supplementary Fig. S1). However, normalizing each gene to itself hides that CAI2 expression is much lower than that of p16 and ARF in many tissues, including fibroblasts. As illustrated in Fig. 2A, when all three genes were normalized to CAI2 in fibroblasts, the low expression of CAI2 in comparison with p16 and ARF was almost ubiquitous with the exception of spinal cord, which expressed CAI2 approximately 200-fold higher than normal fibroblasts, and adrenal gland at approximately 16-fold higher. In contrast, other tissues tested expressed CAI2 at levels similar to or much lower than fibroblasts, with the lowest levels (<0.1-fold) seen in fetal tissue, kidney, and heart. P16 and ARF are independent genes with independent promoters that share correlated expression profiles. Consistent with this, an SRC analysis of ARF and p16 expression showed a significant association (SRC = 0.68, P <
with the control scrambled vector (psCAI2) versus lipid only is shown.

0.001), with an even stronger correlation of CAI2 with both genes (p16 SRC = 0.73, P < 0.0001; ARF SRC = 0.89, P < 0.0001; Fig. 2B). We hypothesized that CAI2 could be a regulator of p16 and/or ARF. No neuroblastoma cell line was low for all three genes, though NMB7, IMR32, and PCL1643 were low for both CAI2 and p16 (Supplementary Table S2). Using the NMB7 cell line, which, unlike the other two cell lines grows rapidly as dispersed cell populations that are easily transfected (Fig. 2C), we overexpressed CAI2 and observed a small induction of both p16 and ARF expression (2–7-fold), consistent with this hypothesis, though the lack of an influence on cell growth (Fig. 2D) suggests that the levels of induction may be too small to be functionally significant.

**CAI2 and neuroblastoma cell line transformation**

The ability to be induced to differentiate is a characteristic of many neuroblastoma cell lines. We observed that NMB7 cells, with serial passage and without additional treatment, morphologically change from cells with a rounded, trapezoidal morphology (Fig. 3A) to a thinner, more elongated phenotype with long processes (Fig. 3B). Tyrosine hydroxylase (TH) expression is known to increase as neuroblastoma cells differentiate (23). However, with increasing passage number, TH expression decreased by more than 100-fold (Fig. 3C), suggesting that the morphologic change did not represent a normal differentiated phenotype. The NMB7 cell line at low-passage number is also one of the few neuroblastoma cell lines that do not appreciably express CAI2 (Supplementary Table S2). However, with serial passage, CAI2, p16, and ARF expression each exhibited greater than 100-fold increases in expression (Fig. 3C, P < 0.001 for CAI2 and ARF, P < 0.01 for p16, ANOVA). In addition, counter to the growth suppressor effects expected of p16 and ARF, high-passage cells proliferated more rapidly than low-passage cells, with a DT of 22 hours versus 29 hours (Supplementary Fig. S2).
RA can induce differentiation and death in neuroblastoma cell lines. Treatment of low-passage NMB7 cells with 5 μmol/L RA resulted in a visible loss of viability within 24 hours, with more than 95% of the cells having detached by 72 hours, whereas high-passage NMB7 cells showed no effect from the RA treatment (Supplementary Table S3). The 24-hour RA treatment had no significant effect on TH, ARF, or p16 gene expression in the high- or low-passage cell lines, though CAI2 expression decreased in the low-passage cell line (0.39-fold, P = 0.02, t test, Fig. 3D). Serum deprivation can also induce neuroblastoma cell line differentiation. Serum-starved low-passage NMB7 showed visible morphologic signs of stress with cells looking “stringy” by 24 hours, rounded by 48 hours (Supplementary Fig. S3), and detached by 72 hours; TH expression increased moderately within 24 hours of serum starvation (8.8-fold, P = 0.05, t test, Fig. 3D), an indication that differentiation was beginning. On the other hand, there were no significant changes in the expression of CAI2, p16, or ARF (P > 0.05, ANOVA). In contrast, high-passage NMB7 cells showed no negative effects from serum starvation, with no visible morphologic changes or changes in the (already elevated) expressions of CAI2, p16, or ARF (Fig. 3D and Supplementary Fig. S3).

Enhanced mRNA levels of CAI2 in advanced-stage neuroblastoma

As most neuroblastoma cell lines are derived from advanced-stage tumors with high-risk disease (24), and many of the neuroblastoma cell lines highly expressed CAI2 (Supplementary Table S2), we considered whether this represented a unique feature of cell lines or reflected a characteristic of advanced-stage tumor. An examination using RNA from primary neuroblastoma tumors revealed that the 33 prognostically favorable tumors (stages 4S, 1, and 2) expressed low levels of CAI2 that were similar to that found in most normal tissues, though an occasional very high expressor was noted (Fig. 4A). In comparison, the 29 high-risk/stage 4 neuroblastoma expressed significantly higher levels of CAI2 than favorable outcome group (Fig. 4A, P = 0.005, Kruskal–Wallis test comparing stage 4S, 1, and 2 vs. stage 3 vs. stage 4). Interestingly, a significant trend could be established when comparing lowest-stage with highest-stage neuroblastoma (SRC = 0.33, P = 0.01), though expression was not significantly associated with MYCN amplification status (Wilcoxon rank sum test, P = 0.18, n = 62 patients at all stages; P = 0.88, n = 29 stage 3 and 4 patients only). CAI2 expression was also significantly associated with age at diagnosis (SRC = 0.39, P < 0.002, Fig. 4B). Age over 1 year

Figure 3. NMB7 cells morphologically change, and p16, ARF, and CAI2 expression increases with serial passage. A, NMB7 cells at pretransformation (shown at passage 10) have a rounded, trapezoid-like morphology. B, after prolonged serial passage (shown at passage 22), cells morphologically change into an elongated structure with long protrusions. C, RNA was extracted from NMB7 during serial passage (P), and CAI2, p16, ARF, and TH expression determined by qRT-PCR. D, low- and high-passage (pass) NMB7 cells were cultured for 24 hours in the presence of complete media (untreated), complete media + 5 μmol/L RA, or media without sera (0% FBS) for 24 hours before being visually assessed and expression determined. Parallel cultures were also continued for 72 hours, but loss of viability in the treated low-passage cells precluded harvesting of RNA. See also Supplementary Fig. S3 and Supplementary Table S3.
is a negative prognostic factor in neuroblastoma, and most of the patients with high levels of CAI2 delineated to the high-risk category. Thus, we were concerned that an association with age could be an artifact of prognostic features. However, when we limited the analysis to only high-risk stage 3 and 4 patients, CAI2 expression remained significantly associated with age of diagnosis (SRC = 0.38, P < 0.05, Fig. 4C).

We next considered survival outcomes, with the three patients who had no follow-up data censored at day 0, and dichotomized CAI2 expression at 35.9, which was the median of CAI2 values from the stage 3 and 4 patients, resulting in a "high CAI2 expression" population that was comprised of 4 of 33 stages 1, 2, and 4s patients, 2 of 9 stage 3 patients, and 13 of 20 stage 4 patients (Fig. 4A). Among all patients, higher CAI2 expression was significantly associated with shorter event-free survival, EFS [HR for higher vs. lower CAI2 expression = 2.88; 95% confidence interval, CI (1.12–7.45), log-rank test P = 0.022, Fig. 5A] and showed a significant association with overall survival, OS [HR, 3.27; 95% CI, (1.1–9.77), log-rank test P = 0.025, Fig. 5B]. However, when we limited our analysis to only stage 3 and 4 patients, the association of CAI2 expression with either EFS or OS was no longer apparent (HR = 1.84 and P = 0.26 for EFS, Fig. 5C; HR = 1.37 and P = 0.58 for OS, Fig. 5D). Thus, CAI2 expression, in this limited data set, was a prognostic factor associated with a poor outcome, but by itself did not predict survival.

MYCN amplification is a very strong prognostic factor in neuroblastoma. Therefore, we wanted to determine if CAI2 expression added prognostic information over and above MYCN amplification status, using Cox proportional hazards regression models that contained both factors. As expected, MYCN amplification status was a strong negative prognostic factor for both EFS and OS, and remained so even when CAI2
expression was added to the model. On the other hand, the association previously observed between higher CAI2 expression and outcome was attenuated in the model containing both factors in the entire population for both EFS [adjusted HR for higher vs. lower CAI2 expression, 2.19; 95% CI, (0.81–5.94), P = 0.12] and OS [adjusted HR, 2.39; 95% CI, (0.76–7.52), P = 0.14]. Similarly, when we limited the analysis to the stage 3 and 4 population only in the model containing both factors, the association of CAI2 expression with either EFS or OS remained unapparent [HR, 1.67; 95% CI, (0.56–4.97), P = 0.36 for EFS; or HR, 1.25; 95% CI, (0.40–3.88), P = 0.70 for OS).

Discussion
The hallmark of neuroblastoma is its clinical and biologic heterogeneity, with treatment based on subgroups defined by age at diagnosis, extent of disease, and tumor biology. Clinically, risk stratification has been very successful. However, it remains true that some patients with identical risk factors who receive the same treatment can have considerably different outcomes. For example, though immunotherapy with ch14.18 has resulted in significant decreases in relapse and a significant improvement in cure rate, almost one third of the children continue to fail therapy and succumb to the disease (18). The challenge, therefore, is to
refine risk stratification through the identification of biomarkers and novel biologic targets.

The over- and underexpression of ncRNAs have been associated with neuroblastoma prognosis. We and others have shown that miRNA expression profiles can differentially identify high- versus low-risk neuroblastoma (25, 26). The IncRNA IncRAN is overexpressed and prognostically significant in neuroblastoma and bladder cancer (27, 28). In a bladder cancer cell line, overexpression of IncRAN enhanced the proliferation, migration, and invasion of the cells, whereas its suppression enhanced chemosensitivity (28). Expression of the ncRNA NDM29 correlated with growth rate of neuroblastoma and other tumor cell lines in an inverse fashion, with low expression correlating with a faster growth, whereas overexpression in the SKNBE2 neuroblastoma cell line led to their differentiation and an increased chemosensitivity (29). The diverse family of genes known as transcribed ultra-conserved regions (T-UCR) are another class of IncRNAs that have been implicated in neuroblastoma through their association with MYCN amplification, outcome of high risk/stage 4 patients, and RA response (30–32). Many of the characteristics of IncRAN, NDM29, and the T-UCRs are similar to what we find with CAI2 in neuroblastoma. In NMB7, cellular "transformation" is accompanied by not only an increase in CAI2 expression, but also a loss of sensitivity to RA. High-passage NMB7 cells also express significantly higher levels of CAI2 and proliferate faster than low-passage NMB7 cells. CAI2 was also expressed highly in most tumor cell lines, but low in normal cells, suggesting a possible correlation with growth rate.

9p21 is a chromosomal region implicated in many different cancers, with deletion/mutation/epigenetic inactivation of p16 and ARF almost universal events in cancer. However, neuroblastoma has been a glaring exception. In fact, it has been more than a decade since we first reported on the paradoxical overexpression of p16 in advanced-stage neuroblastoma and its association with poor outcome (20). As ncRNAs play regulatory roles, aberrant expression of these molecules may contribute to the paradoxical p16 expression. We considered mir24, a regulator of p16 (33). However, we and others have not observed this miRNA to be differentially expressed in neuroblastoma (25, 26). ANRIL has also been shown to negatively regulate p16 expression (15, 16), though the expression status of ANRIL in neuroblastoma remains unknown. With the discovery that CAI2 can modulate p16 and ARF expression in vitro, there is now an alternative pathway of p16 regulation to consider. A role in neuroblastoma is also supported by our findings that CAI2 is overexpressed in advanced-stage neuroblastoma and is an independent prognostic factor.

Despite strong associations, we did not see an effect of CAI2 on NMB7 cell growth, either negative or positive, after transient transfections, and the effect on p16 was only minimal. It is possible that the conditions in which we are evaluating the effects of CAI2 on growth and p16 expression are inappropriate. For example, if CAI2 optimally influences p16 expression under conditions of stress or DNA damage response, p16 levels may not appreciably change when CAI2 is overexpressed in normal, proliferating cell lines. This would be similar to the actions performed by ANRIL, which suppresses p16 and ARF expression in response to DNA damage (16). The observation that the highest levels of CAI2 are seen in spinal cord, a conduit of stress response, supports this idea. Alternatively, if CAI2 is a transcript "stabilizer" like the IncRNA BASE1-AS (34), then overexpression might again only exhibit a minimal effect. Several IncRNAs have been shown to transcriptionally regulate indirectly through epigenetic actions. For example, the recently reported IncRNA ecCEBPA is a regulator of DNA methylation (35), whereas HOTAIR and ANRIL bind and modulate the Polycomb-repressive complex (36), which is required for epigenetic silencing during development and cancer. However, we showed many years ago that p16 is not methylated in neuroblastoma (19, 22), perhaps precluding the observation of a significant influence of CAI2 on p16 expression.

The fact that CAI2 has only a marginal impact on its nearest neighbor genes while seeming to have a considerable impact on tumor cell growth and survival, as evidenced by its preferential expression in advanced-stage neuroblastoma and association with outcome, may suggest that CAI2 acts in a more global manner, and the regulation of p16 and/or ARF is not its primary function. The NMB7 cells during "transformation" undergo a significant change in morphology, growth, and drug sensitivity consistent with advanced stages of neuroblastoma. The inappropriate overexpression of CAI2 may at least facilitate such a "reprogramming." The possibility of a more global function would be consistent with the histone modification actions of HOTAIR and ANRIL (37). Studies have shown that IncRNAs play roles in multiple biologic processes, including dosage compensation, genomic imprinting, chromatin remodeling, alternative splicing, and nuclear organization (38). A recent study has also shown actions on proliferation, apoptosis, metastasis, and autophagy (39). Exploring possible binding partners of CAI2 will give insight into pathways associated with the gene and clues to its function, as will its overexpression and/or silencing in moderate p16/ARF/CAI2 expressing model systems.

In summary, it is clearer than ever that the 9p21 locus, which is undoubtedly important in cancer, represents a complicated locus. It harbors three tumor-suppressor genes p16, ARF, and p15, the alternative spliced p16y (21) and p10 (40), the contiguously transcribed p12 (41), mutation-generated hybrid proteins such as chimeric ARF (chARF) and p16-Alternate Carboxy Terminus (p16-ACT; ref. 42), the metabolic gene MTAP and its variants (43), and the IFN gene cluster. Thus, alterations to this region have a profound impact on cell growth, metabolic activity, and signaling. It also contains the IncRNAs ANRIL, p15AS and now CAI2. As CAI2 is embedded in intron 2 of the p16/ARF gene, alterations to this locus will undoubtedly also influence CAI2 as well. CAI2 was significantly associated with high-risk neuroblastoma, a population known to be the least likely to respond to chemotherapy, and a population where the many of patients will relapse and die (18). Thus, CAI2 could play an additional role as a biomarker of advanced-stage neuroblastoma, but it is premature to implicate it in response to chemotherapy. Nevertheless, it is clear now that IncRNAs may act not only as biomarkers and/or be prognostically significant, but that their modulation may influence growth and response to therapy. Therefore, one must...
now also consider the role of CAI2 when considering how alterations at this locus influence cell behavior. As such, the role CAI2 plays in diseases where the inactivation of p16 and ARF, such as coronary artery disease, is not consistent with the known cell-cycle regulatory functions of the proteins should be considered. On the basis of these data and the central role this locus plays in multiple human diseases, we believe that CAI2 must be considered in any future analyses of the genes of this locus on tumorigenesis specifically, and 9p21-associated disease pathogeneses in general.

Sequence has been submitted to Genbank with a submission ID #1642929, accession no. KF311101.

Disclosure of Potential Conflicts of Interest
The University of California, San Diego has filed a patent on CAI2 in which M.B. Diccianni, A.L. Yu, L.M. Barnhill, and Y. Kim are named as inventors. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
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