ELSEVIER

Cancer Genetics and Cytogenetics 146 (2003) 41–47

Cancer Genetics and Cytogenetics

Caspase 3 and 8 deficiency in human neuroblastoma

Achille Iolascon[a], Adriana Borriello[b], Lucia Giordani[c], Valeria Cucciolla[b], Arcangela Moretti[c], Fausta Monno[c], Vittoria Criniti[b], Andrea Marzullo[d], Maria Criscuolo[b], Fulvio Della Ragione[b,*]

[a]*Institute of Pediatrics, University of Foggia, Foggia, Italy*
[b]*Department of Biochemistry and Biophysics "F. Cedrangolo", Second University of Naples, Naples, Italy*
[c]*Department of Biomedicine of the Evolutive Age, University of Bari, Bari, Italy*
[d]*Department of Pathology and Genetics, University of Bari, Bari, Italy*

Received 28 October 2002; received in revised form 5 March 2003; accepted 7 March 2003

Abstract An altered apoptotic response represents a pivotal feature of cancer and is involved in cancerogenesis and resistance to chemotherapy. So far, however, only a few studies have been devoted to survey caspase content in malignant cell lines and primary tumor specimens. In this report, we investigated the expression of two pivotal caspases, 3 and 8, in 63 neuroblastoma specimens by three complementary techniques (i.e., reverse transcriptase polymerase chain reaction, immunoblotting, and immunohistochemistry). We confirmed the frequent absence of caspase 8 expression. Moreover and most important, we demonstrated, for the first time to our knowledge, that a significant percentage of neuroblastomas lack caspase 3 mRNA and protein. Both caspase alterations do not show any correlation with tumor stage and *MYCN* status. Immunohistochemistry showed a large number of caspase-negative cell islets also present in positive samples. Our findings suggest that the absence of caspases might play an important role in neuroblastoma development and resistance to apoptosis-based treatments.

1. Introduction

The antitumoral activity of most chemo- and immunotherapeutic agents depends on the activation of programmed cell death in susceptible cancers [1,2]. Apoptotic death is morphologically characterized by a series of figures including phosphatidylserine exposure, cell shrinkage, and condensation and fragmentation of chromatin [3], which distinguishes this process from cell necrosis.

The programmed cell death is activated at the molecular level, when cells receive death-inducing stimuli via the following: 1) engagement of specific membrane receptors (Fas/APO-1/CD95 receptor); 2) shortage of obligatory growth factors, and 3) physical or chemical damages. Different initiation events result in the activation of at least two nonexclusive distinct "executioner" pathways. One is related to the activation of caspase 8 (FLICE/Mech-1/Mch-5), which follows the engagement of the so-called death receptors (for example, Fas/Fas ligand interaction). The other relies on the increase of mitochondrial membrane permeability, which causes the release of cytochrome c (and other proteins) from the mitochondrial intermembrane space. Both of these executioner pathways cause the proteolytic activation of caspase 3 (and/or its homologues, caspases 6 and 7), followed by the final cascade with the cleavage of multiple downstream caspase substrates [4]. In this scenario, a key role is also played by p53 protein, which modulates the apoptotic response induced by DNA damage, growth factor deprivation, and hypoxia [5]. Moreover, p53-mediated apoptosis has been identified as a central mechanism by which many antitumor treatments kill cancer cells [6].

Abnormal apoptosis is a hallmark of human cancers, and there is strong evidence that the sensitivity of various tumor types to current therapies critically depends on the expression of apoptosis regulatory proteins. Inactivation of the *p53* gene, down-regulation of the Fas-dependent pathway [7,8], and overexpression of antiapoptotic proteins (like Bcl-2) have all been largely demonstrated in drug-resistant tumors [9]. Conversely, a relatively few number of systematic analyses on the level of different caspases in malignant cell lines and tumor specimens has been reported [7,10–17]. We

* Corresponding author. Department of Biochemistry and Biophysics "F. Cedrangolo", Second University of Naples, Via De Crecchio 7, 80138 Naples, Italy. Tel.: +39-081-5665812; fax: +39-081-441688.
E-mail address: fulvio.dellaragione@unina2.it (F. Della Ragione).

0165-4608/03/$ – see front matter
doi: 10.1016/S0165-4608(03)00125-0

recently investigated the content of different caspases (caspases 1, 3, 4, 6, 8, and 10) in a panel of cell lines established from different neoplasias, including breast cancers, neuroblastomas, pancreatic carcinomas, acute leukemias, osteosarcomas, and rhabdomyosarcomas (Della Ragione et al., unpublished observations, 2001). We found that only neuroblastoma-derived cell lines show a frequent deficiency of different caspases. Therefore, to evaluate the meaning of the findings obtained on cell lines, we moved to analyze the content of caspases 3 and 8 (at the RNA and protein levels) in a large number of human neuroblastoma specimens. For the first time to our knowledge, the data obtained demonstrate the deficiency of caspase 3 in a significant percentage of cases. Moreover, we confirmed the lack of caspase 8 and ruled out any linkage of the deficient status with *MYCN* amplification and specific tumor stage. Our findings suggest that neuroblastoma development and resistance to treatment might depend on the absence of these pivotal enzymes, at least in part.

2. Materials and methods

2.1. Tumor samples

Sixty-three primary neuroblastomas from the Italian Tissue Bank (Centro di Biotecnologie Avanzate, Istituto dei Tumori, Genova, Italy) were selected to represent the distribution of stages found in neuroblastoma. Patients were staged according to the revised International Stage System of Neuroblastoma [18]. After resection, all the tumors were frozen immediately in liquid nitrogen and stored at −80°C until analysis. Diagnosis was established by histological examination of tumor tissue obtained at surgery. To assure that tumor samples used for molecular analyses contained a sufficient proportion of malignant cells, only samples that had been clearly demonstrated to contain more than 95% tumor cells were used in the present study. This ruled out the possibility that the obtained results were due to normal cells present in the analyzed specimens. MYCN status was determined as described [19].

2.2. Antibodies and reagents

Monoclonal antibodies against caspase 3 were from Transduction Laboratories (Lexington, UK). Rabbit polyclonal antibodies against caspase 8 were from Upstate Biotechnology (Lake Placid, NY). All other reagents for immunoblotting were described in detail elsewhere [20–22].

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed using the StrataScript RT-PCR Kit (Stratagene, La Jolla, CA). Briefly, 2.5 μg of total RNA, prepared as reported [23], was reverse-transcribed by StrataScript RNAase H^- RT (25 units) using oligo(dT) primer (150 ng) in a final volume of 25 μL. cDNA samples were diluted 10-fold in a PCR reaction assay to a volume of 50 μL containing, in addition to the DNA template, 30 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 ng of each primer, 0.2 mmol/L of each nucleotide, and 1 unit of Taq DNA polymerase. Primers and temperature conditions for glyceraldheyde 3-phosphate dehydrogenase (GAPDH) were reported previously [24].

Primers used for caspase 3 amplification were as follows: 5′-GGATTATCCTGAGATGGGTTTATG-3′ and 5′-ATAATGAAAAGTT-TGGG-3′ (amplified fragment of 363 base pairs [bp]). Temperature conditions for caspase 3 were as follows: hot start at 95°C for 5 minutes, 30 cycles composed of steps at 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, and a final elongation step at 72°C for 7 minute. The relative amount of caspase 3 PCR product was estimated by means of coamplification with the *GAPDH* reporter gene.

Primers used for caspase 8 were the following: 5′- CACTAGAAAGGAGGAGATGGAAAG-3′ and 5′-CTATCCTGTTCTCT-TGGAGAGTCC-3′. These primers recognized two amplified fragments of 411 and 366 bp corresponding to α1 and α2 transcripts, respectively. Temperature conditions for caspase 8 were as follows: hot start at 95°C for 5 minutes, 30 cycles composed of steps at 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. As with caspase 3, coamplification with GAPDH cDNA was used to estimate the relative amount of caspase 8 PCR products.

Before amplification with each specific primer pair, an aliquot of the cDNA preparation was amplified using GAPDH primers to determine the integrity of the generated cDNA. Moreover, we used five different cDNA concentrations to assure that signals (of GAPDH and the analyzed gene) were proportional to input mRNA. These controls are important for comparison between samples because they ensure that equivalent amounts of RNA are amplified. Finally, each experiment was performed at least in duplicate and, in several cases, in triplicate.

Aliquots of PCR reactions were separated and analyzed by electrophoresis on 2% (wt/vol) agarose gels or nondenaturing 8% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide; 29:1). In the latter case, the amplified products were stained using silver nitrate method [23,24]. In several cases, the resultant RT-PCR products were recovered from the gels and sequenced as reported [23,24]. In all cases, the sequences of the amplified products corresponded to those reported in the literature.

2.4. Western blotting

The preparation of cancer samples from neuroblastoma specimens for immunoblotting was described previously [20–22]. About 40–80 μg of proteins were resolved by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis [20–22]. The proteins were transferred from the gel to a

nitrocellulose membrane, and Western blotting was carried out exactly as described previously [20–22].

2.5. Immunohistochemistry

The analyses were performed on histological slides from formalin-fixed and paraffin-embedded tumor tissues. The deparaffinized sections were treated with 3% hydrogen peroxide for 15 minutes to quench the endogenous peroxidase activity. Thereafter, the sections underwent microwave exposure in 10 mmol/L of citrate buffer, pH 6, for 5–10 minutes to retrieve antigenicity. Goat polyclonal antisera, raised against the carboxyl terminus of human caspases 3 and 8 (Santa Cruz Biotechnology, Santa Cruz, CA), were used as primary antibodies. The epitope mapped at the p20 subunit of caspases 3 and 8. After an overnight incubation at 4°C with the 1:50 diluted primary antibody, a biotinylated secondary antigoat antibody was applied with 1:100 diluition for 30 minutes, followed by the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). For immunostaining, color was developed by 3-amino-9-ethylcarbazole and the sections were lightly counterstained with hematoxylin (DAKO, Copenhagen, Denmark) [24]. The percentage of positive and negative cells was established as reported previously [24].

3. Results and discussion

Recent reports and data from our laboratory indicate that caspase 8 protein is absent in a high percentage of neuroblastoma cell lines [11,14; (Della Ragione et al., unpublished observations, 2001). Thus, we decided to investigate the level of either caspase 3 or 8 in primary neuroblastoma specimens. Caspase 3 was selected because it represents the point of convergence of multiple "executioner" apoptotic pathways.

We studied a total of 63 different neuroblastoma specimens, which were classified into five stages (1–4 and 4S) on the basis of established clinical criteria [18]. Our investigation particularly involved 9 tumors in stage 1, 10 in stage 2, 9 in stage 3, 30 in stage 4, and 5 in stage 4S. All the specimens had been characterized previously for *MYCN* gene amplification [19].

Each cancer sample was analyzed for the expression of caspases 3 and 8 genes by means of reversed PCR methodology, which allowed a semiquantitative estimation. Indeed, we used conditions where the quantity of the amplification product (estimated by laser scanning) was strictly proportional to the input cDNA used. Amplification of glyceraldheyde 3-phosphate dehydrogenase mRNA was used as the internal standard for all specimens analyzed. Finally, in

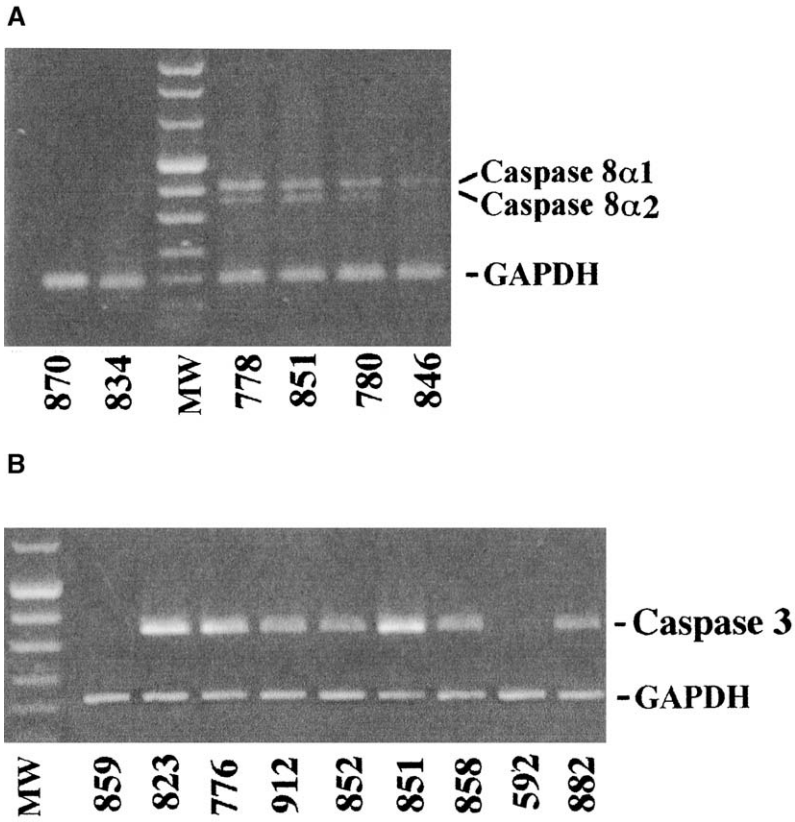

Fig. 1. Analysis of caspase 8 and caspase 3 mRNA in human neuroblastoma specimens by RT-PCR. Total RNA (2.5 μg) was reverse-transcribed as described in Materials and methods. cDNA samples were then diluted in a PCR reaction mixture and amplified by specific primers. Aliquots of PCR reaction (5 μl) were separated on a 2% (wt/vol) agarose gel. (*A*) Reports caspase 8α1 and 8α2 analyses, while (*B*) shows caspase 3 amplification. GAPDH was coamplified in all the samples. The numbers reported represent samples examined. MW, molecular weight standards.

Table 1
Expression of caspase 3 and caspase 8α1 and 8α2 genes in human neuroblastomas of different stages

Stage no.	Sample no.	*MYCN*[a]	GAPDH	Caspase 3	Caspase 8α1	Caspase 8α2	Follow-up[b]
1							
	655	1	+	−	−	−	Remission, 24 months
	591	1	+	−	+	+	Remission, 37 months
	823	1	+	+++	−	−	Clinical remission
	776	1	+	+++	−	−	Clinical remission
	772	1	+	++	−	−	Deceased
	806	1	+	+	+	+	
	859	1	+	−	+	+	Clinical remission
	916	1	+	++	+	+	
	778	1	+	+++	++	++	Clinical remission
2							
	606	1	+	−	−	−	Remission, 33 months
	652	1	+	−	+	+	Remission, 38 months
	647	1	+	++	+	+	Remission; 35 months
	634	1	+	−	+	+	Remission, 29 months
	852	1	+	++	−	−	Clinical remission
	851	1	+	+++	++	++	Clinical remission
	762	1	+	−	−	−	Clinical remission
	764	1	+	++	+	+	Clinical remission
	656	1	+	++	+	+	
	626	1	+	+	−	−	
4s							
	586	1	+	+	+	+	Deceased, 53 months
	809	1	+	++	++	++	Clinical remission
	912	1	+	++	++	++	Relapsed
	803	1	+	++	++	++	Deceased
	887	1	+	++	+	+	
3							
	619	13	+	+	+	+	Deceased, 6 months
	579	1	+	+++	+	+	Remission, 33 months
	592	12	+	−	−	−	Deceased, 10 months
	572	1	+	++	+	+	
	858	1	+	++	+	+	Relapsed
	802	60	+	−	+	+	Deceased
	799	1	+	++	+	+	Clinical remission
	793	1	+	++	+	+	Relapsed
	698	1	+	++	+	+	
4							
	687	20	+	−	−	−	Deceased, 4 months
	640	1	+	−	−	−	Remission, 33 months
	691	1	+	++	+	+	Deceased
	651	7	+	−	−	−	
	947	Ampl	+	+++	+	+	Clinical remission
	718	1	+	++	+	+	Deceased
	811	1	+	+++	−	−	Relapsed
	870	1	+	++	−	−	Relapsed
	871	1	+	+++	++	++	Relapsed
	872	1	+	+++	−	−	Clinical remission
	876	1	+	++	++	++	Relapsed
	882	1	+	++	++	++	Relapsed
	701	1	+	+++	+	+	
	846	1	+	++	+	+	Relapsed
	728	1	+	+++	+	+	Relapsed
	780	1	+	−	+	+	Relapsed
	837	1	+	+++	+	+	Clinical remission
	834	1	+	+++	−	−	Deceased
	321	Ampl	+	+++	++	++	Clinical remission
	292	Ampl	+	−	−	−	Deceased
	505	Ampl	+	+++	+++	+++	Deceased
	350	Ampl	+	++	++	++	Deceased
	690	Ampl	+	++	++	++	Deceased
	664	Ampl	+	++	++	++	Deceased
	597	Ampl	+	++	−	−	Clinical remission
	753	Ampl	+	++	+	+	Deceased
	651	Ampl	+	++	−	−	Deceased
	644	Ampl	+	++	++	++	Deceased
	994		+	+++	++	++	
	1034		+	+++	++	++	

The symbols −, +, ++, and +++ represent the relative amount of the PCR product estimated by laser scanner analysis. These results were a mean of three independent experiments.

[a] *MYCN* reports the number of copies of the gene or if the gene is amplified (Ampl) (19).

[b] Clinical remission refers to patients in remission >5 years, otherwise the number of months are reported.

Table 2
mRNA and protein levels of caspase 3 and 8 in human neuroblastomas of different stages

		Caspase 3		Caspase 8	
Stage no.	Sample no.	mRNA[a]	Protein (% of positive cells)[b]	mRNA[a]	Protein (% of positive cells)[b]
4s					
	809	+	>75	+	>75
	912	+	>75	+	>75
1					
	806	+	>75	+	>75
	591	−	<20	+	>75
2					
	762	−	Absent	−	Absent
3					
	592	−	<20	−	<20
	572	+	>75	+	>75
4					
	834	+	>75	−	20
	651	−	Absent	−	Absent
	882	+	>75	+	>75

[a] Determined by RT-PCR.
[b] Determined by immunohistochemical methodologies.

more than 80% of the samples, the identity of the PCR product was verified by recovering the amplified material from the gel and by direct sequencing [23,24]. No mutations were identified in all the sequenced samples (48 samples).

Fig. 1 reports examples of caspase 3 and 8 (α1 and α2)–positive and –negative specimens. Complete deficiency of caspase 3 and 8 mRNA occurred in neuroblastomas of all stages. Moreover, we were unable to observe any direct correlation between the incidence of deficiency and follow-up or *MYCN* gene amplification (Table 1). The reported findings do not confirm the previous claim of association among neuroblastoma aggressiveness, *MYCN* amplification, and lack of caspase 8 expression [12]. We do not have any explanation for this important discrepancy because both studies have been conducted on a large number of samples.

Table 2 reports the results of an immunohistochemical investigation performed on the available specimens of neuroblastoma (10 samples); these specimens were also studied for mRNA caspase content (see Table 1). The data obtained, which were reported as the percentage of positive cells, confirm, at protein level, the results of RT-PCR studies. Fig. 2 shows examples of caspase 8 negative (Fig. 2A) and positive (Fig. 2B) specimens. In all the positive samples, we detected islets of deficient cells. Finally, we carried out immunoblotting analyses on eight of the samples analyzed by immunohistochemistry (data not shown), and the results agreed with the RT-PCR results. They also demonstrated that the staining of immunohistochemical experiments corresponds solely to caspase 3 and 8 proteins. In conclusion, the data obtained indicate the occurrence of alterations at different steps of the caspase cascade in neuroblastoma.

When our study was almost completed, similar findings were reported by others [11,12]. The authors specifically described the absence of caspase 8 expression in 13 of 18 neuroblastoma cell lines analyzed. Moreover, the observed deficiency was explained by caspase 8 gene hypermethylation and was correlated with *MYCN* gene amplification [11].

Conversely, one of the aims of our investigations was to evaluate whether the lack of caspase expression is a specific feature of neuroblastoma, or if it occurs in other malignant cell phenotypes. In preliminary studies, we demonstrated that caspase deficiency is a phenomenon rarely observable in malignant cell lines and that it is almost specific to neuroblastoma (Della Ragione et al., unpublished observations,

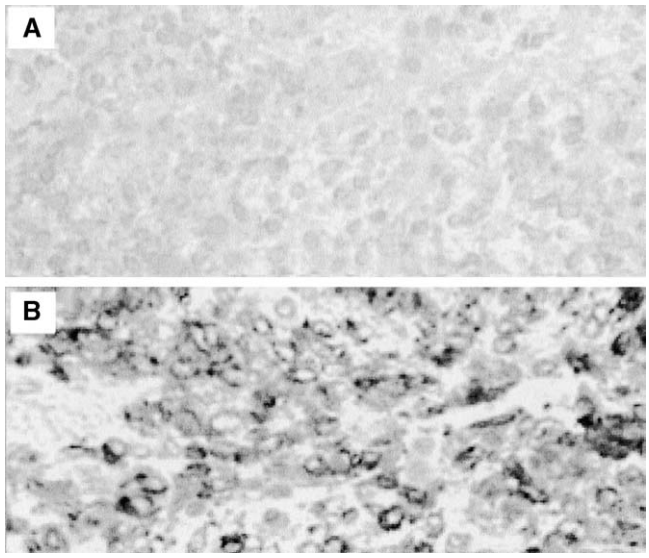

Fig. 2. Immunochemical analysis of caspase 8 protein in human neuroblastoma samples. Specimens of neuroblastomas showing deficiency of caspase 8 protein (*A*, sample 651) or the presence of the protease (*B*, sample 882). Details about the immunohistochemical staining technique are reported in Materials and methods.

2001). We subsequently examined the transcription of caspases 3 and 8 in neuroblastoma specimens. We found that a remarkable percentage of cancer of all stages does not express the two genes (15%–45%), while no 4S neuroblastoma was deficient in the expression of these genes. The data obtained by RT-PCR were confirmed by immunohistochemical analyses and immunoblotting experiments. In addition, immunohistochemistry indicates that positive samples include a remarkable number of negative cell isles. Thus, the percentage of deficient samples might have been underestimated in our experiments. Finally, we stress that this study is the first investigation to our knowledge that demonstrates the absence of caspase 3 in primary cancer samples.

Abnormalities in caspase content suggest that the apoptotic response is partially hampered in neuroblastoma; this could be significant during the development of the cancer and during the response to chemotherapy. Indeed, the use of apoptosis-based treatments could select the caspase-negative (and thus more resistant) cells.

This view is supported by a number of studies demonstrating that cell lines lacking caspase 3 or 8 show defects in the apoptotic process. MCF-7 cells, which have lost caspase 3 expression because of a 47-bp deletion within exon 3 of the respective gene, present an abnormal response to cell death–inducing agents, including tumor necrosis factor and staurosporine [10]. This phenomenon was completely reverted by the forced expression of the cysteine-protease by expression vector transfection [10]. In addition, renal cell lines with a very low caspase 3 protein content were almost resistant to apoptosis because of the intracellular zinc chelator *N*,*N*,*N*′*N*′-tetrakis(2-pyridylmethyl)ethylendiamine [25]. Similarly, loss of caspase 8 expression in neuroblastoma cell lines correlates with resistance to apoptosis induced by the tumor-selective ligand tumor necrosis factor–related, apoptosis-inducing ligand (TRAIL) [14]. The re-expression of the cysteine enzyme enhances the sensitivity to TRAIL [14]. Thereby, the absence of one of the two caspases specifically modifies the mechanism of apoptotic response.

From a mechanistic point of view, while caspase 3 deficiency probably causes an impairment of the entire apoptotic process, the cancer advantage related to caspase 8 absence is less straightforward. It has been demonstrated very recently, however, that caspase 8, not caspase 3, is involved in the transcription-independent death process activated by p53 protein [26]. Thus, the absence of caspase 8 might confer two distinct advantages to neuroblastoma cells: 1) it decreases the antitumoral immunological response because of the engagement of cell death receptors (by Fas ligand, tumor necrosis factor, and TRAIL), and 2) it down-regulates p53-mediated transcription-independent apoptosis. In this context, it is also intriguing that neuroblastomas express a high level of survivin gene [27]. Survivin is an anti-apoptotic protein that inhibits caspase 3 and 7 activation and thus protects neoplasias against anticancer treatment on the basis of cell death mechanisms. Considering the data on caspases 3 and 8 and those reported on survivin gene expression [27], it seems probable that neuroblastomas have developed a large variety of antiapoptotic strategies that may be responsible for the resistance of this cancer to treatment.

In conclusion, this study demonstrates for the first time, to our knowledge, the deficiency of caspase 3 in neuroblastoma specimens. Moreover, we observe that the lack of caspase 8 is not correlated with negative prognostic features, including *MYCN* gene amplification. Future investigations will evaluate the importance of these current observations in neuroblastoma development and in the planning of new therapy based on the forced re-expression of caspase genes.

Acknowledgments

The authors thank G.P. Tonini for some of the neuroblastoma mRNA samples. This work was supported in part by grants from Fondazione Italiana per la Ricerca sul Cancro (FIRC), Associazione Italiana per la Ricerca sul Cancro (AIRC), MURST, Associazione Italiana per la Lotta al Neuroblastoma, and Cluster di Biomedicina - Progetto1 - MURST.

References

[1] Hannum YA. Apoptosis and the dilemma of cancer chemotherapy. Blood 1997;89:1845–53.

[2] Sayers TJ, Brooks AD, Lee JK, Fenton RG, Komschlies KL, Wigginton JM, Winkler-Pickett R, Wintrout RH. Molecular mechanisms of immune-mediated lysis of murine renal cancer: differential contribution of perforin-dependent versus Tas-mediated pathways in lysis by NK and T cells. J Immunol 1998;161:3957–65.

[3] Earnshaw WC. Nuclear changes in apoptosis. Curr Opin Cell Biol 1995;7:337–43.

[4] Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Gree DR, Martin SJ. Ordering the cytochrome c–initiated caspase cascade: hierarchical activation of caspase-2, -3, -6, -7, 8 and –10 in a caspase-9-dependent manner. J Cell Biol 1999;144:281–92.

[5] Gottlieb TM, Oren M. p53 and apoptosis. Semin Cancer Biol 1998;8:359–68.

[6] Housman DE, Jacks T. p53 status and the efficacy of cancer therapy in vivo. Science 1994;266:807–10.

[7] Martinez-Lorenzo MJ, Gamen S, Etxeberria J, Lasierra P, Larrad L, Pineiro A, Anel A, Naval J, Alava MA. Resistance to apoptosis correlates with a highly proliferative phenotype and loss of Fas and CPP32 (caspase-3) expression in human leukemia cells. Int J Cancer 1998;75:473–81.

[8] Strobel P, Nanan R, Gattenlohner S, Muller-Deubert S, Muller-Hermelink HK, Kreth HW, Marx A. Reversible monoclonal lymphoadenopathy in autoimmune lymphoproliferative syndrome with functional FAS (CD95/APO-1) deficiency. Am J Surg Pathol 1999;23:829–37.

[9] Coustan-Smith E, Kitanaka A, Pui CH, McNinch L, Evans WE, Raimondi SC, Behm FG, Arico M, Campana D. Clinical relevance of Bcl-2 overexpression in childhood acute lymphoblastic leukemia. Blood 1996;87:1240–6.

[10] Janicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 1998;273:9357–60.

[11] Teitz T, Wei T, Valentine MB, Vanin EF, Grenet J, Valentine VA, Behm FG, Look AT, Lahti JM, Kidd VJ. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 2000;6:529–35.

[12] Teitz T, Lahti JM, Kidd VJ. Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. J Mol Med 2001;79:428–36.

[13] Takita J, Yang HW, Bessho F, Hanada R, Yamamoto K, Kidd VJ, Teitz T, Wei T, Hayashi Y. Absent or reduced expression of the *caspase 8* gene occurs frequently in neuroblastoma, but not commonly in Ewing sarcoma or rhabdomyosarcoma. Med Pediatr Oncol 2000;35: 541–3.

[14] Hopkins-Donaldson S, Bodmer JL, Bourloud KB, Brognara CB, Tschopp J, Gross N. Loss of caspase-8 expression in neuroblastoma is related to malignancy and resistance to TRAIL-induced apoptosis. Med Pediatr Oncol 2000;35:608–11.

[15] Zuzak TJ, Steinhoff DF, Sutton LN, Phillips PC, Eggert A, Grotzer MA. Loss of caspase-8 mRNA expression is common in childhood primitiveneuroectodermal brain tumour/medulloblastoma. Eur J Cancer 2002;38:83–91.

[16] Fulda S, Kufer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B, Debatin KM. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. Oncogene 2001;20:5865–77.

[17] Shivapurkar N, Toyooka S, Eby MT, Huang CX, Sathyanarayana UG, Cunningham HT, Reddy JL, Brambilla E, Takahashi T, Minna JD, Chaudhary PM, Gazdar AF. Differential inactivation of caspase-8 in lung cancer. Cancer Biol Ther 2002;1:65–9.

[18] Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F. Revisions of the international criteria for neuroblastoma diagnosis, staging and response to treatment. J Clin Oncol 1993;8:1466–77.

[19] Tonini GP, Boni L, Pession A, Rogers D, Iolascon A, Basso G, Cordero di Montezemolo L, Casale F, Pession A, Perri P, Mazzocco K, Scaruffi P, Lo Cunsolo C, Marchese N, Milanaccio C, Conte M, Bruzzi P, De Bernardi B. MYCN oncogene amplification in neuroblastoma is associated with worse prognosis, except in stage 4s: the Italian experience with 295 children. J Clin Oncol 1997;15:85–93.

[20] Della Ragione F, Russo GL, Oliva A, Mastropietro S, Mancini A, Borrelli A, Casero RA, Iolascon A, Zappia V. 5′-Deoxy-5′-methylthioadenosine phosphorylase and $p16^{INK4}$ deficiency in multiple tumor cell lines. Oncogene 1995;10:827–33.

[21] Della Ragione F, Russo GL, Oliva A, Mercurio C, Mastropietro S, Della Pietra V, Zappia V. Biochemical characterization of $p16^{INK4}$- and p18-containing complexes in human cell lines. J Biol Chem 1996; 271:15942–9.

[22] Della Ragione F, Cucciolla V, Borriello A, Della Pietra V, Manna C, Galletti P, Zappia V. Pyrrolidine dithiocarbamate induces apoptosis by a cytochrome c-dependent mechanism. Biochem Biophys Res Commun 2000;268:942–6.

[23] Iolascon A, Giordani L, Borriello A, Carbone R, Izzo A, Tonini GP, Gambini C, Della Ragione F. Reduced expression of transforming growth factor-beta receptor type III in high stage neuroblastomas. Br J Cancer 2000;82:1171–6.

[24] Iolascon A, Giordani L, Moretti A, Basso G, Borriello A, Della Ragione F. Analysis of CDKN2A, CDKN2B, CDKN2C and cyclin Ds gene status in hepatoblastoma. Hepatology 1998;27:989–95.

[25] Kolenko V, Uzzo RG, Bukowski R, Bander NH, Novick AC, His ED, Finke JH. Dead or dying: necrosis versus apoptosis in caspase-deficient human renal cell carcinoma. Cancer Res 1999;59:2838–42.

[26] Ding H-F, Lin Y-L, McGill G, Juo P, Zhu H, Blenis J, Yuan J, Fisher DE. Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. J Biol Chem 2000;275:38905–11.

[27] Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet 1998;351:882–3.