

Identification of a hTid-1 mutation which sensitizes gliomas to apoptosis

G.A. Trentin^a, Y. He^d, D.C. Wu^b, D. Tang^{b,c}, M. Rozakis-Adcock^{a,c,d,*}

^a Faculty of Health Sciences, McMaster University, Hamilton, Ont., Canada L8N 3Z5

^b Father Sean O'Sullivan Research Institute, St. Joseph's Hospital, Hamilton, Ont., Canada L8N 1Y2

^c Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ont., Canada L8N 3Z5

^d Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Ont. Canada

Received 5 October 2004; revised 5 November 2004; accepted 9 November 2004

Available online 25 November 2004

Edited by Vladimir Skulachev

Abstract Human Tid-1 (hTid-1) is a DnaJ chaperone protein with homology to the *Drosophila* tumor suppressor Tid56. We report the first case of a tumor-associated mutation at the human *TID1* locus, which was identified in the SF767 glioma cell line giving rise to aberrantly high levels of a hTid-1_L mutant variant. In this study, we set out to determine whether this change in hTid-1 status influences the response of glioma cells to adenoviral (Ad)-mediated delivery of the two major isoforms of *TID1*, hTid-1_L and hTid-1_S. Ad-hTid-1_S induced apoptosis in hTid-1 mutant SF767 cells, while causing growth arrest in wild-type hTid-1-expressing U373 and U87 cells. By contrast, Ad-hTid-1_L infection had no apparent effect on glioma cell growth. The apoptosis induced by hTid-1_S was accompanied by mitochondrial cytochrome *C* release and caspase activation and blocked by stable overexpression of Bcl-X_L. Our findings suggest that the status of hTid-1 in gliomas may contribute to their susceptibility to cell death triggers.

© 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: human Tid-1; Chaperone; Glioma; Mutation; Apoptosis; Cell cycle; Tumor suppressor

1. Introduction

Human Tid-1 (hTid-1), the human homolog of the *Drosophila* Tid56 tumor suppressor protein, is a member of the DnaJ family of co-chaperones [1,2]. Three alternatively spliced variants of the human *TID1* gene have been described [3], the hTid-1_L and hTid-1_S being the most abundant [3–5]. Expression of the third and shortest splice variant is restricted to the thymus [3], and the corresponding endogenous 38-kDa protein has only been detected in cell lysates from human Thp1 and U937 leukemia lines [6]. Previous studies have

demonstrated that DnaJ proteins function as essential regulatory cofactors to the heat shock 70 (Hsp70) family of molecular chaperones [2] and cooperate in various cellular processes, including intracellular protein folding, trafficking and degradation [7,8]. More recently they have emerged as integral components of signal transduction pathways, in which they function in the assembly, activation or inactivation of multiple key signaling molecules that regulate cell cycle progression and apoptosis and which figure prominently in human cancers. These include the p53 [9,10], retinoblastoma (Rb) [11,12], von Hippel-Lindau (VHL) [13,14] and Wilms (WT1) [15] tumor suppressors, p27^{KIP1} [16], Raf-1 kinase [12,17] and Apaf-1 [18,19].

Drosophila Tid56 is the first member of the DnaJ co-chaperone family to be classified as tumor suppressor [20]. Recently, genetic studies in *Drosophila* have implicated *l(2)tid* as a novel component of the Hedgehog-Patched (Hh-Ptc) signaling pathway, which directs embryonic development and transcriptional regulation of genes such as *wingless* (*wng*) and *Cubitus interruptus* (*Ci*) that control cell proliferation and cell-fate specification [21]. Disruption of this pathway in humans through mutational inactivation of *Ptc* or amplification of *Gli*, the human homolog of *Ci*, predisposes individuals to medulloblastoma [22] and glioblastoma [23], underscoring the potential physiological significance of human Tid-1 in tumor progression processes in the brain.

Loss of heterozygosity (LOH) within the chromosomal region of 16p13.3 to which the human *TID1* gene has been mapped is associated with a number of human malignancies, including tumors of glial origin [24,25]. Ours is the first study to identify a coding region sequence mutation targeting a member of the chaperone family. We found one glioma cell line, SF767, with a heterozygous *TID1* frameshift mutation which yields aberrantly high levels of a 52-kDa hTid-1_L mutant variant. Moreover, we demonstrate that the hTid-1_S isoform can readily stimulate a Bcl-X_L-sensitive cell death program in gliomas harboring a mutant hTid-1 but not in cells proficient in hTid-1. Our results suggest that the status of hTid-1 in gliomas may contribute to cellular susceptibility to apoptotic triggers.

2. Materials and methods

2.1. Cell lines

The human glioma cell lines U87, U373, SF767, SF126 and SF188 were obtained from the University of California at San Francisco Brain Research Center. 293/293T cells and COS-1 were purchased

*Corresponding author. Present address: University of Toronto, Medical Sciences Building, Room 6234, 1 King's College Circle Toronto, Ont., Canada M5S 1A8. Fax: +1 416 978 5959.

E-mail address: maria.rozakis@utoronto.ca (M. Rozakis-Adcock).

Abbreviations: Ci, cubitus interruptus; CMV, cytomegalovirus; FBS, fetal bovine serum; GFP, green fluorescent protein; Hh-Ptc, Hedgehog-Patched; hTid-1, human Tid-1; LOH, loss of heterozygosity; MOI, multiplicity of infection; MMP, mitochondrial membrane permeability; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; PT, permeability transition; Rb, retinoblastoma; RT, reverse transcriptase; TID, tumorous imaginal discs; VHL, von Hippel-Lindau

from the ATCC. All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂.

2.2. Cloning and recombinant expression of hTid-1_L mutant

Total RNA was isolated from cultured SF767 cells using RNeasy Kit (Qiagen) and was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen) and oligo(dT) primers, each according to the manufacturer's instructions. Several overlapping PCRs were then performed using Expand High Fidelity *Taq* DNA polymerase (Roche) that spanned the entire human *TID1* cDNA. Polymerase chain reaction (PCR) products were gel purified by using the QIAEXII DNA extraction kit (Qiagen) for direct sequencing. cDNA encoding the mutant hTid-1_L identified in SF767 cells was subcloned into pCDNA3 (Invitrogen). Transient transfections were performed by calcium phosphate-DNA precipitation.

2.3. Construction and preparation of recombinant adenoviruses

The cDNAs encoding full-length hTid-1_L (amino acid residues 1–480) and hTid-1_S (amino acid residues 1–453) were cloned downstream of the cytomegalovirus (CMV) immediate early promoter of the adenoviral shuttle vector pAdTrack-CMV [26]. HPD mutants of hTid-1_L and hTid-1_S were constructed by site-directed mutagenesis using synthetic oligonucleotides to generate a PCR fragment incorporating a H121Q base substitution of the HPD motif within the J-domain of hTid-1. Recombinant adenoviral stocks were generated and purified as previously described [26]. Glioma cells were infected with a multiplicity of infection (MOI) between 150 and 300.

2.4. Generation of SF767 stable cell lines

High titer retrovirus was generated by transfection of a retrovirus packaging line 293T, with pBabe or pBabe/Bcl-X_L as described previously [27]. SF767 cells were incubated with retrovirus for 1 h at 37 °C, additional medium was then added and cells were cultured for 48 h before beginning selection of infected cells in medium containing puromycin (1 µg/ml).

2.5. Immunoblot analysis

Cells were washed twice with PBS and lysed in 2× SDS sample buffer. For Western blot analysis of PARP cleavage, cells were lysed in SDS reducing buffer (62 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue), sonicated for 20 s, followed by 30 min incubation at 37 °C. Protein concentrations were determined using the Bio-Rad protein assay. A total of 50 µg of protein from each sample was then separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane and subjected to Western blot analysis with rabbit anti-mouse Tid-1 antibody [5], mouse monoclonal anti-human PARP monoclonal antibody (C2-10; BD Pharmingen), or monoclonal anti-Bcl-X_L (H-62; Santa Cruz). Proteins were detected using ECL reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

2.6. Cell growth rate determination

Hoechst 33258 DNA Fluorometric Assay was used to follow the growth of infected glioma cells over a one-week period. Cells were seeded in triplicate in 96-well plates at 1.25×10^3 cells/well 16–20 h prior to infection with control green fluorescent protein (GFP), hTid-1_L- or hTid-1_S-expressing adenovirus at the appropriate MOI. Each day thereafter, the wells of one 96-well plate were washed once with Milli-Q water and frozen at –80°C. Following day 7, all plates were subjected to two cycles of freeze/thawing with 1 h incubation between with Milli-Q water. Cells were stained with Hoechst 33258 (Sigma; 10 µg/ml in 10 mM Tris, 1 mM EDTA, and 2 M NaCl, pH 7.4) and fluorescence measurements were made using a CytoFluor Series 4000 plate fluorescence reader. Cell number was calculated from a standard curve.

2.7. Cell cycle analysis

5×10^5 cells were plated in 60 mm dishes 16–20 h prior to adenoviral infection. At 72 h following infection, cells were harvested by trypsinization and resuspended at a concentration of 10^6 cells/ml in PBS. Cells were incubated for 10 min on ice with propidium iodide (PI) staining

solution [0.25 mg/ml PI (Sigma), 1% Triton X-100, and 0.1 mg/ml RNase A (Sigma) in PBS] and DNA content of GFP-positive cells was then measured using a Coulter Epics XL Flow Center equipped with the Epics XL System II software for profile analysis.

2.8. Propidium iodide exclusion assay

Cells were seeded at 5×10^5 cells per 60 cm dish and 16–20 h later, infected with adenovirus expressing GFP alone, hTid-1_L or hTid-1_S. Cell viability was assessed 72 h post-infection using the PI exclusion assay. Briefly, cells were trypsinized and resuspended in PBS at 10^6 cells/ml. Cells were then incubated with PI (PI; 1 mg/ml) for 5 min on ice. The number of GFP-positive, PI-positive cells was measured using a FACSVantage SE flow cytometer (Becton Dickinson) equipped with CELLQuest software for data acquisition.

2.9. Quantitation of apoptosis in SF767 stables

SF767/pBabe and SF767/pBabe-Bcl-X_L stable cell lines were plated in 60 mm dishes at 5×10^5 cells and the next day infected with control or hTid-1 adenoviruses. At 72 h post-infection, cells were photographed using a Zeiss fluorescent microscope. The percentage of apoptotic cells was quantified by counting the number of cells exhibiting apoptotic cell morphology from more than 200 GFP positive cells in five randomly selected fields and expressing it as a percentage of total GFP positive cells.

2.10. Cytochrome c release

Cytosolic fractions were prepared from SF767 parental, SF767/pBabe and SF767/pBabe-Bcl-X_L cells 72 h following infection with adeno-GFP, -hTid-1_L or -hTid-1_S as previously described [28]. An aliquot (10 µg) was subsequently subjected to Western blot analysis with anti-cytochrome c antibody (7H8.2C12; BD Pharmingen). The membrane was reprobed with antibodies to actin (I-19; Santa Cruz) as a loading control.

2.11. Statistical analysis

Statistical analysis of cell growth data was conducted using the *f* test to determine whether the data curves for GFP control and wild-type or mutant hTid-1_L and hTid-1_S differed significantly from those derived from uninfected cells. An analysis of variance (ANOVA) for a single factor followed by Duncan's multiple range test was also used to determine if the data for hTid-1_L and hTid-1_S were significantly different from control data in the apoptosis assays. A *P* value of <0.05 was considered significant in all cases. Bonferroni correction was used to adjust for any multiple-testing concerns. Statistical analysis of the data was performed using Microsoft Excel software.

3. Results

3.1. Identification of mutant hTid-1 in SF767 glioma

To assess the protein expression levels of hTid-1 isoforms in human glioma tumors, we screened lysates from glioblastoma-derived cell lines by Western blotting with Tid-1 specific antibodies. Intriguingly, although none of the five glioma cell lines surveyed showed loss of hTid-1 protein, an aberrant higher molecular weight species of ~52 kDa was observed in the SF767 cell line, in addition to the 43-kDa hTid-1_L and 40-kDa hTid-1_S endogenous mature forms of hTid-1 (Fig. 1A). To determine the nature of the mutation at the *TID1* locus, the presumptive protein-coding region of the cDNA prepared from SF767 cells was sequenced and found to contain two additional bases, a thymine at nucleotide position 1438 and cytosine at nucleotide position 1449 (Fig. 1B). These mutations alter the reading frame of the hTid-1_L sequence, introducing an additional 71 amino acids following the penultimate threonine residue at position 479, and appear to increase the steady-state abundance of the mutant form of the hTid-1_L (hTid-1_Lmut) protein. Transient

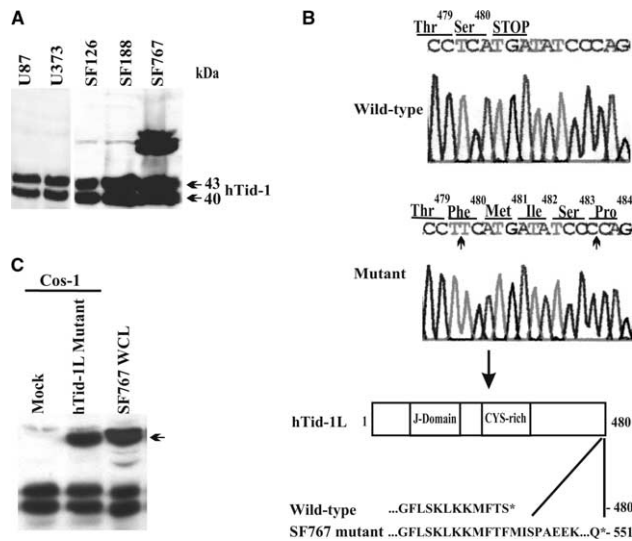


Fig. 1. Identification of a hTid-1 mutant in SF767 glioma cell line. (A) Western blot analysis of hTid-1 expression in human glioblastoma cell lines. Results from five cell lines are shown. Cell lysates were immunoblotted with polyclonal anti-mTid-1 antibodies. Arrows indicate the 43- and 40-kDa isoforms of hTid-1. (B) Sequence analysis of hTid-1 cDNA prepared from SF767 cells with corresponding schematic of reading frame change at the C-terminus. Mutations are underlined in the sequencing chromatogram. (C) Western blot analysis of cell lysates prepared from COS-1 cells ectopically expressing hTid-1_L mutant variant. An aliquot of SF767 cell lysate (50 µg) was also subjected to immunoblotting to show comigration with the 52-kDa aberrant protein indicated by the arrow.

expression of the recombinant hTid-1_Lmut cDNA into COS-1 cells revealed that the 551 amino acid precursor protein, with a predicted molecular mass of 61-kDa, appears to be correctly processed into a mature 52-kDa form by protease cleavage of the amino-terminal mitochondrial leader sequence (Fig. 1C). Moreover, it confirmed that the dicodon frameshift mutation generates a protein product that comigrates with the aberrant endogenous form observed in SF767 cells (Fig. 1C).

3.2. hTid-1_L and hTid-1_S have differential effects on cell viability

We evaluated the effects of adenovirus-mediated transfer of hTid-1_L and hTid-1_S isoforms on growth and survival of glioma cell lines expressing wild-type (U87, U373) or mutant hTid-1 (SF767). Western blot analysis of cell lysates from glioma cells prepared 24, 48, 72 and 96 h following infection with Ad-hTid-1_L or Ad-hTid-1_S showed efficient transduction and strong expression of 43- and 40-kDa bands corresponding to the mature processed protein of each isoform, respectively (Figs. 2A–C). In addition, high levels of 50- and 48-kDa bands representing the cytoplasmic precursors of each splice variant were observed. Cell growth was monitored over one week using a DNA fluorometric assay. No significant inhibition of cell growth was observed in any of the three cell lines over the 7-day period with expression of GFP alone or hTid-1_L with growth rates comparable to mock-infected cells (Figs. 2A–C). By contrast, transduction with Ad-hTid-1_S resulted in pronounced growth suppression of all three cell lines. A modest inhibition of growth is observed in U373 following infection with control virus, indicating a cytotoxic effect of the virus on the cells.

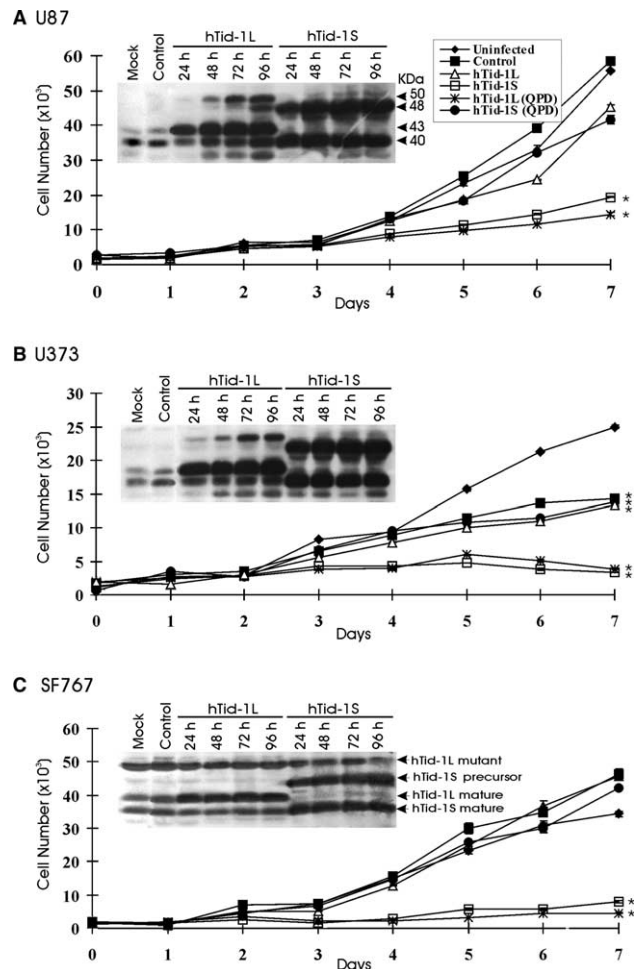


Fig. 2. Effects of exogenous hTid-1 isoforms on the growth of glioma cells. (A) U87, (B) U373 and (C) SF767 glioma cells were infected with adenoviruses expressing GFP alone, hTid-1_L, hTid-1_S or QPD mutants of the wild-type proteins. Cell number was determined at the indicated times using a fluorometric assay. Error bars represent means ± S.D. between three different wells of one representative experiment. Three independent experiments were performed for each cell line. Statistical analysis of data shows that curves indicated by asterisk (*) are significantly different from uninfected curves in each cell line ($P < 0.005$). Exogenous hTid-1_L or hTid-1_S expression was assessed over time in infected U87, U373 and SF767 cells by Western blot analysis of cell lysates with Tid-1 antibodies. The apparent molecular size of the resulting polypeptides representing cytoplasmic precursors and mitochondrial processed forms of hTid-1 are indicated in the margin.

DnaJ protein function is dependent upon a highly conserved histidine-proline-aspartate (HPD) sequence located within the J-domain [29]. Mutation of one or all of the amino acids completely abolishes J-domain-mediated stimulation of Hsp70 ATPase activity. Mutants of the HPD motif in which the histidine has been substituted for glutamine (QPD) were constructed to establish that the effects observed on glioma cell growth by hTid-1 were related to the chaperone function of the protein. Western blot analysis confirmed high levels of exogenous expression of the mutant proteins in infected cells (data not shown). Since these mutations impair ATP hydrolysis and consequently, coupling of Hsp70 to hTid-1 and its substrate without affecting the ability of hTid-1 to interact with the target substrate, they act as dominant-negative forms of

the protein. Interestingly, hTid-1_L and hTid-1_S QPD mutants demonstrated effects on cell growth antithetical to those of their wild-type counterparts. Specifically, QPD mutant of the long isoform, hTid-1_L, had pronounced inhibitory effects on the growth of all three cell lines while in contrast, cells infected with the QPD mutant of the short isoform, hTid-1_S, did not show any discernible changes in cell viability, with growth rates comparable to mock- or control-infected cells (Figs. 2A–C).

3.3. hTid-1_S induces S and G2/M cell cycle arrest

To determine if the growth inhibitory effects observed with exogenous hTid-1 gene expression in glioma cells was caused by cell cycle arrest, the DNA profiles of infected U87, U373 and SF767 cells were analyzed by flow cytometry. Infection with control- and hTid-1_L-expressing adenovirus had no discernible effect on the cell cycle profile of the three cell lines examined at 48 h (data not shown) and 72 h after infection (Fig. 3A). In marked contrast, adenovirus-mediated transfer of hTid-1_S in SF767 cells induced a pronounced accumulation of cells in the S and G2/M phases of the cell cycle observed at 48 h (data not shown) and 72 h of infection (Fig. 3A), an event not observed in wild-type hTid-1-expressing gliomas. Furthermore, a shoulder was also noted in the sub-G1 region at 72 h post-infection reflecting a fraction of SF767 cells with a DNA content less than that of G1 cells, suggesting that cells infected with hTid-1_S were undergoing cell death.

3.4. Ectopic expression of hTid-1_S promotes apoptosis in SF767 gliomas

The apoptosis observed with flow cytometry was quantitatively confirmed by the PI exclusion assay. The viability of SF767 cells infected with hTid-1_S declined significantly over time to less than 60% of the viability of adenovirus control-infected cells at 72 h post-infection (Fig. 3B). By contrast, U87 and U373 cells infected with either hTid-1_L or hTid-1_S or virus control did not show any apparent changes in cell viability. These data correlated with apoptotic morphology characterized by chromatin condensation and formation of apoptotic bodies detected with DAPI staining of floating hTid-1_S-infected SF767 cells (data not shown).

3.5. hTid-1_S induces caspase activation and cytochrome *c* release from mitochondria

To understand how hTid-1_S triggers cell death in SF767 cells, we first examined whether caspases, the major mediators of classical apoptosis pathways, are activated in response to hTid-1_S overexpression. To this end, we assayed for the cleavage of the caspase substrate Poly-(ADP-ribose) polymerase (PARP) in cell lysates prepared from hTid-1_S-infected SF767 cells at 24, 48, 72 and 96 h post-infection (Fig. 4A). As controls, parallel cultures were infected with adenoviruses expressing either GFP or hTid-1_L. Western blot analysis with anti-PARP antibody shows that intact PARP migrates at 116 kDa in control adeno-GFP-infected cells, whereas in cells overexpressing hTid-1_S there is cleavage of PARP to an 89-kDa fragment (Fig. 4A, top panel) observed at 48 h post-infection, demonstrating that hTid-1_S is sufficient in activating one or more caspases in these cells. By contrast, caspase activation was not detectable in cells expressing exogenous hTid-1_L. Intriguingly, hTid-1_S-triggered caspase activation in SF767

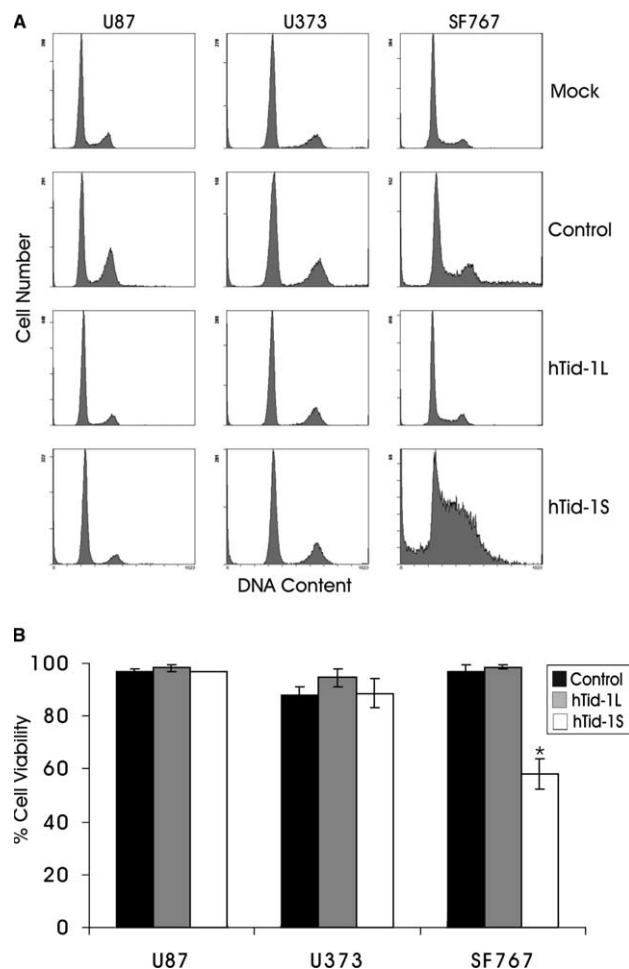


Fig. 3. Effects of exogenous hTid-1_L and hTid-1_S expression on cell cycle regulation. (A) Asynchronously growing U87, U373 and SF767 glioma cells were mock-infected or infected with adenoviruses expressing GFP alone (control), hTid-1_L or hTid-1_S. At 72 h following infection, cells were trypsinized and analyzed for DNA content by PI staining using flow cytometry. Overexpression of hTid-1_S induces apoptosis. (B) Cells were plated at low density and infected with hTid-1_L and hTid-1_S expressing adenoviruses. Cell viability was determined 72 h post-infection using the PI exclusion assay. Shown is the relative cell viability compared with mock-infected control cells. Error bars indicate the range between two independent experiments. Asterisk (*) indicates the data that were found to be significantly different from control data ($P < 0.003$).

cells coincided with proteolytic cleavage of the 52-kDa hTid-1_L mutant (Fig. 4A, lower panel).

Another feature of apoptosis is the collapse of mitochondrial membrane integrity and release of mitochondrial constituents such as cytochrome *c* (cyto-*C*), which potentiates the formation of the apoptosome and activation of effector caspases [30]. To determine whether the apoptotic activity of hTid-1_S is associated with release of cyto-*C*, cytosolic fractions were prepared from parental SF767 cells following infection with control-GFP, hTid-1_L- and hTid-1_S-expressing adenovirus and the extent of cyto-*C* release was assessed by Western blotting with an anti-cyto-*C* antibody (Fig. 4B). Whereas low to undetectable levels of cyto-*C* were observed in control and hTid-1_L-infected cells, hTid-1_S expression in SF767 cells elicited the release of cyto-*C* from the mitochondria (Fig. 4B, lanes 1–3). Probing cell

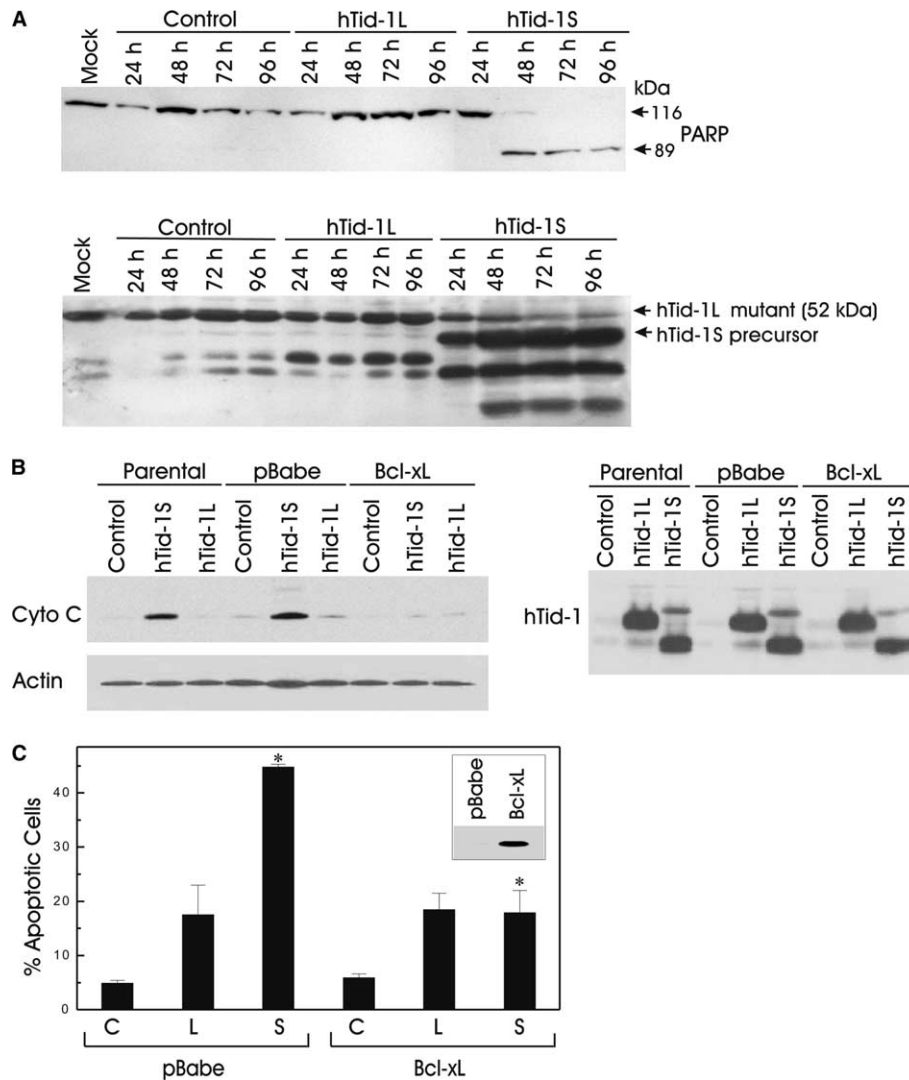


Fig. 4. hTid-1_S induces caspase activation and cytochrome *c* release in SF767 gliomas. (A) Cell lysates were prepared at the indicated time points following infection with adenovirus and subjected to immunoblot analysis with anti-PARP antibody (upper panel) or with anti-Tid-1 antibodies (lower panel). (B) Analysis of cytochrome *c* release in control, hTid-1_L- and hTid-1_S-infected SF767/parental, SF767/pBabe and SF767/pBabe-Bcl-X_L cells at 72 h post-infection. Cytosolic fractions (10 μg) were analyzed by Western blotting using anti-cytochrome *C* antibody. The membrane was reprobbed with antibodies to actin as loading control. Lysates were also immunoblotted for levels of hTid-1 proteins (right panel). (C) Bcl-X_L suppresses apoptosis induced by hTid-1_S. Percentage of apoptotic cells in hTid-1_L- or hTid-1_S-infected SF767/pBabe and SF767/Bcl-X_L stable lines at 72 h post-infection is shown. Error bars represent means ± S.D. between three independent experiments performed in triplicate. Asterisk (*) indicates the data that were found to be significantly different from control data ($P < 0.01$). Inset, Western blot analysis of cell lysates prepared from SF767 cells infected with either empty pBabe or pBabe/Bcl-X_L retrovirus using antibodies to Bcl-X_L, depicting protein levels of exogenous Bcl-X_L.

lysates with anti-Tid-1 antibodies confirmed comparable expression levels of exogenous hTid-1_L and hTid-1_S.

3.6. Bcl-X_L protects cells from hTid-1_S-induced cell death and cytochrome *c* release

It is well established that the expression of either Bcl-2 or Bcl-X_L is known to maintain the integrity of mitochondria and to prevent cyto-*C* release in response to diverse apoptotic stimuli [31]. To determine if cyto-*C* release mediated by overexpression of hTid-1_S in SF767 is inhibited by Bcl-X_L, SF767 cells stably expressing Bcl-X_L were generated by retroviral infection with pBabe-Bcl-X_L. The level of overexpression of Bcl-X_L in the stable cell lines was examined by Western blotting analysis (Fig. 4C, inset). Cytosolic fractions were prepared

from SF767/pBabe-Bcl-X_L and SF767/pBabe control cells 72 h after infection with control, hTid-1_L- and hTid-1_S-expressing adenovirus and the extent of cyto-*C* release was assessed by immunoblot analysis. As shown in Fig. 4B, cyto-*C* was detected in the cytosolic fraction of SF767/pBabe cells following infection with hTid-1_S but not with hTid-1_L and the hTid-1_S-induced release of cyto-*C* from the mitochondria was completely suppressed in SF767 cells overexpressing Bcl-X_L.

To assess whether Bcl-X_L can block apoptosis triggered by hTid-1_S, SF767/pBabe, SF767/pBabe-Bcl-X_L cells were then infected with adenovirus expressing GFP, hTid-1_L or hTid-1_S and apoptosis was visualized and quantified at 72 h post-infection by photomicrography (Fig. 4C), as well as trypan blue exclusion (data not shown). Overexpression of pBabe/Bcl-X_L

but not pBabe was able to antagonize the apoptogenic effects imposed by hTid-1_S on SF767 cells. The extent of cell death inhibition by Bcl-X_L was quantitated and determined to be greater than 50% (Fig. 4C). Taken together, our findings suggest that hTid-1_S regulates apoptotic signaling in SF767 at a convergence point at the mitochondria that targets cyto-C release and is inhibitable by Bcl-X_L.

4. Discussion

Molecular chaperones have emerged as important determinants of cell growth and survival responses and the relative abundance of these proteins can have profound effects on cell transformation processes. Hsp70 and other heat shock proteins have been shown to effectively inhibit both caspase-dependent and caspase-independent apoptotic stimuli and confer immortality in various human cell types [18,32,33]. Consistent with these observations, pathological overexpression of Hsp70 and Hsp90 has been reported in breast tumors, lung cancer and leukemias and is associated with refractory disease and poor prognosis [34], whereas antisense depletion results in the induction of apoptosis in several human tumors [35,36] providing proof of concept evidence that elevated expression of chaperone proteins is important for maintaining tumor cell survival.

This is the first study to identify a coding region sequence mutation targeting a member of the chaperone family. We found one glioma cell line, SF767, with a heterozygous *TID1* frameshift mutation which yields aberrantly high levels of a 52-kDa hTid-1_L mutant variant, although the functional significance of this alteration in relation to the tumorigenic phenotype of SF767 gliomas is presently unknown. One possibility is that tumor-associated mutant forms of hTid-1 could function with other genetic alterations in exerting an anti-apoptotic gain of function activity, which may contribute to survival and progression of malignant gliomas harboring such mutations.

We have demonstrated that the two major splice variants of hTid-1 differentially affect glioma cell growth and viability in vitro. While adenoviral transduction of the long isoform, hTid-1_L, had no discernible effect on the growth properties of glioma cells, we observed marked inhibition of cell proliferation with overexpression of the hTid-1_S splice variant in all glioma cell lines tested (Fig. 2 and data not shown). This observation together with the finding that the transdominant inhibitory QPD mutant of hTid-1_L, which presumably antagonizes endogenous hTid-1_L function, is seen to inhibit tumor cell growth suggests a role for hTid-1_S in growth suppression and conversely, hTid-1_L in promoting the growth and survival of gliomas.

We have found that ectopic expression of hTid-1_S in SF767 glioma cells harboring a mutant *TID1* allele rendered these cells susceptible to apoptosis, while causing growth arrest in wild-type hTid-1-expressing U373 and U87 cells. Along a similar vein, there are reports correlating induction of apoptosis with gene transfer of the p53 tumor suppressor in mutant-p53 but not wild-type p53 glioma cell lines [38,39]. It is noteworthy that in SF767 cells, induction of apoptosis at 72 h by exogenous hTid-1_S coincides with caspase activation and proteolytic cleavage of the hTid-1_L mutant species. This has led us

to speculate that the overabundance of this hTid-1_L mutant could result in novel substrate combinations with Hsps, which may contribute to development of the transformed phenotype by altering the activity, localization or stability of key regulatory substrates that enhance cellular resistance to apoptosis in these tumor cells. In keeping with this idea, caspase cleavage of the hTid-1_L mutant in SF767 cells may activate the tumor-associated death pathway silenced by this mutant or alternatively convert hTid-1_L mutant from an inhibitor of apoptosis to an activator of cell death, a paradigm that has been ascribed to other caspase substrates such as Bcl-2, RIP and Livin [40–42].

Our results describing the pro-apoptotic properties of hTid-1_S in SF767 gliomas appear at variance with those published by Syken et al. [4], in which inducible expression of recombinant hTid-1_S in O2OS osteosarcoma cell lines decreased susceptibility to damaging stimuli such as TNF α [4]. By contrast, we have observed a modest enhancement of etoposide cytotoxicity by hTid-1_S in gliomas, however, only at short exposure times (data not shown). The lack of consistency in the roles of hTid-1 proteins in the regulation of cell growth and apoptosis raises the possibility that the ability of hTid-1 splice variants to influence chemosensitization might vary depending upon the chemotherapeutic agent and schedule employed or may reflect cell-type dependent differences. However, a recent report by the same group [37] revealed that RNAi depletion of both hTid-1_L and hTid-1_S, but not that of hTid-1_L alone, rendered tumor cells completely refractile to apoptotic stimuli supporting our contention that hTid-1_S is endowed with pro-apoptotic properties.

Results from PARP cleavage and subcellular fractionation experiments in SF767 cells revealed that hTid-1_S expression is sufficient for release of cyto-C and effector caspase activation. Moreover, our observation that Bcl-X_L can protect cells from hTid-1_S-induced cell death suggests that hTid-1_S action converges at the mitochondria. The mechanism by which Tid-1 proteins engage the intrinsic pathway remains unclear, however, as Tid-1 proteins reside predominantly in the mitochondria, one possibility is that they are involved in direct regulation of mitochondrial membrane permeability (MMP) by targeting components of the permeability transition (PT) pore implicated in cyto-C release [43]. For example, it will be interesting to determine whether hTid-1_S modulates the interaction between the mitochondrial voltage-dependent anion channel, VDAC2 and the inactive conformer of pro-apoptotic Bak, enabling homodimerization and activation of Bak, which has been shown to induce efflux of cyto-C by accelerating the PT pore opening of VDAC2 [44]. Alternatively, hTid-1 might integrate death signals by impinging on signaling cascades that lie upstream of the mitochondria. Tid-1 proteins have recently been reported to modulate NF- κ B signaling, in association with Hsp70, by repressing the activity of I κ B kinase β (IKK β) thus interfering with I κ B α phosphorylation and degradation and release of NF- κ B transcription factor for nuclear translocation [45]. NF- κ B is a potent modulator of apoptosis as it induces the expression of a number of genes whose products can inhibit apoptosis induced by both death receptors and mitochondria-dependent pathways [46].

In summary, we have shown that hTid-1_S is capable of initiating apoptotic cell death in gliomas that express mutated but not wildtype endogenous hTid-1 proteins. Future studies will have to be directed towards identifying the cellular targets of

hTid-1 isoforms in order to elucidate the molecular mechanisms by which hTid-1 proteins engage the cell death machinery.

Acknowledgments: This work was supported by a grant from the National Cancer Institute of Canada (NCIC) to M.R.-A. G.A.T. is the recipient of a Terry Fox Research Studentship from the NCIC. M.R.-A. is a scholar of the Canadian Institutes of Health Research (CIHR).

References

- [1] Cheetham, M.E. and Caplan, A.J. (1998) Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chaperones* 3, 28–36.
- [2] Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J. and Bukau, B. (1999) Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc. Natl. Acad. Sci. USA* 96, 5452–5457.
- [3] Yin, X. and Rozakis-Adcock, M. (2001) Genomic organization and expression of the human tumorous imaginal disc (TID1) gene. *Gene* 278, 201–210.
- [4] Syken, J., De-Medina, T. and Munger, K. (1999) TID1, a human homolog of the *Drosophila* tumor suppressor l(2)tid, encodes two mitochondrial modulators of apoptosis with opposing functions. *Proc. Natl. Acad. Sci. USA* 96, 8499–8504.
- [5] Trentin, G.A., Yin, X., Tahir, S., Lhotak, S., Farhang-Fallah, J., Li, Y. and Rozakis-Adcock, M. (2001) A mouse homologue of the *Drosophila* tumor suppressor l(2)tid gene defines a novel Ras GTPase-activating protein. *J. Biol. Chem.* 276, 13087–13095.
- [6] Sarkar, S., Pollack, B.P., Lin, K.T., Kotenko, S.V., Cook, J.R., Lewis, A. and Pestka, S. (2001) hTid-1, a human DnaJ protein, modulates the interferon signaling pathway. *J. Biol. Chem.* 276, 49034–49042.
- [7] Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295, 1852–1858.
- [8] Nollen, E.A. and Morimoto, R.I. (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing ‘heat-shock’ proteins. *J. Cell. Sci.* 115, 2809–2816.
- [9] Zyllicz, M., King, F.W. and Wawrzynow, A. (2001) Hsp70 interactions with the p53 tumour suppressor protein. *EMBO J.* 20, 4634–4638.
- [10] Wadhwa, R., Yaguchi, T., Hasan, M.K., Mitsui, Y., Reddel, R.R. and Kaul, S.C. (2002) Hsp70 family member, mot-2/mthsp70/GRP75, binds to the cytoplasmic sequestration domain of the p53 protein. *Exp. Cell Res.* 274, 246–253.
- [11] Inoue, A., Torigoe, T., Sogahata, K., Kamiguchi, K., Takahashi, S., Sawada, Y., Saijo, M., Taya, Y., Ishii, S. and Sato, N. (1995) 70-kDa heat shock cognate protein interacts directly with the N-terminal region of the retinoblastoma gene product pRb. Identification of a novel region of pRb-mediated protein interaction. *J. Biol. Chem.* 270, 22571–22576.
- [12] Nihei, T., Takahashi, S., Sagae, S., Sato, N. and Kikuchi, K. (1993) Protein interaction of retinoblastoma gene product pRb110 with M(r) 73,000 heat shock cognate protein. *Cancer Res.* 53, 1702–1705.
- [13] Feldman, D.E., Thulasiraman, V., Ferreyra, R.G. and Frydman, J. (1999) Formation of the VHL-elongin BC tumor suppressor complex is mediated by the chaperonin TRiC. *Mol. Cell* 4, 1051–1061.
- [14] Melville, M.W., McClellan, A.J., Meyer, A.S., Darveau, A. and Frydman, J. (2003) The Hsp70 and TRiC/CCT chaperone systems cooperate in vivo to assemble the von Hippel-Lindau tumor suppressor complex. *Mol. Cell Biol.* 23, 3141–3151.
- [15] Maheswaran, S., Englert, C., Zheng, G., Lee, S.B., Wong, J., Harkin, D.P., Bean, J., Ezzell, R., Garvin, A.J., McCluskey, R.T., DeCaprio, J.A. and Haber, D.A. (1998) Inhibition of cellular proliferation by the Wilms tumor suppressor WT1 requires association with the inducible chaperone Hsp70. *Genes Dev.* 12, 1108–1120.
- [16] Nakamura, S., Tatuno, I., Noguchi, Y., Kitagawa, M., Kohn, L.D., Saito, Y. and Hirai, A. (1999) 73-kDa heat shock cognate protein interacts directly with P27Kip1, a cyclin-dependent kinase inhibitor, during G1/S transition. *Biochem. Biophys. Res. Commun.* 257, 340–343.
- [17] Song, J., Takeda, M. and Morimoto, R.I. (2001) Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nat. Cell Biol.* 3, 276–282.
- [18] Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M. and Green, D.R. (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat. Cell Biol.* 2, 469–475.
- [19] Saleh, A., Srinivasula, S.M., Balkir, L., Robbins, P.D. and Alnemri, E.S. (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat. Cell Biol.* 2, 476–483.
- [20] Kurzik-Dumke, U., Gundacker, D., Renthrop, M. and Gateff, E. (1995) Tumor suppression in *Drosophila* is causally related to the function of the lethal(2)tumorous imaginal discs gene, a dnaJ homolog. *Dev. Genet.* 16, 64–76.
- [21] Canamasas, I., Debes, A., Natali, P.G. and Kurzik-Dumke, U. (2003) Understanding human cancer using *Drosophila*: Tid47, a cytosolic product of the DnaJ-like tumor suppressor gene l(2)tid, is a novel molecular partner of patched related to skin cancer. *J. Biol. Chem.* 278, 30952–30960.
- [22] Raffel, C., Jenkins, R.B., Frederick, L., Hebrink, D., Alderete, B., Fults, D.W. and James, C.D. (1997) Sporadic medulloblastomas contain PTCH mutations. *Cancer Res.* 57, 842–845.
- [23] Kinzler, K.W., Ruppert, J.M., Bigner, S.H. and Vogelstein, B. (1988) The GLI gene is a member of the Kruppel family of zinc finger proteins. *Nature* 332, 371–374.
- [24] Lininger, R.A., Zhuang, Z., Man, Y., Park, W.S., Emmert-Buck, M. and Tavassoli, F.A. (1999) Loss of heterozygosity is detected at chromosomes 1p35-36 (NB), 3p25 (VHL), 16p13 (TSC2/PKD1), and 17p13 (TP53) in microdissected apocrine carcinomas of the breast. *Mod. Pathol.* 12, 1083–1089.
- [25] Gutmann, D.H., Saporito-Irwin, S., DeClue, J.E., Wienecke, R. and Guha, A. (1997) Alterations in the rap1 signaling pathway are common in human gliomas. *Oncogene* 15, 1611–1616.
- [26] He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W. and Vogelstein, B. (1998) A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95, 2509–2514.
- [27] Wu, D., Ingram, A., Lahti, J.H., Mazza, B., Grenet, J., Kapoor, A., Liu, L., Kidd, V.J. and Tang, D. (2002) Apoptotic release of histones from nucleosomes. *J. Biol. Chem.* 277, 12001–12008.
- [28] Zhang, X.D., Zhang, X.Y., Gray, C.P., Nguyen, T. and Hershey, P. (2001) Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of human melanoma is regulated by smac/DIABLO release from mitochondria. *Cancer Res.* 61, 7339–7348.
- [29] Tsai, J. and Douglas, M.G. (1996) A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. *J. Biol. Chem.* 271, 9347–9354.
- [30] Cory, S. and Adams, J.M. (2002) The Bcl-2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* 2, 647–656.
- [31] Yang, X., Hao, Y., Ferenczy, A., Tang, S.C. and Pater, A. (1999) Overexpression of anti-apoptotic gene BAG-1 in human cervical cancer. *Exp. Cell Res.* 247, 200–207.
- [32] Dias, S., Shmelkov, S.V., Lam, G. and Rafii, S. (2002) VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* 99, 2532–2540.
- [33] Gabai, V.L., Yaglom, J.A., Volloch, V., Meriin, A.B., Force, T., Koutroumanis, M., Massie, B., Mosser, D.D. and Sherman, M.Y. (2000) Hsp72-mediated suppression of c-Jun N-terminal kinase is implicated in development of tolerance to caspase-independent cell death. *Mol. Cell Biol.* 20, 6826–6836.
- [34] Jolly, C. and Morimoto, R.I. (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.* 92, 1564–1572.
- [35] Wei, Y.Q., Zhao, X., Kariya, Y., Teshigawara, K. and Uchida, A. (1995) Inhibition of proliferation and induction of apoptosis by abrogation of heat-shock (HSP) 70 expression in tumor cells. *Cancer Immunol. Immunother.* 40, 73–78.

- [36] Nylandsted, J., Wick, W., Hirt, U.A., Brand, K., Rohde, M., Leist, M., Weller, M. and Jaattela, M. (2002) Eradication of glioblastoma, and breast and colon carcinoma xenografts by Hsp70 depletion. *Cancer Res.* 62, 7139–7142.
- [37] Edwards, K.M., and Münger, K. (2004). Depletion of physiological levels of the human TID1 protein renders cancer cell lines resistant to apoptosis mediated by multiple exogenous stimuli. *Oncogene* advance online publication 24 May 2004, 1–13.
- [38] Gjerset, R.A., Turla, S., Sobol, R.E., Scalise, J.J., Mercola, D., Collins, H. and Hopkin, P.J. (1995) Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. *Mol. Carcinogenesis* 14, 275–285.
- [39] Gomez-Manzano, C., Fueyo, J., Kyritsis, A.P., Steck, P.A., Roth, J.A., McDonnell, T.J., Steck, K.D., Levin, V.A. and Yung, W.K. (1996) Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. *Cancer Res.* 56, 694–699.
- [40] Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K. and Hardwick, J.M. (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278, 1966–1968.
- [41] Lin, Y., Devin, A., Rodriguez, Y. and Liu, Z.G. (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev.* 13, 2514–2526.
- [42] Nachmias, B., Ashhab, Y., Bucholtz, V., Drize, O., Kadouri, L., Lotem, M., Peretz, T., Mandelboim, O. and Ben-Yehuda, D. (2003) Caspase-mediated cleavage converts Livin from an antiapoptotic to a proapoptotic factor: implications for drug-resistant melanoma. *Cancer Res.* 63, 6340–6349.
- [43] Reed, J.C. and Kroemer, G. (2000) Mechanisms of mitochondrial membrane permeabilization. *Cell Death Differ.* 7, 1145.
- [44] Cheng, E.H., Sheiko, T.V., Fisher, J.K., Craigen, W.J. and Korsmeyer, S.J. (2003) VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* 301, 513–517.
- [45] Cheng, H., Cenciarelli, C., Tao, M., Parks, W.P. and Cheng-Mayer, C. (2002) HTLV-1 Tax-associated hTid-1, a human DnaJ protein, is a repressor of I κ B kinase β subunit. *J. Biol. Chem.* 277, 20605–20610.
- [46] Karin, M. and Lin, A. (2002) NF- κ B at the crossroads of life and death. *Nat. Immunol.* 3, 221–227.