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Targeting HSP90/Survivin using a cell permeable structure based peptido-mimetic shepherdin in retinoblastoma

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ABSTRACT

Background: Retinoblastoma (RB) is a childhood retinal malignancy. Effective therapeutic strategies are still being investigated in RB disease management. Here, the anti-cancer effect of shepherdin, a peptido-mimetic inhibiting heat shock protein (HSP90)-Survivin interaction has been analyzed.
Methods: We analyzed HSP (HSP70/90) and Survivin protein expressions by immunohistochemistry (29 archival tumors), qRT-PCR, FACS and Western analysis (10 un-fixed RB tumors). We also analyzed cellular cytotoxicity and anti-proliferative effect in peptide treated RB cells (Y79, Weri Rb1) and MIO-M1 cells.
Results: Heterogeneous expressions of HSP70/90 and Survivin with a significant association between HSP70 and HSP90 ($r^2 = 0.59$, $p = 0.001$) was observed. In RB cells, anti-tumor effects were detected with 0.42 μg/ml of shepherdin at 4 h s of serum starvation. Decreased Survivin, Bcl2, MMP-2 activity with increased Bax, Bim, and Caspase-9 protein expressions were noticed. No significant changes were observed in shepherdin treated non-neoplastic MIO-M1, nor in scramble-peptide treated RB cells.
Conclusion: The presence of HSPs (HSP70/90) and Survivin reveals multiple cellular mechanisms adopted by RB cells during cancer progression. Serum starvation induced HSP90 whose interactions with Survivin were specifically inhibited by shepherdin. The associated molecular shuffling has been reported. These findings strongly implicate the potential of targeting HSP90-Survivin interaction as an adjuvant therapy in RB management.

1. Introduction

Heat shock proteins (HSPs) are highly abundant molecular chaperones in tissues under stress conditions to enhance cell survival [1]. HSPs play a significant role in protein folding, homeostasis, and maturation during proteotoxicity [2]. Also, these foldosomes are involved in numerous cellular signaling pathways such as cell cycle regulations, proliferation, apoptosis and cytoskeleton [3]. Based on their molecular sizes, HSPs have been categorized as HSP100, HSP90, HSP70, HSP60, HSP40 and other small heat shock proteins [4].

HSPs especially HSP90, HSP70, and HSP27 are predominantly over-expressed in several cancer types such as glioblastoma, retinoblastoma [5–7] and significant association with tumor progression has been reported [8–10]. HSP90 accounts for 1–2% of total normal cellular protein, and its level is elevated in approximately 4–6% under stress [11]. Functional activity of HSP90 is attained through its interaction with numerous client proteins mainly like HER2, AKT, CDK4, HIF, MMP-2 and Survivin [12,13].

Among these client proteins, Survivin, an inhibitor of apoptosis (IAP) gene family member is over-expressed in many cancers including retinoblastoma [14]. Survivin exhibits a multifaceted role in apoptosis inhibition, cell division, tumor progression and cell cycle regulation in various clinical cancers [15–17]. The wild type Survivin and its splice variants (Survivin-2B and Survivin-2α with pro-apoptotic activity; Survivin-ΔEx3 and Survivin-3B with anti-apoptotic activity) are reported to be associated with cancer aggressiveness [18,19]. Suppression of Survivin induces

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mitochondrial apoptosis, inhibits cell proliferation and increases the susceptibility of tumor cells to chemotherapy [20–24].

HSP90 controls proteostasis of Survivin by refolding the unfolded/denatured protein to a native state. HSP90-Survivin may serve as a potential target for RB cancer therapy as the percentage of HSP90 and Survivin is reported to be high [7]. Disruption of HSP90/Survivin physical interaction by targeting the ATP-binding pocket of HSP90 inhibits the downstream signaling cascade of Survivin, leading to the ubiquitination and deactivation of Survivin signaling pathways resulting in tumor growth arrest and development [25,26].

Previous reports have implicated the disruption of HSP90/Survivin protein interactions using structure based peptido-mimetic, shepherdin (K79-L87, KHSSGCAFL) [27,28]. Shepherdin was made cell permeable by conjugating protein transduction domains (PTDs) namely helix III (cell-penetrating Antennapedia homeodomain sequence) [29] or second carrier sequence (HIV Tat protein) [30] to its N-terminal end. Anti-cancer effects of shepherdin have been reported in breast and prostate cancer models [27]. In the current study, firstly we evaluated the molecular expression of HSP70/HSP90 and Survivin in advanced retinoblastoma (RB) primary tumors relative to the normal retina. Then, we examined the dose-dependent efficacy of cell permeable shepherdin (Tat peptide conjugated) using *in-vitro* RB cancer cells (Y79 and Weri Rb1) and non-neoplastic cell line, Muller glial cells (MIO-M1). Molecular basis of shepherdin's anti-cancer effects was also investigated.

2. Materials and methods

2.1. Primary RB tissues

The institutional ethics committee (Vision Research Foundation, Sankara Nethralaya, India) reviewed and approved this study in agreement with Helsinki Declaration. RB primary tumor samples were collected from 29 enucleated eyeballs as part of RB management (2010–2012). Histopathological information namely tumor invasion of the choroid, optic nerve, or orbit was obtained from surgical pathology reports (Supplementary Table 1). Among the tumors analyzed, there were 17 invasive tumors and 12 non-invasive tumors with high-risk histopathological features. From these samples, a representative of 10 tumor samples was analyzed for the respective mRNA expression (qRT-PCR) and protein expression (FACS and Western analysis).

Based on patient's clinical presentation, the tumors were classified as per International Intraocular Retinoblastoma Classification [31]. There were 15 tumors in group E, ten tumors in group D and six tumors in group B. In group E, there were nine invasive and four non-invasive cases. There were six invasive and four non-invasive cases in group D tumors. Similarly, there were two invasive and four non-invasive in group B tumors. Normal adult retinae collected from 3 cadaveric eyeballs (C.U. Shah eye bank, Sankara Nethralaya, http://www.sankaranethralaya.org/eye-bank.html) during the year 2011 were included as controls in the study.

2.2. Cell culture

Human RB cancerous cell lines (Y79, Weri Rb1, Riken cell bank, Japan) and human non-neoplastic, Mueller glial cell line (MIO-M1, derived from human neural cells of the retina and a kind gift from G.A. Limb, UCL Institute of Ophthalmology, London, England) were used in the study. The RB cell lines were cultured as reported earlier [32].

2.2.1. Nutrient-deprivation RB cell model

Cells were treated with peptido-mimetic in serum-free condition for an initial period of 4 h and supplemented with 5% growth medium, incubated at 37 °C with 5% CO_2 for 20 h. HSP90 and Survivin levels were analyzed at the end of 4 h of serum deprivation and after 20 h of 5% serum supplementation (Supplementary Fig. 2: a and b). Considering that HSP90 was the target for binding with shepherdin, the 4 h post-starvation was a reasonable time-point for revealing shepherdin-binding effects (if any). As the nutritionally deprived environment imitates one of the stress inducers, this model will help to analyze the potent cytotoxic dose of shepherdin [33,34].

2.3. Antibodies and peptides

Monoclonal antibodies to HSP90 (4874, Cell signaling Technology, Danvers, MA), HSP70 (4872, Cell Signaling Technology, MA), Survivin (sc-17779, Santacruz Biotechnology, USA), Bax (Ab7977, Abcam), Bcl-X S/L (4H33, sc-70418, Santacruz Biotechnology, USA), Bim (H-191, sc-11425, Santacruz Biotechnology, USA), caspase-9 (F7,sc-17784 Santacruz Biotechnology, USA), β-actin (Sigma Aldrich, USA), Anti-mouse-fluorescence isothiocyanate (FITC) conjugated (sc-2010, Santacruz Biotechnology, USA) and anti-rabbit-cy3 conjugated (A10520, Invitrogen, USA) and FITC-conjugated streptavidin (SNN1008, Invitrogen, USA) were used in the study. N-terminal end of shepherdin and scramble-peptides were tagged with biotinylated cell permeable HIV-Tat protein. The amino acid sequence of peptides are shepherdin-peptide; shepherdin^{Tat}:biotin-X-YGRKKRRQRRRKHSSGCAFL-$CONH_2$; scramble-peptide:biotin-X-YGRKKRRQRRRSKLACFSHG-$CONH_2$, where X = EAHX, hexanoic acid spacer [27]. These HPLC purified, synthetic peptides were synthesized commercially (Custom peptide synthesis, Mumbai).

2.4. Immunohistochemistry

HSP70/HSP90 and Survivin protein expressions were studied using immunohistochemistry as reported earlier [35] by using primary antibodies (1:50 dilution).

2.5. Western blotting analysis and flow cytometry

Cells were washed thrice with 1X phosphate buffered saline (PBS) and lysed in RIPA buffer. Fifty micrograms of protein lysate were resolved on 12% SDS polyacrylamide gel, and then electro-transferred onto nitrocellulose, and blocked. They were then probed with antibodies of HSP70/HSP90 (1:500), Survivin (1:50) and β-actin (1:2000) and detected using chemiluminescence (Thermo Scientific, Pierce, and Rockford, USA).

For FACS, PBS washed cells were fixed on ice with 0.01% paraformaldehyde for 15 min followed by permeabilization in 0.1%NP-40 for 15 min. The PBS washed cells were incubated with primary antibodies [anti-HSP70/anti-HSP90 (1:25), and anti-Survivin (1:25), anti-Bcl2 (1:50), anti-Bax (1:50), anti-Bim (1:50)] for 2 h at 4 °C. The washed cells were incubated in dark with secondary antibodies [anti-mouse-FITC conjugated (1:2000); anti-rabbit-cy3 conjugated (1:50) for 1 h at 4 °C. Finally the washed cells were analyzed on a flow cytometer (BD Calibur) using Cell Quest software for FITC uptake. Cells without antibodies and with secondary antibody alone were used as controls.

2.6. Internalization assay

Cells were treated with various concentrations of peptides (0.17, 0.25, 0.34, 0.42 μg/ml) in serum-free media for 4 h. Following this treatment, 5% serum supplementation was provided to the cells and the cellular uptake of peptides at 0, 4, 8, 20 h of serum