Investigation of radio-resistance mechanism in gamma irradiated melanoma cancer

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MASTERS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by **Maryam Shahid** under registration no. NUST201463170MSMME62424F, titled: "Investigation of radio-resistance mechanism in gamma irradiated melanoma cancer" be accepted in partial fulfillment of the requirements for the award of Masters Degree with **A** grade.

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DECLARATION

It is hereby declared that this research study has been done for partial fulfillment of requirements for the degree of Masters of Sciences in Biomedical Sciences. This work has not been taken from any publication. I hereby also declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification in this university or other institute of learning.

Maryam Shahid

Every breath I take in my life and every little drop of blood running in my body, are dedicated to my family to whom I owe everything.

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"In the name of Allah the most Merciful and Beneficent"

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DNA	Deoxyribonucleic Acid
RT	Radiation Therapy
CDKs	Cyclin Dependent Kinases
SSBs	Single Stranded Breaks
DSBs	Double Stranded Breaks
Gy	Gray (Unit of Radiation)
PKC	Protein Kinase C
PARP	Poly ADP ribose polymerase
Caspase	Cysteine-aspartic proteases
MDR	Multi Drug Resistance
MRP	Multi Drug Resistance Protein
DMSO	Dimethyl sulfoxide
DMBA	7,12-Dimethylbenz[a]anthracene
BSA	Bovine Serum Albumin
NC	Nitrocellulose
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
ECL	Enhanced Chemiluminescence
UV	Ultra Violet Radiation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ABSTRACT

Radiation therapy plays a central role in adjuvant strategies for the treatment of both pre- and postoperative human cancers. However, radiation therapy has low efficacy against cancer cells displaying radio-resistant phenotypes. Melanoma or skin cancer is one of the leading cancers worldwide and is the type of cancer that is most often resistant to ionizing radiation. This study was carried out to understand the underlying mechanism of radio-resistance. In order to elucidate an integrated mechanism, BALB-C xenografts were established in order to investigate the potential effect of gamma radiation doses in-vivo. The expression of various proteins was investigated via western blot analysis which include; PARP, PKC-α, Caspase-3 and MRP. There was a prominent change in the expression of these apoptotic and anti apoptotic proteins by their treatment with gamma radiations.

1. INTRODUCTION

1.1 Cancer

Normal functionality of living organisms is maintained by a finely tuned balance between cell proliferation and cell death. If this balance is lost or even one of the cells becomes rebellious and starts uncontrolled proliferation, a tumor will be formed and, if metastasized, can lead to any fatal kind of cancer. This can result from any of the several process like (a) Activation of proto-onco genes, which is turning of normal gene into onco gene via mutation of or over expression, (b) inactivation of tumor suppressor genes, which are there to stop the metastasis, (c) changes in gene repair mechanisms, which are there to minimize the unwanted alterations in gene expression.

Cancers are classified into various types on the basis of their origin and tissue types. (a) Carcinoma is the cancer of epithelial origin. It occurs in all the internal as well as external lining of body parts including skin and gastrointestinal track. Carcinomas has further two subtypes: one of them is Adenocarcinoma which appears in organs or glands and the other one is Squamous cell Carcinoma which appears in squamous epithelium. (b) Sarcoma is the type of cancer which appears in connective of supportive tissues like muscles, bones, cartilage etc. (c) Myeloma originates from plasma cells and bone marrows. (d) Leukemia tends to originate from the blood and flows in blood stream. (e) Lymphoma occurs in the lymphatic system, lymph nodes etc.

1.2 Melanoma

Skin, the largest organ of the body, has three layers. (a) Epidermis is the outer most layer of skin which protects the body from external environment. It is 0.01 inches thick. The

main types of cells present in epidermis include: *Squamous cells* which are flat and outer most cells that shed all the time as the new ones are made; *Basal cells* are present in the lower part of epidermis and they keep on dividing and eventually replacing the squamous cells; *Melanocytes* are the cells that are involved in the brown pigmentation of the skin called melanin and they protect the skin from the harmful effects of sun rays. These cells have the potential to become 'melanoma' cancer. (b) Dermis is the middle layer of the skin which contains the hair follicles, sweat glands, blood vessels etc. (c) Subcutis is the deepest layer of skin which is involved in making a network of collagen and fat cells, conserves energy and protects the body organs from external shocks.

Melanoma skin cancer begins in melanocytes that have become cancerous. They appear brown or black if the melanin is being made and they appear pink or even white is the production of melanin stops because of cancer. Melanoma is one of the most dangerous forms of cancer. It can appear on any part of skin and can spread many bounds if not treated properly. Melanoma has the potential to spread to other parts of the body via dermis and if it goes deeper into the dermis, it can also reach the blood vessels causing way more adversity.

1.3 Radiation therapy

One of the treatments of melanoma cancer is radiation therapy (RT). Radiations are used to destroy the cancer cells by altering their genes which cause their growth and division. The radiation used is called ionizing radiation because it forms ions and deposits energy in the cells of the tissues it passes through. This deposited energy can kill cancer cells or cause genetic changes resulting in cancer cell death. This is how radiations can be used to

kill cancer cells (Baskar et al., 2012). RT is known to be the most effective non-surgical method of locoregional therapy of melanoma cancer (Strojan, 2010).

Ionizing radiations are categorized into two main types. One of them is photon radiation which includes X-rays and gamma rays while the other one is particle radiation which includes electrons, protons, neutrons, alpha and beta particles, etc.

1.3.1 Mechanism of Radiation therapy

The normal life cycle of the cells has 5 phases. The phases of cell cycle are controlled by the proteins known as cyclin dependent kinases (CDKs). (a) The first phase of the cell cycle is *Go phase*. In this phase, the cells are in resting phase. They do not tend to divide and they keep on doing their normal work in the body. It can last from hours to years. (b) The second phase of the cell cycle, *G1 phase*, is the time where the cell gathers the information about when to go the next phase. In this phase, the cells get ready to divide. It lasts for 18-30 hours. (c) The *S phase* of the cell cycle is involved in the synthesis of DNA so that both the cells get enough and same amount of DNA. It takes about 18-20 hours. (d) The *G2 phase* builds up the apparatus for the process of mitosis and gathers the information about the starting point of mitosis. It takes place for 2 to 10 hours (e) *M phase* is the phase where mitosis occurs. One cell divides into two exactly same cells within 30 to 60 minutes in this phase.

The cell cycle is necessary for understanding the radiation mechanism because the actively dividing cells tend to be killed earlier by radiations as compared to the cells in the resting phase or slowly dividing cells. The more speedily the cell divides, the earlier it gets the chance to be killed by radiation. Since the cancer cells are uncontrolled and follow rapid division, they are easily killed by RT. But radiation therapy can also kill the

normally dividing cells which is one of the side effects of RT. The required balance between destruction of cancer cells and the protection of normal cells should be maintained properly. How much the cell is damaged by the radiation is called *radiosensitization*. RT kills the tumor cells by causing double stranded breaks in the DNA of the cells and hence interrupting the cell cycle. The more number of DSBs result by more absorbance of radiation dose(Huber et al., 2013).

1.4 Radioresistance

Melanoma has always been believed to be more radio-resistant as compared to other types of cancers (Chang et al., 2006). The radioresistance of a tumor is determined by innate ability of the cells to repair the DNA damages either by non-homologous end joining or by homologous recombination. Tumor mass is not restored again if the irradiated tumor cells leave the DSBs unrepaired. Radioresistance and hence the survival of the tumor can be a result of cellular stress response caused by ion transports to DNA damage by controlling various cell functions like cell cycle, DNA repair or metabolic adaptations(Huber et al., 2013). Many protein pathways occurring within the tumor cell can be involved in the mechanism of radioresistance which are discussed further.

1.5 Scope of study

Proteins are involved in carrying out all the processes occurring in the cells. Some proteins act as onco-genes which mediate cancers while others act as tumor suppressor genes and control the unwanted proliferation of the cells. *PKC-α* belongs to the family of Protein Kinase C (PKC) which is a family of phospholipid-dependent serine/threonine kinases. It is known to be involved in cell proliferation, apoptosis, survival and drug

resistance. Due to its key role in cell signaling, PKC-α has the potential to be proficient remedial target for several types of cancer(Kang, 2014). *PARP* (Poly(ADP-ribose) polymerase) plays a very active role against the development of tumor by taking care of DNA repair, DNA replication and the cell death is response to any form of stimuli. It also interacts with other proteins and is involved in many cancer pathways (Yap et al., 2011). *Caspase-3* is an apoptotic protein involved in the pathways that lead to apoptosis of the cells when required. It acts within a cell against cancers. It is considered to be linked to the ultimate proceedings in the execution programmed cell death by means of specific as well as extensive DNA fragmentation(Didenko et al., 2002). *MRP* (Multidrug resistance-associated protein) and *MDR* (Multidrug resistance) have commonly been found to confer chemoresistance and radioresistance in cancer. Due to their function and significance, they are the targets of several anticancer efforts(2000).

In this study, we are studying the action of these proteins and their behavior in cancer cells and we are investigating the effect of gamma radiations on DMBA induced melanoma tumor following the mechanism of radioresistance.

1.6 Objectives

The objective of this study is to highlight the mechanism of radioresistance present in tumor cells which would resist the apoptotic activity of cells against the gamma radiations. After getting to know about the levels of various proteins adding to it, we can treat the radioresistance with respective drugs and reduce the effect of radioresistance so that the radiation therapy will be used as a harmless treatment for the cancer.

2. LITERATURE REVIEW

It is well apperceived that it may take very long time afore incipient scientific erudition on the nature of cancer or novel technologies are introduced into clinical application for amended treatment of cancer patients (Baumann and Overgaard, 2016). Tumor metastasis is a perplexing procedure and can be to a great extent separated into three stages: (i) escape of the tumor cells from the mass of essential tumor and spread of the cells through the lymphatics or veins, (ii) entrance through vessel dividers and other tissue obstructions, and (iii) development at the new site. Numerous qualities have been involved in this procedure (Wang et al., 2000).

The incidence of any cancer that is growing most rapidly among men is Melanoma, and this is second rapid among women. According to a study held in 2010, Melanoma is fifth main cause of cancer among men and sixth main cause among women and 68130 cases were reported whereas 8700 deaths occurred because of Melanoma. The estimated occurrence of Melanoma among men is one in 39 and for women, it is one in 58. Melanoma can be diagnosed in between fourth and fifth decade giving the approximated age of 59. After diagnosis, the percentage of localized disease, regional disease and non-regional metastatic disease is 82% - 85%, 10% - 13% and 2% - 5% respectively. Over the decades, it has been noted that the overall survival rates have been changed and fortunately increased to 87% and 92% in 1986 and 2004 respectively (Khan et al., 2011). Genetic and environmental, both are the variables that add to the cause and improvement of cutaneous Melanoma. (Yang et al., 2001) Melanoma is an exceedingly forceful tumor whose occurrence is expanding more drastically than some other sort of growth. Metastatic melanoma predicts a poor anticipation, accordingly proposing that our present treatments are regularly insufficient in taking out the greater part of the melanoma tumor cells(Donato et al., 2014).

Apoptosis is an important module in the development as well as upholding of tissues within the organisms and it provides the mechanism to eradicate injured and redundant cells. (Lewis et al., 2002)

Radiation treatment assumes a focal part in adjuvant procedures for the treatment of both pre-and postoperative human growths. Be that as it may, radiation treatment has low adequacy against growth cells showing radio-safe phenotypes.(Shehzad et al., 2013) Radiation likewise demolishes the cell layer by initiation of sphingomyelinase, a chemical that catalyzes the hydrolyses of sphingomyelin to the lipid second courier ceramide, in this manner inciting the cell demise by apoptosis (Cohen-Jonathan et al., 1999). The core is another basic focus for radiation-incited apoptosis, by means of enactment of ace apoptotic qualities. (Todorovic et al., 2008)

The quantity of electrons per gram is an essential supporter to the constriction properties of a material at the vitality levels where the Compton impact prevails. Compton scattering prevails for substance components with low nuclear numbers, for example, C, N, O and S, which comprise melanin. In the Compton scattering, exchange of photon vitality to matter happens by means of a course of communications, where the vitality of the episode photon is exchanged to high-vitality electrons, and to optional photons of logically lower vitality until the photoelectric impact happens. Along these lines, the presence of structures made out of electron-rich, covalently connected fragrant themes could clarify the radiation dissipating properties of melanins.

The high-vitality electrons produced by Compton scattering are at last in charge of the biologic impacts of gamma radiation by either coordinate connection with DNA or through radiolysis of water in the cells, a procedure that outcomes in the development of responsive fleeting free radicals fit for harming DNA. Melanin may interface with these high-vitality electrons and keep them from entering a phone, in this way empowering its capacity as a radioprotector. The Compton electrons may then experience optional collaborations with other melanin particles, having their vitality steadily brought down by melanin (Dadachova et al., 2007).

Melanoma has long been believed to be radio-resistant and therefore the mainstay of treatment has been surgery. Albeit systemic biologic treatment, for example, interferon, may drag out infection free survival, it no doubt does not improve locoregional control and likely has close to nothing, assuming any, effect on general survival. In spite of the fact that radiotherapy (RT) has a moderately low likelihood of accomplishing long haul

control in patients with not completely resectable locoregional malady, it has been progressively utilized postoperatively to enhance locoregional control in patients at high hazard for lingering subclinical sickness after surgery.(William M. Mendenhall et al., 2008)

The inborn radiosensitivity of tumor cells is an essential element for deciding radiotherapy viability. Amid tumor advancement, changes in apoptosis acceptance, cell cycle control and oncogenes enactment may balance the radiosensitivity of tumor cells. The ability to do repair when DNA is harmed by ionizing radiation is a basic consider deciding cell radiosensitivity. Ionizing radiation creates a wide range of sub-atomic sores to DNA, including single strand breaks (SSBs), twofold strand breaks (DSBs), and an incredible assortment of base harms. DSBs are the most harmful type of DNA harm, in light of the fact that a solitary unrepaired DSB can prompt irregular mitosis with misfortunes of huge sections of DNA. It has been demonstrated that the few DSBs that are created by ionizing radiation connect nearly to the measure of prompted cell demise. DSBs are essentially repaired through homologous recombination repair (HRR) and nonhomologous end joining.(Ibañez, 2015)

(Bush and Li, 2002) says that MDRs play very important role in chemoresistance. The molecular mechanism regarding MDR has not been clarified as such but a few studies have accounted that the mechanism of MDR was associated with the over-expression of P-glycoprotein (P-gp) encoded by MDR1 gene in cancer cells. So, the inhibition of P-gp expression in tumor cells can prove to be one of the very effective ways to turn around MDR and make the tumor cells respond to chemotherapy(Lu et al., 2012).

Multidrug resistance (MDR) depends on other various instruments, one of which is the impact of ATP-restricting tape (ABC) transporters. They are included in the dynamic transport of phospholipids, particles, peptides, steroids, polysaccharides, amino acids, bile acids, pharmaceutical medications and other xenobiotic mixes. ABCB1 (P-glycoprotein, P-gp, MDR1), ABCC1-C6 (MRP1-6) and ABCG2 (BCRP) give imperviousness to cytostatic medications of tumors and add to the disappointment of tumor.(Sertel et al., 2012)

The need to anticipate radio resistance on individual tumor premise is as of late turning out to be more vital on account of the presence of an assortment of treatment modalities including changed fractionation radiotherapy, high LET radiation treatment, a wide scope of chemotherapeutic operators and consolidated chemo-radiotherapy notwithstanding or as another option to surgery.(Takehito Sasaki, 2004) In all likelihood the radio resistance of melanoma is because of the nearness of melanin color, which goes about as endogenous radio protector,(Anna A. Bro_zyna, 2008)

Protein kinase C (PKC) was initially found by Yasutomi Nishizuka in 1977 as a histone protein kinase actuated by calcium and diacylglycerol (DAG), phospholipids and/or phorbol esters. It is realized that the PKC family comprises of serine/threonine-particular protein kinases that contrast in their structure, cofactor prerequisite and substrate specificity. Because of biochemical properties and grouping homologies, PKCs are isolated into three subfamilies: firstly, traditional or customary PKCs (cPKCs; PKCα, PKCβI, PKCβII and PKCγ), which are calcium reliant and actuated by both phosphatidylserine (PS) and DAG. Besides, novel PKCs (nPKCs; PKCδ, PKCε, PKC and PKC), which are calcium free and managed by DAG and PS, lastly, atypical PKCs (aPKCs; PKC and PKC), which are calcium-autonomous and don't require DAG for actuation, despite the fact that PS can control their action.(Marengo et al., 2011)

PKCα, δ, ε, ζ, and λ/ι are the PKC isozymes communicated in melanoma. Among them, PKCα is the most essential as it is included in separation, multiplication, survival, metastasis, and antiapoptosis of melanoma cells. Assist αv-integrin expression and αvmediated relocalization of p53 by PKCα upgrade melanoma cell survival. What's more, articulation of PKCα assumes a basic part in the intrusion and relocation of melanoma cells as it can empower αvβ3-integrin-mediated attack of melanoma cells by expanding the GTPase action of Rac. PKCα likewise prompts melanoma vasculogenic mimicry or the arrangement of again vascular systems by profoundly forceful disease cells along these lines advancing the forceful, metastatic phenotype of tumor cells. In any case, the cell movement of PKCα may shift, even in similar melanoma tissues.(Kang, 2014)

Intriguing studies have exhibited that the α isoenzyme may go about as a tumor promoter or as a tumor silencer. For instance, overexpression of PKCα has been exhibited in tissue tests of prostate, endometrial and high-review urinary bladder, up-or down-direction of PKCα has been watched for hematological malignancies while down-control of PKCα has been portrayed in basal cell carcinoma and colon growths(Marengo et al., 2011)

As a few studies connected PKCα to improved multiplication and hostile to apoptotic signals, there has been noteworthy enthusiasm for this kinase as a potential focus for malignancy treatment. Be that as it may, PKCα had restricted accomplishment as a medication focus for malignancy. In fact, because of its exceptionally perplexing and very tissue-particular capacities, PKCα goes about as a tumor promoter or a tumor silencer relying upon the unique circumstance. To include another level of unpredictability, PKCα is up-controlled in a few growths, (for example, bladder, endometrial, and bosom disease) and down-managed in others, (for example, colorectal tumors and threatening renal cell carcinomas(Garg et al., 2014)

PKC flagging has beforehand been appeared to assume a part in interceding cell reactions to ionizing radiation (IR). The expression of PKCb seems to be enhanced within 1 hour after IR exposure. Moreover, the kinase action of PKC is instigated 5-overlap within 30 seconds of IR, and PKC-particular downstream atomic flag transducers are in this way phosphorylated.(Cerne et al., 2014)

The utilization of the dynamic type of caspase-3 for the recognition of apoptotic occasions has been investigated. This protease has been involved as an "effector" caspase connected with the start of the "passing course" and is along these lines a vital marker of the phone's entrance point into the apoptotic flagging pathway. Caspase-3 is actuated by the upstream caspase-8 and caspase-9, and since it serves as a merging point for various flagging pathways, it is appropriate as a read-out in an apoptosis measur(2012, Jeon et al., 1999). The capacity of caspase-3 inhibitors to anticipate apoptotic DNA discontinuity and consequent cell demise has prompted enthusiasm for the improvement of medications went for controlling the apoptotic procedure through caspase-3 restraint(Didenko et al., 2002).

PARP is known for its part in DNA-repair. In any case, numerous injuries can happen inside a little scope of DNA, known as oxidative bunched DNA sores (OCDLs), which are hard to repair and may prompt the more extreme DNA twofold strand break (DSB)(Wang and Weaver, 2011). Wasteful DSB repair can then result in expanded mutagenesis and neoplastic change. OCDLs happen all the more as often as possible inside an assortment of tumor tissues. Strikingly, PARP is exceptionally communicated in a few human malignancies. Also, perpetual aggravation may add to tumor-genesis through ROS-prompted DNA harm(Aguilar-Quesada et al., 2007). Besides, PARP can adjust irritation through communication with NFκB and managing the statement of incendiary flagging particles. Along these lines, the upregulation of PARP may display a twofold edged sword. PARP is expected to repair ROS-actuated DNA injuries, however PARP expression may prompt expanded aggravation by means of upregulation of NFκB flagging. Here, we talk about the part of PARP in the repair of oxidative harm versus the arrangement of OCDLs and estimate on the attainability of PARP hindrance for the treatment and avoidance of tumors by abusing its part in aggravation.(Swindall et al., 2013)

PARP comprises of a group of 17 proteins, in spite of the fact that to date, PARP1 and PARP2 are the main ones known to be required in DNA repair. The establishing relative PARP1 is an omnipresent atomic compound that distinguishes softens up DNA and flags downstream through its enzymatic movement(Reinbolt and Hays, 2013). What's more, PARP1 initiation brings about adjustment of many atomic proteins, bringing about a monstrous redesign of different cell capacities, which is instigated by dynamic PARP1. PARP2 was found after the perception of lingering DNA-subordinate PARP movement in embryonic fibroblasts acquired from PARP1-insufficient mice. PARP2 is known to connect with PARP1 and both are included with XRCC1, DNA polymerase-b, and DNA ligase III, which are indispensable parts of the BER and DNA SSB repair pathways(Espinoza). The elements of PARP2 don't totally cover with PARP1; notwithstanding considerable PARP2-incited PARP action in PARP1-knockout murine models with genotoxic incitement, genomic flimsiness is watched, recommending that PARP2 can't completely make up for the loss of PARP1.(Yap et al., 2011)

In human growths, expanded articulation of the PARP-1 quality has been accounted for in Ewing's sarcomas, and in threatening lymphomas. Conversely, diminished expression has been seen in a few gastric and colon tumor cell lines, and additionally in some breast cancers (Miwa and Masutani, 2007).

3. RESEARCH METHODLOGY

3.1. MTT Assay

MTT Assay was carried out to find the effect of increasing radiations on the viability of cells. MTT solution (5mg/ml) was first prepared using distilled water and 1x PBS. The solution was filtered and stored at 4c. A 96 well plate was used which contained colon cell lines HCT-15 which were grown for 24 hours. After 24 hours they were treated with different amount of radiations and left for another 24 hours. After that, the media was removed and 50ul of MTT solution was added to each well. After 3 hours the MTT solution was removed and 200ul of DMSO was added. Pipetting was done and the cells were analyzed for percentage of cell viability.

3.2. Animal Models

BALB/C mice were purchased from the National Institute of Health (NIH) Islamabad. 15 female mice were purchased that were 6 weeks old and were divided into 3 groups with 5 mice per group; control, 2 Gy Radiation and 6 Gy Radiation. The mice were given a week to acclimatize.

Figure 1: BALB-C Mice used for experimentation

3.3. DMBA and Croton oil

7,12-Dimethylbenz[a]anthracene (DMBA) was used to initiate the development of skin tumor in mice. DMBA was prepared by dissolving in acetone. 100mg DMBA was dissolved in 100 ml of acetone to get a final solution of 0.1% w/v. This was stored at -20C. 100ul DMBA was used in each application which gave a 400nmol dose of DMBA per application. (Filler et al., 2007)

Croton oil was used as a promoter for the development of tumors. Croton oil was purchased from Sigma Aldrich. 100ul of croton oil was applied to the skin using a brush to ensure maximal application. (Shuryak et al., 2011)

Melanoma appears to develop in 5–40% of its hosts after treatment with DMBA (Broome Powell et al., 1999).

3.4. Induction of tumor

The mice were prepared for DMBA application by first shaving off hair from their back. After shaving they were given a week before the first application of DMBA. After a week mice from each group were given the first application of the prepared DMBA solution. DMBA was applied six times until skin lesions were observed over a time period of 4 weeks. Similarly, Croton oil was applied 2 times per week on alternate days to DMBA application.

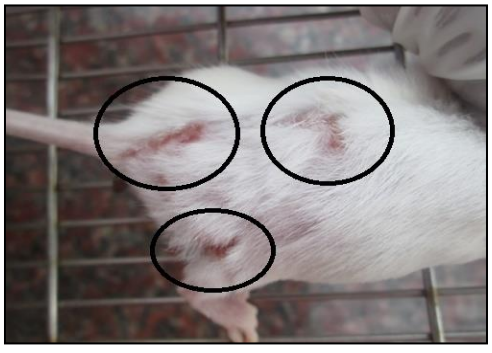

Figure 2: Tumor induced in animal models

3.5. Treatment

Control group was set as standard and it was left untreated. The other two groups were treated with gamma radiations from Pinstech Islamabad. One group was treated with the amount of 2 Gy of radiation while the other one was treated with 6 Gy of radiation.

3.6. Tumor Isolation

The mice were sacrificed and the tumor was isolated from within the skin and kept in liquid nitrogen. It was then transferred and stored in the cryopreservative freezer at -80C for further use. The samples needed preparation before being used for protein quantification and western blot analysis.

3.7. Sample Preparation

Samples from each group were taken and 2µg sample was immersed in 1ml of RIPA lysis solution and was homogenized to extract protein. 1ml of lysis solution was taken in a falcon tube along with 2µg sample and rapid pipetting was used to lyse the cells to extract proteins. The resulting solution was then centrifuged at 10,000 rpm for 20 minutes. The supernatant was extracted using a pipette while the pellet was discarded. The extracted protein was then stored at -80C to be used in protein quantification and western blot analysis.

3.8. Bradford Assay

Protein quantification was carried out using the Bradford protein assay. A standard curve is first drawn using BSA (Bovine serum albumin) to make different protein concentrations. 6 samples are made, with the following concentrations; 0, 2, 4, 6, 8, 10ug/ml. 0.1mg/ml stock solution of BSA was used to make these concentrations.

	H_2O (µl)	BSA (µl)	Bradford Reagent (µl)
0	800	0	200
2	780	20	200
4	760	40	200
6	740	60	200
8	720	80	200
10	700	100	200

Table 1: BSA Assay concentrations

The samples were vortexed after preparation and were incubated at 37°C for 5mins. Absorbance was then checked at 595nm using UV spectrophotometer. A standard curve was prepared which was then used to check the concentration of proteins in our samples.

A standard curve was prepared using the absorbance values and the regression line was plotted with an R^2 value of 0.99.

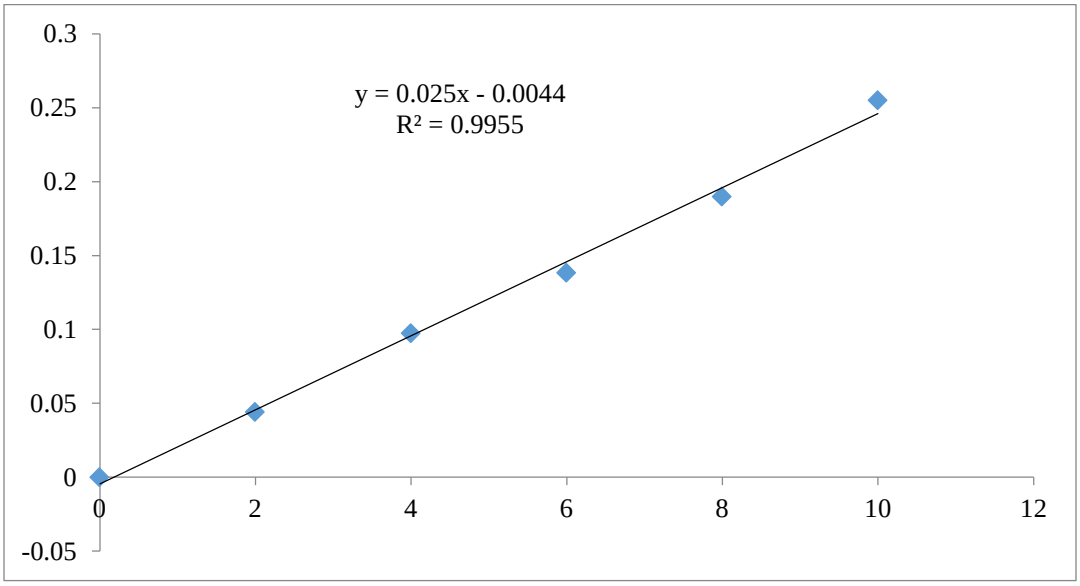

Figure 3: BSA Assay

3.9. Protein Quantification

Protein quantification was then carried out using the tumor samples. 3 samples from each group were taken for quantification. The samples were prepared at a concentration of 2µg/ml. For each sample 200µl of Bradford reagent, 798µl of H_2O and 2µl of sample was added in separate Eppendorf tubes. The tubes were then vortexed and incubated at 37°C for 5 minutes. Absorbance for each sample was checked at 595nm.

Using the equation y = 0.025x - 0.0044 obtained from the regression line obtained above, values for sample loading were calculated where y is the absorbance while x is the amount of our sample.

	Absorbance	**2ug**	**1ug**	**30 ug load**	**H_2O**	**4X**	**Total**
Control	0.11	4.45	2.23	13.48	1.52	5	20
2 Gy	0.25	10.14	5.07	5.91	9.09	5	20
6 Gy	0.18	7.40	3.70	8.10	6.90	5	20

Table 2: Sample load preparation

3.10. Western Blotting

Western blot analysis was carried out to identify proteins of interest. Mini Trans-Blot cell from BIO-RAD was used. The regression line equation was used to calculate the loading values for the samples.

3.10.1 SDS-PAGE electrophoresis

The concentration of the running gel was optimized and it was found that the best results were obtained at 12% gel. The samples were prepared by mixing with distilled water and 4X loading buffer. They were then kept at 95°C for 5–10 minutes and were then centrifuged. They were then loaded onto 12% SDS polyacrylamide gel. The gel was run

for 2 hours first at 90 volts and then at 120 volts using 1X running buffer in the electrophoresis chamber. A broad range protein marker (Santa Cruz) was run alongside the tumor samples.(Pak et al., 2004)

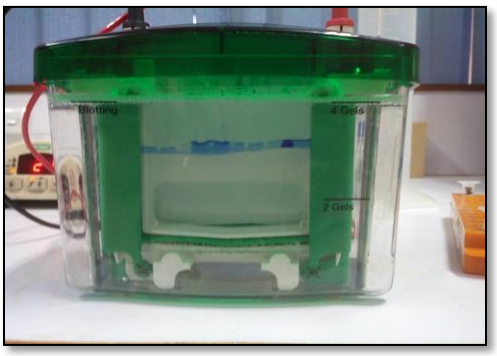

Figure 4: Gel running in Electrophoresis chamber

3.10.2 Transfer to NC membrane

After running the samples on the gel, the transfer of proteins onto Nitrocellulose membrane was carried out. The gel was sandwiched between two sponges, blotting papers and NC membrane. Transfer was carried out for 25 minutes for a single gel and 50 minutes for two gels at 90V. When two gels were being transferred simultaneously, their sides were switched at 25 minutes. 1X transfer buffer was used in the chamber for this process.

3.10.3 Ponceau S Staining

Ponceau S stain (Merck chemicals) was prepared using acetic acid and was used to stain the NC membranes. The stain made the bands and the protein marker visible for marking them before moving onto the next step i.e. blocking. The membranes were marked on the base of different molecular weights of proteins at specific places using the protein marker to identify the proteins of interest.

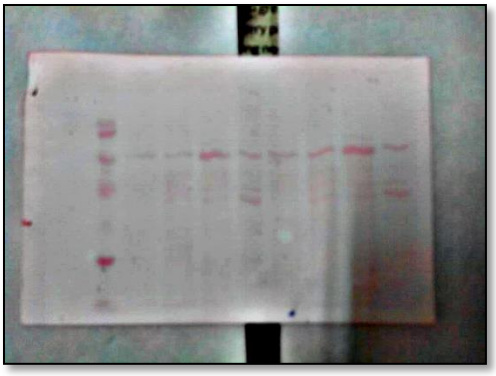

Figure 5: NC membranes stained by Ponceau S Stain

3.10.4 Blocking with non-fat milk

5% non-fat milk prepared in 1X TBS that was prepared by dissolving Tris and NaCl in distilled water. The membranes were marked and cut into strips. These strips were then kept in 5% non-fat milk for 2 hours at 4°C on a rocking platform. Blocking is done to prevent non-specific binding of the primary and secondary antibodies with the membrane.

3.10.5 Preparation of Antibodies

Antibodies were prepared using 1X TBS. Primary antibodies were prepared in the ratio of 1:1000 and secondary antibodies were prepared in the ratio of 1:2000. Primary antibodies included PKC-α, Caspase-3, PARP and MRP. Actin was also used as a loading agent. The secondary antibodies included those of rabbit, mice and goat.

3.10.6 Primary Antibody Incubation

The strips were removed from the non-fat milk and were incubated in primary antibody overnight at 4°C on a rocking platform. The next day, they were removed from the primary antibody solution and washed with TBST for 5, 5, 10, 10 and 10 minutes before being incubated in secondary antibody.

3.10.7 Secondary Antibody Incubation

After washing, the strips were then incubated in secondary antibody for two hours with shaking. After the two-hour incubation period was over they were washed again with TBST for 5, 5, 10, 10 and 10 minutes and were then used for signal detection.

3.10.8 Signal Detection using X-ray development

Signal detection was done using the ECL kit (Santa Cruz). The NC strips with our proteins were laid onto a clean paper and ECL reagent was dropped onto each strip. The strips were then transferred onto the plastic sheet and laid inside the X-ray cassette. X-ray sheets were fixed in the cassette and the cassette was properly closed which was then left for 30 – 40 minutes in a dark room. After fixing, the sheets were then used to detect the proteins by using the developer and fixer buffers. One X-ray sheet was left over-night and was developed after 24 hours for better results.

4 RESULTS

4.1. Cell Viability Assay

In the results of cell viability assay, the control group was set at 100% as standard. Comparative to that, the group which was given the radiation of 2 Gy showed 80% viability and that of 6 Gy showed 60% viability. This showed that the radiations we used were actually affective and they were killing the cells they were exposed to. The graph of cell viability assay is shown below.

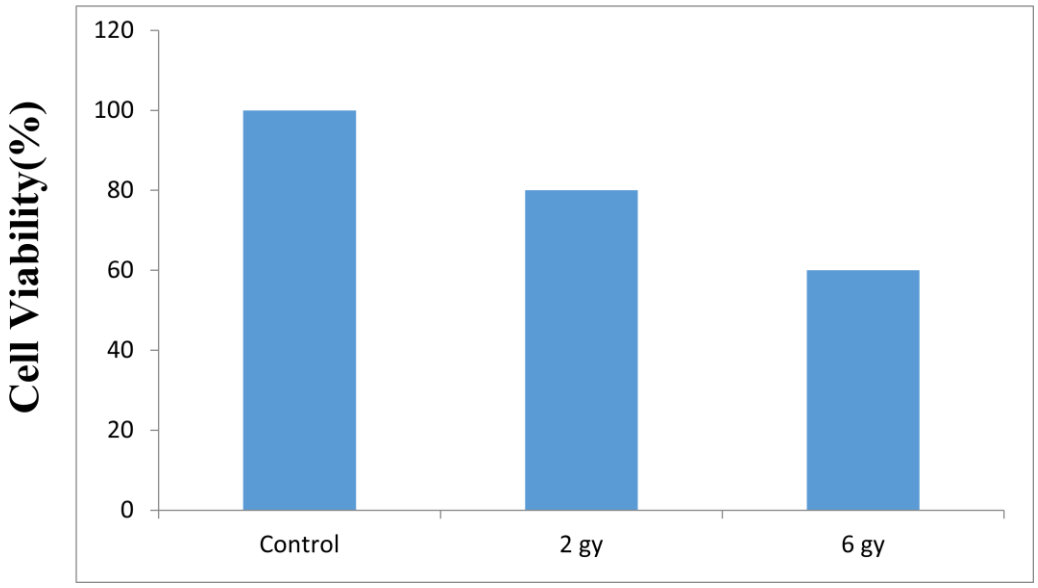

Figure 6: Cell Viability Assay

4.2. Tumor Volume

As the DMBA was applied to the skin of mice, the tumor started to appear in the form of white lesions. With the passage of time and continuous application of carcinogen DMBA followed by the promoter croton oil, the size of the tumor began to increase. The radiation was given to the mice in 5th week, after sufficient size of tumor was developed. The graph of tumor size increasing with time is shown below.

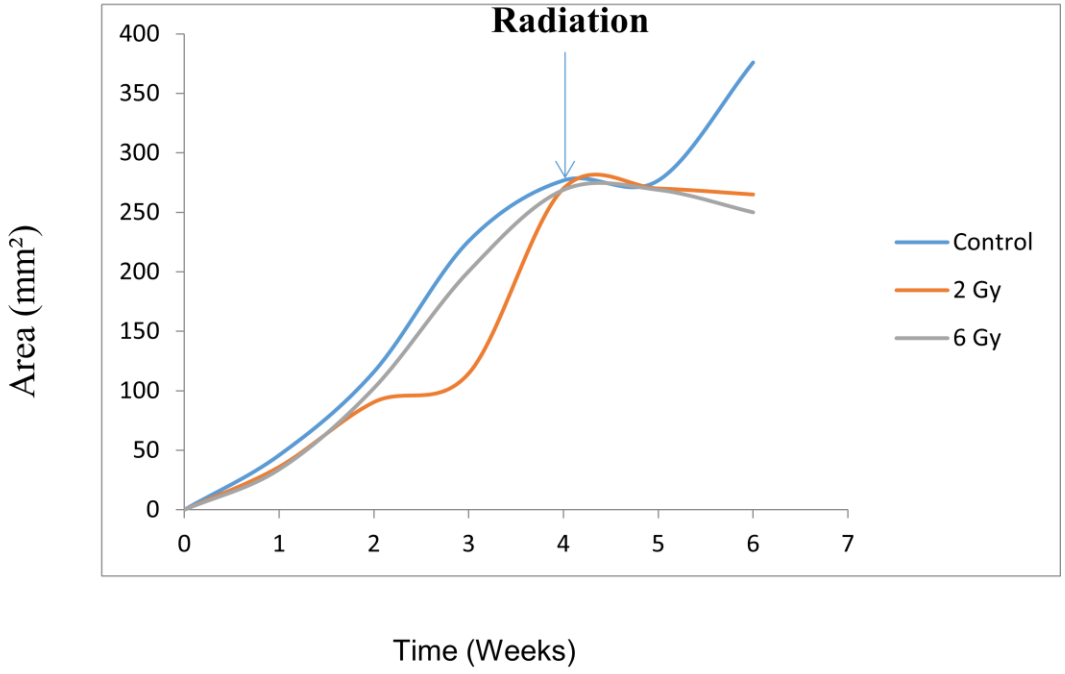

Figure 7: Tumor Volume

4.3. Western Blot Analysis

PKC-α, which is an onco-gene involved in cell cycle check points, showed maximum results in the control group and least in the 6 Gy group. The 2 Gy group had the results between control group and 6 Gy group. This shows that the amount of PKC-α was decreasing with the increase in the quantity of the radiation. *Caspase-3*, the apoptotic protein, showed the biggest protein band in 6 Gy group, comparatively smaller band in 2 Gy group and least visibility in control group. This shows that quantity of apoptotic proteins are being increased by the radiation therapy which is helpful for the treatment of cancer. *PARP* showed its behavior similar to that of caspase-3. It was increased with the increase in the amount of radiation. *MRP* was present in most quantity in the control group and least in 6 Gy group. By this we can say that resistance was developed in the tumor cells by the DMBA and the increase in PKC-α also promoted MRP and it was then inhibited by the inhibition of PKC-α by the radiations.

The X-ray results of the western blot analysis are shown in figure 8.

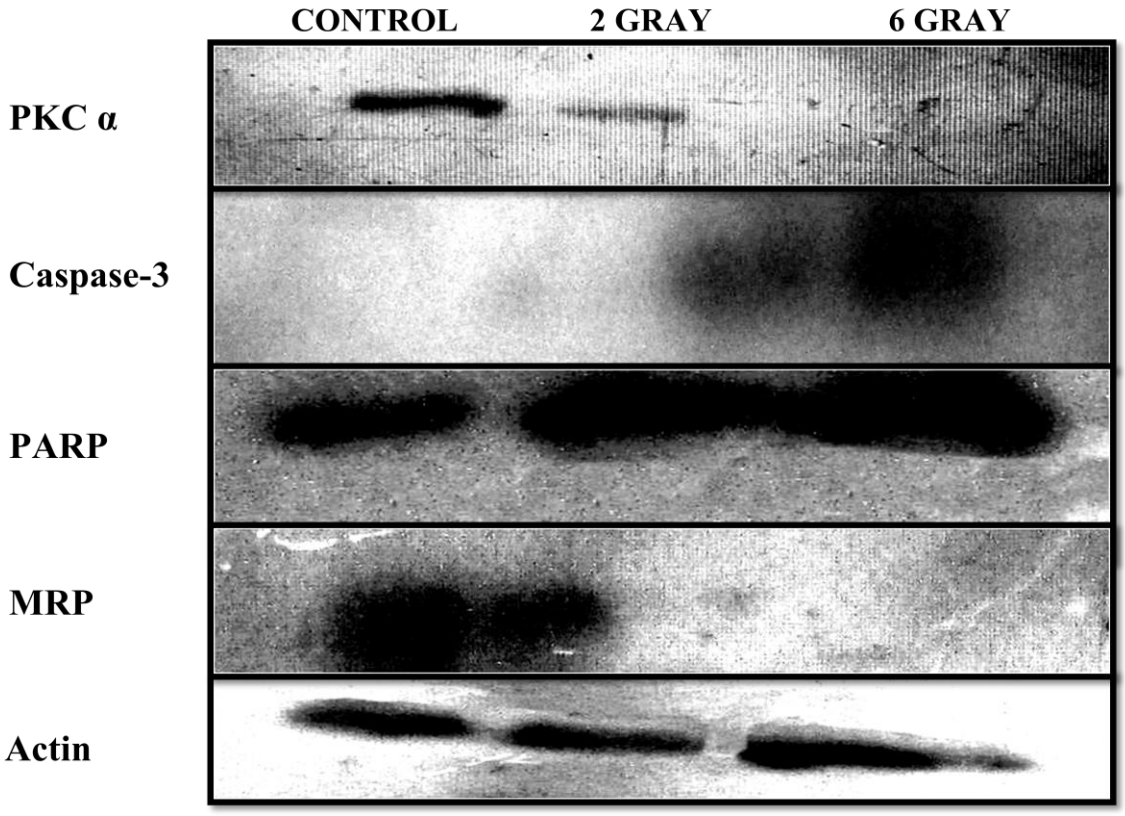

Figure 8: Western Blot results

5. CONCLUSIONS

By carrying out this study we got to know about the behavior of various proteins in cancer and the way they responded to the radiation therapy. When melanoma was induced by DMBA, the amount of PKC-α and MDR MRPs was elevated. The increase in the expression of PKC-α resulted in the decrease of tumor suppressor genes Caspase-3 and PARP. This is how the melanoma was induced in the mice. This is shown in the figure below.

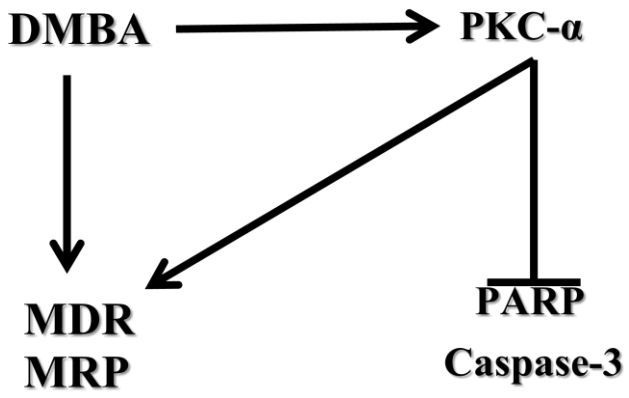

Figure 9: Effect of DMBA on PKC-α, PARP, Caspase-3 and MRP

After the radiations were given to the tumor induced mice, the expression of PKC-α was inhibited. As a result, the inhibition mechanism of PARP and Caspase-3 by PKC-α was also deceased. That is why, the PARP and Caspase-3 showed their most expression in 6 Gy group while the PKC-α showed its maximum expression in control group. This mechanism is shown in the following figure.

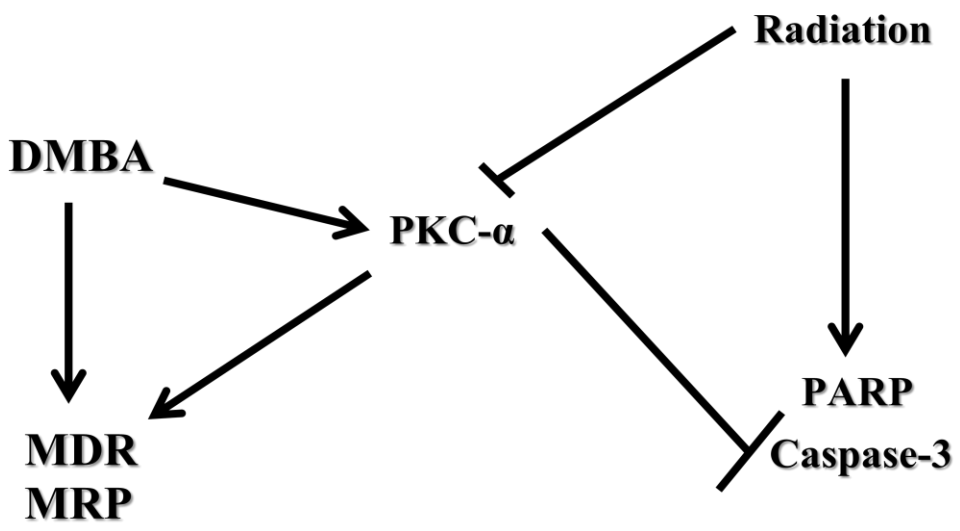

Figure 10: Effect of radiation on PKC-*α*, PARP, Caspase 3 and MRP

We clearly see that the MRPs are there to produce the resistance against the treatments of cancer. If they are not there at the first place, the treatment we provide to the subject could be more effective. Various drugs can be used as an adjuvant therapy along with radiations to minimize the effect of radioresistance. In this way, if radiation is treating the cancer, and the drug is opposing the effect of radioresistance, the tumors will be cured way early and easily.

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