Regulation of Apoptosis and Caspase-8 Expression in Neuroblastoma Cells by Isoforms of the *IG20* Gene

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Abstract

The *IG20* gene undergoes alternative splicing resulting in the differential expression of six putative splice variants. Four of these (IG20pa, MADD, IG20-SV2, and DENN-SV) are expressed in virtually all human tissues. However, investigations examining alternative splicing of the *IG20* gene to date have been largely limited to nonneural malignant and nonmalignant cells. In this study, we investigated the expression of alternative splice isoforms of the *IG20* gene in human neuroblastoma cells. We found that six *IG20* splice variants (*IG20*-SVs) were expressed in two human neuroblastoma cell lines (SK-N-SH and SH-SY5Y), highlighted by the expression of two unique splice isoforms (i.e., KIAA0358 and IG20-SV4). Similarly, we found enriched expression of these two *IG20*-SVs in human neural tissues derived from cerebral cortex, hippocampus, and, to a lesser extent, spinal cord. Using gain-of-function studies and siRNA technology, we determined that these "neural-enriched isoforms" exerted significant and contrasting effects on vulnerability to apoptosis in neuroblastoma cells. Specifically, expression of KIAA0358 exerted a potent antiapoptotic effect in both the SK-N-SH and SH-SY5Y neuroblastoma cell lines, whereas expression of IG20-SV4 had proapoptotic effects directly related to the activation of caspase-8 in these cells, which have minimal or absent constitutive caspase-8 expression. These data indicate that the pattern of expression of these neural-enriched IG20-SVs regulates the expression and activation of caspase-8 in certain neuroblastoma cells, and that manipulation of *IG20*-SV expression pattern may represent a potent therapeutic strategy in the therapy of neuroblastoma and perhaps other cancers. [Cancer Res 2008;68(18):7352–61]

Introduction

Neuroblastoma is one of the most frequently occurring solid tumors in children, particularly in the first year of life, when it accounts for 50% of all tumors (1). Although improvement in outcome has been observed in small, well-defined subsets of patients over the past several years, the outcome for patients with a high-risk clinical phenotype has not improved, with long-term survival <40% (2). A characteristic feature of neuroblastoma is its remarkable clinical and biological heterogeneity (3). Whereas advanced stage neuroblastoma in older children typically responds poorly to aggressive chemotherapy regimens, certain tumors in patients below 1 year of age may spontaneously regress or differentiate into benign ganglioneuromas (2, 3). This spontaneous regression likely represents the activation of an apoptotic and/or differentiation pathway, and the prognosis in neuroblastoma patients may be related to the level of expression of molecules involved in the regulation of apoptosis.

The *IG20* (insulinoma-glucagonoma) gene has been implicated in cancer cell survival and apoptosis (4–10), neurotransmission (11, 12), and neurodegeneration (13). We have previously shown that various splice isoforms of the *IG20* gene (*IG20*-SVs), including IG20pa, MADD/DENN, and DENN-SV, act as negative or positive regulators of apoptosis, and their levels of expression can profoundly affect cell survival in nonneural cells (6–10). *IG20*-SVs are believed to act, in part, by modulating inflammatory and apoptotic signaling pathways, effects mediated through interactions with tumor necrosis factor receptor 1 (TNFR1). TNFα interacts with TNFR1 to trigger proinflammatory actions through various stress-activated protein kinases, such as c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase (14, 15). *IG20* interacts strongly with TNFR1, and all putative *IG20*-SVs contain the death domain homology region required for this binding. We have shown that expression of MADD/DENN is required and sufficient for cancer cell survival in nonneuronal cancer cells and mediates its effects by acting as a negative regulator of caspase-8 activation (16). Overexpression of IG20pa, on the other hand, results in enhanced apoptosis and activation of caspase-8 through enhanced death-inducing signaling complex formation (9). The caspase-8 (*CASP8*) gene encodes a key enzyme at the top of the apoptotic cascade. In neuroblastoma cell lines and tumor samples, CgG methylation of *CASP8* at the 5′ end has been associated with inactivation of the gene (17), and recent hypotheses have proposed that *CASP8* may act as a neuroblastoma tumor suppressor gene (17–20). Furthermore, neuroblastoma cell lines that do not express caspase-8 are resistant to TNF-related apoptosis-inducing ligand–induced apoptosis (19), and suppression of caspase-8 expression has been shown to occur during establishment of neuroblastoma metastases *in vivo* (21).

In this study, we show the preferential expression of two unique splice isoforms (KIAA0358 and IG20-SV4) of the *IG20* gene in selected nervous system tissues and in two neuroblastoma cell lines known to be deficient in the expression of caspase-8. Through gain-of-function studies and using siRNA technology, we show that expression of IG20-SV4 enhances cellular apoptosis and leads to the expression and activation of caspase-8 in SK-N-SH and SH-SY5Y neuroblastoma cells, thereby sensitizing these cells to the proapoptotic effects of TNFα. In contrast, expression of KIAA0358 effectively renders cells resistant to apoptosis, even when IG20-SV4 is coexpressed, and down-modulation of this

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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isoform causes markedly enhanced apoptotic cell death and activation of caspase-8.

Materials and Methods

Cell culture. SK-N-SH, SH-SY5Y, and SK-N-BE(2)-C human neuroblastoma cell lines were purchased from American Type Culture Collection and cultured according their instructions. Briefly, SK-N-SH cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 0.1 mmol/L nonessential amino acids, 1.5g/L sodium bicarbonate, 1.0 mmol/L sodium pyruvate, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. SH-SY5Y and BE(2)-C cells were cultured in a 1:1 mixture of Eagle's MEM with nonessential amino acids and Ham's F12 medium (Invitrogen) supplemented with 10% FBS, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. The cell lines were maintained at 37°C in a humidified chamber with 5% CO_2.

Design of small inhibitory RNAs. The small inhibitory RNAs (siRNA) used in this study are shown in Table 1. The siRNAs targeting exons 13L, 16E, and 15 ("Mid") and SCR (negative control shRNA) are identical to those previously described (10). The siRNA targeting exon34 was designed using OligoEngine Workstation 2 and purchased from OligoEngine, Inc. These siRNAs were screened in SK-N-SH cells and the most efficient were used to construct the 34E-shRNA lentivirus.

Plasmid construction. The siRNAs were cloned into the pSUPER vector using *Bgl*II and *Hin*dIII sites (22) to generate pSup-34 plasmids. The shRNA cassettes (including the H1 RNA promoter and the shRNA) were excised from pSup-34 using *Xba*I and *Cla*I sites and ligated into the pNL-SIN-CMV-GFP vector to generate 34E lentivirus constructs. The pcTat, pcRev, and pHIT/G plasmids were gifts from Drs. B.R. Cullen (Howard Hughes Medical Institute and Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC) and T.J. Hope (Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL). The YFP-IG20pa plasmid (16) was used as a backbone to subclone YFP-KIAA0358 from the corresponding pBKRSV plasmid (6) using the *Bst*Z171 and *Bsi*WI sites. The YFP-KIAA0358 and YFPIG20-SV4 Mid-shRNA resistant mutant constructs were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, the primers 5′-CGGAACCACAGTACAAGCTTTAGCCTCTCAAACCT-CACACTGCC-3′ (forward) and 5′-GGCAGTGTGAGGTTTGAGAGGC-TAAAGCTTGTACTGTGGTTCCG-3′ (reverse) were used to insert four silent mutations (underlined letters) in the cDNAs without affecting the amino acid sequence. *Hin*dIII restriction sites in the mutants, generated due to base substitutions, were used to identify positive clones that were further confirmed by sequencing. The caspase-8 promoter luciferase vector was constructed by PCR amplification of a 1.2-kb fragment from pBLCAT-Casp8 vector and cloning into Promega pGL4.17 luciferase vector at *Kpn*I and *Xho*I sites. The pBLCAT3 vector containing fragment −1,161/+16 of the caspase-8 promoter was a gift from Dr. Silvano Ferrini's lab (Laboratory of Immunological Therapy, Istituto Nazionale per la Ricerca sul Cancro, University of Insubria, Genoa, Italy; ref. 23).

Preparation of lentivirus stocks. Lentivirus stocks were prepared as described previously (22). Briefly, subconfluent 293FT cells grown in 100-mm plates were cotransfected with 10.8 mg lentivirus vector, 0.6 mg pcRev, 0.6 mg pcTat, and 0.3 mg pHIT/G using calcium phosphate. Culture medium was replaced after 16 h, and the supernatant was harvested at 40 h and filtered using a 0.45-mm filter. The optimal viral titer for each cell type was determined as the least amount of viral supernatant required to transduce at least 50% of target cells without apparent cytotoxicity.

RNA preparation. Total RNA extracted from human cerebral cortex, hippocampus, and cerebellum and human thyroid, skeletal muscle, lung, and liver were purchased from BD Clontech. Total RNA extracted from primary neuroblastoma was a gift from Dr. Jill Lahti's lab of St. Jude's Children's Research Hospital (Memphis, TN). For testing the efficiency of down-modulation of *IG20*-SVs by different siRNAs, the transduced green fluorescent protein (GFP)–positive SK-N-SH cells were sorted on the MoFlo High-Performance Cell Sorter (DAKO Denmark). Total RNA was extracted from 1×10^6 GFP-positive neuroblastoma cells and other described cell lines using Trizol reagent (Invitrogen Life Technologies).

Reverse transcription-PCR. We used 1 μg of RNA for reverse transcription-PCR (RT-PCR) using the SuperScript III One-Step RT-PCR system (Invitrogen Life Technologies). Briefly, the cDNAs were synthesized at 50°C for 30 min followed by incubation at 94°C for 2 min. Subsequently, 30 cycles of PCR were carried out with denaturation at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for variable time periods (as described below), followed by a final incubation at 72°C for 7 min. For amplifying exons 13L and 16, F-1 and B-1 primer pairs (5′-CGGGACTCT-GACTCCGAACCTAC-3′ and 5′-GCGGTTCAGCTTGCTCAGGAC-3′, respectively) were used, with 1-min extension time. For amplifying exon 34, F4824 and B5092 primer pairs (5′-CTGCAGGTGACCCTGGAAGGGATC-3′ and 5′-TGTACCCGGGTCAGCTAGAGACAGGCC-3′, respectively) were used, with 30-s extension time as described previously (8). The sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has previously been published (9). The PCR products were then separated on a 5% polyacrylamide gel.

Cell proliferation assay. Cell proliferation assays were done according to the Vybrant 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (V-13154, Molecular Probes, Invitrogen) instructions. Briefly, 24 h posttransduction, 1×10^4 sorted GFP-positive SK-N-SH cells were plated onto 96-well plates. Every other day, cells were washed with PBS and labeled with 10 μL of 12 mmol/L MTT stock solution in each well, incubated at 37°C for 4 h, and washed with PBS. Fifty microliters of DMSO were added to each well and mixed thoroughly with a pipette, and absorbance was recorded at 540 nm.

CFSE dilution assay. Twenty-four hours posttransduction, 1×10^5 SK-N-SH cells were stained with 2 mmol/L SNARF-1 carboxylic acid, acetate, succinimidyl ester (S-22801, Molecular Probes, Invitrogen) for 15 min at 37°C. Cells were washed and either used immediately for FACS analysis or plated onto six-well plates. Every other day, cells were collected and washed, and carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution, as an indicator of cell division, was determined in GFP-positive cells by FACS analysis at excitation/emission of 480/640 nm.

1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide staining. SK-N-SH cells (1.5×10^5) were plated onto six-well plates. Twenty-four hours later, cells were treated with different shRNA-expressing lentiviruses for 4 h, washed, and replenished with fresh warm medium immediately and every other day thereafter. At 5 d, the transduced cells were trypsinized with 0.05% trypsin, 0.53 mmol/L EDTA and suspended in 1-mL warm PBS. Then, 5 μL of 10 μmol/L 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide (DiIC;

Table 1. Nucleotide sequences of exon-specific siRNAs against *IG20*

siRNA	Target sequence	Targeting exon	Targeting isoforms
SCR	5′TTTAACCGTTTACCGGCCT-3′	None	None
MID	5′GTACCAGCTTCAGTCTTTC-3′	15	IG20pa, KIAA0358, MADD, IG20-SV2, DENN-SV, IG20-SV4
34E	5′AGAGCTGAATCACATTAAA-3′	34	IG20pa, MADD, IG20-SV2, DENN-SV
13L	5′CGGCGAATCTATGACAATC-3′	13L	IG20pa, KIAA0358, MADD
34E + 13L	5′AGAGCTGAATCACATTAAA-3′ 5′CGGCGAATCTATGACAATC-3′	34 and 13L	IG20pa, KIAA0358, MADD, IG20-SV2, DENN-SV

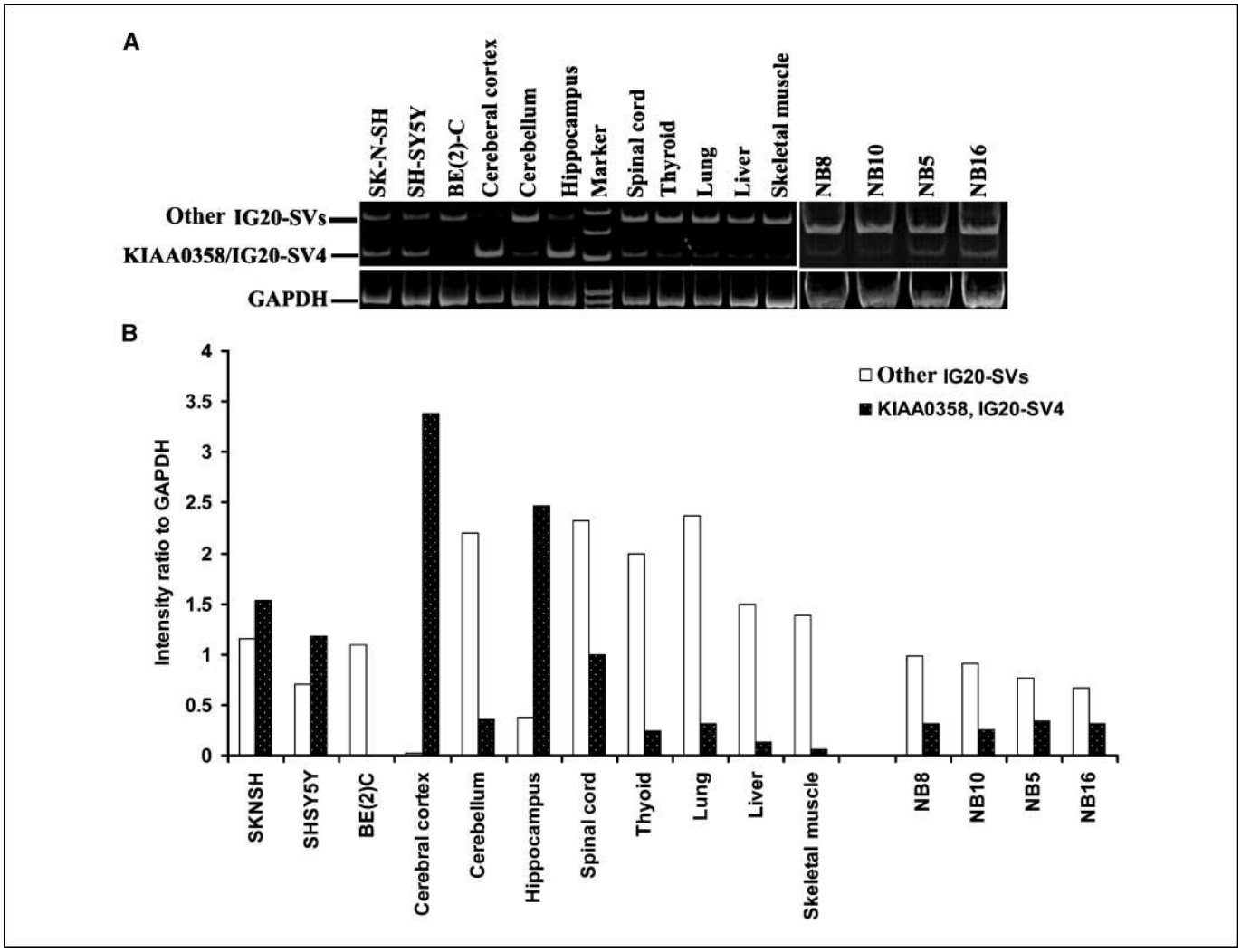

Figure 1. Expression of IG20 splice isoforms in human neuroblastoma cell lines, primary neuroblastoma tumor lines, and various human tissues. One microgram of total RNA was used for RT-PCR using the SuperScript III One-Step RT-PCR system (Invitrogen Life Technologies). *A,* amplification of exon 34 region of *IG20*-SVs using F4824 and B5092 primers. *B,* quantification of relative intensities of bands in relation to the housekeeping gene *GAPDH* from *A* using ImageJ (http://rsb.info.nih.gov/ij/).

Molecular Probes, Invitrogen) were added and the cells were incubated at 37°C, 5% CO_2 for 20 min. Cells were washed once by adding 2 mL of warm PBS and resuspended in 500 μL of PBS. DiIC-stained cells were analyzed on a CyAn ADP Flow Cytometer (DAKO Denmark). Only GFP-positive cells were gated and analyzed.

Apoptosis assay. Annexin V-phycoerythrin (PE)/7-amino-actinomycin D (7-AAD) labeling was done according to the manufacturer's instructions (BD PharMingen) and samples were analyzed by flow cytometry. Neuroblastoma cells (1.5×10^5) were plated onto six-well plates. Twenty-four hours later, cells were treated with different shRNA-expressing lentiviruses for 4 h, washed, and replenished with fresh warm medium immediately and every other day thereafter. At 5 d, the transduced cells were trypsinized and washed twice with cold PBS and then resuspended in 1× assay binding buffer. Annexin V-PE/7-AAD labeling was done at room temperature for 15 min before analysis by flow cytometry (BD FACScan). Only GFP-positive cells were gated and analyzed.

Caspase-8 inhibition. At 3 d posttransduction with different shRNAs, SK-N-SH cells were treated with 40 and 80 μmol/L of Z-IETD-FMK (BD PharMingen) for an additional 2 d or with 10 μg/mL cycoheximide (Sigma) for an additional day. Collected cells were subjected to Annexin V-PE/7-AAD staining followed by FACS or Western blot analysis to determine active caspases.

Western blot analysis. Different shRNA-expressing, lentivirus-transduced neuroblastoma cells were trypsinized and washed with PBS and lysed at 0°C for 30 min in a lysis buffer [20 mmol/L HEPES (pH 7.4), 2 mmol/L EGTA, 420 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μ/mL aprotinin, 1 mmol/L Na_3VO_4, and 5 mmol/L NaF]. The protein content was determined with a dye-binding microassay (Bio-Rad), and after boiling the samples for 2 min in a 1× SDS protein sample buffer, 20 μg of protein per lane were loaded and separated on 10% SDS-polyacrylamide gel. The proteins were blotted onto Hybond ECL membranes (Amersham Biosciences). After electroblotting, the membranes were blocked with TBS with Tween 20 [TBST; 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% milk and were incubated with antibodies diluted in a 5% BSA-TBST buffer that can detect cleaved caspase-8 (Santa Cruz, C-20), caspase-9 (Cell Signaling), and full-length caspase-3 (R&D System, 84803) overnight. The primary antibody dilutions were those recommended by the manufacturer. The membranes were then washed, incubated with the appropriate secondary antibodies (1:5,000) in a blocking buffer for 1 h, and repeatedly washed. Proteins were detected by an enhanced chemiluminescence plus Western blotting detection system (Amersham). The anti-GAPDH-horseradish peroxidase (Abcam) antibodies were used as loading controls.

Transient transfections and luciferase assays. SK-N-SH cells (1.5×10^5 per well) were seeded in 12-well plates and cotransfected with 1.6 μg of pEYFP-C1 or pEYFP-IG20-SV4, 1 μg of pGL4.17 (a promoterless control), or 1 μg of pGL4.17-caspase-8 promoter. Twenty nanograms of pSV40-Renilla luciferase vector were cotransfected as a normalizing control. Transfections were carried out in triplicate. After 48 h of incubation, cells were collected and analyzed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

Dominant-negative Fas-associated death domain (pcDNA-DN-FADD) or control vector (pcDNA3.1) was transfected (5ug each) into 6×10^6 SK-N-SH cells and distributed into six-well plates. To increase the transfection efficiency of DN-FADD, nucleofection from Amaxa Biosystems was used. After 24-h culture, the cells were transduced with either SCR or 34 + 13L shRNA. At 3 d posttransducion, the cells were untreated or treated with 10 ng/mL TNFα for 48 h. The cells were trypsinized and stained with Annexin V-PE/7-AAD for FACS analysis. Only GFP-positive cells were gated and analyzed.

Statistical analysis. All results are expressed as mean ± SE. Student's *t* test was used to determine the *P* values using Microsoft Excel Software (version 2003).

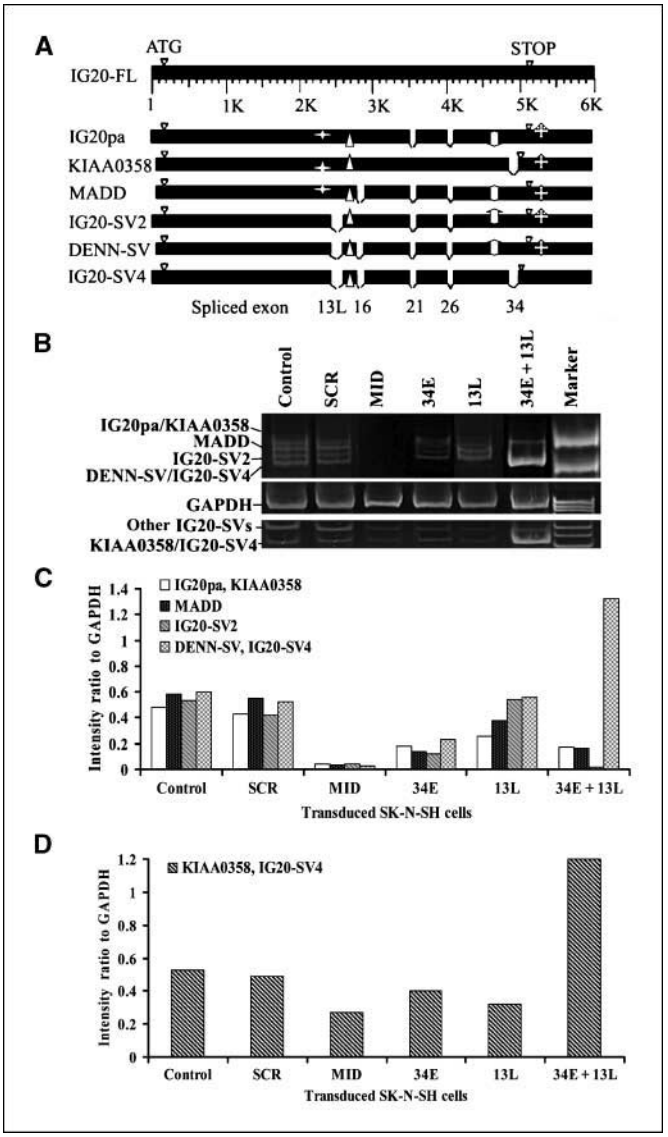

Figure 2. IG20-SVs and down-modulation effect of exon-specific siRNAs directed against specific isoforms on endogenous IG20-SVs in SK-N-SH cells. *A,* human *IG20*-SVs generated by alternative mRNA splicing. *Solid bars,* regions of complete cDNA sequence homology between variants. *Empty areas,* spliced exons 13L, 16, 21, 26, and 34, which, when spliced in different combinations, can give rise to the six *IG20*-SVs. *B,* effect of down-modulation of endogenous IG20-SVs by exon-specific siRNAs in SK-N-SH cells. One microgram of total RNA obtained from GFP-positive SK-N-SH cells obtained by fluorescence-activated cell sorting (FACS) at 5 d posttransduction was used for RT-PCR. The products were separated on a 5% PAGE. Amplification of *IG20*-SVs using F1-B2 primers (*top*) and F4824-B5092 (*bottom*) is shown. *C,* quantification of relative intensities of bands from *B* (*top*) using ImageJ. *D,* quantification of relative intensities of bands from *B* (*bottom*) using ImageJ.

Results

Expression of IG20-SVs in Neuroblastoma Cell Lines and Nervous System Tissues

To begin to understand the potential relevance of *IG20* alternative splicing in the control of apoptosis in neuroblastoma cells, we first tested the constitutive expression patterns of *IG20*-SVs in several neuroblastoma cell culture lines. We used RNA extracted from the SK-N-SH, SH-SY5Y, and SK-N-BE(2)-C human neuroblastoma cell lines and performed RT-PCR using multiple sets of *IG20*-specific primers as described in Materials and Methods. Figure 1 shows the expression pattern of *IG20*-SVs in the tested tissues and cell lines.

Although only one representative sample for each tissue type is shown, we used RNAs from multiple samples of each tissue type to validate the RT-PCR results. In agreement with previous studies (6, 7, 16), we confirmed that different *IG20*-SVs are expressed in different patterns and levels in various human tissues. In addition, we found that two isoforms, KIAA0358 and IG20-SV4, which are not significantly expressed in nonneural tissues, are highly expressed in two (SK-N-SH and SH-SY5Y) of the three human neuroblastoma cell lines tested and in human cerebral cortex, hippocampus, and, o a lesser extent, spinal cord (Fig. 1). In addition, these two isoforms were expressed in both caspase-8–expressing (NB5 and NB16) and caspase-8–deficient (NB8 and NB10) primary neuroblastoma tumor lines. The levels of expression of KIAA-0358 and IG20-SV4 did not correlate with the constitutive expression of caspase-8 in these cells.

SiRNAs Effectively Down-Modulate the Expression of Endogenous IG20-SVs in Neuroblastoma Cells

To analyze the effects of *IG20*-SVs on neuroblastoma cell survival and apoptosis, we designed siRNAs to selectively down-modulate specific *IG20*-SVs as shown in Fig. 2*A*. We had previously identified the most effective siRNAs targeting all isoforms and targeting exons 13L in studies using HeLa cells and PA-1 cells (16, 22, 24). We similarly screened several siRNAs targeting exon 34 and chose the most effective for use in this study. We cloned each siRNA in lentiviral vectors to allow for stable expression of the siRNAs that could be detected through GFP expression.

The targeted exons and resulting down-modulated IG20 isoforms for each siRNA used in this study are summarized in Table 1. We cloned shRNAs into a self-inactivating lentivirus vector (pNL-SIN-GFP; ref. 25) and generated 13L, Mid-, 34E, and SCR (negative control shRNA) constructs. Using GFP, this enabled us to monitor the expression of double-copy cassettes likely resulting in enhanced silencing (25). The transduction efficiency was >50% as determined by GFP expression. For testing the down-modulation efficiency, total RNA from transduced and GFP-positive SK-N-SH cells was used for RT-PCR. The results are shown in Fig. 2*B* and *D*. As expected, SK-N-SH cells expressing Mid-shRNA showed decreased expression levels of all *IG20*-SVs relative to control (SCR). 13L-shRNA caused down-modulation of IG20pa, MADD, and KIAA0358; 34E-shRNA caused down-modulation of IG20pa, MADD, IG20-SV2, and DENN-SV; and

34E + 13L shRNA caused down-modulation of all of these *IG20*-SVs with the addition of KIAA0358. Interestingly, when all isoforms except IG20-SV4 were down-modulated, the expression of this sole isoform seemed to be increased at 5 days posttransduction (Fig. 2*B–D*).

Down-Modulation of KIAA0358 in Neuroblastoma Cells Leads to Spontaneous Apoptosis but Has No Apparent Effect on Cellular Proliferation

Down-modulation of IG20-SVs has no effect on cellular proliferation of neuroblastoma cells. To assess the influence of *IG20*-SVs on neuroblastoma cell growth and proliferation, various shRNA-expressing viable cells were counted using a MTT assay and CFSE dilution. Relative to controls, a significant decrease in the numbers of viable cells expressing Mid-, 34E, 13L, and 34E + 13L shRNA was observed (Supplementary Fig. S1). However, there was no difference in CFSE dilution (SNARF-1 carboxylic acid, acetate, succinimidyl ester) over time among the SCR control, Mid-, 34, 13L, and 34 + 13L shRNA–treated cells, suggesting that the differences in cell numbers were not due to decreased cellular proliferation (Supplementary Fig. S1*B*). Further, shRNA-treated cells failed to show significant differences in cell cycle progression (data not shown). Together, these results indicated that manipulation of the expression patterns of *IG20*-SVs had little or no effect on cell proliferation or cell cycle progression.

Down-modulation of KIAA0358 induces apoptosis in SK-N-SH neuroblastoma cells. Because there is no single method that can conclusively show cellular apoptosis, we determined spontaneous cell death using both mitochondrial membrane potential DiIC staining (Fig. 3*A* and *B*) and Annexin V-PE/ 7-AAD staining (Fig. 3*C*) to ensure the reliability of our findings. We observed that down-modulation of all *IG20*-SVs with Mid-shRNA resulted in a significant increase in spontaneous apoptosis. Down-modulation of IG20pa, MADD, and KIAA0358 (by targeting exon 13L) and down-modulation of all isoforms with the exception of IG20-SV4 (by targeting exons 13L and 34E) also resulted in significantly increased spontaneous apoptosis. These results were consistently observed using both methods of apoptosis determination and after repeating all experiments at least thrice. This suggested to us that certain *IG20*-SVs may act as prosurvival factors because their knockdown resulted in spontaneous apoptosis. The most likely candidates for this prosurvival function were MADD/DENN and KIAA0358 based on the proapoptotic results of down-modulation of these two IG20-SVs. Interestingly, the selective expression of KIAA0358 and IG20-SV4 in the absence of other isoforms (targeting exon 34) resulted in markedly reduced apoptotosis. This finding strongly indicated that expression of KIAA0358 had a pronounced antiapoptotic effect because expression of IG20-SV4 alone (in the absence of all other isoforms including KIAA0358) resulted in very high levels of spontaneous apoptosis (Fig. 3*A–C*), which were suppressed by DN-FADD overexpression (see below).

Enhanced apoptosis in SK-N-SH cells depleted of KIAA0358 is due to expression and activation of caspase-8. To identify the mechanism of enhanced apoptosis induced by *IG20*-SV down-modulation, we examined whether specific caspases were activated in transduced SK-N-SH cells. Cells depleted of KIAA0358 (Mid, 13L and 13L + 34E cells) showed enhanced expression of cleaved caspase-8. There was accompanying evidence for processing of caspase-3 (slightly reduced expression of procaspase-3) but no change in caspase-9 (Fig. 3*D*).

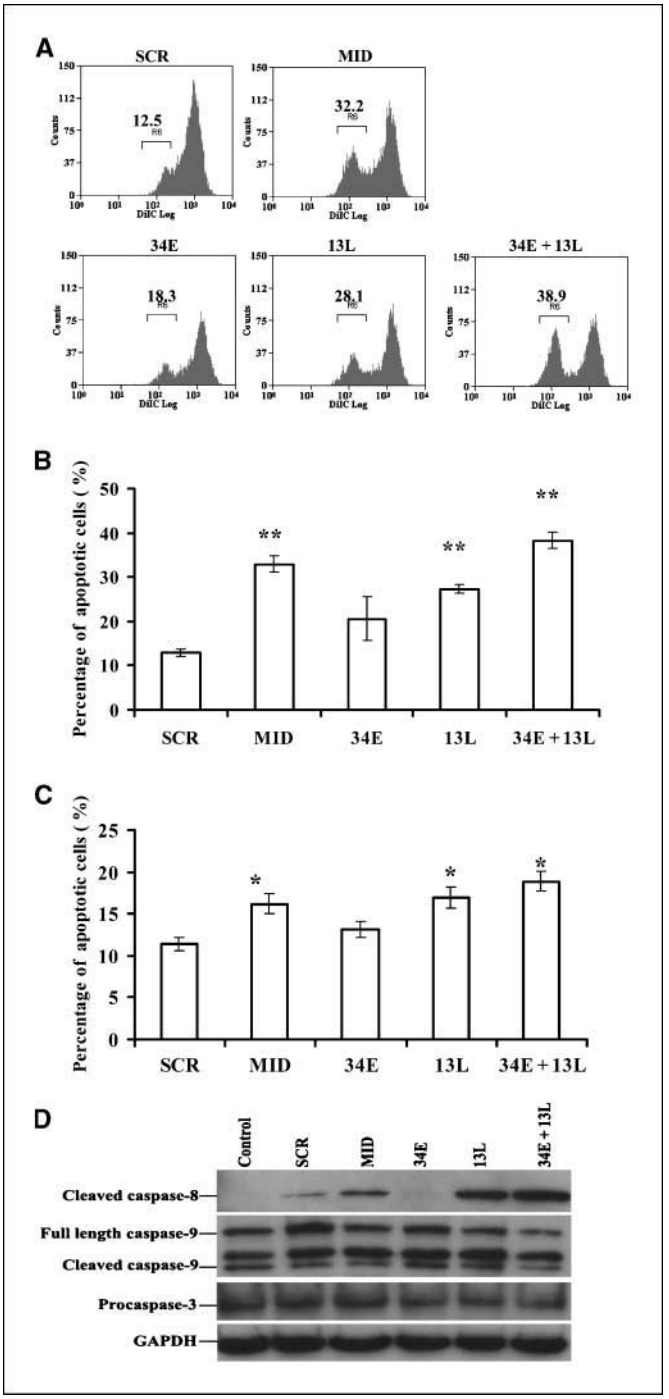

Figure 3. Apoptotic effects and caspase-8 activity with down-modulation of IG20-SVs in SK-N-SH cells. *A,* representative data showing mitochondrial depolarization as determined by DiIC staining. Five days posttransduction, SK-N-SH cells were collected and one third of the collected cells were stained with 50 nmol/L of DiIC. Loss of staining (as a marker of mitochondrial depolarization) was detected by FACS analysis. Percentages of apoptotic cells are indicated on the histograms. *B,* summary of the results showing percentages of cells with increased mitochondrial depolarization as measured by DiIC staining from three independent experiments. **, $P < 0.01$, test groups versus SCR. *C,* summary of results showing percentage of cells with increased apoptosis as determined by Annexin V-PE/7-AAD staining. Another one third of the collected cells as described in *A* were stained with Annexin V-PE/7-AAD and detected by FACS. *, $P < 0.05$, test groups versus SCR. The data were gated from GFP-positive cells only. *D,* caspase-8 activity in neuroblastoma cells transduced with siRNAs. The final one third of cells from *A* were lysed and subjected to Western blot analysis for caspase-8, caspase-9, and caspase-3. Representative of three separate experiments.

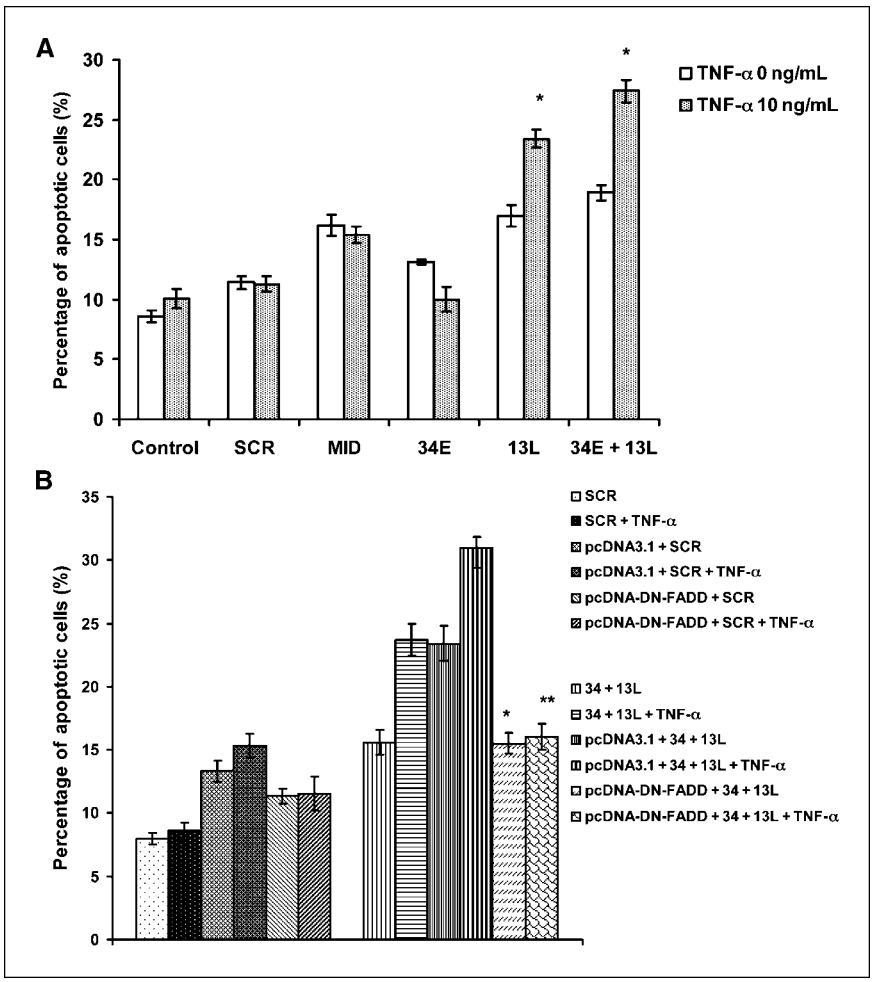

Figure 4. Effects of TNFα treatment on apoptosis of siRNA-transduced SK-N-SH cells. Three days posttransduction, SK-N-SH cells were treated with 10 ng/mL TNFα for 2 d, and cells were collected and stained with Annexin V-PE/7-AAD. *A,* summarized results showing percentage of cells with increased apoptosis from three independent experiments. *, $P < 0.05$, TNFα-treated cells versus untreated cells. *B,* summarized results showing percentage of apoptosis in transduced neuroblastoma cells; cells were untreated or treated with 10 ng/mL TNFα after transfection with pcDNA or pcDNA-DN-FADD. Results from three independent experiments. *, $P < 0.05$; **, $P < 0.01$, pcDNA-DN-FADD–transfected cells versus pcDNA3.1-transfected cells. The data were collected from GFP-positive cells only.

Manipulation of IG20-SVs in other neuroblastoma cell lines [SH-SY5Y and SK-N-BE(2)-C]. Similarly, SH-SY5Y cells transduced with 13L and 13L + 34E siRNAs showed enhanced apoptosis associated with prominent expression and activation of caspase-8 (Supplementary Fig. S2). Because we determined that SK-N-BE(2)-C cells did not express KIAA0358 and IG20-SV4, we obviously did not expect that the siRNAs targeting these isoforms would be relevant in this cell line. Instead, we overexpressed IG20-SV4 and KIAA0358 in SK-N-BE(2)-C cells and examined the effect on caspase-8 activation. Introduction of these isoforms had no effect on the expression/activation of caspase-8 (Supplementary Fig. S3), which was expressed at very low baseline levels in these cells, consistent with previous reports (26, 27).

Treatment with TNFα Enhances Apoptosis in Neuroblastoma Cells Expressing IG20-SV4 in a FADD-Dependent Manner but Does Not Attenuate the Antiapoptotic Effect of KIAA0358

As a binding partner for the TNFR1, the *IG20* gene promotes both proapoptotic and antiapoptotic signals in HeLa cells (6, 7). Therefore, we tested the apoptotic effect of TNFα on SK-N-SH cells. Treatment with TNFα enhanced apoptosis in cells transduced with shRNAs targeting the 13L exon and the combination of exons 13L and 34E (Fig. 4*A*). This induced sensitization to TNFα was significantly suppressed by DN-FADD overexpression (Fig. 4*B*). However, cells transduced with shRNA targeting exon 34 that did not alter endogenous expression of KIAA0358 and IG20-SV4 continued to be resistant to apoptosis even after TNFα treatment (Fig. 4*A*).

Overexpression of KIAA0358 Can Rescue SK-N-SH Cells from Spontaneous Apoptosis Induced by Down-Modulation of All IG20-SVs by Dampening Caspase-8 Activation

We created silent mutations in cDNAs encoding KIAA0358 at sites corresponding to the 5th, 7th, 11th, and 14th nucleotides of the Mid-shRNA target sequence. These mutations affected neither the amino acid sequence nor the protein expression. We generated SK-N-SH cells stably expressing YFP-KIAA0358-Mut. We confirmed that the Mid-shRNA was unable to down-modulate YFP-KIAA0358-Mut but effectively down-modulated the expression of all endogenous *IG20*-SVs (Fig. 5*A*). Interestingly, expression of this KIAA0358 mutant was sufficient to rescue SK-N-SH cells from spontaneous apoptosis caused by Mid-shRNA transduction (Fig. 5*B*), confirming the antiapoptotic properties of KIAA0358. These prosurvival effects were associated with nearly complete dampening of caspase-8 activation (Fig. 5*C*).

Down-Modulation of KIAA0358 and Selective Expression of IG20-SV4 Modulate the Expression of Caspase-8 in Caspase-8–Deficient SK-N-SH Cells

To determine whether the increased apoptosis induced using 34 + 13L shRNA was due to modulation of the expression of

caspase-8, we measured the expression of caspase-8 transcripts in SK-N-SH cells treated with the different combinations of siRNAs. We found that SK-N-SH cells in which all isoforms were down-modulated, leaving expression of IG20-SV4 unperturbed (13L + 34E), expressed increased levels of caspase-8 mRNA compared with control cells (Supplementary Fig. S4). To confirm that the increased expression of caspase-8 was due to induction of gene expression, we exposed the cells to 10 μg/mL of cycloheximide as an inhibitor of new protein synthesis. This inhibited the expression of caspase-8 protein (Fig. 6*A*), suggesting that the effects of *IG20*-SV manipulation were mediated at the level of *CASP8* gene expression. This result was further confirmed by using a luciferase assay, in which overexpression of IG20-SV4 caused a significant (4-fold) increase in activation of the CASP8 promoter compared with control or pEFYP-c1 (empty vector; Fig. 6*B*).

Inhibition of Caspase-8 Effectively Decreases Apoptosis in 13L- and 34E + 13L–Transduced SK-N-SH Cells in a Dose-Dependent Manner

We then pretreated the cells with the specific caspase-8 inhibitor Z-IETD-FMK (40 and 80 μmol/L), which significantly attenuated the apoptotic effect caused by down-modulation of KIAA0358 in a dose-dependent fashion (Fig. 6*C*). The inhibitory effect of Z-IETD-FMK on caspase-8 expression was confirmed by Western blot analysis (Fig. 6*D*). Inhibition of caspase-8 did not significantly affect apoptosis in cells treated with shRNA targeting 34E (Fig. 6*C*).

Discussion

Previously reported cDNAs, designated MADD and DENN, share the same location on human chromosome 11p11 as *IG20*, which comprises 36 exons (4, 5, 14, 15). Other proteins with high homology and almost identical death domains have been discovered and include rat Rab3-GEP, a GDP/GTP exchange protein (28, 29), and KIAA0358, a human brain protein of unknown function (30). Since the publishing of these reports, it has become clear that IG20, MADD, DENN, and KIAA0358 are different isoforms of the same gene (6) that stem from alternative splicing of exons 13L, 16, 21, 26, and 34. In fact, a total of seven putative *IG20*-SVs have been identified (i.e., IG20pa, MADD, DENN-SV, IG20-SV2, KIAA0358, IG20-SV4, and IG20-FL; ref. 6, 7). *IG20*-SVs

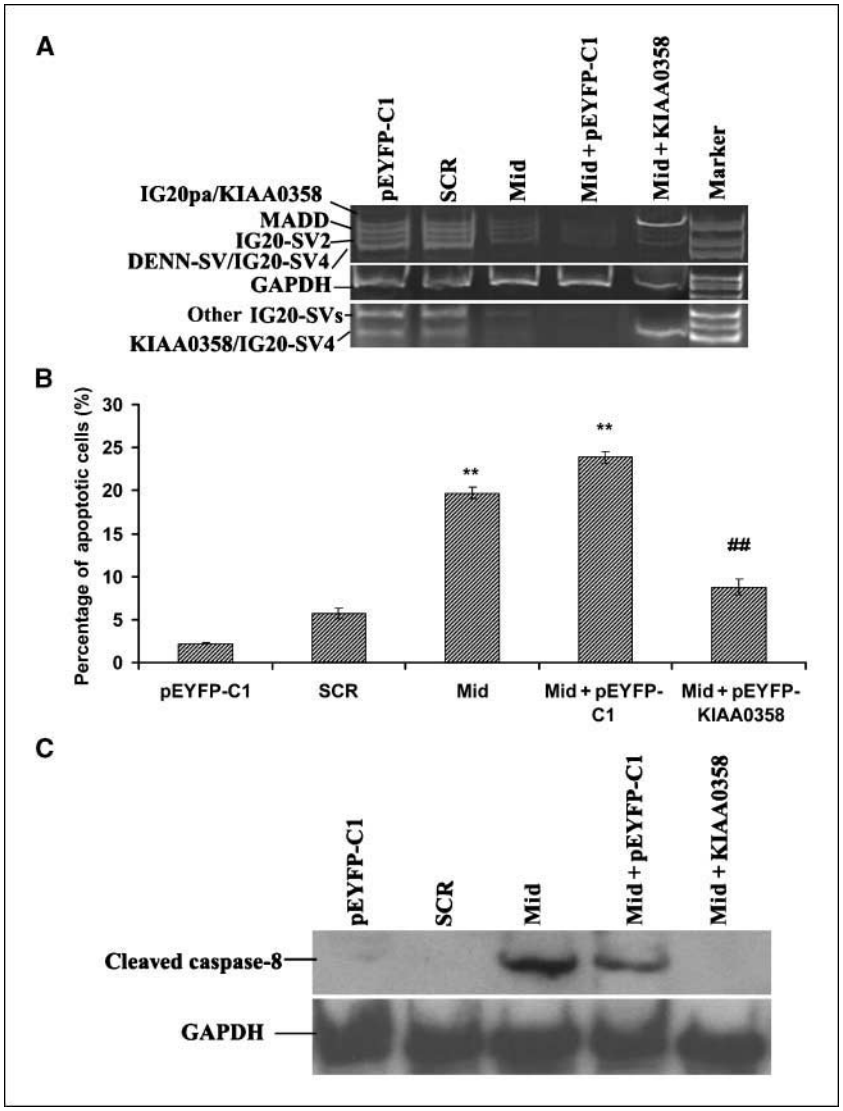

Figure 5. Expression of KIAA0358 in isolation can prevent apoptosis and suppress caspase-8 activity in SK-N-SH cells. *A,* RT-PCR of *IG20*-SVs from stable cells expressing control vector (*pEYFP-C1*) or YFP-KIAA0358-Mut and infected with Mid-shRNA for 5 d. *B,* mitochondrial depolarization assay. SK-N-SH cells were stained with DiIC to determine spontaneous apoptosis. Representative of three independent experiments. **, $P < 0.01$, versus SCR; ##, $P < 0.01$, versus Mid + pEYFP-C1. The data were collected from YFP and GFP double-positive cells only. *C,* Western blot showing caspase-8 activity. Cell lysates were subjected to Western blot analysis of caspase-8. Representative of three individual experiments.

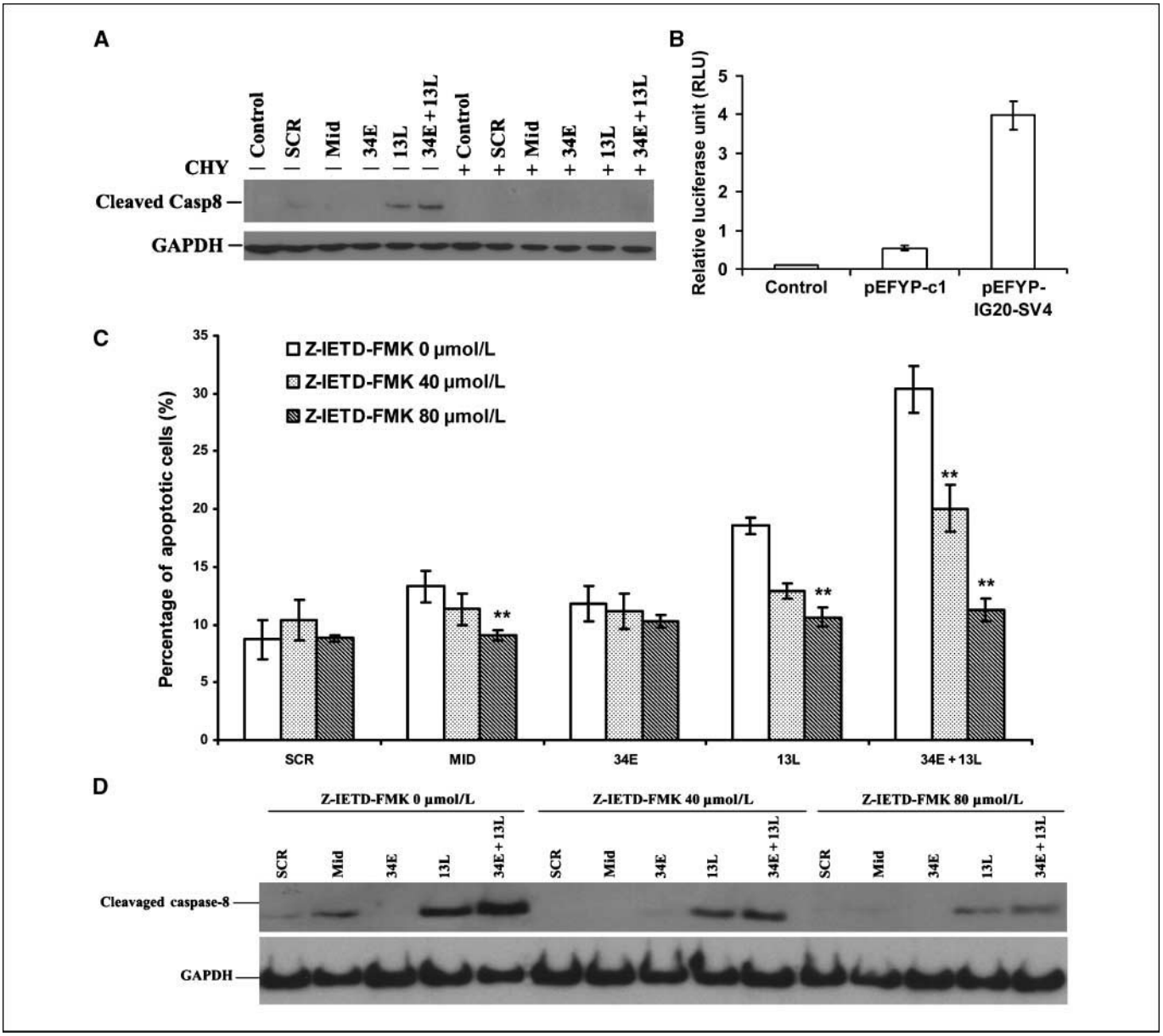

Figure 6. Down-modulation of KIAA0358 or selective expression of IG20-SV4 enhances apoptosis through expression/activation of caspase-8 in SK-N-SH cells. *A,* effects of cycloheximide on the expression/activation of caspase-8. Three days posttransduction with shRNA-expressing virus, SK-N-SH cells were treated with 10 μg/mL cycloheximide (a protein synthesis inhibitor) for 2 d. Whole-cell lysates were subjected to Western blot analysis. *B,* caspase-8 reporter assay. SK-N-SH cells were cotransfected with pGL4.17-caspase-8 promoter vector, pSV40-Renilla luciferase vector, and pEYFP-C1 or pEYFP-IG20-SV4 using Lipofectamine 2000; 48 h later, cells were collected and analyzed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega). *C* and *D,* effects of caspase-8 inhibition. Three days posttransduction with shRNA-expressing virus, SK-N-SH cells were treated with 40 and 80 μmol/L of Z-IETD-FMK (a caspase-8 inhibitor) for 2 d. Collected cells were subjected to Annexin V-PE/7-AAD staining for FACS analysis (*C*) or Western blot analysis (*D*). *C,* percentage of apoptosis in cells transduced with different shRNAs in the presence or absence of the caspase inhibitor. **, $P < 0.01$, Z-IETD-FMK treated versus untreated. *D,* Western blot showing the inhibitory effect of Z-IETD-FMK on caspase-8 activity. Representative data from three independent experiments.

can differentially regulate cell proliferation and death in non-neuronal cell lines (4–10).

In the current study, we first observed that *IG20*-SVs are highly expressed in select nervous system tissues and discovered that KIAA0358 and IG20-SV4, which are not highly expressed in nonneural cells, were significantly expressed in cerebral cortex, hippocampus, and, to a lesser extent, spinal cord. We therefore designated IG20-SV4 and KIAA0358 as "neural-enriched" IG20-SVs. These neural-enriched isoforms were also found to be expressed in two neuroblastoma cell lines (SK-N-SH and SH-SY5Y) known to be deficient in caspase-8 expression, but not in the SK-N-BE(2)-C neuroblastoma cell line, which is known to express caspase-8. There was relatively little mRNA expression of neural-enriched IG20-SVs in human cerebellum or skeletal muscle. The differential presence of these neural-specific *IG20*-SVs is consistent with current evidence of tissue-specific differences in alternative splicing of pre-mRNAs (31, 32).

To investigate the physiologic relevance of the expression of the neural-enriched *IG20*-SVs in neuroblastoma cells, we down-modulated select combinations of *IG20*-SVs using siRNAs in SK-N-SH and

SH-SY5Y neuroblastoma cells. We found that down-modulation of MADD/DENN using shRNA targeting exon 13L enhanced spontaneous apoptosis (SK-N-SH and SH-SY5Y) and TNFα-induced apoptosis (SK-N-SH). It is important to understand that the 13L siRNA will also down-modulate KIAA0358 expression. As expected, down-modulation of all IG20-SVs also resulted in enhanced apoptosis of neuroblastoma cells in SK-N-SH cells, although not significantly in SH-SY5Y cells. However, selective down-modulation of IG20pa, MADD, IG20-SV2, and DENN-SV, allowing for unaltered endogenous expression of IG20-SV4 and KIAA0358, resulted in markedly enhanced cellular survival in both neuroblastoma cell lines. In contrast, knockdown of all splice isoforms except for IG20-SV4 caused a significant enhancement of apoptosis in both SK-N-SH and SH-SY5Y cells. These results suggested that KIAA0358 exerts a predominant suppressive effect on IG20-SV4 in certain neuroblastoma cells.

Because previous studies have shown that MADD/DENN acts as a negative regulator of caspase-8 activation in nonneural cells (33), we hypothesized that the mechanism responsible for enhanced apoptosis in neuroblastoma cells mediated by manipulation of IG20-SV4 and KIAA0358 expression involves activation of caspase-8. Whereas the expression of KIAA0358 and IG20-SV4 in selected primary neuroblastoma tumor lines did not suggest a reciprocal relationship between IG20-SVs and caspase-8, we hypothesized that these IG20-SVs may be involved in the regulation of caspase-8 activation in neuroblastoma cells.

We first noted that caspase-8 expression was increased in cells in which KIAA0358 was down-modulated (treated with 13L and 34E + 13 siRNAs and, to a lesser extent, in cells in which all *IG20*-SVs were knocked down). When transduced SK-N-SH cells were treated with cycloheximide, the induced caspase-8 was inhibited, consistent with it being newly synthesized protein, and suggesting that the pattern of IG20-SV4 and KIAA0358 expression may be involved in the regulation of *CASP8* gene expression. This was confirmed by showing the effect of IG20-SV4 on the activation of the *CASP8* promoter using a luciferase assay. The marked activation of the *CASP8* promoter by IG20-SV4 is direct evidence that IG20-SVs may exert their effects through regulation of *CASP8* gene expression. As expected, inhibition of caspase-8 protected cells from undergoing apoptosis only when KIAA0358 was down-modulated (i.e., using 13L, 34E + 13L, and Mid-siRNAs).

This argued strongly that the mechanism of enhanced apoptosis in these cells was related to caspase-8 expression and activation. Furthermore, the selective expression of IG20-SV4 sensitized neuroblastoma cells to the proapoptotic effects of TNFα, and this sensitization was suppressed by DN-FADD, offering further support for the mechanistic role of caspase-8 in the enhancement of both spontaneous and TNFα-induced apoptosis mediated by selective overexpression of IG20-SV4. Whether these effects occur at the level of the death-inducing signaling complex or possibly after dissociation from TNFR1 (i.e., complex II; ref. 34) will be explored in future studies.

Whereas the levels of apoptosis and caspase-8 activation were very high in neuroblastoma cells in which all IG20-SVs except IG20-SV4 were down-modulated, selective expression of KIAA0358 in the presence of IG20-SV4 (or in the setting of down-modulation of all other isoforms) effectively prevented apoptosis and caspase-8 expression, suggesting that KIAA0358 may have a dominant-negative effect on IG20-SV4. To further confirm the prosurvival effects of KIAA0358 on neuroblastoma cell survival, we generated SK-N-SH cells stably expressing a mutant KIAA0358, which contained silent mutations that did not affect protein expression but prevented down-modulation of KIAA0358 by Mid-shRNA; the cell was transduced with Mid-shRNA for 5 days. As expected, SK-N-SH cell lines expressing this KIAA0358 mutant were largely resistant to apoptosis compared with control cells treated with Mid-shRNA. This effect was accompanied by a nearly complete dampening of caspase-8 activation. Whereas the effects of manipulation of neural IG20-SVs were similar in the SK-N-SH and SH-SY5Y cell lines (both deficient in caspase-8), we observed no effect of introduction of either IG20-SV4 or KIAA0358 on caspase-8 expression in the SK-N-BE(2)-C cell line, which has constitutive expression of caspase-8, suggesting that the effects of IG20-SV expression are likely tissue or cell dependent (33).

Silencing of the *CASP8* gene may play a role in neuroblastoma tumor progression by the induction of tumor cell resistance to apoptosis induced by cytotoxic agents, or by death-inducing ligands such as TNFα or TNF-related apoptosis-inducing ligand (17–19, 35, 36). Induction of caspase-8 expression by gene transfer in neuroblastoma cells has been shown to confer sensitivity to death receptor ligands and to their combinations with cytotoxic agents (23, 37). Further, IFN-γ can sensitize neoplastic cells to apoptosis through up-regulation of caspase-8 (18), and an IFN-sensitive response element in the caspase-8 promoter may play a role in this IFN-γ–driven regulation of caspase-8 expression in cancer cells (23). The regulation of caspase-8 expression likely involves other complex interactions involving the *CASP8* gene. Our results suggest that the expression of IG20-SVs may play a role in determining caspase-8 expression/activation and susceptibility to apoptosis in neuroblastoma cells.

In summary, we show for the first time that proapoptotic signaling caused by down-modulation of KIAA0358 or overexpression of IG20-SV4 effectively induces spontaneous apoptosis and sensitization to TNFα-induced apoptosis through expression and activation of caspase-8 in neuroblastoma cells known to be deficient in caspase-8. Furthermore, enhanced expression of IG20-SV4 alone can overcome the transcriptional inhibition of the *CASP8* gene and up-regulate its expression, whereas KIAA0358 acts as a negative regulator of caspase-8 expression and activation in these cells. Further study examining the precise mode of action of *IG20*-SVs in modulating the expression of caspase-8 in neuroblastoma and other cancer cells may elucidate novel targets that can be manipulated to enhance apoptosis (both spontaneous and in response to cytotoxic drugs) in cancer cells and is currently under investigation.

Disclosure of Potential Conflicts of Interest

B.S. Prabhakar, L.C. Li, and M.N. Meriggioli have a patent application pending related to the use of siRNAs targeting the neural-enriched IG20-SVs in neurodegeneration and cancer. The other authors disclosed no potential conflicts of interest.

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