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Short Communication

Identification and Characterization of Novel Survivin Splice Variants with Anti-Apoptotic Activities

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Abstract

Survivin is a member of the endogenous inhibitor-of-apoptosis (IAP) family. Several alternative Survivin splice variants have been described and while their function is not fully understood, several have been shown to have diagnostic and prognostic significance. We have identified two novel alternative splice variants, Survivin-3γ and Survivin-3γ-V that differ by 4 bases resulting in proteins with very different C-termini. We characterized their expression in normal hematopoietic stem and progenitor cells (HSPC) and in cancer cells and evaluated their anti-apoptotic function. Both splice variants are highly expressed in primary umbilical cord blood CD34+ HSPC. While growth factor stimulated proliferation of CD34+ cells is associated with increased wild-type Survivin expression, the expression of the Survivin-3γ+3γV splice variants decrease relative to Survivin-WT, suggesting that they may be associated with HSPC maintenance/quiescence. In contrast to neoplastic cell lines and most cancers, where Survivin-WT is the predominant transcript, Survivin-3γ+3γV are expressed at higher levels in benign prostate hyperplasia tissue and at equivalent levels in normal breast tissue. In prostate and breast cancer cells where Survivin-WT expression increases with stage of disease, expression of Survivin-3γ and Survivin-3γV expression does not change with increasing severity of disease. Over expression of Survivin-3γ and Survivin-3γV enhances resistance to the chemotherapeutic agents Taxol and Etoposide, and imparts growth factor independent growth to normal HSPC, suggesting that the Survivin-3γ and Survivin-3γV splice variants possess similar anti-apoptotic function as Survivin-WT.

ABBREVIATIONS

WT: Wild Type; MDM2: Murine Double Minute 2; HI-FBS: Heat Inactivated Fetal Bovine Serum; SNP: Single Nucleotide Polymorphism; UCB-Umbilical Cord Blood

INTRODUCTION

Survivin is a multifunctional protein linked to apoptosis, angiogenesis and cell growth and division [1,2]. While primarily developmentally expressed, it is over-expressed in virtually every human cancer. Survivin transcription is primarily cell cycle regulated, peaking during G_2/M phase [3], however cell cycle-independent Survivin gene expression has been noted [1]. Survivin interacts with prominent signaling pathways associated with cell growth and proliferation, tumor suppressor genes, oncogenes, stress response proteins and growth stimulatory cytokines. It is one of the few genes actively repressed by wild-type p53 [4,5]. Survivin over-expression is linked to resistance to apoptosis, bypass of cell cycle checkpoints and resistance to chemotherapy and numerous molecular profiling studies and retrospective analysis of patient responses have identified Survivin as a risk factor for disease progression and poor prognosis [1,6-9]. As a result of its nodal properties and expression patterns, Survivin has been pursued as a therapeutic target [1,2,6].

While Survivin's main transcript is the predominant transcript in most cancers, alternatively spliced Survivins having both similar and opposing function have been identified and linked to differential diagnosis and prognosis [10, 11]. In this report, we describe the alternative splice variants Survivin-3γ and -3γV and characterize their biological activity relative to wild-type Survivin. Both variants have anti-apoptotic activity similar to

Survivin-WT, imparting resistance to chemotherapy in neoplastic cells and growth proliferation in non-transformed cells. Both are expressed at higher levels than Survivin-WT in primary HSPC and do not increase with cell proliferation, suggesting a role in stem cell maintenance.

MATERIALS AND METHODS

Cell lines, primary cells and plasmids

HeLa cells were grown in DMEM with 10% HI-FBS (Hyclone Sterile Systems, Logan, UT). Murine Yac-1 lymphoma cells, HL-60 human promyelocytic leukemia cells and K562 malignant multipotent hematopoietic cells were maintained in RPMI-1640 with 10% HI-FBS. Mo7e-bcr-abl megakaryocytic leukemia cells were maintained in the presence of 100 units GM-CSF. Survivin-WT, -Flag-tagged WT, -3γ and -3γV were amplified by PCR and cloned into MIEG-3 [12]. All plasmids were transfected into phoenix cells [13] and the retrovirus produced used to infect Yac-1 cells. After 48 hours infection, GFP^+ expression was reconfirmed. Primary umbilical cord blood cells were obtained under IRB approval.

Reverse transcription and PCR

Total RNA was obtained using the absolutely RNA purification kit (Stratagene, La Jolla, CA). A constant amount of RNA was reverse transcribed with random primers (Promega, Madison, WI) and MMLV-reverse transcriptase (Promega) in 50μl with 1mM dNTPs and RNase inhibitor [12]. Primers for SYBR Green RT-PCR were designed to produce an amplicon size of 75-150 bp. PCR was performed using Platinum SYBR Green qPCR supermix UDG with Rox (Invitrogen, Carlsbad, CA) in an ABI-7000 (Applied Biosystems, Carlsbad, CA), with an activation step of 50°C for 2 minutes, denaturation at 95°C for 2 minutes and amplification for 45 cycles at 95°C-15 sec, 50°C-30 sec, 72°C-30 sec. Total RNA from HL60 cells was reverse transcribed using oligo-dT primers (Roche, Indianapolis, IN) and the cDNA library used as a template to amplify full-length cDNA using the primers: Flag-Sur-Forward 5'-G*GAATTC*GCCGCCACC<u>ATGGACTACAAAGACGATGACGACAAGGG ATCC</u>ATGGGTGCCCCGACG-3' and Sur-3B-Reverse-5'-CCG*CTCGAG*CTATCATTCTGCTAACAGAGC-3'and *Pfu* Turbo polymerase (Stratagene) and the products cloned into MIEG-3; (restriction site is shown in italics, Flag-tag sequence is underlined and sequence present in Survivin-WT is shown in bold) Transformed *E.coli*c lones were picked and sequenced. To confirm variant sequence, specific primers were synthesized and PCR was carried out on cDNA. PCR products were gel purified by Qiaquick gel purification kit (Qiagen, Valencia, CA) and cloned into Zero blunt Topo PCR cloning kit (Invitrogen) and sequenced.

Rapid amplification of cDNA ends (RACE)

To obtain the complete 5' and 3' Survivin-3γ, RACE was performed using the SMART RACE cDNA amplification kit (Clonetech, Mountain view, CA) as recommended by the manufacturer. PCR products were cloned and sequenced as described above.

Expression analysis using breast and prostate cancer arrays

Survivin-3γ+3γV expression in breast and prostate cancers was evaluated using a tissue scan real-time array (OriGene Technologies, Rockville, MD). SYBR Green RT-PCR was carried out using primers recognizing only the intended Survivin variant and hypoxanthine phosphoribosyl transferase (HPRT). Relative expression levels of 3γ+3γV were calculated and expressed relative to Survivin-WT ($2^{-\Delta\Delta Ct}$ method).

Cell lysis and western blot analysis

Western blot onYac-1 cell lysates was performed as we described [14]. Membranes were probed using rabbit anti-SurvivinpAb (Novus Biologicals, Boulder, CO) or mouse anti-Survivin mAb (DAKO, Tucson, AZ) and peroxidase conjugated secondary antibody. Bands were visualized using ECL-plus reagent (Amersham, Piscataway, NJ).

Cytotoxicity assays

Log phase Yac-1 cells were plated at $1x10^5$ cells/well and treated with Paclitaxel (Sigma) for 96 hours or Etoposide (Sigma) for 24 hours. Following incubation, MTS reagent (Promega) was added, incubated at 37°C for ~ 3 hours and absorbance (OD 490nm) measured.

RESULTS AND DISCUSSION

While amplifying the Survivin-3B variant from HL-60 leukemia cells, we observed a slower migrating band, which we excised, cloned and sequenced (Figure 1A), revealing a full length protein with a unique C-terminal 13 amino acids, including the stop codon (Figure 1B). We amplified the cDNA using a polydT reverse-transcribed cDNA library from three hematopoietic leukemia cell lines as previously described [15], resolved the products on an agarose gel (Figure 1C) and excised, cloned and sequenced the ~ 400bp band. Analysis showed a novel splice variant, which we named Survivin-3γ, and unexpectedly, an alternative variant that differed by 4 bases introduced at the beginning of the novel-3γ exon (shown in larger font in Figure 1D) that we named Survivin-3γV.

The addition of 4 bases (ACAG) in Survivin-3γV results in a frame shift generating a novel C-terminus, likely due to an alternate consensus 3' splice site ("AG") 4 bases upstream of the 3γ-exon. Survivin-3γ and -3γV nucleotide sequences are highly homologous to Alu sequences known to introduce alternate splicing signals. Moreover, Chromosome 17, where Survivin localizes, contains a high density of Alu sequences [16]. Interestingly, the base "A" in the 3'splice acceptor site of Survivin-3γ is a SNP site with "A" having a frequency of 60% and "G" 40% (refSNP ID: rs2467572). Thus, in individuals homozygous for "G", Survivin-3γ may not be made since the consensus 3'splice site "AG" would be "GG".

Since a Survivin-3γV stop codon was not found in the sequenced region, we performed 3' RACE using a primer that recognizes all splice variants and an anchor primer (Figure 2A). A second round of RACE was performed with a Survivin-3γ specific primer and the anchor primer, resulting in amplification of a specific ~500bp product (Figure 2B). Translation from the longest insert revealed that Survivin-3γ had 12 new amino acids at the C-terminus (Figure 2C), while Survivin-3γV was 162 amino acids long compared to 142 for Survivin-WT (Figure 2D). Secondary structure prediction using Jpred [17] suggests that Survivin-3γ

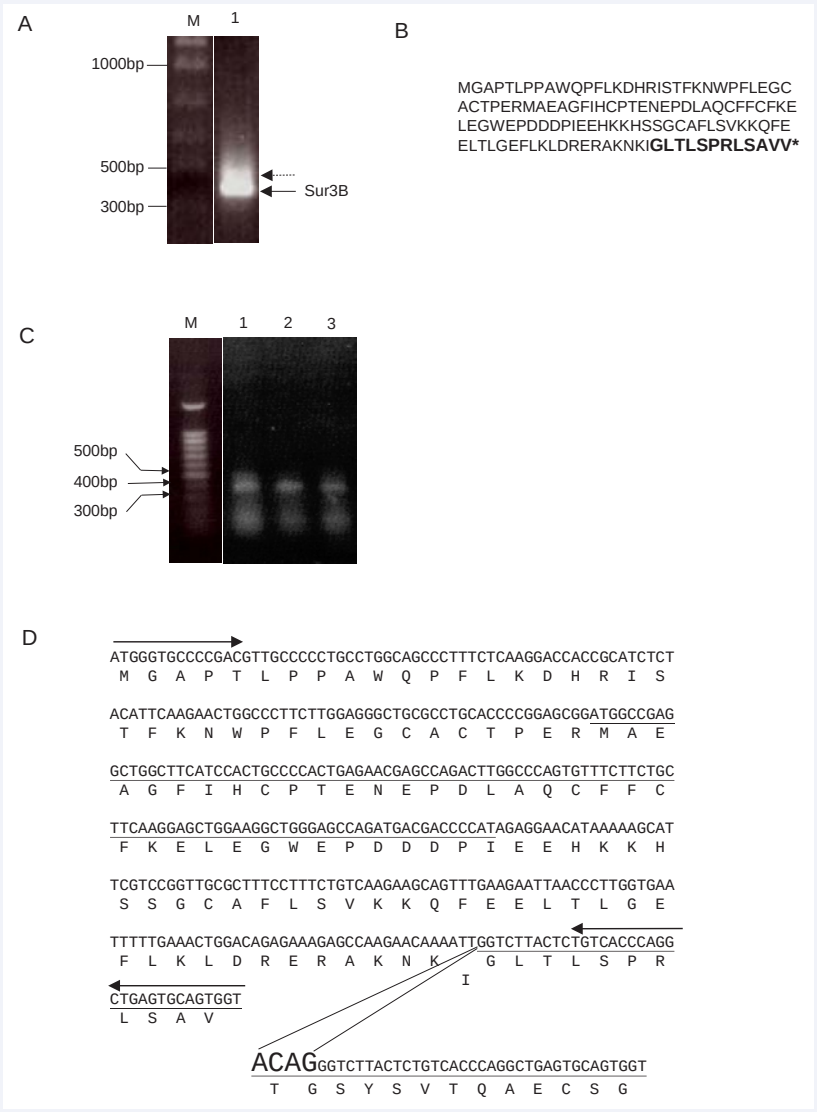

Figure 1 Identification of a novel splice variant of Survivin: (A). Agarose gel electrophoresis of cDNA amplified from HL60 cells (lane 1). The Survivin-3B variant is indicated by a solid arrow, the dotted arrow points to the slower migrating band. (B). Amino acid sequence of Survivin-3γ. Novel amino acids identified in Sur-3γ are indicated in bold, with the stop codon indicated by a *. (C). Survivin-3γ was amplified from cDNA obtained from HL60 (lane 1), K562 (lane 2) and Mo7e-Bcr-abl (lane 3) cells using a forward primer specific for all splice variants and a putative splice variant specific reverse primer (shown as arrows in Figure 1D). (D). were designed Nucleotide and amino acid sequence of Survivin 3γ with the 4 base insertion shown as a projection in larger font size, leading to altered reading frame (shown at the bottom). Each exon is identified.

is structurally similar to Survivin-WT (Figure 2E), with the new amino acids predicted to form a C-terminal helix that could form the coiled-coil domain, albeit smaller than Survivin-WT. Secondary structure prediction for Survivin-3γV (Figure 2F) showed that the terminal helix would not form, suggesting that its localization/function may differ from Survivin-WT and Survivin-3γ, which is supported by PSORT II analysis [18].

Genomic analysis indicates that ~60% of human genes have alternative splice forms [19], resulting in isoforms of different length and domains that result in altered sub cellular localization, expression patterns and interaction affinity. Five Survivin variants having similar or opposing function have been identified (see [10,20]), with additional variants found in the *est* database (reviewed in [10]). The identification of Survivin splice variants has sparked interest in their clinico-pathological significance, and diagnostic, prognostic and therapeutic value. While not meant to be inclusive, numerous studies on variant expression and value have been reported. The -2α variant carrying a truncated BIR domain is apoptotic and can attenuate Survivin-WT activity [21]. It is highly expressed in malignant stages of breast cancer [22] and likely a marker for breast cancer diagnosis and prognosis [23]. It has been linked to shorter disease free progression and overall survival in some cancers and childhood acute myelogenous leukemia (AML) [24]. The significance of high expression of pro-apoptotic -2α but association with adverse outcome is unclear. Lower expression of Survivin-2α is associated with thyroid malignancy [25].

The –ΔEx3 variant with a modified C-terminus and pro-anti-

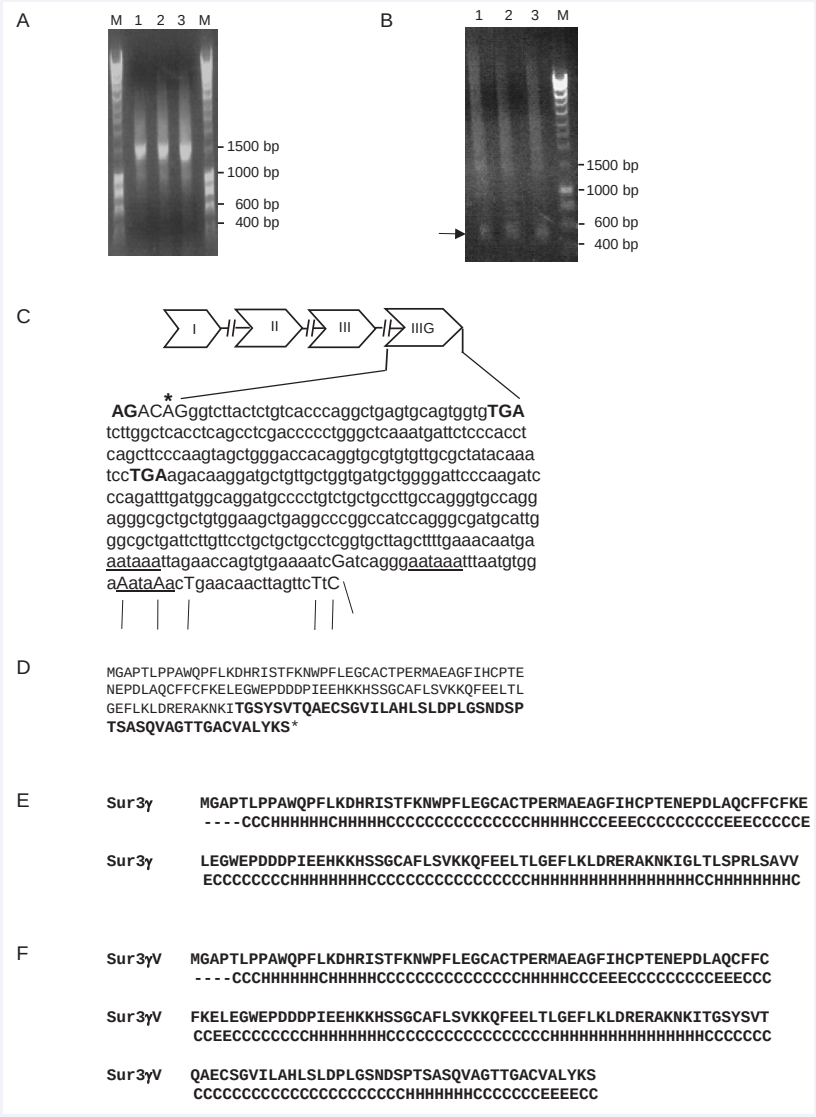

Figure 2 Identification of the 3' end of Survivin-3γ and Survivin-3γV: (A). 3' RACE was performed using the specific and anchor primers: Survivin-3γ–5'RACE-5'-GCACTCAGCCTGGGTGACAGAGTAAGACC-3', Survivin-3γ–3'RACE-5'-GGTCTT ACTCTGTCACCCAGGCTGAGTGC-3', Survivin-WT-5'RACE-5'-CAAGTCTGGCTC GTTCTCAGTGGGGC-3', Survivin-WT-3'RACE-5'-GCCCCACTGAGAACGAGC CAGACTTG-3'. Amplification products obtained from HL60 (lane 1), K562 (lane 2) and Mo7e-Bcr-abl (lane 3) are shown. (B). Amplification of Sur-3γ and Sur-3γV cDNA ends. Samples obtained from the reaction in (A) were used as templates and PCR performed with Sur-3γ and -3γV specific primer and anchor primers, HL60 (lane 1), K562 (lane 2) and Mo7e-Bcr-abl (lane 3) cells. (C). Exon organization of new Survivin variants. The exon structure of Sur-3γ and Sur-3γV is shown (not drawn to size) with introns as broken lines. Nucleotide sequence obtained from the longest 3'RACE clone is shown with the last base of the shorter clones indicated in upper case and by an arrow. The first TGA (shown in bold) is the stop codon for Survivin-3γ and the second TGA (shown in bold italics) is the stop codon for Sur-3γV. The first "AG" (shown in upper case bold) is the 3'ss of Sur-3γV, resulting in inclusion of "ACAG" in the sequence and the second "AG" (shown in upper case with a * on top of A) is the 3'splice site of Sur-3γ. The site of SNP in 3'ss is shown as *. The poly-adenylation signal sequence (aataaa) is underlined. (D). Amino acid sequence of Sur-3γV obtained after including the 4 base, "ACAG", in the splice junction of exon 3 and exon 3γ. (E). The amino acid sequence of Sur-3γ was used to predict its secondary structure, H=helix, E=β-sheet and C=unstructured region. (F). Predicted secondary structure of Survivin-3γV. H=helix, E=β-sheet and C=unstructured region.

apoptotic activity [26] has been most widely studied. It is a dependable diagnostic marker of advanced bladder, prostate and breast cancers and of tumor invasiveness/metastasis in bladder and colorectal cancer [20]. Survivin–ΔEx3 is highly expressed in thyroid carcinomas and a diagnostic marker of papillary thyroid carcinoma [27]. Survivin-2B having a truncated BIR domain has predicted attenuated anti-apoptotic function [28] and may undergo dimerization with -ΔEx3 to promote apoptosis, particularly in breast cancer where it has been associated with favorable prognosis [29]. However, silencing of -2B results in apoptotic induction [30] and expression of -2B with -Ex3 is believed to be causative for some high grade carcinomas [31,32]. In contrast, Survivins-2B and -ΔEx3 play no role in pituitary tumorigenesis [33]. Thus the role of these variants are not clear and nature and stage of disease are likely dependent variables. Survivin-3α is a truncated variant [34] not widely studied. It is expressed in most

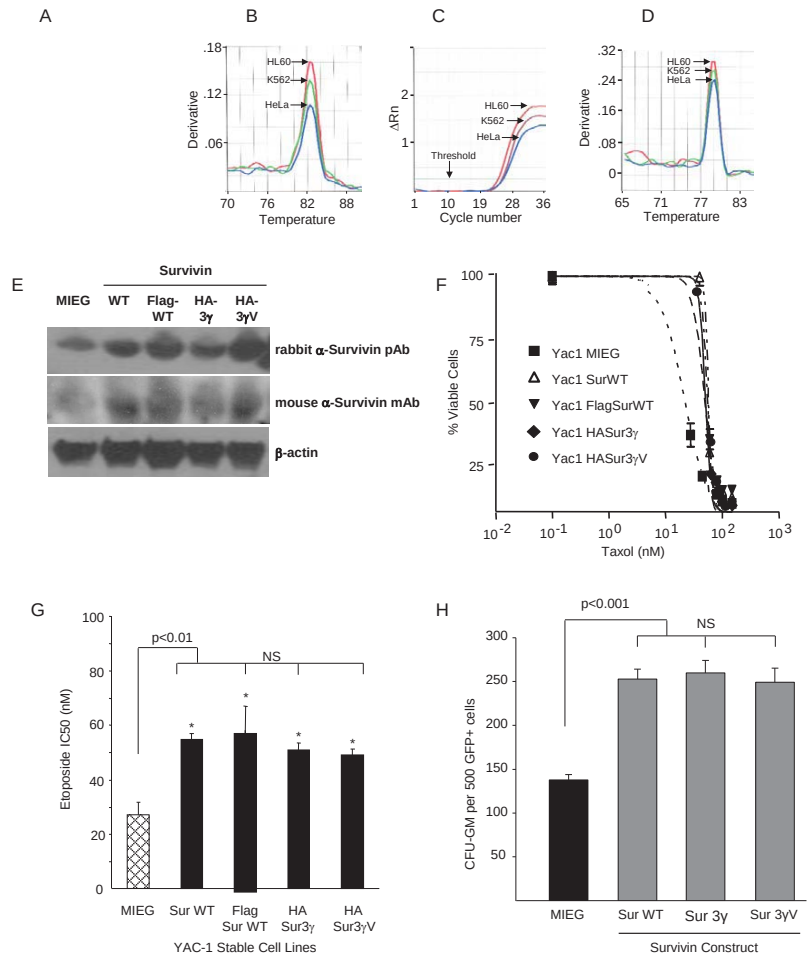

Figure 3 Specificity of the Survivin-WT and Survivin-3γ+3γV primers, expression of Survivin transcripts in Yac-1 cells and chemotherapy resistance: Amplification plots of Survivin-3γ+3γV (A) and Survivin-WT (C) from one well each of HL60, K562 and HeLa cells. Dissociation plot for Survivin-3γ+3γV (B) and Survivin-WT (D) generated from the same wells as in (A) and (C). Western blot analysis of stable Yac-1 cells expressing Survivin transcripts (E). SYBR green qRT-PCR validated expression of all constructs. Representative data from 1 of 3 experiments. HuSurvivin-WT was expressed at 31-fold (Range: 29-33), Flag-HuSurvivin-WT at 76-fold (Range: 71-82), HA-Survivin-3γ at 63-fold (Range: 59-68) and HA-Survivin-3γV at 37-fold (Range: 35-38) higher than MIEG-1 vector control cells at the RNA level. (F). Dose response curve of transduced Yac-1 stable cells treated with various doses of Taxol. Each point represents mean ± sem of quadruplicate cultures/point (G). Mean IC50 ± SD for each Yac-1 cell line stably transduced with Survivin constructs. Data from 3 separate experiments. * p<0.01, ANOVAwith Tukey's post hoc analysis. NS=not statistically significant. (H). Mean CFU-GM ± sem of FACS sorted human cord blood GFP-CD34+ cells transduced with MIEG vector alone or containing Survivin constructs. Quadruplicate plates per point; * p<0.001, ANOVAwith Tukey's post hoc analysis.NS=not statistically significant.

breast cancers, but also in benign tumors, but not in surrounding normal tissues [23]. Survivin -3B containing a complete BIR domain [35] is anti-apoptotic as expected, regulates cell cycle and augments chemoresistance [36]. Five splice variants are found in colorectal cancers with –ΔEx3and -3B possibly associated with tumor progression [37] and decreased -2B linked to tumor progression [38].

To begin to understand the expression pattern of Survivin-3γ+3γV we first evaluated non-hematopoietic cells. Design of primers detecting variants differing by 4 consecutive bases was not possible; therefore we designed primers that detected both variants. Amplification plots (Figures 3A,C) and dissociation curves (Figures 3B,D) confirmed primer specificity. Survivin-WT was the predominant transcript (Table 1A), with Survivin-3γ+3γV transcripts present at ~0.7-6%. In breast cancer samples, increased Survivin-WT expression at stages 1, 2 and 3 was seen (Table 2A), with little change related to advanced stage. Ct values for Survivin-WT and -3γ+3γV in stage 0 samples indicated that they were expressed at the same level as Survivin-WT (29.2 ± 0.7vs 29.3 ± 1.0, n=7). However, Survivin-3γ+3γV expression remained constant at all stages, resulting in lower relative expression to Survivin-WT in stages 1 and 2. Survivin-3γ+3γV was elevated in stage 3, however this was skewed by very high expression in 1/11 samples. In prostate cancers (Table 2B), Survivin-WT expression was higher in stage 1 than benign prostate hyperplasia (BPH), however sample size was small. No clear overexpression of Survivin-WT was seen at advanced stages. The expression of Survivin-3γ+3γV was higher than Survivin-WT in BPH tissue and expression of Survivin-3γ+γV transcripts remained higher than Survivin-WT at all stages except stage 1.

Table 1: A. Expression of Survivin-WT and Survivin 3γ + 3γV in cell lines.
B. Expression of Survivin-WT and Survivin 3γ + 3γV in primary hematopoietic cells.

Cells	Origin	Ct		
		Sur WT	Sur-3γ+3γV	Δ vs Sur WT [1]
HeLa	Cervical Carcinoma	24.5±0.3	31.6 ± 0.2	*.007*
HL60	APL	23.5± 0.1	29.12 ± 0.1	*.020*
K562	CML	25.2±0.2	31.11 ± 0.3	*.016*
Mo7e	Megakaryocytic leukemia	29.2±0.3	36.06 ± 0.2	*.014*
Molt4	ALL	28.4±0.3	32.41 ± 0.2	*.060*
Jurkat	T-cell leukemia	30.9±0.1	35.34 ± 0.3	*.045*
C1R	B-lymphoblastic leukemia	31.4±0.2	35.82 ± 02	*.016*

Cells	Origin	Ct		
		Sur WT[2]	Sur-3γ+3γV [2]	Δ vs Sur WT [1]
Fresh CD34$^+$	UCB	1.10 ± 0.17	1.04 ± 0.10 [ns]	26.74 ± 2.5
CD34$^+$ 24 hr GF	UCB	8.45 ± 0.43 [*]	0.18 ± 0.01 [*,†]	0.55 ± 0.33
CD34$^+$ 48 hr GF	UCB	9.54 ± 0.40 [*]	0.22 ± 0.01 [*,†]	0.59 ± 0.03

[1]Fold change in mRNA expression relative to Survivin-WT
[2]Fold change[X±SEM] of SurWT or Sur-3γ+3γV relative to fresh CD34$^+$calculated by the $2^{-\Delta\Delta Ct}$ method
[3]Fold change [X±SEM] of Sur-3γ+3γV compared to SurWT at each treatmentcalculated by the $2^{-\Delta\Delta Ct}$ method
* p<0.00005 vs.SurWT or Sur-3γ+3γV in fresh CD34$^+$ cells; † p<0.00005 vs. treated SurWT;
[NS] not significant. Analyzed using Students T-test

Table 2: A. Expression of Survivin-WT and Survivin 3γ + 3γV in primary breast cancer.

Breast Cancer Stage [N]	Sur WT [1]	Sur 3γ+3γV [1]	Δ Sur 3γ+3γV vs WT [2]
0	1.1 ± 0.21	1.08 ± 0.18	1.15 ± 0.20
[7]	(0.49-1.91)	(0.62-2.06)	(0.68-1.85)
I	3.27 ± 0.79[*]	1.17 ± 0.21 [†]	0.79 ± 0.36 [§]
[10]	(0.22-6.73)	(0.34-2.25)	(0.14-3.56)
II	2.81 ± 0.33[*]	1.14 ± 0.26 [†]	0.50 ± 0.10 [§]
[20]	(0.91-5.0)	(0.32-4.51)	(0.07-1.92)
III	3.42 ± 0.79[*]	3.11 ± 2.34[†]	1.61 ± 086
[11]	(0.16-8.23)	(0.41-24.13)	(0.06-7.06)

B. Expression of Survivin-WT and Survivin 3γ + 3γV in primary prostate cancer

Prostate Cancer Stage [N]	Sur WT [1]	Sur 3γ+3γV [1]	Δ Sur 3γ+3γV vs WT [2]
BPH	1.17 ± 0.21	1.14 ± 0.18	4.38 ± 1.15 [§]
[12]	(0.35-2.49)	(0.40-2.59)	(1.16-13.83)
I	2.78 ± 0.50	1.31 ± 0.36	1.49 ± 0.15
[2]	(2.29-3.28)	(0.95-1.68)	(1.34-1.65)
II	1.02 ± 0.12	1.66±0.64	6.17 ± 2.61[§]
[25]	(0.24-2.98)	(0.31-15.94)	(1.53-65.34)
III	1.64 ± 0.42	1.37 ± 0.24	3.48 ± 0.91 [§]
[8]	(0.42-4.01)	(0.32-2.56)	(1.44-8.28)

[1]Fold change (normalized to HPRT) were used to calculate fold change over stage 0 by the $2^{-\Delta\Delta Ct}$ method
[2]Fold change relative to Survivin-WT calculated by $2^{-\Delta\Delta Ct}$ method
All data are Mean ±SEM for the number of samples indicated
*p<0.05 vs. stage 0;[†]p<0.05 vs.Survivin-WT at each stage;
[§] p<0.005 vs. Sur-WT at the same stage of the disease, Analyzed by Students-T test

In consensus with most reports, we found that Survivin-WT was the major transcript expressed. The lack of change in Survivin-WT with prostate disease severity is consistent with one study [39] where Survivin expression was detected by immune histochemistry. However, other reports show increased Survivin with disease severity either at the RNA level [40,41] or by immune histochemistry [41]. This could be due to methodology; SYBR green qRT-PCR is more sensitive and efficient than standard PCR in amplifying a ~150bp amplicon. We also show that Survivin-WT and Survivin-3γ+3γV expression were comparable in normal breast tissues and expression of Survivin-WT increased at stage 1 and remained high at all stages. This contrasts with one report [42] that Survivin increased gradually with disease severity. This difference may also reflect different methodologies. Interestingly, expression of Survivin-3γ+3γV was constant with only a minor increase in stage 1 breast cancer.

Survivin-3γ+3γV expression in UCB $CD34^+$ cells enriched for HSPC (Table 1B) was >26-fold higher than Survivin-WT. Growth factor stimulation induced Survivin-WT expression, as we described [43-45]. In contrast, Survivin-3γ+3γV expression increased only slightly after 48 hours; however HPRT expression also increased (28.4 ± 0.2 to 24.4 ± 0.1, $p < 0.0002$), indicating that the relative levels of -3γ+3γV transcripts decreased ~2-fold compared to Survivin-WT. To our knowledge these are the first Survivin variants expressed higher than Survivin-WT in normal cells, suggesting they might play an important role in normal prostate tissue and HSPC. Analyses of Survivin function *in vitro* and *in vivo* clearly indicate that Survivin is required for normal hematopoietic function and maintenance [46,47].

It is interesting that the BIR domain of Survivin is disrupted in all variants excerpt -3B. Since theSurvivin-3γ and 3γV variants were identified while attempting to clone -3B, we expected that they would show anti-apoptotic function. To investigate function we created Yac-1 lymphoma cells stably expressing human Survivin-WT with/without an N-terminal Flag-tag or Survivin-3γ or Survivin-3γV, each with N-terminal HA-tags. Since crystal structure of Survivin-WT indicates that the C-terminal helix could be involved in protein-protein interaction, whereas the N-terminus was unordered [48], we engineered N-terminal tags into all Survivinc DNAs. Western blots using a rabbit anti-Survivin polyclonal antibody that recognizes human and mouse Survivin and a selective mouse anti-human Survivin monoclonal antibody showed a background amount of murine Survivin as well as the presence of each of the transduced human Survivins, indicating that each variant was translated (Figure 3E). Survivin-WT was expressed at the highest level followed by HA-3γV and Flag-WT with HA-3γ expressed at a lower level, despite being the highest expressed transcript. Drug resistance to Etoposide and Taxol were evaluated at concentrations and duration of incubation predetermined to be optimal. Overexpression of either Survivin-WT or Flag-Survivin-WT provided 2.74- and 2.81-fold resistance to Taxol-induced cell death (Figure 3F). Ectopic expression of HA-Survivin-3γ or -3γV also provided 2.59- and 2.37-fold resistance, respectively. In three experiments, the $IC_{50's}$ for Survivin-WT, Flag-Survivin-WT, HA-Survivin-3γ and HA-Survivin-3γV were significantly higher than control. Similarly, Yac-1 cells expressing Survivin-WT also showed resistance to Etoposide with an IC_{50} of 0.27 ± 0.06nM (Figure 4G). Flag-Survivin-WT, HA-Survivin-3γ and HA-Survivin-3γV all conferred similar resistance. Expression of murine Survivin-WT and the only known murine Survivin splice variant, Survivin-121 were unchanged (not shown), indicating that changes in endogenous murine Survivins were not involved in the increased drug resistance. Since Yac-1 cells over-expressing either Survivin-3γ or -3γV showed the same level of resistance as Survivin-WT to two different classes of cytotoxic drugs, Taxol, a microtubule destabilizer, and Etoposide, a topoisomerase-II inhibitor, it is likely that Survivin-WT, -3γ and -3γV proteins possess equivalent anti-apoptotic function. To further evaluate Survivin-WT, -3γ and -3γV function, we transduced primary cord blood CD34+ cells and measured CFU-GM proliferation in vitro. Transduction with all 3 constructs significantly enhanced proliferation of HSPC, indicating equivalent growth promoting activity (Figure 4H). We have not yet evaluated the effects of Survivin-3γ and -3γV proteins on cytokinesis, cell cycle or long term HSPC function. Furthermore, we have evaluated chemotherapy resistance in murine cell lines in the context of endogenous Survivin. Future analysis in conditional Survivin deletion models, as we described for Survivin-WT [49], will provide more definitive information.

CONCLUSION

We identified two Survivin splice variants with anti-apoptotic activity similar to Survivin-WT. Survivin-3γ and Survivin-3γV expression do not change with increasing severity of primary prostate and breast cancers. Unlike other Survivin splice variants, Survivin-3γ and -3γV are expressed at higher levels than Survivin-WT in primary HSPC, and their lack of increase with cell proliferation suggests that they may play a role in HSPC maintenance. Since these splice variants were cloned from and found to be highly expressed in hematopoietic tissue, it will be particularly interesting to evaluate their expression and role in hematopoietic malignancies, particularly maintenance of leukemic stem cells. In this regard, a recent report has also linked Survivin splice variant expression to pluripotency in human embryonic stem cells [50].

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