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Assessment of mRNA Splice Variants by qRT-PCR

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Abstract

Alternative splicing is an essential process for the generation of protein diversity. The physiological role, cellular localization, and abundance of splice variant products compared to the wild-type protein may be completely different. This is illustrated by the five splice variants of the antiapoptotic protein survivin that are more abundant in cancerous cells compared with normal tissues. Interestingly, some survivin splice variants have been associated with drug resistance. Herein, we describe a SYBR green I-based real-time PCR method to assess the messenger RNA levels of the human survivin splice variants in taxane-sensitive versus taxane-resistant ovarian cancer cells and in human ovarian cancer samples. Furthermore, in this chapter, we describe the quantification of survivin splice variants by real-time quantitative PCR (qPCR) after *in vitro* and *in vivo* small interference RNA (siRNA)-mediated silencing of survivin splice variants.

Keywords

SYBR green I-based PCR; Small interference RNA; Real-time PCR; Splice variants; Drug resistance; Ovarian cancer; Survivin

1 Introduction

Survivin, a protein highly expressed in human cancers, is associated with chemotherapy resistance [1–5]. Survivin plays a dual intracellular role as an antiapoptotic protein and as a regulator of mitosis [1, 6]. Alternative splicing of the human survivin gene (*BIRC5*) generates five splice variants, including wild-type (WT) survivin (142 aa), survivin 2α (74 aa), survivin 2B (165 aa), survivin Ex3 (137 aa), and survivin 3B (120 aa) (*see Fig. 1*) [7–10]. The expression levels and the subcellular localization patterns of each survivin isoform have been shown to be associated with their functional properties [1, 7, 11, 12]. For example, it has been reported that, compared with their taxane-sensitive counterparts, taxane-resistant ovarian cancer cells express higher survivin 2B messenger RNA (mRNA) levels [13]. *In vitro* and *in vivo* small interference RNA (siRNA)-mediated silencing of survivin 2B induced similar effects as siRNA-mediated targeting of all of survivin splice variants [13]. The antitumor effect of the survivin-directed siRNAs was further enhanced in combination with chemotherapy [13]. A significant association was observed between survivin 2B expression and progression-free survival in epithelial ovarian cancers [13]. Currently, several therapies targeting survivin are being investigated, some of them in clinical trials [14]. However, studies proposing survivin as a prognostic or diagnostic marker

or a therapeutic target should consider the differential expression levels of individual survivin splice variants, not only in ovarian cancer but also in many other cancers.

Real-time polymerase chain reaction (PCR) has the capacity to detect the amount of PCR product at every cycle by fluorescence [15]. Previously, we used real-time PCR to identify survivin splice variants after siRNA-based silencing experiments [13]. Here, we describe a real-time PCR-based method to assess the in vitro and in vivo levels of survivin splice variants in ovarian cancer cells and human ovarian cancer tissues.

Two common methods used to perform real-time PCR experiments are Taqman assays and SYBR-I-based PCR [15]. While the Taqman probes use a dual specific labeled probe, in addition to the pair of primers per gene, the SYBR-I-based PCR rely on the intercalation of the SYBR-I dye into the double-strand DNA produced during the PCR reaction [15]. Although Taqman probes are more specific than SYBR PCR, the ability to view melt curves with the SYBR method is an added advantage to determine the specificity of amplification [15]. In this study, we used SYBR-based PCR to assess the alternative splicing variability of gene transcripts because it is also relatively simple and less expensive compared to labeled Taqman probes [15].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. When RNase-free conditions are necessary, all buffers must be prepared with RNase-free reagents and DEPC treated water.

2.1 Cell Culture

The specific taxane-sensitive and taxane-resistant ovarian cancer cell lines (SKOV3ip1, SKOV3.TR, HEYA8, and HEYA8.MDR) have been described previously [16, 17] and are not commercially available. However, other ovarian cancer cells available in the American Type Culture Collection (ATCC) or in the European Collection of Cell Cultures (ECACC) can be used, including A2780, A2780CIS, SKOV3, OV-90, and NIH:OVCAR-3. Researchers can also make their own drug-resistant ovarian cancer cells.

1. Roswell Park Memorial Institute-1640 (RPMI) supplemented with 10 % Fetal Bovine Serum (FBS). Store at 4 °C.
2. Phosphate-Buffered Saline buffer (PBS).
3. Trypsin solution (0.25 %) and ethylenediaminetetraacetic acid (EDTA, 1 mM).
4. Cell culture materials (15 and 50 ml conical tubes, 1, 5, 10, and 25 ml pipettes, culture flasks, or plates).

2.2 RNA Extraction

1. RNaseZAP.
2. TRIzol reagent.

3. Dounce homogenizer.
4. Isopropanol.
5. Chloroform.
6. Ethanol 75 %.
7. 1.5 ml nuclease-free tubes.
8. RNase-free water.
9. Filter tips (0.5–10, 10–100, and 100–1,000 µl).

2.3 Complementary (c)DNA Synthesis (see Note 1)

1. 500 µg/ml oligo (dT)_{12–18}.
2. dNTP mix: 10 mM each dNTP at pH 7.
3. 5× First-strand buffer: 250 mM Tris–HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3.
4. 0.1 M Dithiothreitol (DTT).
5. SuperScript II.
6. Nuclease-free water.
7. Nuclease-free microcentrifuge tubes.

2.4 Real-Time PCR Amplification

1. 2× SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA).
2. 10 µM forward primers and 10 µM reverse primers.
3. 96-well real-time PCR plate.
4. 96-well plate adhesive plastic lid.

2.5 In Vitro siRNA-Based Silencing of Specific Survivin Splice Variants

1. Optimem + GlutaMax 1×.
2. Negative control siRNA, total survivin, and survivin 2B siRNAs (*see* Note 2).
3. HiPerFect transfection reagent (Qiagen, Valencia, CA) (*see* Note 3).
4. Six-well plates.

¹-To synthesize cDNA, we used reagents purchased from Life Technologies (former Invitrogen, Grand Island, NY). However, any other well-optimized cDNA synthesis kit can be used. All reagents must be stored at –20 °C.

²-To simultaneously silence all survivin splice variants, the siRNA should bind to any region encompassing the exon 1 and exon 2 (accession number, NM_001168). These exons are common to all survivin isoforms (*see* Fig. 1). The siRNA sequence used here was 5'-CAGACTTGCCCGAGTGTTT-3'. To target survivin 2B specifically (accession number, NM_001012271) (*see* Fig. 1), the following sequence was used: 5'-AATACCAGCACTTTGGGAG-3'.

³-All our procedures have been optimized with the use of HiPerFect transfection reagent. We recommended this reagent, because it has been shown to cause less toxicity in our cell lines.

2.6 In Vivo Silencing of Survivin Splice Variants with Liposomal-Incorporated siRNAs

1. Female athymic nude mice (NCr-nu, 8–12 weeks old; Taconic, Germantown, NY).
2. Syringes 30G.
3. PBS.
4. Hank's Balanced Salt Solution (HBSS) serum free (with Ca^{2+} and Mg^{2+}).
5. 1,2-Dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC)
6. *t*-Butyl alcohol.
7. Tween 20.

2.7 Equipment and Software

1. Laminar flow bench and cell culture incubator.
2. Mini plate spinner MPS 1000 (Labnet, Woodbridge, NJ) or A-2-MTP rotor 5430/5430R centrifuge (Eppendorf, Hauppauge, NY).
3. Centrifuge 5430 R (Eppendorf) or any equivalent centrifuge with capability to reach $12,000 \times g$ at 4 °C.
4. NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) or an equivalent spectrophotometer with high accuracy and reproducibility.
5. Heating blocks.
6. StepOne plus real-time PCR thermal cycle system (Applied Biosystems) or any equivalent real-time PCR instrument (please read carefully the instrument parameters and settings before starting).
7. StepOne software v2.1 (each PCR instrument includes its own software).
8. Acetone/dry ice bath.

3 Methods

3.1 Cell Culture Conditions

Maintain ovarian cancer cell lines by culturing in RPMI-1640 medium supplemented with 10 % FBS in 5 % CO_2 /95 % air at 37 °C. Perform all in vitro assays at 70–80 % cell density. Procedure for cell culture and passaging:

1. Wash the cell monolayers with PBS without Ca^{2+} or Mg^{2+} .
2. Pipette trypsin solution onto the washed cell monolayer using 1 ml per 25 cm^2 of surface area. Place flask in the incubator (37 °C) for 5–10 min.
3. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.

4. Resuspend the cells in equal volume of serum-containing RPMI to inactivate the trypsin, and centrifuge at 1,000 rpm for 5 min.
5. Discard the supernatant and resuspend cells in the appropriate media for cell passage or for siRNA transfection.

3.2 RNA Extraction

Before starting, clean the working area and all the reagents and materials with RNaseZAP. Always wear gloves, avoid speech over open tubes, and use aerosol-barrier tips and fresh solutions (maintain all of these precautions for cDNA synthesis and real-time PCR procedures). Please read the protocol enclosed with the TRIzol reagent for full details.

1. For RNA extraction from tissue, remove the tissue samples from -80°C freezer and thaw slowly on ice. Take approximately 35 mg of tumor tissue (mouse or human tumor samples). For RNA extraction from cell growth cultures, wash cells (approximately 1×10^7 cells) with chilled PBS twice.
2. Add 1 ml of TRIzol and homogenize. For homogenizing tumor tissue, use a dounce homogenizer (20 strokes).
3. Place the sample in a 1.5 ml microcentrifuge tube and incubate for 5 min at room temperature.
4. Add 200 μl of chloroform and shake for 15 s. Incubate for 3 min at room temperature.
5. Centrifuge the sample at $12,000 \times g$ for 15 min at 4°C .
6. Transfer the upper aqueous phase (containing the RNA) into a new 1.5 ml microcentrifuge tube.
7. Add 500 μl of isopropanol and incubate for 10 min at room temperature. This step allows precipitation of the RNA.
8. Centrifuge the samples at $12,000 \times g$ for 15 min at 4°C . Carefully, observe a gel-like pellet in the bottom of the tube.
9. Remove the supernatant, wash the pellet with 1 ml of 75 % ethanol, and mix the sample by vortex.
10. Centrifuge the sample at $7,500 \times g$ for 5 min at 4°C .
11. Remove the supernatant and air-dry the RNA pellet for 10 min.
12. Dissolve the RNA pellet using RNase-free water; incubate for 10 min at 60°C .
13. Determine the quality and concentration of the RNA with the NanoDrop. A 260/280 ratio of 1.8:2.1 is necessary for cDNA synthesis (*see* Note 4).

⁴.There are other methods to measure the RNA quality: (a) separating an aliquot of the sample on a denaturing polyacrylamide gel, staining with ethidium bromide, and visualizing the RNA in an UV transilluminator; (b) with the use of an Agilent 2100 bioanalyzer.

3.3 cDNA Synthesis

Before starting, read carefully the protocol enclosed with the SuperScript II reverse transcriptase. The main points are listed here (*see* Note 1):

1. In a nuclease-free microcentrifuge tube, combine 1 µl of oligo (dT)_{12–18}, 1.0 µg of total RNA, 1 µl of 10 mM dNTPs, and nuclease-free water up to 13 µl of total volume.
2. Heat the reaction to 65 °C for 5 min; briefly centrifuge the tube, and quickly place the tube on ice.
3. Add 4 µl of 5× First-strand buffer and 2 µl of 0.1M DTT, mix, and incubate for 2 min.
4. Add 1 µl (200 units) of SuperScript II RT and mix gently by pipetting. The final total volume in the tube will be 20 µl.
5. Incubate to 40 °C for 2 h.
6. Stop the reaction by heating the sample to 70 °C for 15 min (*see* Note 5).

3.4 Real-Time PCR Primer Design

Primer design is the most important part of qPCR analysis [15]. As SYBR green I dye assay will detect all double-stranded DNA, including nonspecific reaction products, a well-designed primer is essential for accurate quantitative results. An ideal pair of primers for SYBR-I-based qPCR should meet some important criteria including the amplicon length (60–200 bp), primer length (19–23 bp), GC content (35–65 %), melting temperature (50–68 °C), complementarities (avoiding primer self- or cross-annealing stretches greater than 4 bp), specificity (BLAST primer sequence against entire mRNA database), single-nucleotide polymorphisms (SNP) (primer sequences should not include known SNPs), and 3'-end stability (runs of three or more of C's or G's at the 3' end of the primer should be avoided). Herein, to amplify all survivin splice variants at the same time, we designed primers in the region containing exons 1 and 2 that are commons to all survivin isoforms (*see* Fig. 1). To amplify survivin WT, survivin 3B, and survivin EX3, we used a common forward primer described by Knauer et al. [10]. The reverse primer for survivin WT was designed in the exon 3 and 4 junction, and for survivin EX3, in the 3'-UTR region (*see* Fig. 1). The reverse primer for survivin 2B and the primers for survivin 3B were described by Knauer et al. [10]. The primers to amplify survivin 2α were from Caldas et al. [8]. Primers were designed with the Internet-free Primer3 v.0.4.0 software at <http://fokker.wi.mit.edu/primer3/> [18] with the following parameters (*see* Note 6):

- Product size range 100–200
- Primer size minimum 19, optimum 20, maximum 21

⁵The cDNA can be stored at –20 °C (1–2 weeks).

⁶Other Internet tools can be used to design primers including the primer designing tool-NCBI (<http://www.idtdna.com/Primerquest/Home/Index>), the IDT PrimerQuest (<http://www.idtdna.com/Scitools/Applications/Primerquest/a>), and the primer3web version 4.0 (<http://frodo.wi.mit.edu/>) tool that allow to visualize primers positions in the mRNA sequence and select primers for certain splice variants.

- Primer T_m minimum 59, optimum 60, maximum 61
- Other parameters as default values

Table 1 includes the accession number (which identifies each gene in The National Center for Biotechnology Information, NCBI) and the oligonucleotide sequences to specifically amplify each survivin splice variant. In addition, a pair of primers for an internal standard or control gene should be used [15]. Here, β -actin is used as the internal standard (*see* Table 1 and Note 7). For all primers a BLAST with the primer designing tool-NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was performed to ensure that each pair of primers amplifies only one survivin splice variant.

3.5 Real-Time PCR Amplification

We describe here the use of SYBR-I PCR master mix. As we mentioned above, SYBR-I is a dye which binds nonspecifically to double-stranded DNA [15]. Any unspecific DNA product in the PCR reaction (including primer-dimers) contributes to increases in fluorescence, which is detected by the real-time PCR instruments. Thus, before setting up the real-time PCR reaction, it is necessary to optimize the annealing temperature and concentration for each pair of primers (*see* Note 8). Therefore, perform a real-time PCR reaction (in triplicate) followed by a melt-curve analysis (*see* Note 9) following steps 4–9.

1. Set up the PCR machine program with the following thermal settings: 1 cycle of 15"/95 °C; 40 cycles of 15"/94 °C, 30"/X °C ($X = 60$ °C for total survivin, $X = 58$ °C for wildtype survivin, survivin 2B, and Ex3; 50 °C for survivin 2 α , 54 °C for survivin 3B; and 60 °C for β -actin), for 30"/72 °C. For melt-curve analysis, perform 1 cycle at 55–95 °C (in 0.5 °C increments) for 30".
2. Prepare a master mix by adding (per reaction) 12.5 μ l of 2 $\times$ SYBR green, 1 μ l of forward primer, 1 μ l of reverse primer, and 3.5 μ l of water. Adjust volumes according to the number of replicates and samples (including negative controls) (*see* Note 10).
3. In a 96-well real-time PCR plate, mix 18 μ l of master mix (step 2) and 2 μ l of cDNA (from Subheading 3.2, step 6) to obtain 20 μ l total volume. Once all reactions are completed, cover the plate with an adhesive plastic lid.
4. Centrifuge the plate in a mini plate spinner.

⁷The internal standard for PCR reactions should be amplified simultaneously with the gene of interest. In addition, the expression of the internal standard should not be altered by the experiment and should be amplified at the same level or more than the gene of interest. Finally, the amplification product of the internal standard and the gene of interest should be very similar in size.

⁸The optimal primer concentration is determined by independently varying forward and reverse primer concentrations. Generally, three different concentrations of each primer are tested, which generate a matrix of nine different PCR reactions of primer forward and primer reverse ratios (all in nM concentrations) as follows: 50/50, 50/200, 50/600, 200/50, 200/200, 200/600, 600/50, 600/200, and 600/600. For the first three PCR reactions, decrease 2 °C, and for the last three reactions, increase 2 °C in the annealing temperature. This strategy will identify the concentration and annealing temperature for each pair of primers.

⁹The melting temperature (T_m) of each product is defined as the temperature at which the corresponding maximum peak occurs. The melt-curve should display a single sharp peak in each reaction. The StepOne plus and other real-time PCR instruments include default protocols, which contain the melt-curve analysis at the end of the PCR cycles. Primer concentrations should also be optimized for real-time PCR experiments. The optimal primer concentration for each survivin splice variant is 200 nM (final concentration).

¹⁰Negative controls should include PCR reactions without cDNA (for detecting contamination or nonspecific amplification) and without enzyme (to determine that any amplification that occurs in the sample is derived from the synthesized cDNA and not genomic DNA).

5. Place the plate in the PCR machine and start the program. The software must be in active mode to collect the amplification and melt-curve.
6. With the Step One Software version 2.1, determine the best concentration and annealing temperature for each pair of primers, based in the threshold cycle (Ct) values (*see* Note 11) and melt-curve graphs. The melt-curve should display single sharp peaks (*see* Note 12).
7. Repeat steps 4–9 with the real samples.
8. Using the Step One Software version 2.1, or equivalent software, determine the threshold cycle corresponding to each sample and analyze the relative expression using the Ct method (*see* Note 13).
9. The relative expression of each survivin splice variant can be calculated mathematically or in the excel program with the Ct values. The following steps describe the use of the Ct method to calculate the relative abundance of total survivin in a panel of six ovarian cancer cell lines. Table 2 shows the results of one SYBR real-time PCR experiment to assess the survivin mRNA expression levels in a panel of six ovarian cancer cell lines.
10. Normalize the average Ct of the gene of interest (total survivin) to the internal control (β -actin) for each sample (*column 2 minus column 1, see* Table 2 and Note 14):

$$Ct = Ct(\text{Total survivin}) - Ct(\beta\text{actin})$$

11. Calculate the differences between the Ct of each sample and the Ct of the control sample (*column 3, Table 2*). This is Ct (*column 4, Table 2*):

$$Ct = Ct(\text{cell line}) - Ct(\text{SKOV3ip1})$$

12. Calculate the relative quantification (RQ) or fold change (*column 5, Table 2*) (*see* Fig. 2).

$$RQ = 2^{-Ct}$$

3.6 In Vitro siRNA-Based Silencing of Survivin Splice Variants

Here we used siRNAs to silence total survivin and survivin 2B. Specific siRNAs should be designed to silence other survivin splice variants (*see* Note 15):

1. Resuspend ovarian cancer cells in RPMI-1640 supplemented with 10 % FBS at 3×10^4 cells/ml.

¹¹The Ct values are defined as the number of cycles required for the fluorescent signal to exceed the background level. Therefore, the more cDNA is present in a sample, the lower the Ct values, as the threshold is reached earlier.

¹²The primer concentrations selected should provide the lowest Ct and highest fluorescence intensity values. Strong amplification of negative control reactions (generally, Ct higher than 35, i.e., 32) indicates that significant nonspecific amplification is occurring. Equally, more than one pick in the melt-curve analysis is indicative of PCR artifacts including primer dimers or unspecific PCR products. In general, these artifacts have lower melting temperatures than the target amplicon.

¹³The Ct method allows calculation of the relative abundance of each transcript [19]. Another method, commonly used to calculate the mRNA expression levels, is a standard plot of serial cDNA dilutions of each transcript versus the Ct values, followed by interpolating in the graph, the Ct values for each unknown sample [19].

¹⁴In real-time PCR, the control sample is known as the calibrator. This is the sample taken as reference, and its relative abundance is expressed as 1 or 100 %. Here, the calibrator was the SKOV3ip1 cell line.

2. Plate 2 ml of cells in six-well plates. Incubate plates to 37 °C for 24 h.
3. The siRNA/HiPerFect ratio should be optimized for each cell line. For HEYA8.MDR cells a 1:3 siRNA/HiPerFect ratio was used (*see* Note 16). Carefully read the protocol enclosed with the HiPerFect transfection reagent with the following indications:
4. Resuspend siRNAs, negative control, and each survivin splice variant siRNAs, at a stock concentration of 1 µg/µl with DNA-free water.
5. In a 1.5 ml tube, mix 3 µl each siRNA, 9 µl of HiPerFect, and 88 µl Optimem. Mix using vortex (30 s) and let stand 10–20 min at room temperature.
6. Take the six-well plate containing the cells (step 2), remove the RPMI medium, and add 1,900 µl of Optimem medium.
7. Add 100 µl of siRNA transfection mix (step 5) to the cells drop by drop. Mix by gentle rotation of the plate. Incubate the plate for 48 h in 5 % CO₂/95 % air at 37 °C.
8. Collect the cells and isolate total RNA as described in Subheading 3.2. Perform the cDNA synthesis and real-time PCR as described in Subheadings 3.3 and 3.4.
9. Calculate the changes in expression of each survivin splice variant using the relative value of control siRNA as 100 % of expression.

3.7 In Vivo Silencing of Survivin Splice Variants with siRNA-Loaded Liposomes

1. Use female immunocompromised athymic nude mice (NCR-nu, 8–12 weeks old) to establish ovarian cancer xenografts. Nude mice should be kept in barrier animal facilities maintained at a 12 h light/dark cycle. House the mice at 5 per cage with free access to food and water.
2. Trypsinize ovarian cancer cells (60–80 % confluence) and centrifuge at 1,000 rpm for 5 min at 4 °C.
3. Wash two times with PBS.
4. Dilute cells at a concentration of 5×10^5 – 1×10^6 cells/ml in HBSS (*see* Note 17). HEYA8.MDR cells were used at 1×10^6 cells/ml.
5. Inject 200 µl of cells per mouse into the peritoneal cavity.

¹⁵ Many pharmaceutical companies have their own tools to design siRNAs. Other Internet tools are also available: <http://sirna.wi.mit.edu/> or <https://www.genscript.com/ssl-bin/app/rnai>. However, these tools, in general, use the mRNA sequence of the most frequently occurring transcript. For example, the commercially available siRNAs for survivin (BIRC5) are directed to WT survivin (NCBI accession number NM_001168). For silencing specifically other splice variants, use their own NCBI accession numbers (i.e., for survivin 2B, NM_001012271). Then, by aligning the sequences of all splice variants, corroborate that the siRNA sequence is not complementary to the mRNA of any other splice variant. If the siRNA targets more than one splice variant, design the siRNA manually. Rules for selecting siRNA targets on mRNA sequences are available at http://www.rnaiweb.com/RNAi/siRNA_Design/.

¹⁶ Some cell lines are difficult to transfect with HiPerFect or any other transfection reagent. Electroporation is commonly used to transfect those cells.

¹⁷ The number of inoculated cells depends on the rate of cell growth and in the tumorigenic capacity of the cells.

6. After 3–4 weeks of the tumor implantation, proceed with the liposomal-siRNA injections. At these stage cells have formed tumors, which can be assessed by palpation of the peritoneal area (*see* Note 18).
7. Mix siRNA and 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) in the presence of excess *t*-butyl alcohol at a ratio 1:10 (w/w) siRNA/DOPC.
8. Add Tween 20 to the mixture at a ratio of 1:10 (v/v) (Tween 20:siRNA/DOPC).
9. Vortex the mixture and freeze in acetone/dry ice bath. Lyophilize the mixture.
10. Resuspend the siRNA-lipid mixture to a final siRNA concentration of 25 µg/ml in PBS without Ca²⁺ and Mg²⁺.
11. Inject 200 µl (5 µg siRNA) of liposomal siRNA into the peritoneal cavity of nude mice bearing tumors.
12. Two to three days after injection, sacrifice the animal, remove the tumor, and store at –80 °C for total RNA isolation. Perform RNA isolation, cDNA synthesis, and real-time PCR as described in Subheadings 3.2, 3.3 and 3.4.
13. Calculate changes in the expression of each survivin splice variant using the control group of mice (mice injected with liposomal-control siRNA) as 100 % relative mRNA expression levels (*see* Fig. 3).

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Fig. 1.

Splicing of the human survivin pre-mRNA produces five different splice variants. The *empty boxes* on the survivin WT cartoon indicate the approximate location of the siRNAs to inhibit all survivin splice variants (Total siRNA). The *black box* on survivin 2B cartoon indicates the location of the survivin 2B-targeted siRNAs. Reproduced from Vivas-Mejía et al. [13]

Fig. 2.

Relative survivin mRNA levels in a panel of ovarian cancer cells. RNA isolation, cDNA synthesis, and SYBR green I-based PCR were performed as described in Subheadings 3.2, 3.3, and 3.4, respectively. β -actin was used as the internal standard. Survivin expression levels were calculated with the C_t method. Total survivin mRNA values were expressed relative to the SKOV3ip1 cells. Columns represent the means of triplicates $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$. Reproduced from Vivas-Mejía et al. [13]

Fig. 3. Real-time PCR with total RNA isolated from tumor tissues. Mice were treated as described in Subheading 3.6. RNA isolation, cDNA synthesis, and SYBR green I-based PCR were performed as described in Subheadings 3.2, 3.3, and 3.4, respectively. Survivin expression levels were calculated with the C_t method and expressed relative to the control siRNA-treated mice. Columns represent the means of triplicates $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Reproduced from Vivas-Mejía et al. [13]

Table 1

Oligonucleotides (primers) used for SYBR-I-based real-time PCR experiments

Gene	Reference sequence	Forward primer	Reverse primer
Survivin WT	NM_001168	GACCACCGCATCTCTACATTC	TGCTTTTATGTTCTCTATGGG
Survivin 2B	NM_001012271	GACCACCGCATCTCTACATTC	AAGTGCTGGTATTACAGGCGT
Survivin Ex3	NM_001012270	GACCACCGCATCTCTACATTC	ATTGTTGGTTTCCTTTGCATG
Survivin 3B	AB154416.1	GAGGCTGGCTTCATCCACTG	GCTCTCTCAATTTTGTCTTG
Survivin 2α	AY927772	GCTTTGTTTGAAGTGAAGTTGTCAA	GCAATGAGGGTGGAAGCA
Total survivin	NM_001168	AGCCCTTTCTCAAGGACCAC	CAGCTCCTTGAAGCAGAAGAA
β-actin	NM_001101.3	ATAGCACAGCCTGGATAGCAACGTAC	CACCTTCTACAATGAGCTGCGTGTG

Table 2

Example of a SYBR real-time PCR experiment

	1	2	3	4	5
Cell line	Ct (β -actin)	Ct (T survivin)	Ct	Ct	RQ
SKOV3ip1	23.04	25.19	2.15	0	1
SKOV3.TR	22.95	24.78	1.83	-0.32	1.25
HEYA8	22.89	25.77	2.88	0.7	0.62
HEYA8.TR	21.79	23.99	2.2	0.05	0.97
A2780PAR	22.6	24.69	2.09	-0.06	1.04
A2780CP20	21.52	23.56	2.4	0.25	0.84