Differential Expression of S-Phase Kinase-Associated Protein 2 (Skp2) mRNA in Phenotypically Distinct Neuroblastoma Cell Lines

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Abstract

Neuroblastoma, one of the most common pediatric cancers, develops from the neural crest during early childhood. A defining characteristic of neuroblastoma is the existence of three distinct cell phenotypes in the tumor: N-type neuroblastic/neuroendocrine precursors, I-type stem cells, and S-type Schwannian/substrate-adherent cells. Recent data suggest increased Notch1 activity in I-type cells, which are the most tumorigenic phenotype of the three. In this study we investigated, by RT-PCR, the mRNA expression of S-phase kinase-associated protein 2 (Skp2), a putative downstream target of the Notch1 pathway. Three cell lines were used, one from each cell phenotype. Increased expression of Skp2 was found in the I-type CB-JMN line as compared to the N-type SH-SY5Y, which had intermediate expression, and S-type SH-EP-1, which had little expression of the transcript. No significant expression differences were found between the two reported isoforms of Skp2. The results indicate that Skp2 may play a role in neuroblastoma tumorigenicity. Further studies must be performed to better evaluate the links between Notch1, Skp2, and cell phenotype.

Introduction

Neuroblastoma is a cancer which develops from the neural crest during early childhood development. It is one of the most common cancers in children, accounting for approximately 10% of all pediatric cancers (Biedler et al., 1997). Diagnosis typically involves categorizing a neuroblastic tumor according to its location, the patient's age at diagnosis, the presence and degree of metastasis, and the extent of cellular maturation and homogeneity. Neuroblastoma cells can be separated into three unique phenotypes: neuroblastic/neuroendocrine precursor N-type cells, I-type stem cells, and Schwannian/substrate-adherent S-type cells. These cell types display distinct morphological features and growth patterns in tissue culture. N-type cells appear to be immature neuroblasts, possessing small refractile cell bodies, a high nuclear:cytoplasmic ratio, and the presence of short neuritic processes. S-type cells adhere tightly to substrate (in contrast to N-types, which preferentially form cell aggregates) and appear as large flattened cells with an abundant cytoplasm. I-type cells are intermediate in morphology to N- and S-type cells, with equal ability to attach to cells and substrate, an

intermediate nuclear:cytoplasmic ratio, and the occasional presence of neurites. Among the various other features distinguishing these phenotypes, it has been shown that malignancy, as assessed by both tumor formation in nude mice and anchorage-independent growth in soft agar, is significantly more pronounced in I-type cells than in both N-type and S-type cells. Tumorigenicity studies involving both nude mice and soft agar have revealed that S-type cells are non-tumorigenic, forming few to no tumors in soft agar and no tumors in nude mice. N-type cells possess intermediate malignancy, forming tumors in nude mice with a frequency of 33-100% and having colony-forming efficiencies of 4-32%. I-type cells have the greatest malignancy potential, forming tumors in 100% of mice and exhibiting plating efficiencies 3-fold higher than that of N-type cells (Ross et al., 2003).

Previous studies have shown that Notch1, a signaling protein involved in cellular differentiation during embryonic development, is capable of inducing embryonic stem cells to pursue a neural lineage (Lowell et al., 2006). Preliminary studies have further suggested that Notch1 expression may be another distinguishing factor between the three neuroblastoma phenotypes. I-type cells have been shown to express greater Notch1 mRNA and protein levels compared to N-type and S-type cells (Walton et al., 2006). Notch1 has been linked in previous studies to the cell cycle machinery by its ability to regulate entry into S-phase. This is accomplished through induction by Notch1 of transcription of the S-phase kinase-associated protein 2 (Skp2) mRNA. Skp2 serves as an F-box protein subunit in the SCF ubiquitin-ligase complex. The F-box subunit binds particular substrates to the complex, allowing it to target specific proteins for proteasome-mediated degradation (Kipreos & Pagano, 2000). Skp2 binds the cyclin-dependent kinase inhibitor $p27^{Kip1}$, targeting it for degradation. This CKI has been shown to play an important role in timing of the G_1-S phase transition by inhibiting cellular progression into the S-phase (Sarmento et al., 2005). Thus, an up-regulation of Skp2 (resulting from an increased presence of Notch1) brings about $p27^{Kip1}$ degradation, which in turn leads to entry into S-phase and hence uncontrolled cell replication.

The purpose of this current investigation is to determine whether the up-regulation of Notch1 in I-type cells corresponds to higher levels of Skp2 mRNA in I-types than in N-type or S-type cells. Confirmation of this link may aid in the search for the cause of tumorigenicity in neuroblastoma. Additionally, differential expression between the two reported transcriptional

isoforms of Skp2 was also examined. These results lay the groundwork for future studies to determine whether there are functional and regulatory differences between the two isoforms.

Materials and Methods

Cell Lines

CB-JMN, SH-EP-1, and SH-SY5Y cells were kindly provided by Barbara Spengler and Dr. Robert Ross. The cell lines were cultured in MEM with 10% fetal calf serum, 10 μg/mL penicillin and 10 μg/mL streptomycin at 37°C, with 5% CO_2 and humidity. The choice of cell lines was determined based on two primary factors: 1) The cell lines had significant differences in Notch1 mRNA levels, in order to better expose a link between Notch1 and Skp2 in neuroblastoma. 2) Only N-myc non-amplified lines were chosen. Amplification of this proto-oncogene is generally associated with a poor prognosis in neuroblastoma patients (Grandinetti et al.), raising the possibility that if Skp2 is indeed an important player in tumorigenicity, then N-myc protein may be interacting with and affecting the Skp2 pathway. Choosing non-amplified lines ensured that N-myc would not interfere with the results of this investigation.

Table 1. Neuroblastoma cell lines studied in this project

Cell Line	Phenotype	N-myc Amplification
CB-JMN	I	Non-amplified
SH-EP-1	S	Non-amplified
SH-SY5Y	N	Non-amplified

Primers

Primers specific for two regions of Skp2 were ordered from Invitrogen Life Technologies. The first primer pair (Skp2-5) was designed to amplify a region spanning the 5' UTR and exon 1, resulting in a 240 bp product for cDNA and approximately 700 bp product for genomic DNA. The second primer pair (Skp2-M) was designed to amplify a region spanning exons 2 through 4, with the forward primer in exon 2 and the reverse primer in exon 4. The primers result in a predicted product size of 220 bp generated from cDNA, and an approximately 5 kb product size from genomic DNA. A second group of primers specific for each of the two isoforms of Skp2 mRNA was ordered from Integrated DNA Technologies, Inc. The first primer pair (Skp2-V1) was designed to amplify a region within exon 10, whose sequence is unique to isoform 1 (GenBank Accession No. NM_005983), resulting in a product size of 232 bp for both cDNA

and genomic DNA. The second primer pair (Skp2-V2) was designed to amplify a region spanning exons 9 and 10, with the 10th exon having a sequence unique to isoform 2 (GenBank Accession No. NM_03263). The predicted product sizes were 256 bp for cDNA, and approximately 12 kb for genomic DNA. A third set of primers amplifying a region of GAPD was ordered from Invitrogen Life Technologies and was used as a control, generating a predicted product of 225 bp for cDNA, and a genomic DNA product of approximately 2.1 kb.

Table 2. Primers used in RT-PCR

Primer	Sequence (5'→ 3')		Position*	Predicted size from genomic DNA
Skp2-5	Forward	TGGAACGTTGCTAGGCTTAG	39-58	700 bp
	Reverse	GCAGTTCAGAAGTCTTGCTG	250-269	
Skp2-M	Forward	GAGCTGCTAAAGGTCTCTG	528-546	5 kb
	Reverse	AGGTCCATGTGCTGTACAC	727-745	
Skp2-V1	Forward	CAGATGGTACCCTTCAACTG	1297-1316	232 bp
	Reverse	TACTCCAGCAATTGGGCTTC	1509-1528	
Skp2-V2	Forward	GCGGCTACAGAAAGAATCTC	1048-1067†	12 kb
	Reverse	CTGTATGTTTGAGGGCATCC	1284-1303†	
GAPD	Forward	AACGGATTTGGTCGTATTGG	100-119	2.1 kb
	Reverse	TTTGGAGGGATCTCGCTCCT	308-327	

* Skp2 mRNA, GenBank Accession No. NM_005983; GAPD mRNA, GenBank Accession No. NM_002046.
† Skp2 Isoform 2 mRNA, GenBank Accession No. NM_03263.

Total RNA Extraction

Total RNA was prepared from the CB-JMN, SH-SY5Y, and SH-EP-1 cell lines using the standard organic RNA isolation procedure developed by Chomczynski and Sacchi (1987) with slight modifications as outlined by Farrell (1989). After removal of the media from the cells, the cells were either first treated with 5 mL PBS buffer followed by treatment with 3 mL Accutase (SH-EP-1), or treated directly with 3 mL Accutase for approximately 5 min (CB-JMN, SH-SY5Y). 5-7 mL of culture medium (fetal calf serum) was added, and for each line the entire solution containing freed cells was collected in an individual 15 mL conical tube and centrifuged at 1,500x g for 6 min. After removal of the supernatant, the pellet was resuspended in 1 mL of PBS and centrifuged again at 1,500x g for 15 min. The supernatant was again discarded and each pellet was resuspended in 4 mL of a GT buffer solution containing 4M guanidine thiocyanate with 668 µL β-mercaptoethanol per 40 mL solution added just prior to resuspension of the pellets. The cell lysate was passed through an 18 gauge needle and syringe

until no longer viscous, and then treated with 400 µL of 2M sodium acetate (pH 4.0) and vortexed briefly. 4 mL of phenol was added, followed by 1 mL of chloroform:isoamyl alcohol (49:1 ratio). The solution was vortexed approximately 30 sec., left on ice for 15 min. to allow for complete phase separation, then centrifuged at 10,000 rpm at 4°C for 30 min. The upper phase was then added to 8 mL of 100% ethanol pre-chilled on ice, and the solutions were allowed to precipitate at -20°C for approximately 4-5 hours. The tubes were again centrifuged at 10,000 rpm at 4°C for 30 min. The supernatant was discarded, and the pellet resuspended in 400 µL of GT buffer. This resuspension was added to 800 µL of pre-chilled 100% ethanol in a microcentrifuge tube. The tubes were left at -20°C overnight to allow further RNA precipitation, then centrifuged again at 10,000 rpm, 4°C for 30 min. The RNA pellets were resuspended in 70% ethanol, vortexed, left at room temp. for 10 min., and centrifuged again for 15 min. The supernatant was removed, and residual ethanol was allowed to evaporate from the tubes for approximately 15-20 min. Finally, 50 µL of RNAse-free deionized water was added to each dry pellet, and the RNA concentration in each sample was quantified using a spectrophotometer (260 nm). Since each RNA sample varied in its concentration, aliquots of equal RNA concentrations (20 ng/µL) were prepared using RNAse-free deionized water.

RT-PCR

RT-PCR was performed on the total RNA samples prepared from the three neuroblastoma cell lines using the Qiagen OneStep RT-PCR kit. For each set of reactions, a master mix was prepared using 3 µL of 5x RT buffer, 0.6 µL of dNTP's, 0.6 µL of enzyme mix, and 8.8 µL of ddH_2O per reaction sample. 13 µL of the master mix was added to each sample tube, along with a specific primer pair and RNA sample. 0.75 µL of forward primer (10 pmol/ µL), 0.75 µL of reverse primer (10 pmol/ µL), and 0.5 µL (10 ng) of total RNA was added to each sample. 12 µL of mineral oil was added to each sample to minimize evaporation. The samples were placed in a thermal cycler and heated according to the following protocol: RT, 50°C for 30 minutes, activation of the Taq Polymerase enzyme, 95°C for 15 minutes, PCR - 94°C for 20 sec. (denaturation), 56°C for 30 sec. (annealing), 72°C for 30 sec. (extension), followed by a final hold at 4°C. For the Skp2-5 primers, amplification was performed for both 29 and 28 cycles. For the Skp2-M primers, amplification was performed for both 28 and 27 cycles. For the Skp2-V1 and Skp2-V2 primers, amplification was performed for both 32 and 30 cycles each. For the

GAPD primers, amplification was performed for 22 cycles. Final extension was unnecessary, given the small size of the PCR products. After completion of the RT-PCR reaction, 4 μL of loading dye was added to each sample. 8 μL of each product was then added to a 1% agarose gel containing ethidium bromide, and electrophoresis was performed at 140V for approximately 35-40 minutes. Band intensities were quantified using SigmaGel Scanner.

RT-PCR product purification

PCR products for the 5' Skp2 target, the central Skp2 target, and both isoform-specific targets were purified for one of the cell lines (CB-JMN) using Marligen's Purification Kit as per the manufacturer's instructions with slight modifications. 400 μL of binding solution (H1) was added to each RT-PCR product and mixed thoroughly. The mixtures were centrifuged at 13,000 rpm for 1 min. 700 μL of ethanol-containing wash buffer (H2) was then added to each cartridge, followed by a second centrifugation at 13,000 rpm for 1 min. Flow-through was discarded, and a third centrifugation was performed at 13,000 rpm for 1 min to remove residual wash buffer. The contents of each cartridge were then eluted using 35 μL of deionized water warmed to 70°C. Incubation at room temp. for 1 min. followed by centrifugation at 13,000 rpm for 2 min. yielded purified products.

Sequencing

DNA sequencing by Sanger's dideoxy method was performed using the AmpliCycle Sequencing Kit supplied by Applied Biosystems. For each PCR product, a master mix was generated containing 4 μL of 10x cycling buffer, 2 μL of primer, approximately 150 fmol of DNA, 0.5 μL of α-^{33}P dATP, and deionized water yielding a total volume of 30 μL. 6 μL of this solution was added to four tubes. In each tube 2 μL of either ddGTP, ddATP, ddTTP, or ddCTP was then added. All the tubes were then given 15 μL of mineral oil to minimize evaporation. The tubes were placed in a thermal cycler and heated according to the following protocol: 94°C for 3 min., 35 cycles of 94°C for 30 sec. (denaturation), 58°C for 30 sec. (annealing), 72°C for 1 min. (extension). Upon completion of the thermal cycling, 4 μL of stop solution was added to each reaction tube. The tubes were heated to 94°C for 3 min. to denature the samples and were then immediately loaded onto a polyacrylamide gel. 3 μL of each sample was added to the sequencing gel.

Results

The expression of four distinct Skp2 mRNA regions was studied via RT-PCR. Differential expression of the 5' end of the Skp2 transcript (Skp2-5) versus the central region (Skp2-M) was first examined to determine whether previously reported mRNA sequences encoding Skp2-like proteins alpha, beta, and gamma (GenBank accession AB050979, AB050980, AB050981) were present in neuroblastoma cell lines. These three mRNA sequences are homologous to Skp2 but lack a 5' UTR and each possesses a unique 3' sequence, suggesting possible regulatory and/or functional differences from Skp2. The missing 5' region prevents amplification of these three sequences when using the Skp2-5 primers, allowing a comparison of expression levels between Skp2-5 and Skp2-M to determine whether these unusual mRNA sequences are present. The expression levels of Skp2-5 and Skp2-M were analyzed on a 1% agarose gel (fig. 1 and 2, respectively). Of the three cell lines examined, the I-type cell line CB-JMN expressed higher levels of Skp2 regardless of which region (5' versus central) was amplified. N-type SH-SY5Y expressed intermediary levels, while S-type SH-EP-1 expressed little Skp2. GAPD expression was relatively uniform across the samples, although SH-SY5Y consistently showed moderately higher levels of GAPD, indicating a higher initial amount of total RNA. A slightly greater amount of mRNA expression was uniformly seen in the central versus the 5' amplified region, suggesting the possible presence of Skp2-like mRNA. However, the differences were slight, and the expression of mRNA between the three cell lines was the same regardless of which primer pair was used. PCR products from the CB-JMN samples were purified and sequenced using Sanger's dideoxy method. A BLAST analysis of the 107 bp sequence derived from Skp2-5 and the 116 bp sequence derived from Skp2-M (figure 3) showed 98-100% homology to the predicted Skp2 mRNA sequences, confirming that the expected regions of Skp2 were amplified in the PCR reactions.

Next, differential expression of the two Skp2 isoforms was examined via RT-PCR followed by analysis on a 1% agarose gel (figures 4 and 5). Isoform 1 of Skp2 was expressed in both CB-JMN and SH-SY5Y in about equal amounts, with little expression in SH-EP-1. Isoform 2 displayed results similar to Skp2-5 and Skp2-M, with CB-JMN expressing the greatest levels of mRNA, followed by SH-SY5Y and then SH-EP-1. GAPD was expressed in approximately equal amounts, and was not overexpressed in SH-SY5Y since new aliquots of

SY5Y RNA had been generated prior to this series of RT-PCR reactions. PCR products from the CB-JMN samples were again purified and sequenced using Sanger's dideoxy method. The BLAST comparison of the 71 bp sequence derived from Skp2-V1 and the 80 bp sequence derived from Skp2-V2 (figure 6) showed 98% homology to the predicted Skp2 mRNA sequences for both segments, confirming that the expected regions of Skp2 were amplified in the PCR reactions.

The data for the above experiments were quantified via SigmaGel scanner and compiled in figure 7. The use of SigmaGel allowed for computation of numerical values for Skp2 versus GAPD expression in each sample. The data were normalized using EP-1 as the base level of 100% Skp2 expression, allowing for a semi-quantitative view of the expression levels. Overall, Skp2 showed about a 2-fold increase in expression in the N-type cell line SH-SY5Y over the S-type SH-EP-1, and a 4 to 5-fold increase in expression in the I-type CB-JMN versus EP-1. This difference was found when amplifying either the 5' region or the central region of the mRNA. For isoform 1 expression, a 1.5 to 2-fold increase was seen in JMN versus EP-1, and a 2 to2.5-fold increase was seen in SY5Y versus EP-1. For isoform 2, JMN was expressed about 2.6-fold over EP-1, while SY5Y was expressed about 2.4-fold over EP-1.

Discussion

The hypothesis set out by this project was that the S-phase kinase-associated protein 2, having a potential role in the malignancy of I-type stem cells in neuroblastoma, should have greater mRNA expression in the I-type phenotype and particularly in cell lines that express a greater level of the upstream regulator of Skp2 transcription, Notch1. CB-JMN, with a high expression of Notch1 mRNA, does appear to have an up-regulated expression of Skp2 when compared to the less malignant N-type SH-SY5Y and the non-malignant S-type SH-EP-1. A greater sample size is necessary to better establish a correlation between levels of Notch1 and Skp2 mRNA expression. Further, the correlation between phenotype and Skp2 cannot be clearly established for two reasons: 1) a single sample from each phenotype is not enough to establish statistical significance based on cell type, and 2) the three cell lines studied in this experiment were chosen largely because their differences in Notch1 mRNA expression were more pronounced than in many of the other cell lines. Since Notch1 levels vary even within a single phenotype, in order to correlate Skp2 with phenotype a wider sample size must be examined.

It appears that neuroblastoma cells do not express the Skp2-like transcripts whose sequences appear in GenBank. The relative expression of Skp2 in JMN, SY5Y, and EP-1 was the same when both the 5' region-amplifying primers and the central region-amplifying primers were used. Curiously, the PCR reaction set at 28 cycles for Skp2-M had approximately equal expression levels to the Skp2-5 reaction run at 29 cycles. Thus, the central region primers displayed a uniform 1.5 to 2-fold increase in expression compared to the 5' region primers (data not shown). Several hypotheses can be posited as to why these results were seen. It is possible that a certain amount of Skp2 mRNA has its 5' UTR cleaved, degraded, or otherwise made unavailable for primer hybridization during the PCR process. Since relative phenotypic expression is unaffected by the decrease in 5' region amplification, this cleavage/degradation is most likely unrelated to any tumorigenicity associated with Skp2. Another possible explanation is that Skp2-like mRNA is being expressed in neuroblastoma but that it is expressed according to phenotype in the same fashion as Skp2 itself. Third, the transcription of Skp2 pre-mRNA may be up-regulated as a function of Notch1 levels and/or cell phenotype, but subsequent splicing events then generate the variety of mRNA sequences reported.

There does not appear to be a significant difference in expression between the two reported isoforms of Skp2. Even though the first isoform expresses equal levels of Skp2 in JMN and SY5Y (differing from the expression levels found using the Skp2-5 and Skp2-M primers), the possibility of genomic DNA contamination cannot be eliminated from these particular data. Suitable primers could not be found which selectively amplified the first isoform and simultaneously spanned an intronic sequence, so any genomic contamination would have been amplified by the Skp2-V1 primers. The selection of primers in general for the Skp2 mRNA proved particularly difficult and, given the many possible transcript forms generated, RT-PCR is somewhat limited in its usefulness for studying Skp2. Future studies may include a Northern blot analysis, which would readily distinguish differing Skp2 transcripts by size, as well as a Western blot analysis to confirm whether the increase in Skp2 transcript corresponds to increased expression of the Skp2 protein. Experiments altering the reported upstream regulation of Skp2 via transfection of activated Notch1 cDNA or DAPT-induced inhibition of the Notch1 signaling pathway would also aid in confirming or disproving correlations between Skp2 and Notch1. The possibility that overexpression of N-Myc protein affects the Notch1/Skp2 pathway and alters Skp2 transcription should also be accounted for by

including cell lines with amplified MYCN gene. Finally, sample sizes overall should be increased in order to better elucidate any links between Notch1, Skp2, and phenotype in neuroblastoma.

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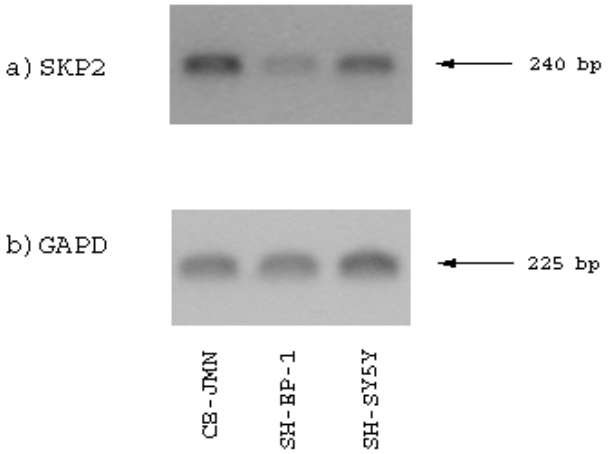

Figure 1. RT-PCR amplification of total RNA extract from three neuroblastoma cell lines: I-type line CB-JMN (lane 1), S-type SH-EP-1 (lane 2), N-type SH-SY5Y (lane 3). Comparison to a 100 bp ladder confirmed predicted product sizes. a) Amplification in the 5' region of Skp2 mRNA. b) Amplification of GAPD mRNA as a loading control.

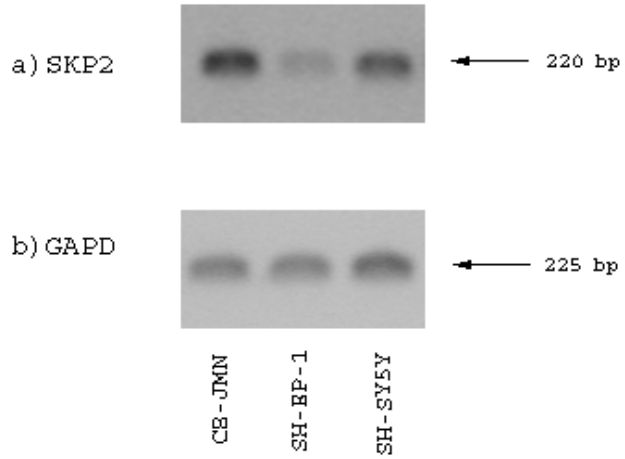

Figure 2. RT-PCR amplification of total RNA extract from CB-JMN (lane 1), SH-EP-1 (lane 2), and SH-SY5Y (lane 3) for a) the central region of Skp2 mRNA, and b) GAPD as a loading control.

Figure 3. Partial sequence alignment of purified RT-PCR product with reported Skp2 mRNA sequence (NCBI). Alignment for the 5' region primer product (top) shows 100% homology to a 107 bp region of Skp2. Alignment for the primer product amplifying a central Skp2 region (bottom) shows 98% homology to a 116 bp segment.

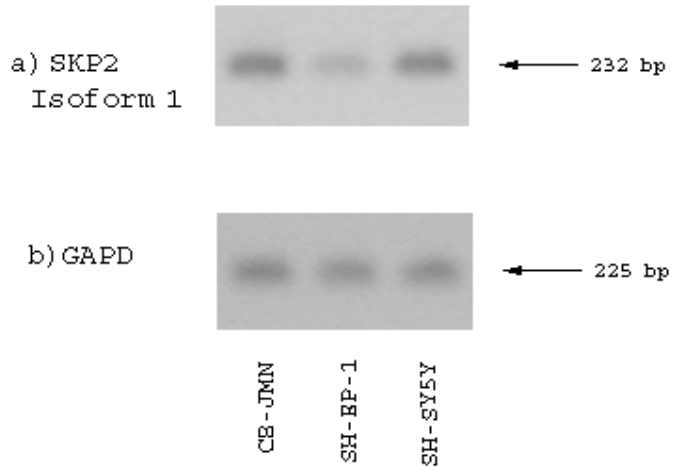

Figure 4. RT-PCR amplification of total RNA extract from CB-JMN (lane 1), SH-EP-1 (lane 2), and SH-SY5Y (lane 3) for a) the isoform-specific exon 10 located at the 3' end of Skp2 mRNA (here, specific for isoform 1), and b) GAPD as a loading control.

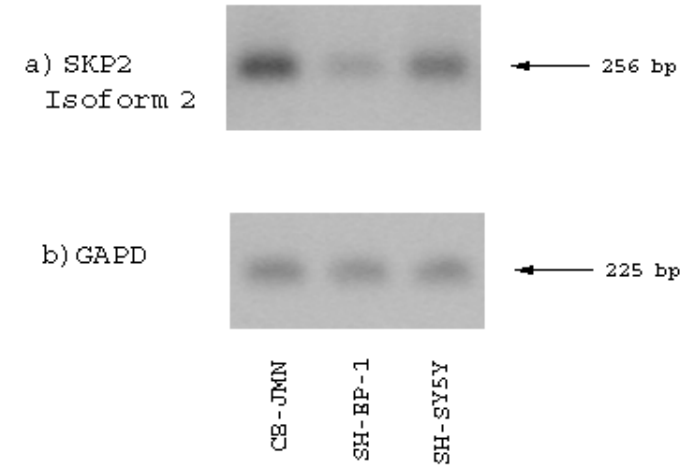

Figure 5. RT-PCR amplification of total RNA extract from CB-JMN (lane 1), SH-EP-1 (lane 2), and SH-SY5Y (lane 3) for a) the isoform-specific exon 10 located at the 3' end of Skp2 mRNA (here, specific for isoform 2), and b) GAPD as a loading control.

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Isoform 1 RT-PCR Product  GATTAATTGCTCCCATTTCACCACCATTGCCAGGCCAACTATTGGCAACATAAAGAACCA  60
                          ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Skp2 mRNA                 GATTAATTGCTCCCATTTCACCACCATTGCCAGGCCAACTATTGGCAACAAAAAGAACCA  1402

Isoform 1 RT-PCR Product  GGAGATATGGG  71
                          ||||||||||||
Skp2 mRNA                 GGAGATATGGG  1413


Isoform 2 RT-PCR Product  TCTCTCTACTTTAGTTAGAAGATGCCCCAATCTTGTCCATCTAGACTTAAGTGATAGTGT  60
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Skp2 mRNA                 TCTCTCTACTTTAGTTAGAAGATGCCCCAATCTTGTCCATCTAGACTTAAGTGATAGTGT  1138

Isoform 2 RT-PCR Product  CATGCTAAAGAATGACTGCT  80
                          ||||||||||||||||||||
Skp2 mRNA                 CATGCTAAAGAATGACTGCT  1158
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Figure 6. Partial sequence alignment of purified RT-PCR product with reported Skp2 mRNA sequence (NCBI). Alignment for the isoform 1 primer product (top) shows 98% homology to a 71 bp region of Skp2. Alignment for the primer product amplifying isoform 2 (bottom) shows 98% homology to an 80 bp segment.

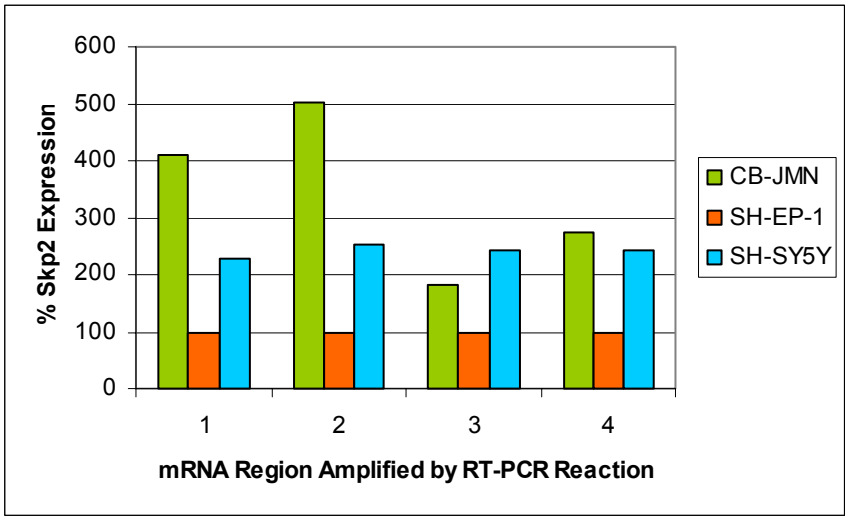

Figure 7. Compiled data (N=2) for RT-PCR amplification of Skp2 mRNA versus GAPD in CB-JMN, SH-EP-1, and SH-SY5Y. Four regions of Skp2 mRNA were amplified: the 5' region (1), a central region (2), the 3' region unique to the first isoform (3), and the 3' region unique to the second isoform (4). Data were normalized to EP-1, which was set at 100% expression.