RESEARCH COMMUNICATION

Survivin Deficiency Leads to Imparalization of Cytokinesis in Cancer Cells

Raju Vivek[1], Soundarapandian Kannan[1]*, Shanmugam Achiraman[2], Ramasamy Thirumurugan[3], Devaraj Sankar Ganesh[2], Muthukalingan Krishnan[2]

Abstract

Background: Survivin has been implicated in cancer progression and is known to be over-expressed in a variety of human malignancies. Positive regulation of survivin expression provides a connecting link between cell cycle and tumorigenesis or perhaps tumour maintenance. Methods: An experiment was designed to analyse survivin expression in cell lines (MCF 7, Zr751, A549, HepG2) using SDS-PAGE, Western blots, RT-PCR, AGE and heamatoxylin-eosin staining were done. Results: SDS-PAGE revealed the presence of 16.5 kDa protein. Subsequent western Blot and cytological analysis showed down-regulation of survivin expression in cancer cells. Conclusion: Therefore, the study allows the conclusion that survivin is essential for proper chromosome segregation and cytokinesis. It seems reasonable to suspect that abnormal expression or function of survivin might contribute to multinucleated and apoptotic conditions.

Keywords: Survivin - anti-apoptotic protein - apoptosis - cancer

Asian Pacific J Cancer Prev, **12**, 1675-1679

Introduction

Survivin is an acidic, 16.5 KDa protein with 142 amino acid residues. It is composed of a single Baculovirus IAP repeat (BIR), evolutionary conserved domain at the N-terminal region and an extended C-terminal α- helix turn helix domain; Further the occurrence of ring finger domain is a unique feature of other IAPs also reported in survivin (Ambrosini et al., 1997). Survivin was originally described as an anti-apoptotic protein (IAP) overexpressed in a variety of human cancers and hence designated as a cancer-specific protein. During fetal development and rarely expressed in normal healthy adult tissues; however, upregulated in majority of cancers (Wimmershioff et al., 2010).

Survivin perfectly regulates the cell cycle, particularly at G2/M phase in dividing cells. Cytologically survivin is incorporated into centrosome and mitotic spindles and finally relocated to midbodies during late telophase (Susanne et al., 2006). Survivin-deficient cells initiate cell division process but failed to complete cytokinesis. Apparently, the absence of spindle midzone and midbody microtubuloes during late mitosis impose defective cytokinesis (Dun et al., 2004). Gassmann et al. (2004) noticed the survivin in nuclear as well as cytoplasmic compartments, such as, the mitotic apparatus, centrosomes, kinetochores, mitotic spindle microtubules, spindle poles, central spindle midzone and midbodies, cytosol, mitochondria and nuclei (Colnaghi et al., 2006). In several studies, it is clearly emphasizing that the survivin is reported to have a regulatory effect on cell division. Yeast cells with IAP gene knockouts did not show any symptoms for cell death, however, it showed defects in mitosis characterized by improper chromosome segregation or imparalized cytokinesis (Chan, 2000).

The chromosomal passenger complex (CPC) is a key regulator of Chromosome segregation and cytokinesis. The CPC was first localized in centromeres and later associated with the central spindle and midbody (Earnshaw 2005).. Survivin appears to be a component of the chromosomal passenger protein complex that participates in multiple facets of cell division (Whealey et al., 2001). Targeting survivin results in improper mitotic cell division (Sha et al. 2006). Hence, an investigation was carried out to find out the possible reason for improper mitotic cell division and formation of multinucleated cells.

Materials and Methods

Cell Cultures and Reagents

The cancer cell lines namely, MCF7, ZR751, A549, HepG2 and normal HBL100 cell line were procured from National Center for Cell Science (NCCS, Pune) and revived in 20% Rosewell Park Memorial Institute medium (RPMI) containing 10% foetal bovine serum. The culture medium was reconstituted with 2 mM L-glutamine, 1 mM

[1]Proteomics and Molecular Cell Physiology Lab, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore, [2]Department of Environmental Biotechnology, [3]Department of Animal Science, Bharathidasan University, Tiruchirappalli, TN, India
**For correspondence: *sk_protein@buc.edu.in*

Sodium Pyruvate and 1mM pencillin and streptomycin. The cultures were maintained in t-25mm flask with the growth condition at 37˚C and 5 % CO_2 in a air jacketed CO2 incubator. After the cells attained 70-80% confluency subjected to trypsinization. Then the cells were seeded into 96mm petriplates (Tarsons) containing fresh medium for further experimentation. The medium was changed to cells a day after revival of cells. RPMI medium containing 10% FBS was used after sub-culturing. When the cells attained above 80% confluency, the medium was gently removed. Cells were then washed twice with PBS EDTA (pH-7.2). Fresh medium containing 10% FBS was added and resuspended gently using a Pasteur pipette. Cells were counted using haemocytometer. A fixed number of cells were seeded into polylysine coated plastic petriplates and maintained for further studies.

Cell Fractionation, SDS-PAGE and Western Blotting

Whole-cell extracts were prepared by scraping cells from Petri dishes, followed by washing cell pellets twice in PBS, and then resuspending cell pellets in two-packed cell volumes of RIPA buffer [150 mM NaCl/ 50mM Tris-HCl, pH 7.5/0.25% (wt/vol) deoxycholate/1% Nonidet P-40/ 5mM sodium orthovanadate-2mMsodium fluoride-protease inhibitor mixture]. Nucleus and cytoplasmic components were isolated after washing cell pellets in buffer A (10 mM Hepes, pH 7.5/ 10mM KCl/ 1.5mM Mg2Cl/ 0.5mM NaF-1mM glycerol phosphate-protease mixture), then lysis in buffer A / B (buffer A plus 0.5% Nonidet P-40) in a 2:1 ratio. After centrifugation (12,000 rpm for 10 min), supernatant was collected (cytoplasmic fraction). The Pellets were washed in ice cold PBS (pH-7.2) again, centrifuged (12,000 rpm for 10 min) at 4 ºC, and then flash-frozen in dry-ice–ethanol in buffer C (20 mM HEPES, pH, 7.5/ 420mM NaCl/ 1.5mM MgCl2/ 0.5mM NaF/ 0.5mM DTT/ 1mM glycerol phosphate-protease mixture), followed by a slow thaw on ice. The supernatant was centrifuged (12,000 rpm) and collected (nuclear fraction). Protein concentrations were determined by following a modified Bradford method. Equal amounts of proteins (50 μg) were resolved by 12% SDS-PAGE under reducing conditions. Proteins were then transferred to nitrocellulose membrane. The protein transfer efficiency and equal loading of proteins were checked by Ponceau S staining of the nitrocellulose membrane. Membranes were blocked for 1 h in TBS (25mM Tris/HCl, pH7.4/ 150mM NaCl/ 2.7mM KCl) containing 4% (wt/vol) low-fat milk or 3% BSA (wt/vol). Membranes were then probed with specific Anti-human survivin antibody recognizing target proteins and proteins were visualized with the Super Signal West Femto maximum sensitivity substrate kit (Pierce).

RT-PCR Detection of Survivin mRNA Expression

The expression of survivin mRNA was analyzed by semi-quantitative RT-PCR. The correlation between band intensity and dose of cDNA template was linear under the conditions described below. Total RNA was extracted from those cells using TRIzol reagent (Invitrogen) and RNA of 2 μl (1 μg/ μl) was used to synthesize cDNA using Superscript First-Strand Synthesis Kit (Strategene, India) following the manufacturer's protocols. The cDNA was used to amplify the survivin mRNA fragment, while the house keeping gene b-actin was also amplified as an internal standard. The corresponding primer sequences were as follows: survivin, forward: 5'-TTCTCAAGGACCACCGCATC-3', reverse: 5'-AGAGGCCTCAATCCATGG-3'. β-actin, forward: 5'-AGCAACCGGGAGCTGGTGG-3', reverse: 5' CATTTCCGACTGAAGAGT-G-3'. The cycling program was performed as follows: 1 cycle of 94 ºC for 3 min; 35 cycles of 94 ºC for 40 s, 52 ºC for 40 s, and 72 ºC for 90 s; followed by a final elongation step at 72 ºC for 10 min. Then RT-PCR products were electrophoresed through 1.5% agarose gel with ethidium bromide. Signals were quantified by densitometric analysis.

Agarose Gel Electrophoresis

The survivin amplicon gene DNA was examined in 0.7% Agarose gel. 5 μl isolated DNA solutions were run on 0.9% agarose at 90V for 45 min, subsequently stained with ethidium bromide, and documented by using a UVP Bio-imaging System. An LM-20E Ultraviolet Bench top Transilluminator was used in conjunction with a Nikon Digital Camera with a UV filter and lens. Documentation was completed by using the DOC-IT system software.

HE Staining

To visualize number 1 nuclei, control and cancer cells were fiexed, embedded in paraffin wax and sectioned (3-5μm) and finally stained with haematoxylin and eosin for 3 minutes before observing under light microscope at 40X magnification.

Results

The present investigation revealed that anti-apoptotic function of survivin become useful for anti-cancer treatment. Since, survivin detected mainly in cancer cells. The present study clearly evaluated and compared the prognostic value of survivin in cancer cells and standardization of immunohistochemical assays. These results showed down-regulation of survivin expression in cancer cells and produced a phenotype of aberrant mitotic progression, failure of cytokinesis, generation of multinucleated and apoptotic cells. Therefore, nuclear expression of survivin is essential for proper chromosome segregation and cytokinesis, because it act as a CPP, it has an important role in both the segregation of sister chromatid and the assembly or stabilization of microtubules in mitosis. Finally the present study clearly demonstrated survivin is essential for tumour cell proliferation as well as survival because of its regulation of cell cycle function. Hence, survivin is target for development of cancer therapy.

Agarose gel electrophoresis was performed to indicate the isolated survivin DNA 15kb bands were determined in the cancer cell line MCF7, A549, ZR751 and HepG2 (Figure 1). There was no band in the normal cell line HBL100 because the survivin expression in the normal cells was undetectable. The expression of survivin mRNA done by RT-PCR showed a high level in all the four cancer

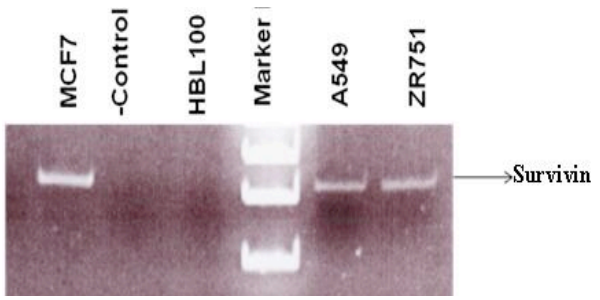

Figure 1. AGE for Isolated Survivin DNA. A 426 bp was obtained in the cancer cell lines (MCF7, A549 and ZR751) and no bands in the normal HBL100 cell line

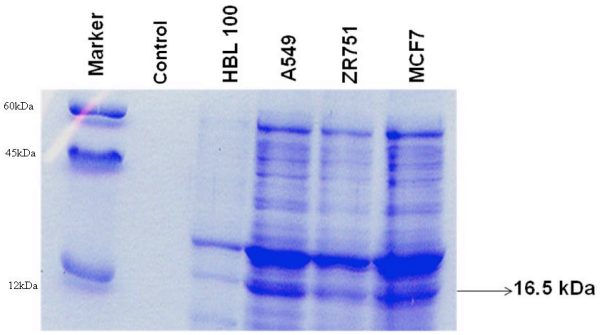

Figure 2. Survivin 16.5 kDa Protein Identified by 12% SDS-PAGE. Bands were obtained in the cancer cell lines and no bands in the normal HBL100 cell lines

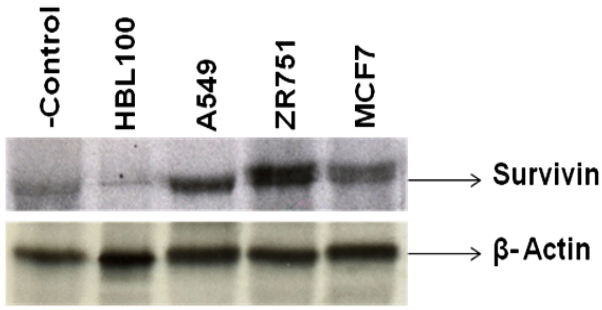

Figure 3. Western Blot of Anti-apoptotic Protein Survivin Expression in Cancer Cell Lines and β-actin Used as an Internal Control

cell lines (MCF-7, A549, HepG2 and ZR751). Moreover the mRNA of survivin was expressed depending on cancer cell lines and higher expression was noted in HepG2 cells.

Survivin was over expressed in all the selected cancer cell lines and it was found to be absent in normal and non-malignant cells. Further survivin overexpression in most of the cancer cells contributes to change in the phenotype of the cancer cells and found to be resistant to apoptotic stimuli as well as inhibition of caspases activity. The cancer cell lines MCF7- Breast cancer, ZR751-Adeno carcinoma, A549-Lung carcinoma and HepG2-Liver carcinoma cells contained a 16.5 kDa protein. This protein band was absent in control HBL100 normal cell line (Figure 2). It has been suggested that survivin might be expressed by all respective cancer cell lines and it was clear that the normal cells are efficient to synthesis survivin, hence this band was absent. Western blot was performed and reconfirmed the survivin expression level using novel rabbit polyclonal antibody raised against survivin protein (Figure 3). The β-Actin was used as an

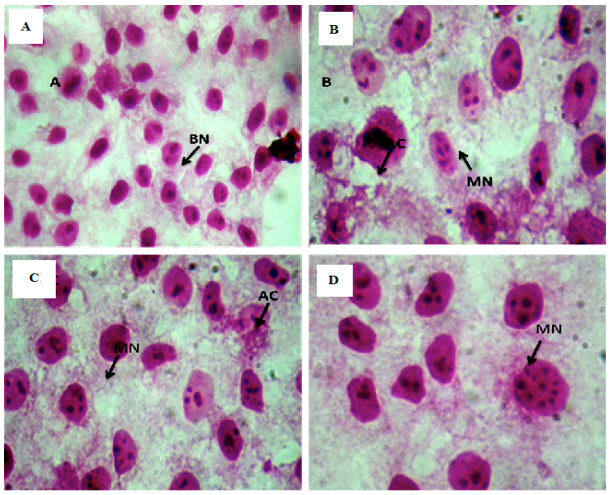

Figure 4. Immunocytochemical Staining of Cultured Cell Lines. Observation of multinucleated conditions in the cancer cell lines (A-MCF7), (B-A549), (C-ZR751) and (D-HepG2) there was no cytokinesis occurs in these cells because of the down-regulation of survivin causes failure of cytokinesis and apoptosis. MN-mono nucleated, BN-bi nucleated, MN-multi nucleated, AC-apoptotic cells

internal standard for western blot analysis.

The present investigation showed that all the selected cancer cells leads to the multi nucleated conditions with condensed DNA. Cells showed two nuclei or one bilobed nucleus (multinucleated conditions) including mitotic arrest and there was no telophase, lead to failure of cytokinesis. The fraction of MCF7, A549, HepG2 and ZR751 cancer cells with 4N DNA content (N is the haploid DNA content), tripled at the expense of cells with 2N DNA content >4N or <2N, in contrast with depletion of survivin in cancer cells caused abnormality in chromosome segregation and polyploidy conditions, to blockage of mitotic arrest, failure of chromosome segregation or failure of cytokinesis. Immunohistochemcial staining is illustrated in Figure 4).

Discussion

Cell division involves coordinated chromosomal and cytoskeleton rearrangements to ensure the faithful segregation of genetic material into the daughter cells (Endoh et al. 2001). The chromosomal passenger complex (CPC), survivin has emerged as a central player at several steps in this process (Angell et al., 2008). Survivin act as a chromosomal passenger protein, ensuring the proper alignment of chromosomes during mitosis and allowing for equal and complete cell division (Vagnarelli et al., 2004). Studies have shown that survivin interacts with both aurora-B and INCENP forming a CPP complex thought to be essential for cytokinesis. To investigate this possibility, we examined the effect of survivin in normal and selected cancer cells during mitosis (Uren, 2000). The down-regulation of survivin expression in cancer cells displayed abnormalities in chromosome segregation including lagging chromosomes and DNA bridges (Castedo et al., 2004). The DNA bridges failed to resolve during telophase and this failure was presumably a means by which cells with multinuclei produced. This

report is in consistent with our finding which is shown in (Figure 4) in which the multinuclear condition is an index of expression of survivin. Depletion of survivin leads to chromosomal nuclear multiplication and lack of cytokinesis (Lampson et al., 2004). Further more our results pointed out that the lagging chromosomes represent sister chromatid that have been left behind, rather than the product of sister chromatid non-disjunction.

Survivin is a well known regulator of cell cycle. It enhances the levels of cell cycle inhibitors in cancer cells (Wang et al., 2004). Interestingly, the western blot date gains support from earlier findings of Koppler et al., 2005 in which they stated that level of p53 was suppressed in the presence of survivin. The mitochondria-targeted survivin has been involved in the formation of tumours, in contrast cytosolic survivin inhibits tumour growth (Liu et al., 2004). Similarly, in the present investigation different cancer cell lines that vary in their multinucleated conditions when compared with normal cell line. It seems reasonable that abnormal expression or function of survivin might contribute to apoptosis (Vong et al., 2005). Besides this, the outcome of the present study may provide a possible reason to elucidate the mechanism of action of survivin in cells. Since survivin was known to regulate the cell cycle and appeared as an essential protein for the completion of cytokinesis. Results from our study demonstrated the down-regulation of survivin also inhibit cytokinesis. Earlier evidence report that depletion of survivin in cancer cells causes of decrease cytokinesis activity and increased karyokinesis further supports our speculation.

Survivin has been implicated in human cancer development and is known to be over expressed in a variety of human cancers. Positive regulation of survivin may provide a connection between cell cycle regulation and tumorigenesis or perhaps tumor maintenance (Fengzhi et al., 2006). The data acquired in the present investigation proved survivin function in segregation of chromosome and its importance in cancer cell cytokinesis and karyokinesis. In conclusion, our observation in this study shows that survivin support as well as proliferation of cancer cells. Therefore the present work indicates the failure of cytokinesis due to down-regulation of survivin expression in selected cancer cell lines tend to form multinucleated cells; hence survivin has to investigate as an anti-cancer target protein (Endoh et al., 2005).

In conclusion, absence of survivin, certain euploid human cells suffer missegregation of chromosomes, abortive assembly of microtubules late in mitosis, failure of cytokinesis. We attribute the defects in mitosis and cytokinesis by disruption of the CPP complex might be due to the expression of survivin down-regulation. These findings are in contrast with reports about the role of survivin in cell survival, cytokinesis, and segregation of sister chromatids, centrosome duplication and the mitotic spindle. It appears that survivin is essential to both chromosome segregation and cytokinesis in various mammalian cells. It seems reasonable to suspect that abnormal expression or function of survivin might contribute to tumorigenesis and multinucleated condition. Future studies will expand on these insights into fundamental mechanisms of action of survivin cell division and cell survival; it will help in validating the survivin network as a viable target for rational cancer therapy in humans

Acknowledgements

We are grateful to the University Grants Commission and Department of Science and Technology, Govt. of India for providing partial financial assistance to carry out this work and Faculties of Zoology Department, Bharathiar University, Coimbatore-46 for their critical comments.

References

Ambrosini H, Matsuzawa C, Altieri DC (1997). A novel anti-apoptotic gene,survivin expressed in cancer and luymphoma. *Nat Med*, **3**, 917-21.

Angell (2008). A study in to the potential role of survivin localization in resistance to drug-induced apoptosis. *Biosci Horizons*, **1**, 2.

Ryan BM, O'Donovan N, Duffy MJ (2009). Survivin: A new target for anti-cancer therapy. *Cancer Treat Rev*, **12**, 84-96.

Castedo M, Perfettini J L, Roumier T, Andreau K, Kroemer G (2004). Cell death by mitotic catastrophe: A molecular definition. *Oncogene*, **23**, 2825-37.

Chan J (2000). Down-regulation of survivin by antisense oligonucleotides increase apoptosis, inhibits cytokinesis and anchorage independent growth. *Neoplasia*, **2**, 235-41.

Colnaghi R, Connel CM, Barrett RM (2006). Separating the anti-apoptotic and mitotic roles of survivin. *J Biol Chem*, **281**, 33450-6.

Duffy J, O'Donovan N, Donal J, Gallagher WM, Brind M (2007). Survivin: A promising tumor biomarker. *Cancer Lett*, **247**, 49-60.

Dun Yang, Alane welm and Michael Biohop (2004). Cell division and cell survival in the absence of survivin. *Proc Natl Acad Sci USA*, **101**, 15100-5.

Earnshaw WC (2005). Cell biology keeping survivin nimble at centromers in mitosis. *Science*, **310**, 1443-4.

Endoh A, Asanuma K, Moriai R, Yamada M, Koyanagi Y and Sato T (2001). Expression of survivin mRNA in CD4+ cells. *Clin Chim Acta*, **306**, 149-51.

Endoh T, Tsuji N, Asanuma K, Yagihashi A, Watanabe N (2005). Survivin enhance telomerase activity via up-regulation of specificity protein 1 and c-Myc- mediated human telomerase reverse transcriptase gene transcription. *Exp Cell Res*, **305**, 300-11.

Gassmann R, Carvalho A, Henzing A J, Borealin (2004). A novel chromosomal passenger required for stability of the bipolar mitotic spindle. *J Cell Biol*, **166**, 179-91.

Kaitna S, Mendosa M, Jantch-Plunger V, Glotar M (2000). INCENP and Aurora like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis. *Curr Biol*, **10**, 1072-81.

Koppler M, Taubert H, Bertel F, et al (2005). Radio sensitization, after a combined treatment of survivin by siRNA and irradiation is correlated of pancreatic cancer caspase 3 and 7 in a wt-p53 sarcoma cell line, but not in a mt-p53 sarcoma cell line. *Oncol Rep*, **13**, 167-72.

Lampson MA, Renduchitala K, Khodhakuv A, Kapoor TM (2004). Correcting improper chromosome-spindle attachment during cell division. *Nat Cell Biol*, **?**, 798-812.

Lens SMA, Vader G, Medema RH (2006). The case of survivin as mitotic regulator. *Curr Opin Cell Biol*, **18**, 616-22.

Li F, Ling X (2006). Survivin study: An update of "What is the Next Wave". *J Cell Physiol*, 208, 476-86.

Liu T, Brouha B, Grossman D (2004). Rapid induction of mitochondrial events and caspase- independent apoptosis in survivin targeted melanoma cells. *Oncogene*, **23**, 39-48.

Reiko H, Ronan K, Erich AN (2003). Exploring the functional interaction between Aurora B, INCEP and survivin in mitosis. *Mol Cell Biol*, **23**, 3325-41.

Resnick TD (2006). INCEP and Aurora B promote in meiotic sister chromatid cohesion through localization of the shugoshin ME1-S332 drosophila. *Dev Cell*, **11**, 57-68.

Sha N K, Khan GJ, Bisen P S (2006). Structural, functional and therapeutic biology of survivin. *Cancer Lett*, **244**, 164-71.

Uren AG (2000) Survivin and the INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr. Biology*, **10**, 1319-28.

Vader G, Kauw JJ, Medema RH, Lens SM (2006). Survivin mediates targeting of the chromosomal passenger complex to the centromer and midbody. *EMBO Rep*, **7**, 85-92.

Vagnarelli P, Earnshaw WC (2004). Chromosomal passenger: the four dimensional regulation of mitotic events. *Chromosoma*, **113**, 211-22.

Vong QP, Cao K, Li HY, Lglesia PA, Zheng Y (2005). Chromosome alignment and segregation regulated by ubiquitination of survivin. *Science*, **310**, 1499-504.

Wang Z, Fukunda S, Pelus LM (2004). Survivin regulates the p53 tumor suppressor gene family. *Oncogene*, **23**, 8146-53.

Whealey S, Carvalho P, Vagnarelli P, Earnshaw W (2001). INCEP is required for proper targeting of survivin to the centromeres and proper anaphase spindle during mitosis. *Curr Biol*, **11**, 886-90.

Wheatly P, Meneish A (2005) Survivin: A protein with dual role in mitosis and apoptosis. *Int Rev Cytol*, **247**, ??

Wimmershioff J, Polkinghorne A, Grest P, et al (2010). Immunohistochemical detection of survivin in canine lymphoma. *J Comp Path*, **??**, 1-12.