Expression of Caspase-9 in Neuroblastoma Cell Lines

Bo Liu
Department of Biological Sciences, Fordham University, Bronx, NY10458
Email: bliu@fordham.edu

Abstract

Neuroblastoma is one of the most common pediatric solid tumors originating from the sympathoadrenal lineage of the neural crest. The tumors found in infants exhibit a high rate of spontaneous regression, a phenomenon in which apoptosis is likely involved, while those in children over one year of age are very aggressive and eventually kill the patients. Caspase-9, an initiator caspase that plays a crucial role in intrinsic apoptosis pathway, has been suggested to be important in controlling tumor development. In this study, we investigated the expression of caspase-9 in three different cell lines on both mRNA levels, by RT-PCR amplification, and protein levels, by western blot analysis. Caspase-9 was detected in all cell lines studied. Lower expression of caspase-9 was found in a MYCN amplified I-type cell line and as compared to a MYCN amplified N-type cell line and a MYCN nonamplified I-type cell line. These results indicate that both the cell phenotypes and MYCN amplification status may have the regulatory effect on expression of caspase-9 in neuroblastoma cells.

Key words: neuroblastoma, apoptosis, caspase-9

Introduction

Human neuroblastoma, one of the most common solid tumors of infancy, is a cancer in the neural crest, and is commonly categorized by their location, age at diagnosis, spread or metastasis, and degree of cellular maturation and heterogeneity (1). Histopathological examination of tumors has revealed the presence in neuroblastomas of a variety of cellular phenotypes, especially neuroblasts and nonneuronal (Schwann cell, glia, melanoblast) precursor cells (2). Scientists have shown that cell diversity also characterizes cultured human neuroblastoma cell lines and have defined three prominent cell types. Most common are sympathoadrenal neuroblasts (N-type), which grow as poorly attached aggregates of small, rounded cells with neuritic processes. A second cell type (S-type) resembles neural crest-derived, nonneuronal precursor cells. These large, flattened cells attach strongly to the substrate, and show contact inhibition of cell growth. A third cell type (I-type), an intermediate stem cell from which the first two may arise, is morphologically intermediate between N-type and S-type (1, 2). Recently, these three cell types have been demonstrated to occur in human neuroblastoma tumors as well. The three cell types display different tumorigenic potentials. S-type nonneuronal cells are nonmalignant, whereas N-type cells and I-type cells are malignant. I-type cells have the highest malignant potential among these three cell types (2, 3). Amplification of the oncogene MYCN in neuroblastoma strongly correlates

with unfavorable outcome and is used as a marker for poor prognosis (4). However, it has also been reported that malignant potential of the neuroblastoma is defined by the malignant stem cell phenotypes, not by the amplification of MYCN or the level of expression (3, 5).

Apoptosis is a process of programmed cell death that depends on a number of genes highly conserved. Multiple pathways tightly control the coordinated action of these genes and ultimately lead to cell death. Proper regulation of apoptosis is essential for normal homeostasis and tissue development (6). Aberrations in apoptosis may contribute to the pathogenesis of cancer, and therefore are likely to be important in the neuroblastoma progression. The apoptotic program involves activation of a group of cysteine proteases termed caspases, which are present in cells as inactive or low active proenzymes (7). Caspase-9 is an initiator caspase that plays an important role in many forms of drug-induced cell death triggered by release of cytochrome c from the mitochondria into the cytosol. In the cytosol, procaspase-9 is activated through the formation of a multi-protein complex, termed an apoptosome, by the binding of cytochrome c to the activating factor Apaf-1 (6, 8). Once the apoptosome is formed and caspase-9 is activated, downstream effector caspases, such as caspase-3 and caspase-7, can then be activated to promote the cell death program (6, 7). It has been reported caspase-9 and its cofactor Apaf-1 play an important role in controlling tumor development (9). Recent studies also found the differential expression of caspase-9 gene in neuroblastoma tumors with unfavorable versus favorable biology (10). Considering these, caspase-9 seems a potential therapeutic target in neuroblastoma.

In this study, we examined expression of caspas-9 in three neuroblastoma cell lines (Table 1), on both mRNA levels and protein levels, to determine whether the expression of caspase-9 gene is correlated with distinct cell phenotypes or amplification status of MYCN gene.

Table 1. Neuroblastoma cell lines studied in this project.

Cell line	Phenotype	MYCN amplification status
BE(2)-C	I	Amplified
BE(2)-M17	N	Amplified
SK-N-ER	I	Nonamplified

Materials and methods

Cell lines and RNA extracts

BE(2)-C, BE(2)-M17 and SK-N-ER RNA cells, and RNA isolated from these three neuroblastoma cell lines were kindly provided by Barbara Spengler and Dr. Robert Ross. All three cell lines were cultured in MEM with 10% FCS, 10 μg/ml penicillin and 10 μg/ml of streptomycin at 37℃, with 5% CO_2 and humidity.

Antibodies

A monoclonal caspase-9 (C9) mouse antibody, which detects endogenous levels of the human procaspase-9 as well as cleaved fragments, was purchased from Cell Signaling. An antibody (4 μg/μl) generated against human GAPDH in mouse was purchased from Ambion. An

anti-mouse IgG (1μg/μl) conjugated to alkaline phosphatase was purchased from Promega

Primers

Two pairs of primers were designed to amplify a segment of human caspase-9 mRNA and a segment of human GAPDH mRNA (Table 2). Caspase-9 primers were predicted to amplify a product of 241 bp from caspase-9 RNA and a product greater than 10.4 kb from genomic DNA. GAPDH primers were predicted to generate a product of 228 bp and a product greater than 2.1 kb

Table 2**.** Primers used in RT-PCR.

Primer		Sequence (5'→3')	Position*	Intron in between the primers
Caspase-9	Forward	GAGTCAGGCTCTTCCTTTG	293-311	10.2kb
	Reverse	CCTCAAACTCTCAAGAGCAC	514-533	
GAPDH	Forward	AACGGATTTGGTCGTATTGG	100-119	1.9kb
	Reverse	TTTGGAGGGATCTCGCTCCT	308-327	

* Caspase-9 mRNA, GenBank Accession No. NM_001229; GAPDH mRNA, GenBank Accession No. NM_002046.

RT-PCR

Twenty nanograms of RNA was amplified in 15 μl RT-PCRs (3μl 5×RT buffer, 0.6μl 10mM dNTPs, 0.75μl 10pmol/μl caspase-9 forward primer, 0.75μl 10pmol/μl caspase-9 reverse primer, 0.6μl enzyme mix, 2μl 10ng/μl RNA and 7.3μl dH_2O) using the One Step RT-PCR Kit (Qiagen). One-step RT-PCR was carried out as follows: one cycle of 50℃×30min and 95℃×15min, followed by 35 cycles of 94℃×30s, 56℃×30s, and 72℃×30s, and then a final extension of 72℃×5min. To control for the amount of RNA present in the samples, RT-PCR amplification of GAPDH was performed on all RNA samples. One nanogram of RNA was amplified in 15 μl RT-PCRs (3μl 5×RT buffer, 0.6μl 10mM dNTPs, 0.75μl 10pmol/μl caspase-9 forward primer, 0.75μl 10pmol/μl caspase-9 reverse primer, 0.6μl enzyme mix, 1μl 1ng/μl RNA and 8.3μl dH_2O) using the One Step RT-PCR Kit (Qiagen). One-step RT-PCR was carried out as follows: one cycle of 50℃×30min and 95℃×15min, followed by 24 cycles of 94℃×30s, 56℃×30s, and 72℃×30s, and then a final extension of 72℃×5min. RT-PCR products were analyzed on a 1% agarose gel.

Sequencing

PCR products were purified by using Rapid PCR Purification System (Marligen) according to the manufacturer's protocol. The concentrations of DNA were measured by UV absorbance at 260 nm (A260) with a UV spectrophotometer. Fifty fmol of purified DNA was Sequenced by Sanger's dideoxy method using AmpliCycle Sequencing Kit (Applied Biosystems), following the manufacture's protocol. Sequencing reaction conditions were: one cycle of 94℃×2min, followed by 35 cycles of 94℃×30s, 58℃×30s, and 72℃×1min. Following the sequencing reactions, 4 μl of stop solution was added to each tube and samples were boiled for 3 min prior to loading and electrophoresis on a sequencing gel. The gel was then dried for two hours and exposed overnight to x-ray film. Sequence read from the film was used for a BLAST

search against NCBI database

Western blot analysis

Cultured cells were washed by PBS twice and then detached from flask by EDTA-trypsin. After centrifuged to remove the medium and trypsin, cell pellets were lysed in lysis buffer (25mM Tris-phoshpate, 2mM DTT, 1mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 1% Triton X-100) and stored at -80℃. Bio-RAD Protein Assay Kits was used to analyze protein concentration, according to the manufacturer's protocol, using 0.5-5μg/μl BSA to make standard curve. Fifteen nanograms of protein fractionated by SDS-PAGE in an 8% acrylamide slab gel were blotted onto a nitrocellulose membrane. After incubated in 15ml blocking buffer (1×TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 2 hours and washed three times for 5 minutes each with 15ml TBST (1×TBS, 0.1% Tween-20), the membrane was incubated with a mouse caspase-9 monoclonal antibody (Cell Signaling) 1:1000 diluted in 1×TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk at 4℃ for 2 days. The blot membrane was then washed three times for 5 minutes each with 15 ml TBST and probed with an anti-mouse secondary antibody (Promega) conjugated to alkaline phosphatase (1:4000 diluted in TBST), followed by detection with 4ml Western Blue Substrate solution (Promega). To control for the amount of protein present in the samples, western analysis of GAPDH was performed on all cell lysates using a mouse GAPDH antibody (Ambion) 1:2000 diluted in TBST as described above.

Result

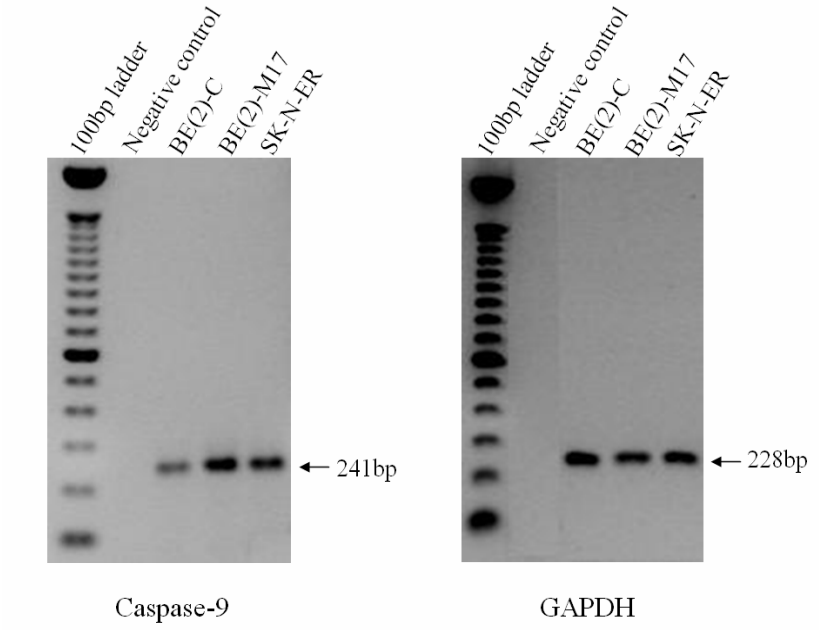

Figure 1. Expression of caspase-9 mRNA in different neuroblastoma cell lines. RT-PCR, using primers located in exon 2 and exon 3, was performed on RNA isolated from three different cell lines. The resulting amplified products were fractionated on a 1% agarose gel. RT-PCR amplification of GAPDH mRNA performed on all three cell lines was used to monitor the amount of RNA present in the samples.

To study the differential expression of caspase-9 mRNA in these three different

neuroblastoma cell lines with different cell phenotypes and MYCN amplification status, RNA isolated from three cell lines were amplified by RT-PCR using primers located in exon 2 and 3 of caspase-9 gene. RT-PCR amplification of GAPDH was used to monitor the amount of RNA in samples. PCR products of caspase-9 mRNA and GAPDH mRNA were then analyzed on 1% agarose gel (Figaure 1). Caspase-9 mRNA was detected in all three neuroblastoma cell lines. Significant difference in caspase-9 mRNA expression was found among these cell lines, while no clear difference was found in GAPDH mRNA expression. With same MYCN amplification status, I-type BE(2)-C cells have lower expression of caspase-9 mRNA than N-type BE(2)-M17 cells. Between the two I-type cell lines, MYCN amplified BE(2)-C cells express caspase-9 RNA at a lower extent as compare to MYCN nonampified SK-N-ER cells. This result indicates that the expression of caspase-9 mRNA may be regulated by both cell phenotypes and MYCN amplification.

PCR product was purified and sequenced by Sanger's dideoxy method. A BLAST search using the sequencing result of 179 bases was performed against NCBI database. One hundred percent homology between 179 bases of PCR-product and caspase-9 mRNA (NM_001229) confirmed the PCR product was amplified from the expected segment of caspase-9 mRNA (Figure 2).

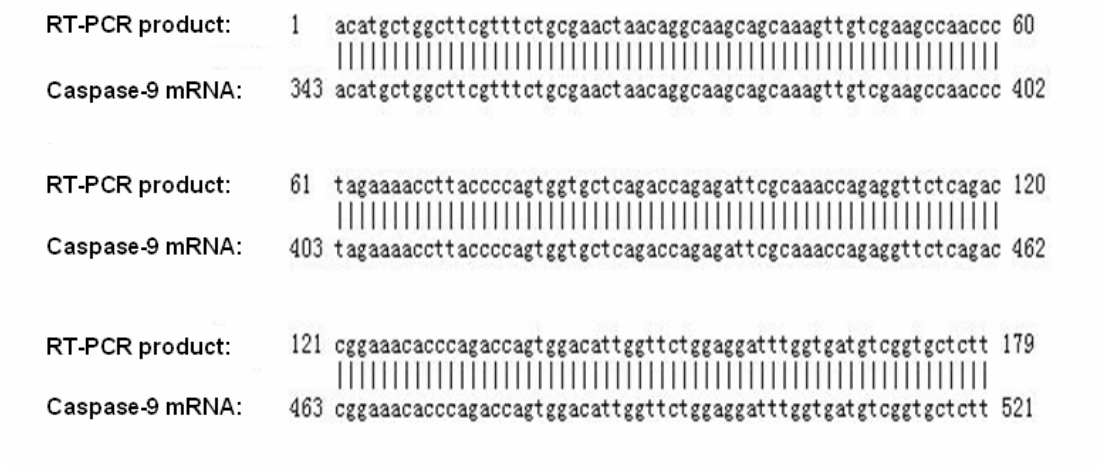

Figure 2. BLAST alignment of RT-PCR product with caspase-9 mRNA (NM_001229) from NCBI.

Western blot analysis was performed on cellular extracts prepared from three neuroblastoma cell lines, using a monoclonal antibody against both procaspase-9 and processed caspase-9 (Figure 3). Expression of procaspase-9 (47kD) was found in all three cell lines. No significant difference in the expression of GAPDH (35kD) was found among three cell lines, whereas procaspase-9 was found to be expressed to a lower extent in BE(2)-C cells as compared to BE(2)-M17 cells and SK-N-ER cells. The consistent results in expression analysis on both mRNA levels and protein levels indicate the expression of caspase-9 may be regulated by both cell phenotype and MYCN amplification status in neuroblastoma cells. Since only three neuroblastoma cell lines were studied in this project, another possibility is that expression of caspase-9 is specifically lower in the BE(2)-C cells, without correlation with cell phenotypes

or MYCN amplification. A further study on a larger number of neuroblastoma cell lines with different phenotypes and different MYCN amplification is needed to confirm the correlation between expression of caspase-9 and cell phenotypes or MYCN amplification.

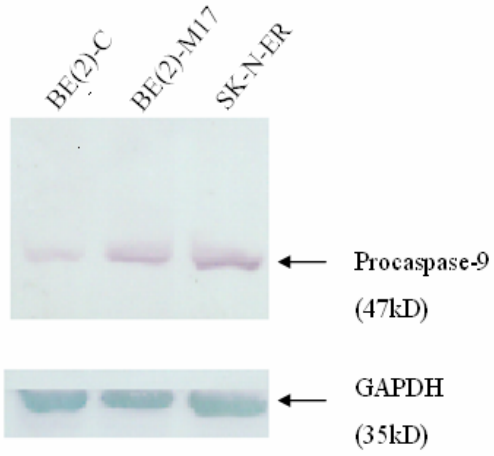

Figure 3. Western blot analysis of procaspase-9 in different neuroblastoma cell lines using a monoclonal antibody against procaspase-9. An antibody against GAPDH was used to detect GAPDH as an internal control.

Discussion

It has been proposed that apoptosis plays an important role in neuroblastoma progression (10). In a tumor, a balance between cell proliferation and apoptotic cell death is a crucial determinant of its net growth rate (11). Accordingly, repressed apoptosis may lead to tumor progression, and enhanced apoptosis may lead to tumor regression. Expression of caspase-9 was found in all three cell lines. Although the N-type cells and I-type cells are both malignant, expression of caspase-9 in these neuroblastoma cells is not silenced. This may be responsible for the possible spontaneous regression in neuroblastoma. This study appears to show differential expression of caspase-9 in different neuroblastoma cells lines. Both the cell phenotypes and MYCN amplification status may have the regulatory effect on expression of caspase-9 in neuroblastoma cells. Lower expression of caspase-9 in I-type BE(2)-C cells comparing to N-type BE(2)-M17 cells may be one possible reason for the higher malignant potential of I-type cells than N-type cells. Since advanced staged neuroblastoma tumors have a higher frequency of I-type cells (3), our result is consistent with an earlier study in which scientists found lower expression levels of caspase-9 in advanced tumors (10).

We also found that the MYCN amplified I-type BE(2)-C cells have lower expression of caspase-9 than I-type cells without MYCN amplification (SK-N-ER). Amplification of MYCN is a frequent event in advanced stages of human neuroblastoma and correlates with poor prognosis (12). MYCN amplification results in high level of N-myc protein, a transcription factor, which perturbs the finely tuned interplay of N-myc and Max and eventually induces abnormal expression of target genes (13). Caspase-9 gene may be among the target genes, and downregulated by N-myc protein. Another possible reason is the frequently loss of heterozygosity of

chromosome region 1p36, where caspase-9 gene is located, in MYCN amplified neuroblastomas (14). Loss of one allele leads to the reduction in caspase-9 expression.

Although a further study on a larger number of neuroblastoma cell lines is required to confirm the correlation of expression of caspase-9 with the cell phenotypes and MYCN amplification status, the present study indicates that caspase-9, the initiator caspase in the mitochondrial apoptosis pathway, may play an important role in regulation of apoptosis in neuroblastoma cells. Experiments have found that caspase-9 was a necessary response to the cytotoxic drugs, such as doxorubicin and cisplatin, within the drug-treated neuroblastoma cells (15). Recently, the specific activation of the mitochondrial apoptotic pathway using cyclooxygenase-2 inhibitors effectively induces apoptosis of neuroblastoma cells both in vitro and in vivo (16). The low expression of caspase-9 may be responsible for drug resistance in advanced neuroblastoma. An apparent imbalance between mitochondrial pro-apoptotic and anti-apoptotic mediators found in advanced stages of neuroblastoma tumors suggests that the mitochondrial apoptotic pathway might have a decisive effect on the development and aggressive behavior of advanced neuroblastoma (10). Future studies on other members in mitochondrial apoptotic pathway, including Apaf-1 and Bcl-2 family proteins, are required to fully explore the role of mitochondrial apoptotic pathway in neuroblastoma. Research on mitochondrial apoptotic pathway in neuroblastoma cells should have significance with regard to the design of novel therapies for neuroblastoma.

Acknowledgement

I would like to thank Jinsong Qiu and Brian Fox for their continuous help through this project. I am grateful to Barbara Spengler and Dr. Robert Ross for the RNA samples, cells and their helpful advices. Sincere thanks to Dr. Berish Rubin for his guidance and providing the opportunity to do the present project.

References

1. Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA, Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages, Cancer Res. 1989 Jan 1;49(1):219-25.

2. Ross RA, Spengler BA, Domenech C, Porubcin M, Rettig WJ, Biedler JL, Human neuroblastoma I-type cells are malignant neural crest stem cells, Cell Growth Differ. 1995 Apr;6(4):449-56.

3. Walton JD, Kattan DR, Thomas SK, Spengler BA, Guo HF, Biedler JL, Cheung NK,Ross RA, Characteristics of stem cells from human neuroblastoma cell lines and in tumors, Neoplasia. 2004 Nov-Dec;6(6):838-45.

4. Brodeur GM, Neuroblastoma: biological insights into a clinical enigma, Nat Rev Cancer. 2003 Mar;3(3):203-16.

5. Ross RA, Biedler JL, Spengler BA, A role for distinct cell types in determining malignancy in human neuroblastoma cell lines and tumors, Cancer Lett. 2003 Jul 18;197(1-2):35-9.

6. Thornberry NA, Lazebnik Y, Caspases: enemies within, Science. 1998 Aug

28;281(5381):1312-6.
7. Fuentes-Prior P, Salvesen GS, The protein structures that shape caspase activity, specificity, activation and inhibition, Biochem J. 2004 Dec 1;384(Pt 2):201-32.
8. Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW, Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation, Mol Cell. 2002 Feb;9(2):423-32.
9. Soengas MS, Alarcon RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW, Lowe SW, Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition, Science. 1999 Apr 2;284(5411):156-9.
10. Abel F, Sjoberg RM, Nilsson S, Kogner P, Martinsson T, Imbalance of the mitochondrial pro- and anti-apoptotic mediators in neuroblastoma tumours with unfavourable biology, Eur J Cancer. 2005 Mar;41(4):635-46.
11. Townson JL, Naumov GN, Chambers AF, The role of apoptosis in tumor progression and metastasis, Curr Mol Med. 2003 Nov;3(7):631-42.
12. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM, Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage, Science. 1984 Jun 8;224(4653):1121-4.
13. Facchini LM, Penn LZ, The molecular role of Myc in growth and transformation: recent discoveries lead to new insights, FASEB J. 1998 Jun;12(9):633-51.
14. Abel F, Sjoberg RM, Ejeskar K, Krona C, Martinsson T, Analyses of apoptotic regulators CASP9 and DFFA at 1P36.2, reveal rare allele variants in human neuroblastoma tumours, Br J Cancer. 2002 Feb 12;86(4):596-604.
15. Bian X, Giordano TD, Lin HJ, Solomon G, Castle VP, Opipari AW Jr, Chemotherapy-induced apoptosis of S-type neuroblastoma cells requires caspase-9 and is augmented by CD95/Fas stimulation, J Biol Chem. 2004 Feb 6;279(6):4663-9. Epub 2003 Nov 14.
16. Johnsen JI, Lindskog M, Ponthan F, Pettersen I, Elfman L, Orrego A, Sveinbjornsson B, Kogner P, Cyclooxygenase-2 is expressed in neuroblastoma, and nonsteroidal anti-inflammatory drugs induce apoptosis and inhibit tumor growth in vivo, Cancer Res. 2004 Oct 15;64(20):7210-5.