Survivin 2α: a novel Survivin splice variant expressed in human malignancies
Hugo Caldas¹, Laura E Honsey¹ and Rachel A Altura*¹,²

Address: ¹Center for Childhood Cancer, Columbus Children’s Research Institute Columbus, OH, USA and ²Department of Pediatrics, The Ohio State University, Columbus, OH, USA

Email: Hugo Caldas - caldash@ccri.net; Laura E Honsey - lehonsey@hillsdale.edu; Rachel A Altura* - alturar@ccri.net

* Corresponding author

Abstract

Background: Survivin and its alternative splice forms are involved in critical cellular processes, including cell division and programmed cell death. Survivin is expressed in the majority of human cancers, but minimally in differentiated normal tissues. Expression levels correlate with tumor aggressiveness and resistance to therapy.

Results: In the present study, we identify and characterize a novel survivin isoform that we designate survivin 2α. Structurally, the transcript consists of 2 exons: exon 1 and exon 2, as well as a 3’ 197 bp region of intron 2. Acquisition of a new in-frame stop codon within intron 2 results in an open reading frame of 225 nucleotides, predicting a truncated 74 amino acid protein. Survivin 2α is expressed at high levels in several malignant cell lines and primary tumors. Functional assays show that survivin 2α attenuates the anti-apoptotic activity of survivin. Subcellular localization and immunoprecipitation of survivin 2α suggests a physical interaction with survivin.

Conclusion: We characterized a novel survivin splice variant that we designated survivin 2α. We hypothesize that survivin 2α can alter the anti-apoptotic functions of survivin in malignant cells. Thus survivin 2α may be useful as a therapeutic tool in sensitizing chemoresistant tumor cells to chemotherapy.

Background

Alternative splicing is estimated to occur in 40–60% of all human genes, accounting for some of the discrepancies between the large number of known proteins and the three-fold lower number of human genes in the genome. Alternative splicing generates a multitude of isoforms that have overlapping but distinct functions during embryonic development and that also contribute to maintaining homeostasis in adult differentiated tissues (reviewed in [1]). Alternative splice forms of key proteins in cancer, TP53, MDM2 [2] and c-MYC [3], have been shown to play a role in oncogenesis.

Survivin was originally identified by structural homology to IAPs in human B-cell lymphoma [4]. It is composed of a single BIR domain and an extended carboxy-terminal coiled coil domain [5]. Transcription from the Survivin locus gives rise to alternatively spliced transcripts identified in both human and mice [6-8]. To date, three alternatively spliced isoforms have been described in humans [6-8]. Survivin-2B is generated by the insertion of an alternative exon, exon 2B; Survivin-ΔEx3 arises from the removal of exon 3 resulting in a frameshift and translation of part of the 3’UTR generating a unique carboxy-terminus; Survivin-3B results from the introduction of a novel exon 3B
resulting in a frameshift and premature termination of the protein (Figure 1A).

Survivin has 2 main functions; one as a chromosomal passenger protein [9] and the other as an inhibitor of apoptosis [10]. Survivin 2B has been shown to be a pro-apoptotic protein that sensitizes resistant leukemia cells to chemotherapy in a p53-dependent fashion [11]. Survivin-ΔEx3 functions as an anti-apoptotic protein and is upregulated in malignancies (Mahotka et al., 1999). No function has yet been described for survivin-3B.

In this report we identify and characterize a novel isoform of survivin, survivin 2α. We show that survivin 2α is expressed at high levels in malignant cells, co-localizes with survivin and has the potential to attenuate the anti-apoptotic effect of survivin.

Results and Discussion

Structural Characteristics of Survivin 2α

In this work, we characterized a novel isoform of the survivin locus. We surveyed the aligned survivin EST sequences available in the UCSC Human Genome Program.
Molecular Cancer 2005, 4:11

Browser and identified an EST from a human breast tumor cDNA library (I.M.A.G.E. clone 1631662). We sequenced the entire cDNA and designated it Survivin 2α. The complete cDNA sequence is shown in Figure 1B. The protein contains the coding sequences from exon 1 and exon 2, and one additional amino acid before termination (Figure 1B). This 74 amino acid protein, with a predicted molecular weight of 8.5 kDa, contains a truncated BIR domain and lacks the carboxy-terminal coiled-coil domain in its entirety (Figure 1A). There are no defined localization signals in the protein, and PSORTII predicts localization within the nucleus and the cytoplasm (Table 1). Alignment with the known human survivin isoforms shows that the sequence of Survivin 2α is identical to exons 1 and 2 of the other survivin splice variants, with the exception of the last amino acid. Alignment of Survivin 2α with the three mouse survivin isoforms also reveals some similarity with survivin40, a 40-amino acid mouse splice variant (not shown). The 3D predicted structure of Survivin 2α shows the absence of the alpha-helical coiled-coil domain, present in survivin (Figure 1C). It also shows minor predicted rearrangements in the structure that may occur to stabilize the protein (Figure 1C, yellow arrows). These re-arrangements occur within the BIR domain, and could have functional implications for the role of Survivin 2α in apoptosis.

The BIR domain has been shown to be important for homodimerization and coordination of the zinc atom cofactor [12]. In the survivin protein, Histidine 80 (H80) is required for zinc atom coordination and homodimerization. Expression constructs containing mutations at this residue within the Survivin protein have previously been shown to accelerate PCD (Programmed Cell Death) in vitro. Similarly, mutations in Cytosine 84 (C84) enhance PCD, as a result of displacement of the wild type Survivin protein [13]. The Survivin 2α protein, truncated at amino acid 74, lacks both of these amino acid residues. Additionally, Survivin 2α lacks the third alpha helix in the BIR domain. As the anti-apoptotic function of Survivin is mediated both by the BIR domain and by the interaction of its C-terminal coiled coil domain with microtubules of the mitotic spindle [10,14,15], it would be predicted that Survivin 2α might not have anti-apoptotic properties.

Survivin 2α is highly expressed in tumor cells
Survivin is critical for global normal embryonic development, as demonstrated by the early embryonic lethality of mice with homozygous deletions in the survivin gene locus [16]. Survivin proteins are virtually absent from most normal differentiated tissues, however these proteins are expressed in certain highly proliferative areas within normal tissues [17-19]. In contrast, survivin is highly expressed in the majority of human malignancies, derived from different cell origins. We evaluated the expression of survivin 2α in 7 different cancer cell lines, 2 non-transformed tissues and 7 primary medulloblastoma tumors by quantitative PCR. We designed primers that will specifically amplify Survivin 2α after selection of polyadenylated RNA. Survivin 2α expression in tumor cells and primary medulloblastoma tumors varied from 2–100 fold above non transformed cells (Table 2). The

| Localization       | Survivin | Survivin 2α |
|--------------------|----------|-------------|
| Cytoplasm          | 56.5%    | 39.1%       |
| Nucleus            | 17.4%    | 34.8%       |
| Cytoskeleton       | 0%       | 4.3%        |
| Golgi Apparatus    | 0%       | 0%          |
| Plasma membrane    | 4.3%     | 0%          |
| ER                 | 4.3%     | 4.3%        |
| Peroxysome         | 0%       | 0%          |
| Mitochondria       | 13.0%    | 13.0%       |
| Lysosomes          | 4.3%     | 4.3%        |

| Features            | Survivin | Survivin 2α |
|---------------------|----------|-------------|
| BIR                 | 1        | Partial     |
| Coiled-Coil         | 1        | 0           |
| Protein Size        | 142 aa   | 74 aa       |
| Predicted Molecular Weight | 16.4 kDa | 8.5 kDa |

| Cell Type                        | Relative Increase |
|----------------------------------|--------------------|
| Normal Cerebellum                | 1.00               |
| Normal Breast (MCF10A)           | 0.97               |
| Breast Carcinoma (MCF7)          | 8.17               |
| Osteosarcoma (U2OS)              | 39.06              |
| Lung (A549)                      | 3.03               |
| ALL (Jurkat)                     | 1.84               |
| Soft Tissue Sarcoma (RH28)       | 94.90              |
| Cervical Carcinoma (HeLa)        | 58.22              |
| Medulloblastoma (Daoy)           | 34.23              |

| Primary Tumors                  | Relative Increase |
|---------------------------------|--------------------|
| Medulloblastoma #1              | 4.68               |
| Medulloblastoma #2              | 154.55             |
| Medulloblastoma #3              | 93.24              |
| Medulloblastoma #4              | 5.69               |
| Medulloblastoma #5              | 8.54               |
| Medulloblastoma #6              | 9.81               |
| Medulloblastoma #7              | 75.10              |

Table 1: Table of the predicted localization and structural features of survivin and the novel isoform survivin 2α.

Table 2: Survivin 2α expression (relative to normal tissue).
levels of Survivin 2α transcripts are comparable to those of Survivin ΔExon3 (Table 3). Like Survivin, Survivin 2α is expressed at increased levels in transformed cells compared to non-transformed cells, and therefore it suggests that it could have a role in tumorigenesis. Additionally, we detected expression of endogenous Survivin 2α protein in HeLa cells, suggesting that the transcript is translated (Figure 1D).

### Functional Properties of Survivin 2α

To characterize a function for Survivin 2α, we transfected Daoy cells with Survivin 2α and a combination of Survivin 2α and Survivin. To induce apoptosis in the Daoy cells, we treated them with 2 µM of the chemotherapeutic agent vincristine. Vincristine is a vinca alkaloid that binds to tubulin, inhibiting microtubule polymerization. It kills Daoy cells in culture by PCD. We analyzed early apoptotic events in vincristine-treated transfected cells by Annexin V staining. Survivin 2α antagonized the anti-apoptotic effect of Survivin in co-transfection assays with or without a cell death stimulus (not shown and Figure 2A). As inhibition of apoptosis by Survivin involves activation of the caspase pathway [20], we assayed Survivin 2α transfected cells for caspase 3 activation. Caspase-3 was strongly activated in vector control and Survivin 2α transfected cells in the presence of vincristine. Much lower levels of caspase-3 activation were observed in Survivin-transfected cells (Figure 2B). In the absence of an apoptotic stimulus we observed a 35% increase of caspase-3 activity in Survivin 2α cells, as well as a 46% increase in early apoptosis, as assessed by Annexin V staining. We also performed electron microscopy analysis of Survivin 2α transfected and non-transfected cells. We sorted transfected cells from non-transfected cells by FACS based on GFP fluorescence, and processed each population for EM analysis (Figure 2C). Overall, there was a 43% increase in incidence of apoptosis in Survivin 2α-expressing cells versus non-expressing cells. Our results suggest that Survivin 2α can attenuate survivin’s anti-apoptotic activity and sensitize tumor cells to chemotherapy. These findings have important therapeutic implications in the treatment of chemoresistant tumors.

### Survivin 2α alters the subcellular localization of survivin

To characterize the subcellular localization of survivin 2α we performed direct fluorescence assays in HeLa cells transfected with a GFP-survivin 2α construct. Survivin 2α localized to the nucleus and the cytoplasm in interphase cells (Figure 3A). In cells undergoing mitosis, survivin 2α was confined to the cytoplasmic compartment (Figure 3B). Interestingly, when co-expressed with survivin, survivin 2α co-localized with survivin to the centromeres of the chromosomes in prometaphase (Figure 3C) and metaphase (Figure 3D), and at the midbody during late telophase/cytokinesis (Figure 3E). Moreover, the normal cytoplasmic localization of survivin shifted to the nucleus in interphase cells. This data suggests a direct interaction between the two proteins, as well as a potential mechanism for the attenuation of survivin’s anti-apoptotic activity by survivin 2α.

### Survivin 2α physically interacts with survivin

To further investigate the possibility that survivin 2α interacts with survivin we performed co-immunoprecipitation experiments. We co-transfected HeLa cells with constructs encoding a Flag-survivin fusion protein and a myc-survivin 2α fusion protein. We used a Flag antibody to precipitate protein complexes, and a myc antibody to detect myc-tagged survivin 2α. We detected survivin 2α-myc in the complexes precipitated with the Flag antibody, substantiating a physical interaction of survivin with survivin 2α (Figure 4).

### Conclusion

We characterized a novel survivin splice variant that we designated survivin 2α. We hypothesize that survivin 2α can alter the anti-apoptotic functions of survivin in malignant cells. Thus, survivin 2α may be useful as a therapeutic tool in sensitizing chemoresistant tumor cells to chemotherapy.

### Methods

#### Patient samples

Seven fresh frozen primary medulloblastoma tumor samples were obtained from the Cooperative Human Tumor Network (CHTN), after approval through the Columbus Children’s Hospital IRB.
Sequencing
IMAGE clone 1631662 (Invitrogen) was sequenced using primers that flanked the multiple-cloning-site.

Plasmids and Cloning
The cDNA for survivin 2α was amplified from the EST clone (Invitrogen) and cloned into the KpnI-BamHI sites of pcDNA4/TO/myc-HisB (Invitrogen) generating an in-frame fusion with the C-terminal myc-tag, or into the KpnI-BamHI sites of pEGFP-N3 generating an in-frame fusion with the C-terminal GFP tag. The start codon in both constructs corresponds to the naturally occurring start codon in the cDNA transcript. The resulting clones were confirmed by sequencing.

Cell Culture and Transfection
HeLa (cervical adenocarcinoma), Daoy (medulloblastoma), Jurkat (acute lymphoblastic leukemia) and MCF-7 (breast adenocarcinoma) cells (ATCC) were grown in DMEM supplemented with 10% FBS at 37°C, 5% CO2;
U2OS osteosarcoma cells (kindly donated by Dr. Greg Otterson) were grown in McCoy's 5A medium supplemented with 10% FBS at 37°C, 5% CO₂; RH28 (alveolar rhabdomyosarcoma, kindly donated by Dr. Steve Qualman) and A549 (lung carcinoma) (ATCC) were grown in RPMI1640 supplemented with 10% FBS at 37°C, 5% CO₂. MCF10-A, a non-transformed breast cell line (ATCC) was grown in MEGM, Mammary Epithelial Growth Medium, Serum-free, (Clonetics) supplemented with 100 ng/ml cholera toxin (Sigma Aldrich) at 37°C, 5% CO₂.

Transient transfections were performed using Effectene transfection reagent (Qiagen) at a DNA: Effectene ratio of 1:10.

**Drug Treatment**

Induction of apoptosis by vincristine was done by treatment of cells with complete growth medium supple-
mented with vincristine sulfate at a final concentration of 2 µM.

**RNA isolation and Real Time PCR**

RNA was isolated from 10⁶ proliferating cells or frozen tumor tissue using Trizol reagent (Invitrogen) as recommended by the supplier. Poly(A) RNA was purified using Oligotex dT kit (Qiagen). 100 ng of poly(A) purified RNA was used as a template in a reverse transcription reaction using random hexamers and Omniscript Reverse transcriptase (Qiagen) and performed according to manufacturer's instructions. Quantitative real-time PCR reactions using Taqman probes (FAM/TAMRA) were run in triplicate on an ABI Prism 7700 Real-time PCR machine (Applied Biosystems). Control GAPDH reactions (Applied Biosystems) were run to normalize ΔCt values. Relative change was calculated by the comparative Ct method, 2^(-ΔΔCt). The survivin 2α specific primers consist of: Forward 5’GCATTGATTGCAAAGACACTTAGTATGGAGGG TAMRA; Reverse 5’GCAATGAGGGTGGAAAGCA; and Probe: 6FAM AGATTGAGTTGCAAAGACACTTAGTATGGAGGG TAMRA

**Apoptosis Assays**

Two apoptosis assays were performed: Caspase-3 assay and Annexin-V FLUOS. For caspase assays 2,000 cells from each experimental condition were subjected to the caspase-3 assay, Caspase 3/7 GLO (Promega) and analyzed on a Victor3 plate reader (Applied Biosystems). Experiments were performed in triplicate.

Annexin V/propidium iodide staining was carried out using the Roche Annexin-V-Fluos Staining Kit following the manufacturer's instructions. Fluorescein and propidium iodide fluorescence measured with a Coulter EPICS XL flow cytometer. Experiments were performed in triplicate.
Microscopy
Proliferating HeLa cells, grown on glass coverslips, were transiently transfected with a GFP-tagged survivin 2α expression construct or co-transfected with GFP-tagged survivin 2α and HcRed-tagged survivin. 24 hours post-transfection the cells were fixed in 4% paraformaldehyde and stained with 50 µg/ml Hoechst dye. Cells were analyzed on a Zeiss LSM510 META confocal microscope, using a 63x PlanApochromat objective. For electron microscopy analysis, proliferating HeLa cells were transfected with GFP-tagged survivin 2α construct for 12 hours. The cells were aseptically sorted by FACS based on green fluorescence from GFP-survivin 2α for positive and negative populations. This was done in order to separate an enriched population that consisted of >90% GFP expressing cells. 10 cells for each condition were fixed in 2.5% gluteraldehyde for 24 hours and processed for EM. For cell analysis, 10 to 12 fields containing 8–10 cells per field at a magnification of 3500× were used. At least 100 cells were counted for each experimental condition and the morphological appearance, including nuclear integrity. Image collection was performed on a Hitachi H-600 transmission electron microscope equipped with a GATAN image acquisition system.

Co-Immunoprecipitation
HeLa cells transfected with Flag-survivin and survivin-2α myc were collected in Cell Lysis Buffer (100 mM Tris-HCl pH8.0, 100 mM NaCl, 0.5% Triton X-100, 0.2 µM PMSF) and incubated at 4°C for 30 min. The cell lysate was clarified by centrifugation and the clarified supernatant dissolved 1:5 in Co-IP buffer (50 mM Tris-HCl pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 1x protease inhibitors cocktail, 1 mM DTT, 1 mM PMSF). The equivalent of 400 µg of lysate total protein was incubated with 2 µg of anti-Flag M2 antibody at 4°C for 1 h with constant rotation. As a control the same amount of lysate protein was incubated in the absence of antibody. Fifty microliters of agarose-conjugated protein A (Invitrogen) were added and the mixture incubated for a further hour in the same conditions. The protein-antibody-protein A complexes were pulled down by centrifugation and subjected to 3 washes with co-IP buffer. The proteins were analyzed through electrophoretic separation in a 20% SDS-PAGE, electroblotted onto nitrocellulose and immunoprobed with an antibody against myc-tag. Detection was performed using the ECL kit (Amersham). Protein standards were used for size determination.

Bioinformatics
Subcellular localization predicted by PSORTII program. Coiled-Coil domain predicted by Coils and PairCoil programs

Authors' contributions
HC performed bioinformatic analysis, subcellular localization, functional studies, co-immunoprecipitation and drafted the manuscript. LH performed quantitative real time PCR in cell lines and primary tumors. RA conceived the study and participated in its design and coordination, and was responsible for overseeing the final version of the manuscript. All authors have read and approved the final manuscript.

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