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RESEARCH ARTICLE

INVESTIGATE THE INTERACTION BETWEEN P53 AND RETINOBLASTOMA DURING HSV-1 INFECTION.

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Manuscript Info

Abstract

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Introduction:-

Cancer refers to an uncontrolled abnormal growth of cells which is a major cause of mortality over the globe [1]. According to GLOBOCAN estimate, 14.1 and 8.2 million new cancers cases and deaths due to cancer respectively occurred in 2012 globally [2]. For the way of explanation, cancer is increasing worldwide, contributing to an enormous burden on society through morbidity, mortality and economic loss, even in the developed countries [2]. However, early diagnosis and in-time proper management with chemotherapy, radiotherapy or surgery can reduce this burden.

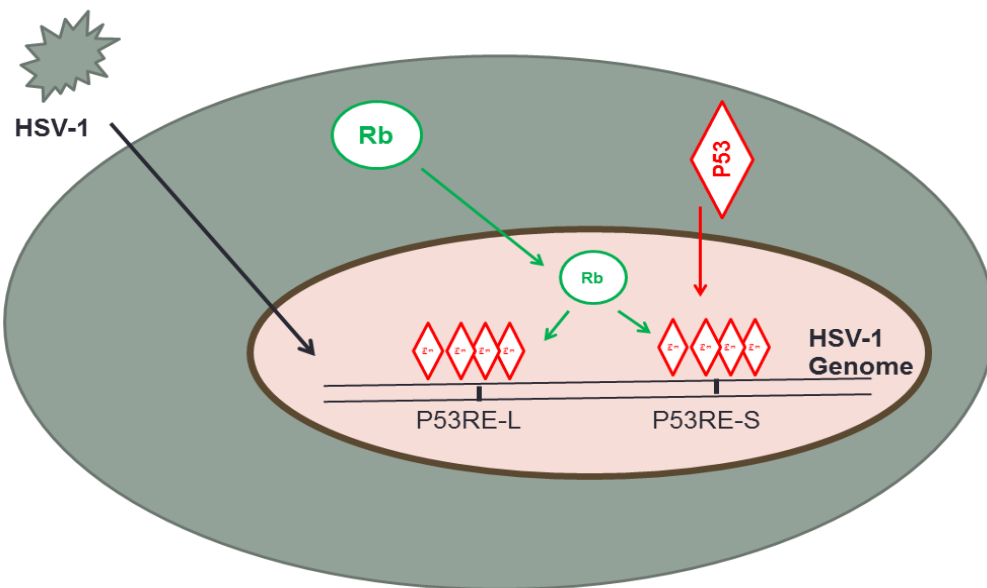
Retinoblastoma is a rapidly growing the most common primary intra-ocular malignancy, usually affecting children below 5 [3]. It develops from the cells having mutant copies of RB1 gene which encodes for a tumor suppressor protein called retinoblastoma protein (pRb) [4]. It accounts for 10-15 percent cancers occurring in the first year of life, and is diagnosed in about 8000 children each year globally [5,6]. Management options for Rb include local ophthalmic treatment, systemic chemotherapy, radiotherapy and surgery [7]. Early detection of intra-ocular tumors offer 95% cure, but extra-ocular expansion increases the mortality [8].

Tumorigenesis sequentially affects the regulatory biological processes which help cell proliferation and their survival. In general, most of the human tumors affect Rb and p53 regulatory pathways [9]. Previously, it was suggested that tumorigenesis of retinoblastoma bypasses or does not involve sequential lesions in p53 pathway [10]. On the contrary, Laurie et al. [9] has challenged this notion of bypassing p53 pathway in retinoblastoma.

Belonging to herpesviridae family, herpes simplex virus (type 1 and 2) is an enveloped double stranded DNA virus, found all over the world causing oral lesions (cold sores) or genital sores [11]. Herpes simplex virus type 1 (HSV-1) is predominantly found in orofacial lesions and is transmitted through mucosal membranes and non-intact skin [12]. It has been suggested that HSV-1 has ability to control the role of p53 in a balance and to cause cell death through HSV-dependent apoptosis (HDAP) to which oncogenic pathway is susceptible [13]. Being neurotropic DNA virus, HSV-1 has been reported to be oncolytic as it can work as a vector for cancer therapeutic genes [14]. It has been studied that HSV-1 modulates Rb gene and this very notion can affect the viral replication, pathogenesis, and

oncogenesis [15]. Therefore, as HSV-1 modifies Rb and p53 pathways, it may play a positive or negative role in the tumorigenesis of retinoblastoma.

Because both p53 and Rb are tumor suppressor proteins, in this experimental study we aim to investigate if there is an interaction between p53 and Rb proteins together during HSV-1 infection.



Material and methods:-

In order to test our hypothesis if these two proteins may interact together, we performed the following experiments using the protocols from ABCAM website [16]:

Transfection experiment. The purpose of this experiment to prepare HEK293 lysates for co-immunoprecipitation. Cell line: HEK293, using new prep p53 (1.9µg/µl). Transfection reagent: Express-IN; (270,000 cells/100mm plate). Transfection Protocol for per 100mm plate: 1. Add to the first tube 5µg plasmid plus 200µl MEM serum free medium. 2. Add to the second tube 15µl Express-IN plus 200µl MEM serum free medium. 3. Mix the two tubes and incubates for 20 min at room temperature. 4. Transfect 400µl mixture to each 100mm plate.

BCA protein assay. It is divided into three steps. First, preparing standers and it include the following protocol: label six eppendorf tubes (0µg/µl, 0.125µg/µl, 0.250µg/µl, 0.500µg/µl, 1.00µg/µl and 2.00µg/µl), pipette 1ml of nanopure H₂O into the tube labeled 0µg/µl, pipette 100µl of BSA stock into tube labeled 2.00µg/µl, pipette 50µl of nanopure H₂O into tubes labeled 0.125µg/µl, 0.250µg/µl, 0.500µg/µl and 1.00µg/µl, pipette 50µl of BSA from the 2.00µg/µl tube into the 1.00µg/µl tube and vortex, pipette 50µl of the combined BSA and dH₂O from the 1.00µg/µl tube into the 0.500µg/µl and vortex, pipette 50µl of the combined BSA and dH₂O from 0.500µg/µl tube into the 0.250µg/µl and vortex, pipette 50µl of the combined BSA and dH₂O from the 0.250µg/µl tube into the 0.125µg/µl and vortex. Second, preparing 96-well plate and it include the following protocol: use 96-well plate cover to mark which wells each sample and standards will be placed, mark standards in either duplicate or triplicate to insure precision, pipette 10µl of blank/standards into designated well, the first row of standards should be just water and the prepared samples in the following rows, pipette 9µl of dH₂O into the rest of the wells which will hold sample, pipette 1µl of sample into designated wells for sample used. Third, preparing BCA reagents and it include the following protocol: calculate amount needed for reagent A then add to falcon tube as following equation ($\# \text{ wells} + 1$) $\times 200 =$ _____ µl reagent A, calculate amount needed for reagent B the add to the same falcon tube as following equation ($\text{_____} \mu\text{l reagent A}$) $\div 50 =$ _____ µl reagent B, vortex solution, each well of the plate will receive 200µl of the BCA reagent, add 200µl of A/B reagent to all wells, incubate at 37°C for 15 minutes. After these three steps we used Tecan Safari2 for reading and it has own protocol.

Co-immunoprecipitation. Co-immunoprecipitation (Co-IP) is a popular technique for the analysis of protein interaction. The procedure includes: 1) An antibody specific to the protein of interest is added to a cell lysate. 2) The antibody-protein complex is then precipitated usually using protein-G or protein-A sepharose which binds most antibodies. If there are any protein/molecules that bind to the first protein, they will also be precipitated. 3) Co-precipitated protein can then be identified by Western blot analysis or by sequencing a purified protein band. After this brief introduction about Co-IP, the following is the protocol: on ice, in a micro centrifuge tube add 30µg cell lysate plus 5µl p53 antibody (DO-1), incubate the sample with antibody 1-12 hr (overnight) at 4°C and under gentle rotation, meanwhile prepare the Sepharose beads (A/G-coupled Sepharose beads), mix the slurry well and add 70-100µl of the beads to each sample and always keep samples on ice, beads will tend to stick to the sides of the tip so try to minimize the movement in the pipette and use a tip cut 5mm from the top, incubate the lysate beads mixture at 4°C under gentle rotation for 2hr, the lysate-beads/antibody incubation time over centrifuge the tube, remove the supernatant from the beads and discard, the complex of interest should now be specifically bound to the antibody coating the beads, wash the beads with washing buffer or lysis buffer 3 times to remove non-specific binding, for each tube wash and mix the beads gently with wash buffer, centrifuge at 4°C and remove the supernatant which can be discarded, ensure to carefully remove as much wash buffer as possible from the beads, the complex is now ready for elution from beads, following steps regarding SDS buffer elution, in SDS elution the Ag-Ab complex is eluted from the beads by heating and boiling samples, in loading buffer with denaturant SDS, this method is advantageous because the extraction method is highly efficient and the resulting sample is more concentrated, it include 4 steps as following, elute 50µl of beads by heating in 50µl of 2×SDS without DTT for 10 minutes at 50°C, pellet beads transfer supplement to a new tube and add DDT at 100mM, add 50µl 2×SDS buffer with DDT to pelleted beads, boil the elution samples for 5 minutes and analyze content of the sample by western blot.

Western blotting. Western Blotting, Western blot or immunoblotting (Western blotting was dubbed because it is similar to Southern blotting which was invented by and named after the inventor E. M. Southern) allows investigators to determine, with a specific primary antibody, the relative amounts of the protein present in different samples. Briefly, 1) Samples are prepared from tissues or cells that are homogenized in a buffer that protects the protein of interest from degradation; 2) The sample is separated using SDS-PAGE and then transferred to a membrane for detection; 3) The membrane is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the membrane. A primary antibody is then added to the solution which is able to bind to its specific protein; 4) A secondary antibody-enzyme conjugate, which recognizes the primary antibody is added to find locations where the primary antibody bound. After this brief introduction about western blot, the following is the protocol: set up a std mini gel with 7.5% SDS/PAGE plus adding double dose of Temed 15µl plus leveling by isopropanol, load 10µg extract protein plus 7µl Bio-Rad pre-stained protein marker, run mini gel at 120 Volt on Bio-Rad power PAC 200 supply, transfer blot by a Bio-Rad box at constant mA at 250mA (9V) for 90 minutes-2hrs using Bio-Rad power PAC 3000 supply, blocking membrane by TBST 5% milk for 1hr, set up the glass plate wrapped by parafilm and add p53-primary Ab 0.5µl in a big Tip Box and incubate in a 4°C refrigerator not in cold room, next day, washing membrane with TBST for 1hr, change a new parafilm for glass plate and incubate with 2nd Ab 0.2µl at room temp for 1hr, washing membrane for 1hr, turn on x-ray film machine, detecting p53 by mix 1ml sol'n A and 25µl sol'n B in a microtube and at membrane on the plastic wrap and incubate for 5 minutes, dry and transfer membrane to cassette and expose to different time (1", 2" or 5" ...).

Membrane stripping and re-probing. Stripping is the term used to describe the removal of primary and secondary antibodies from a Western blot membrane. Stripping is useful when one wants to investigate more than one protein on the same blot, for instance a protein of interest and a loading control. When probing for multiple targets, stripping and re-probing a single membrane instead of running and blotting multiple gels have the advantage of saving samples, materials, and time. After this brief introduction about membrane stripping and re-probing, the following is the protocol: add 280µl of β-mercaptoethanol per 40ml of stripping buffer to a final concentration of 100mM, wash membrane in TBST (1×5 minutes), incubate membrane in stripping buffer for 30 minutes at 50-60°C, wash in TBST (3-4×10 minutes), block membrane for 1hr or O/N in 5% blocking milk, follow normal protocol for developing western blots.

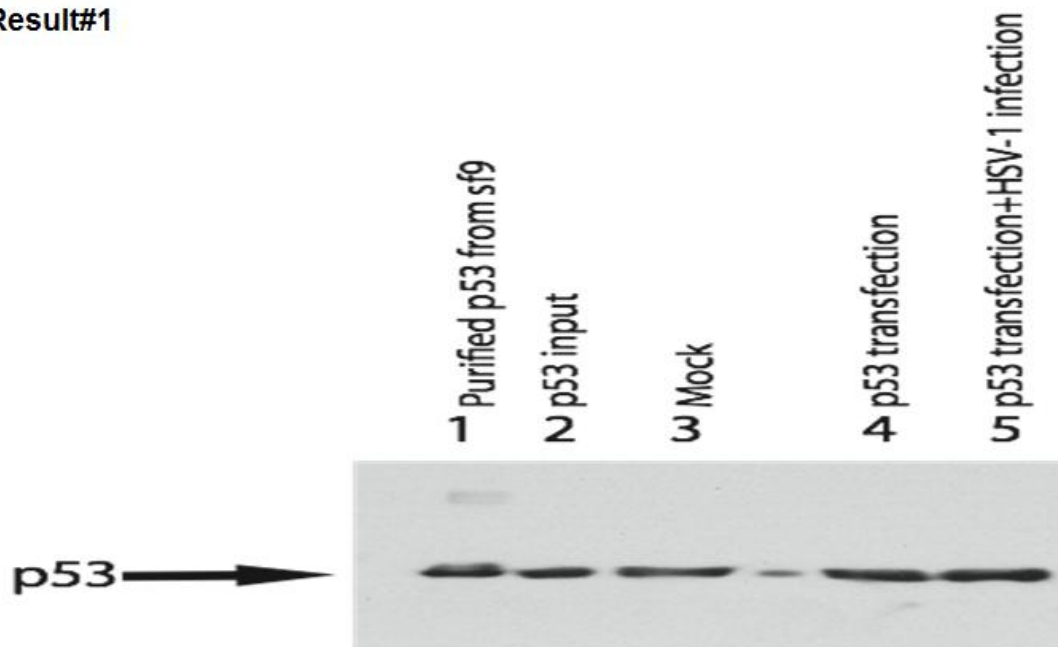
Results:-

Result #1:-

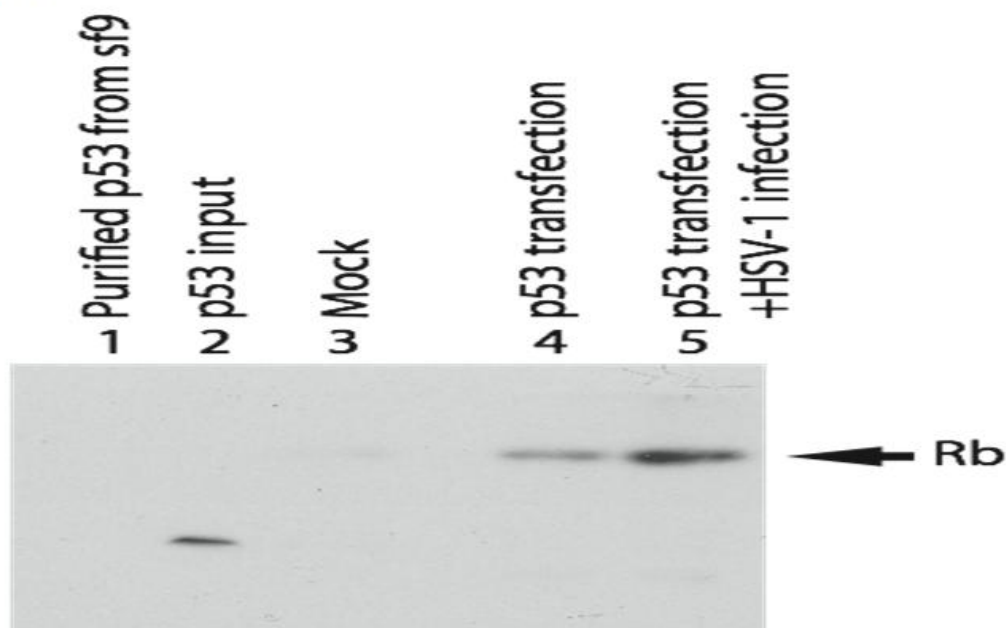
Co-IP exp was carried out with lysates prepared from Vero (exp#1) and HEK293 (exp#2) cell lines. The 1st group was mock; the 2nd group was HEK293 transfected with p53 plasmid and 3rd group was also infected with HSV-1 with MOI=5.

Western blotting using anti-p53 antibody

Lane1 is a purified p53 protein used for positive control (Allow us to know the exact p53 protein migration position in the polyamide gel and we bought it from BD company). Lane2 is a p53 transfection input used for another positive control (Showed our p53-transfected HEK293 cells containing p53 in the cell lysate). Lane3 is co-immunoprecipitated complex from Mock group. Lane4 is co-immunoprecipitated complex from p53 transfection group. Lane5 is co-immunoprecipitated complex from p53 transfection and HSV-1 infection group.

Result#1**Result#2:-**

We stripped the first WB membrane, and Re-probing with Rb antibody.

Result#2

Discussion:-**Regarding result#1:-**

The p53 band was detectable. And p53 band is slightly increased in lane4 and lane5. Which means HSV-1 infection enhanced the interaction. And this result confirms that there is interaction over the response element of the p53.

Regarding result#2:-

The Rb protein was detectable in lane4 and lane5. Which indicate that there is interaction between p53 and Rb (Lane4). And we can see that after the HSV-1 infection the interaction between p53 and Rb is enhanced (Lane5). And this result support that there is interaction between p53 and Rb proteins during HSV-1 infection.

Conclusion:-

The main two conclusions are: (1) Our Co-IP result suggests that p53 interacts with Rb in HEK293 cells. (2) P53-Rb interaction enhanced by HSV-1 infection in HEK293 cells. In order to prevent the carcinogenesis of HSV-1 we recommend to understanding the nature of the interactions between p53 and Rb proteins during HSV-1 infection by further studies.

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