

Expression and Regulation of Survivin in Prostate Cancer

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Biology



Abstract

Survivin is an Inhibitor of Apoptosis Protein (IAP), which makes it responsible for cell proliferation and survival by inhibiting regulated cell death and promoting mitosis. The exact role that it plays in prostate cancer is not yet fully understood; expression and regulation of Survivin is the primary focus of my project. The way Survivin was studied was through fractionating various cancer cells lines (LaPC-4, PC-3 E+, VCAP, C4-2 and DU145-Spindle, Round and Tight) and isolating the nuclear and cytosolic components. These fractionated samples were then treated and analyzed using Western Blot analysis being probed for nuclear and cytoplasmic Survivin, respectively. The two main goals of my project were to see if there is a difference in expression between nuclear and cytosolic Survivin as shown in previous immunohistochemical staining and also to test if movement of the Androgen Receptor from the nucleus to the cytoplasm occurs when the cell components are treated with charcoal-stripped media. The found results were that there is in fact a difference between nuclear and cytoplasmic Survivin as indicated by band definition in Western Blot analysis for all lines used. There was also movement seen in the Androgen Receptor of two cells lines (LaPC-4 and C4-2) with charcoal-stripped media. Since little is known about Survivin other than the fact that it is most readily seen in cancerous cells in humans, the hope is that through my results we will be able to inhibit expression of the protein by understanding what functions the two forms have.

Introduction

Survivin is a protein that functions as a promoter of cell proliferation and survival in tumors and/or regularly proliferating cells. The protein counteracts apoptosis by endogenously inhibiting caspases and has been found in “almost virtually every human tumor that has been studied”¹. Survivin is not usually found in normal adult tissues² but it is seen in large concentrations in more aggressive cancers. Survivin is classified as an Inhibitor of Apoptosis Protein, also known as an IAP. Survivin is the smallest known IAP and is approximately 16.5kiloDaltons big⁸. Structurally, it has a single Baculovirus IAP Repeat (BIR) and an extended carboxy-terminal alpha-helical coiled-coil, but no RING-finger or other identifiable domain⁷. The main function of Survivin is to control mitotic progression⁵ and has maximal expression during the G2/M Phase; it is essential for cell division⁵. However, Survivin has rarely, if ever, been studied in prostate cancer.

An IAP is an Inhibitor of Apoptosis Protein and functions to inhibit regulated cell death. Structurally, IAPs consist of 1-3 copies of a 70-amino-acid zinc-finger fold, which is a designated BIR. Certain IAPs can also include a caspase-recruitment domain (CARD), and a carboxy-terminal RING finger⁶. There have been a total of eight members of the IAP gene family identified in humans; Survivin is one of them. The most well studied human IAPs include: XIAP, c-IAP1 and c-IAP2⁸. IAPs work by preventing the processing of initiator caspase-9 from the apoptosome, which is responsible for activating effector caspases and death substrates⁸.

There are three different identifiable forms of Survivin⁸. Survivin Wild-type: 3-intron-4-exon structure, Survivin-2B: Insertion of an alternative exon 2, and Survivin-ΔEx3: Removal of exon 3 (results in frameshift). As well as two different regulation mechanisms; cell cycle dependent and cell cycle independent, respectively. These forms are illustrated in Figure 2.

Cell cycle dependent regulation of Survivin functions at the transcriptional and post-transcriptional level to control Survivin expression and involves CDE/CHR G1 repressor elements in the Survivin promoter with increased protein stability by phosphorylation on Thr34, respectively⁸. Cell cycle independent regulation of Survivin functions in response to haematopoietic and vascular remodeling cytokines, STAT3-dependent signal transduction and phosphatidylinositol 3-kinase activity¹. The known pathways for cell-cycle dependence and cell-cycle independence are shown in Figure 3.

From previous immunohistochemical (IHC) staining performed by Dr. Knudsen at the Fred Hutchinson Cancer Research Center in Seattle, the two distinct functions of Survivin were readily seen in human prostates. However, no analyses of Survivin have been previously conducted using human prostate tissue and therefore the distinction between cytosolic Survivin (cell-cycle independent) and nuclear Survivin (cell-cycle dependent) must be made in order to confirm that two regulatory paths do exist. Analyzing expression of the primary antibodies used in the previous IHC experiment must do this.

Methods

The guiding questions behind this study were: Do the antibodies for nuclear and cytosolic Survivin used in immunohistochemistry bind to distinct forms of Survivin? As well as: Does treatment of cell lines with charcoal-stripped media cause the Androgen Receptor to move from the nucleus into the cytoplasm? These two questions are important to understanding the function of Survivin in prostate cancer because confirming that there are indeed two distinct functions of Survivin and where they are localized in aggressive cancers can help form treatment plans according to each individual. Also, confirming that Survivin shows movement during Androgen Ablation shows that Survivin, much like the Androgen Receptor will move from a method of proliferation in the nucleus to one of survival in the cytoplasm during commonly used prostate cancer treatments.

In order to test the first hypothesis, LaPC-4, PC-3 E+, VCAP, C4-2 and DU145-Spindle, Round, and Tight human cancer cell lines were used. All of the cells were plated from reserves kept in liquid nitrogen and ranging from 1980-present. After plating, the cells were kept in the 37°C incubator and fed every other day with passage done until there were six plates of each cell line and all plates were the same passage number and dilution. Four plates of each cell line were then taken when eighty percent confluent and a fractionation protocol was followed for harvesting, while the other two plates were kept as reserves in the incubator and continually fed and passed accordingly.

For fractionation, pre-labeled eppendorf tubes, pre-labeled ultracentrifuge tubes and buffers, reagents, rotor of the ultracentrifuge machine, etc. used during the experiment were taken into the cold room (4°C). Twenty-five milliliters of *Cell Lysis Buffer (Mannitol Lysis Buffer)* was made on the day of the experiment from the stock solution and placed on ice for thirty minutes before beginning. Three hundred micro liters per plate *Cell Lysis Buffer (Mannitol Lysis Buffer) w/inhibitors* was also prepared the day of the experiment in the cold room and placed on ice along with *Nuclei Resuspension Buffer* and *2X RIPA Buffer with protease and phosphatase inhibitors*.

The four 10-cm plates of cells were taken from the incubator and media was immediately removed by suction in the tissue-culture hood. The plates were immediately put on ice and taken to the cold room. In the cold room, the plates were washed once with five milliliters of ice-cold PBS/EDTA and subsequently poured off. The plates were then washed once with two milliliters of ice cold *Cell Lysis Buffer* and immediately poured off. The plates were placed on ice at a 30° angle to allow the residual liquid to drain before aspirating all the remaining liquid. Three hundred micro liters of *Cell Lysis Buffer (w/inhibitors)* was then added to each plate and laid flat on ice for thirty minutes.

After thirty minutes, the dishes were set at an angle once again on ice. Tilting the dish, the cells were scraped with a cell scraper vigorously for 3-4 times, collecting the lysate along the bottom edge of the plate with a P1000 pipette. Lysates were transferred to pre-chilled eppendorf tubes, pre-labeled as P0 (nuclear fraction) and spun at 3000 rpm for ten minutes in the centrifuge. On ice, the supernatant was then transferred to an empty eppendorf tube, labeled as P1 and the pellet was kept in P0 tubes. The P0 tubes then had 300 micro liters of *Nuclei Resuspension Buffer* and three micro liters of Dnase I added, combining all tubes. The pellets were Resuspended by vortexing until the pellet went into solution. Finally, 200 micro liters of *2X RIPA Buffer (w/inhibitors)* was added and a nuclear wet mount was prepared to check for appearance of nuclei and little to no debris.

The P1 eppendorf tubes were then spun at 14000 rpm for twenty minutes. On ice, the supernatant was transferred with a 1000 micro liter pipette tip from the P1 tube to a pre-chilled *ultracentrifuge tube*, labeled as P100. All supernatants were combined in one ultracentrifuge tube (Beckman Polycarbonate 13x51mm 3mL tubes). After completing isolation of the nuclear and cytosolic matter of each cell line, the tubes were stored in the -80°C freezer until needed.

After storing and isolating all cell lines, concentration of the protein in the nuclear and cytosolic components had to be determined. Doing a Bradford Assay using known dilutions of photosensitive liquid and dilutions of either cytosolic or nuclear components from the cell lines did this. Each cell line concentration was determined for both components and using a predetermined excel sheet, the necessary amounts of Western Blot reagents were added to the protein in either 10, 25, or 50 micro liter dilutions.

From this step, Western Blot analysis was then performed using a typical “Western Blot with Wet Transfer” protocol and developing using chemiluminescent reagent and radiographic film.

The Western Blots used to test for differences in cytoplasmic and nuclear Survivin used a mouse monoclonal and a rabbit polyclonal primary antibody, respectively. The gels used for both components were 12% Bis-Tris with 10 micro liters of ladder loaded and 15 micro liters of protein into each well. The Western Blot was run at 200mV for twenty minutes or until a one inch space remained at the bottom of the gel. Transfer was done by blocking the gel with a nitrocellulose membrane for 90 minutes at 300mA in the cold room. The membrane was then cut and marked to designate the ladder and each component was probed separately. The nuclear fraction was probed with milk overnight followed by a second milk-blocking step with secondary antibody in order to reduce background noise. The cytoplasmic fraction was probed with a milk overnight primary step followed by a 5% BSA blocking step with secondary antibody and was repeated until radiographic films appeared as clear to read as possible. These final Western analyses are shown in Figures 4 and 5.

The second hypothesis was tested by using a similar method as the above mentioned, however, only cell lines for LaPC-4 and PC-3 E⁺ were used. Each cell line was grown using a medium made from charcoal strip in order to remove any hormones. The plates were grown for six days and four plates each were obtained from the same passage and dilution number. Cells were then harvested using the same method as above for cell fractionation. Western Blot analysis for these cells was conducted using the AR 441 primary antibody and an anti-horse secondary antibody with milk blocking overnight and 5% BSA blocking on the secondary antibody. The Western Blot for this experiment is shown in Figure 6.

Results

From Western Blot Analysis, I was able to confirm that the antibodies used for nuclear and cytosolic Survivin are indeed distinct from each other. When probed for nuclear Survivin, only bands on nuclear fractions are visible and are located at approximately 16.5 kDa on the ladder. However, when probed with cytoplasmic Survivin, bands are located for nuclear and cytosolic fractions, but at different locations on the molecular weight ladder. It is believed that the lower band seen is one of the non-wild type forms of Survivin and the cytosolic fractions at approximately 16.5 kDa are the wild type. Further testing must be done in order to confirm these results and identify the second visible band in the cytosolic probe. However, a distinction between the two antibodies used is obvious.

For the Androgen Receptor, movement of Survivin from the nucleus to the cytoplasm is clearly seen for LaPC-4 cells that were treated with androgen ablation and bands appear at the known molecular weight. This is an important finding because it shows that Survivin, like the Androgen Receptor can be seen in adult human cancer cells as well as the fact that it has two distinct regulations. When androgen is readily available in the nucleus, Survivin is able to control mitotic progression in a cell and therefore proliferate. However, when androgen is taken away from the host and it moves to the cytoplasm, Survivin goes into a somewhat “dormant” state and moves into the cytoplasm as well with the main goal of just “surviving.” The Western Blot for the AR 441 is shown in Figure 6.

Discussion

The findings of this research are extremely important. Since Survivin is known to have a detrimental effect in patients with any aggressive cancer, it is important to understand the different regulations and forms that it has. Being able to confirm previous results that there are two distinct forms and that the antibodies used to probe for them can be used independently are a huge step in furthering our understanding of the protein. Since analysis of prostate cancer tissues is currently only able to happen after the patient has died from the disease, this allows us to be able to selectively study one form or the other as well as use immunohistochemistry to study which form led to a more serious or lethal case of prostate cancer. In doing this, there is the possibility that prostate cancer can be controlled better and studied before the demise of the patient.

Additionally, by studying the movement of the Androgen Receptor from the nucleus to the cytoplasm upon androgen ablation, helps to understand the processes and forms that Survivin might take as a result of common prostate cancer treatment therapies. If Survivin is moved from a proliferating stage in the nucleus when androgen is readily available and then into a “survival” mode when androgen ablation is done, it may appear that the patients’ cancer has gone into remission or been cured. This however, is not the case as every human prostate with cancer has been seen to have Survivin expression and more so in the nucleus of more aggressive cancer. If Survivin can be better regulated or its pathway in the nucleus blocked this can easily be prevented.

Overall, there is more work needing to be done on this research, as it is only the beginning of finding a possible treatment plan for prostate cancer patients. The above findings need to be confirmed further as well as more in-depth biochemical processes of Survivin need to be studied in order to understand all regulations and possible inhibitors of the protein.

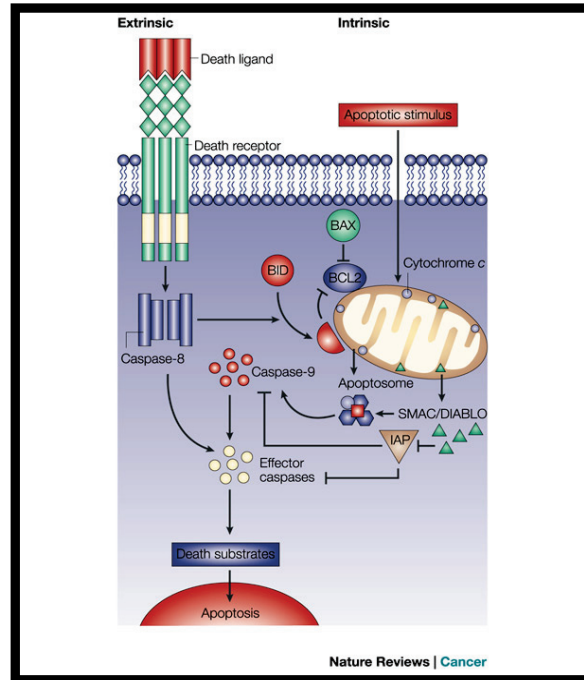


Figure 1. Schematic illustrating apoptotic activation of caspases from both extrinsic and intrinsic stimulus. Survivin is responsible for inhibiting the intrinsic stimulus and therefore silencing apoptosis.

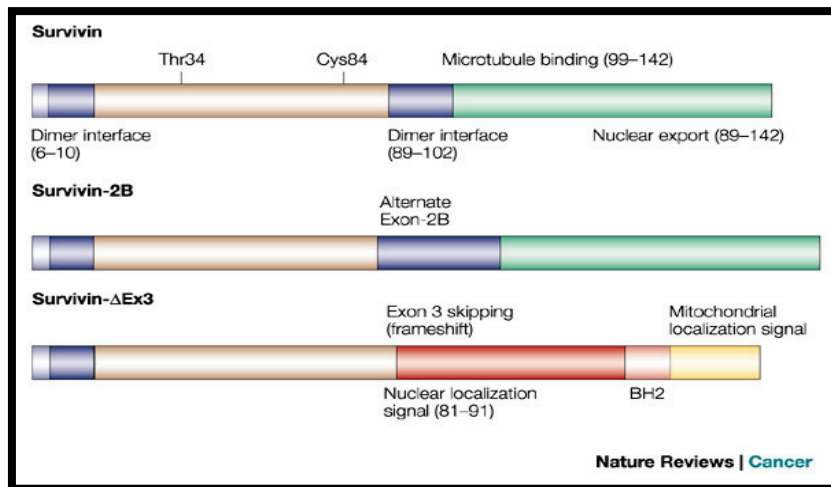


Figure 2. Representation of the three forms of Survivin with an alternate exon 2 and removal of exon 3 in the non-wild type forms. The removal of exon 3 results in a frameshift mutation of the protein.

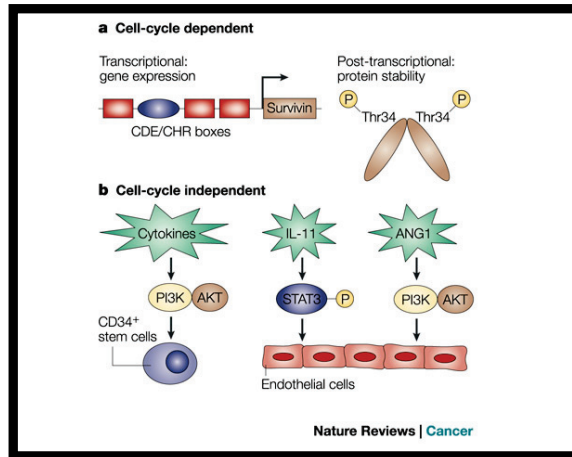


Figure 3. Schematic showing the two ways Survivin functions: either in a cell-cycle dependent manner, or in a cell-cycle independent manner, which leads to activating of cytokines.

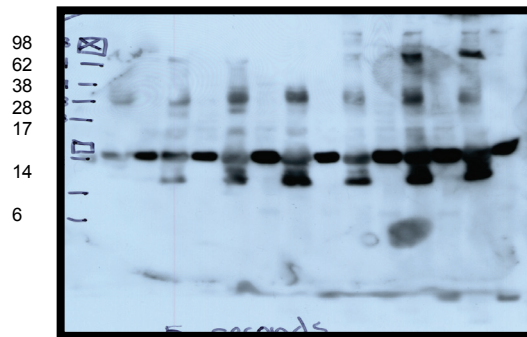


Figure 4. Western Blot probing for nuclear Survivin. Distinct bands are seen only in fractions of nuclear cells for all cell lines at the expected 16.5 kDa molecular weight. Order of loading: P0-S, C+M-S, P0-R, C+M-R, P0-T, C+M-T, P0 LaPC4, C+M LaPC4, P0 PC3, C+M PC3, P0 C4-2, C+M C42, P0-VCAP, C+M-VCAP.

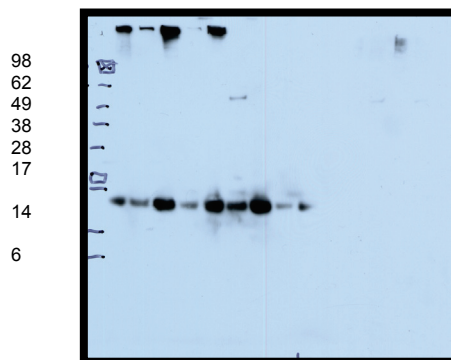


Figure 5. Western Blot probing for cytoplasmic Survivin. Distinct bands are seen for all fractions at the expected molecular weight with lower bands being seen below 16.5 kDa, believed to be a mutation in the Survivin protein. Order of loading: P0-S, C+M-S, P0-R, C+M-R, P0-T, C+M-T, P0 LaPC4, C+M LaPC4, P0 PC3, C+M PC3, P0 C4-2, C+M C42, P0-VCAP, C+M-VCAP.

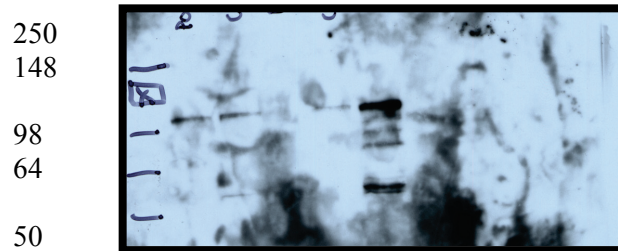


Figure 6. From left to right: Ladder indicating molecular weight in kDa, Nuclear fraction without androgen ablation, Cytosolic fraction without androgen ablation, Nuclear fraction with androgen ablation, Cytosolic fraction with androgen ablation, Positive LnCAP control. When androgen is available to cells, bands are seen in the nuclear and cytosolic fractions of LaPC-4 cells, however when androgen has been stripped from the cells, only a band is seen in the cytoplasm. The large band seen to the far right is a control for the AR 441 primary antibody.

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