A. The Heidelberg carbon-ion gantry, presently the only operating gantry for carbon ion treatment.
B. The Gantry 2 treatment room at the Paul Scherrer Institute. Gantry is used to refer a dedicated particle beam transport line that is designed to rotate around an axis for medical purposes. Adapted from [24].
CHAPTER 6: MATERIALS & METHODS
6.1 MATERIALS

6.1.1 Bio-chemicals

Bio-chemicals used in this study were: Bovine serum albumin (BSA), bromophenol blue, ethidium bromide, foetal bovine serum (FBS), hoescht 33258 dye, paraformaldehyde, propidium iodide and RNase A from Sigma-Aldrich, USA; Low melting agarose, normal agarose, sodium dodecyl sulphate (SDS) and Proteinase K from Boeringer Mannheim, Germany; Cell culture medium MEM, DMEM, trypsin, hygromycin B, ampicillin, and LB medium for bacteria from HiMedia, India; Triton X-100 and glycine from SRL, India; Rhodamine 123 and ProLong® Gold antifade reagent with DAPI from Molecular Probes, Life Technologies, USA; Nonidet P40 from Amresco, USA; MLV Reverse transcriptase with 1st strand buffer, dNTPs, oligo-dT primers, RNasin from Promega, USA; Taq DNA polymerase, 10X assay buffer, MgCl₂ from HiMedia, India; Gene specific primers for gene expression studies from Eurofins, MWG/Operon, USA; ApoAlert® Caspase 3 Fluorescence Assay Kit, ApoAlert® Caspase 3 Colorimetric Assay Kit & ApoAlert® Caspase 8 Fluorescent Assay Kit from Clontech, USA; PVDF membrane from Millipore, USA & GE Healthcare, USA; VisGlow plus Chemiluminescent Substrate, HRP from Visual Protein Biotechnology Corp, Taiwan. Other bio-chemicals and reagents of molecular biology grade used in this study were purchased locally.

6.1.2 Antibodies

The primary antibodies used in this study were: mouse anti-PARP-1 (sc-74469) & mouse anti-caspase-8 (sc-73526) from SantaCruz, USA; rabbit anti-CASP3 (700182), mouse anti-AIF (456200), rabbit anti-p21 (710182), rabbit anti-Ku80 (A13966), mouse anti-E2F-1 (32-1400) & mouse anti-NFkB (p65) (436700) from Invitrogen, USA; Alexa Fluor 488 conjugated custom made anti-Caspase-9 (ab32539) & rabbit anti-beta actin (ab151526) from Abcam, UK; FITC mouse anti-p53 (557026) from BD Pharmingen™, USA; mouse anti-PAR (10H) (ALX-804-220) from Enzo Life Sciences, USA; rabbit anti-MRE11 (4847), rabbit anti-Rad52 (3425), rabbit anti-phospho-H2A.X (Ser139) (9718), rabbit anti-phospho-Chk1 (Ser345) (2348), rabbit anti-phospho-Chk2 (Thr68) (2661) & mouse anti-phospho-p53 (Ser15) (9286) from Cell Signaling Technology, USA.

Secondary antibodies used for IF studies were goat anti-mouse IgG, FITC (sc-2078), goat anti-mouse IgG, FITC (sc-2081), donkey anti-mouse IgG, FITC (sc-2099) and donkey anti-rabbit IgG, R (sc-2095) from Santa Cruz Biotechnology, Inc., USA. For western blotting, anti-rabbit IgG, HRP-linked (7074) was used from Cell Signaling Technology, USA.
6.1.3 Cell lines

Human cervical epithelial carcinoma HeLa and HEK 293T cell lines were obtained from National Centre for Cell Sciences, Pune, India. These cell lines were used to prepare PARP-1 knocked down cells by siRNA mediated gene silencing.

6.1.4 Plasmid vector for knocked down experiments

For PARP-1 knocked down experiments, pRNA-U6.1/Hygro mammalian siRNA expression vector (shown in Figure 6.1.4) with and without siRNA insert designed for silencing PARP-1 gene expression was kindly provided by Prof. Nitai P. Bhattacharyya’s Lab, Crystallography & Molecular Biology Division, Saha Institute of Nuclear Physics (SINP), Kolkata. The DNA sequence of PARP-1 used for the designing of siRNA was

\[ \text{AGAGCGATGCGCTATTACTG} \]

from NCBI Reference Sequence: NM_001618.2; GI: 11496989. This was done with the help of online software in GenScript (https://www.genscript.com/ssl-bin/app/rna). The complete sequence cloned into the BamH1 and Hind III restriction enzymes cutting sites within the pRNA-U6.1/Hygro vector was

\[ \text{AGAGCGATGCGCTATTACTGTCAAGAGACAGTATAGGCAGCTCTTTTTTTCCAA} \]

Pink highlighted portion was for loop formation. Sequences in green highlight were the reverse sequence, while sky blue region was designed for termination. The vector carries Hygromycin resistance gene for establishing stable transfected cell line and uses U6.1 promoter for siRNA expression.

6.2 METHODS

6.2.1 Propagation of plasmid vectors

To get the good amount of vector plasmid for transfection purposes, pRNA-U6.1/Hygro (GenScript, USA) plasmid was propagated through bacterial transformation followed by plasmid isolation. E. coli DH5α cells (Clontech, USA) were made competent using CaCl₂ following the standard method as described in Cohen
et al [1] with slight modification. 50 ng of pRNA-U6.1/Hygro plasmid (5247 bp) with and without siRNA insert targeting PARP-1 gene was used for transformation and the transformed cells were grown in the presence of 100 mg/ml ampicillin in LB broth medium. Then the plasmid was isolated from the overnight culture of transformed E. coli DH5α using the standard method of mini-preparation by alkaline lysis with SDS as described in Sambrook et al [2]. Isolated plasmid was treated with RNase A (20 mg/ml) to avoid the RNA contamination. Finally the plasmid DNA was purified by a standard phenol–chloroform extraction procedure and checked through 1% agarose gel electrophoresis [2]. The purity and concentration of the isolated plasmid DNA were checked by a spectrophotometer. This pure plasmid DNA was used in all the transfection experiments.

6.2.2 Cell culture

Culture condition. Human cervical cancer cell line HeLa and HEK 293T were grown in MEM/DMEM supplemented with 10% foetal bovine serum (FBS) in presence of penicillin, neomycin and streptomycin (complete medium) at 37°C in humidified atmosphere containing 5% CO₂.

Preparation of PARP-1 knocked down cells. PARP-1 knocked down HeLa (designated as HsiI) and PARP-1 knocked down HEK 293T (designated as HEKsiI) cells were prepared by stable transfection of HeLa and HEK 293T cells with siRNA insert of PARP-1 containing plasmid pRNA-U6.1 using Lipofectamine™2000 according to the manufacturer (Invitrogen, USA) protocol and also described in elsewhere [3]. In brief, ~ 1 µg of plasmid DNA and 3 µl of lipofectamine were mixed in 250 µl of serum free MEM/DMEM in microcentrifuge tubes separately and kept it at room temperature for 10 min. Then both were mixed and allowed to incubate at room temperature for another 15 min. Now, the mixture was added to 20- 30% confluent HeLa cells in 60 mm plate containing 2.5 ml serum free MEM/DMEM medium. After incubation in CO₂ incubator at 37°C for 6h with occasional shaking (one hour interval), the medium was replaced with fresh complete medium (with serum) for overnight. Then the cells were grown in presence of 600 µg /ml of hygromycin B for at least one week to obtain the stable transfectants. For control both HeLa and HEK 293T cells were transfected with vector plasmid pRNA-U6.1 without siRNA insert of PARP-1 and designated as H-vector & HEK-vector respectively. These cells were used throughout the experiments along with the respective PARP-1 knocked down cells. All experiments using transfected cells were done within the 3-4 passages of cells.
6.2.3 Irradiation

**Low LET Gamma.** The cells were irradiated with various doses of gamma rays [Co\(^{60}\)] at Saha Institute of Nuclear Physics (SINP), Kolkata (Figure 6.2.3a). The dose rate was 57 rad/min. We used different doses at the range of 0 - 4 Gy. After irradiation immediately medium was replaced with fresh complete medium and incubated for 24 h as required for the experiments done.

**High LET Carbon Ion Beam (CIB).** The irradiation was carried out at the dedicated Radiation Biology Beam line of the 15UD Pelletron of Inter-University Accelerator Centre (IUAC), New Delhi, India (Figure 6.2.3b). In this experiment, 62 MeV [5.2 MeV/u] \(^{12}\)C beam with corresponding entrance LET 290 keV/μm was used. The cells were irradiated in a sterilized closed chamber which is a computer controlled automated irradiation system called ASPIRE (Figure 6.2.3c) installed at the Radiation Biology Beam Line. The system has the facility to place the 35 mm petri dishes in front of a 40 mm diameter window in order to be irradiated by the heavy ion beam having flux of about \(2 \times 10^5\) particles/cm\(^2\)/sec. The petri dishes without the lead were stacked and immersed in a tank filled with serum free medium during the entire duration of irradiation. The dishes were picked up remotely one by one for irradiation with preassigned dose and after exposure the plate was reimmersed back into the tank containing medium. The radiation field uniformity was better than 95% as ascertained during prior experiments using Solid State Nuclear Track Detectors. All the samples were irradiated within 13 - 18 h growth condition and after irradiation fresh complete medium was added into plates and incubated for the time period as required in the specific experiments. The dosimetry is done using the particle counts from a pair of Silicon Surface Barrier Detectors, out of which one is placed at the atmosphere in the exact sample position and the other inside beam line as a monitor. For measuring the particle fluence online as well as controlling the irradiation protocol, the monitor detector is calibrated prior against the counts from
the detector placed at the sample position. The dose in Gray (Gy) is calculated from the particle fluence using the relation:

\[
\text{Dose [Gy]} = 1.6 \times 10^{-9} \times \text{LET [keV/μm]} \times \text{Fluence [particles/cm}^2]\]

6.2.4 Clonogenic Cell Survival Assay

Cell death was measured by colony forming ability of adherent cells. After irradiation (0-4 Gy) both cells H-vector and PARP-1 knocked down HeLa (HsiI) were detached by trypsin, counted by Countess (Invitrogen, USA) or under haemocytometer (SIGMA), serially diluted, and finally plated at a density of 300 cells/tissue culture petri dishes (60 mm). For each dose, cells were seeded into 4 plates each with 4 ml of growth medium and allowed to grow for 10 days at 37°C in humidified CO\textsubscript{2} incubator. After 10 days of growth the visible colonies (approximately 50 cells in each colony) were fixed and stained with 50% ethanol having 0.25% methylene blue for at least 30 min. Then the colonies were visually counted. Surviving fraction was calculated by dividing the number of colonies found in irradiated samples by the number of seeded cells and the plating efficiencies of the un-irradiated control cells of each cell type.

6.2.5 DNA damage analysis

**Comet Assay.** DNA damage was measured using the alkaline single cell gel electrophoresis assay as described by Singh et al [5] with some modifications. In brief, H-vector and HsiI cells were exposed to \textsuperscript{12}C ion beam or gamma at different doses (varied from 0-4 Gy) and allowed to grow at 37°C for 24 h. Washed twice with phosphate buffered saline (PBS), \textasciitilde1 \times 10^4 cells were suspended in 0.6% low melting agarose and layered over a frosted microscopic slide previously coated with a layer of 0.75% (w/v) normal melting agarose. The slides were then immersed in a lysing solution of pH 10 (NaCl 2.5 M, Na\textsubscript{2}-EDTA 0.1 M, Tris 10 mM, and NaOH 0.3 M) and left overnight. The slides were then transferred to a horizontal electrophoresis chamber containing alkaline solution (300 mM NaOH, 1 mM Na\textsubscript{2}-EDTA) and allowed to soak for 20 minutes for unwinding of DNA. Electrophoresis was then carried out for 20 min (300 mA, 20 V). Slides were then washed thrice with neutralizing buffer (Tris 0.4 M, pH 7.5), stained with ethidium bromide (final concentration 40 μg/ml), and observed under a Zeiss fluorescent microscope with a proper filter. The extent of DNA damage was quantified by tail moment measurement, calculated by multiplying the total intensity of the comet tail by the tail length measuring from the centre of the comet head. % of tail DNA and % of head DNA of comets were also measured using the OpenComet (vi.3) plugin [6] in ImageJ software [7]. At least 200 cells were counted randomly for each slide and it was done for three times for each dose of each cell type.
**Immunofluorescence detection of γH2AX foci.** γH2AX foci formation is the marker of DSBs detection. DSB by ionising radiation or by any means results in rapid phosphorylation of the histone H2A family member H2AX at Ser 139 by ATM and known as γH2AX [8, 9]. This occurs within minutes and thousands of phosphorylated H2A.X localize to the sites of DSBs forming γH2AX foci [10]. For the measurement of DSBs and the kinetic study of DSB repair, detection of γH2AX foci by IF has been widely recognised as a standard method and described in elsewhere [11, 12].

For γH2AX foci detection, cells were grown over cleaned and sterile glass cover slips (10 mm diameter) inside the 35 mm petri plates for 14-18 h and irradiated with CIB having desired doses within 0-4 Gy followed by 24 h incubation. For time kinetic studies cells were incubated for 0.5 h, 2.5 h, 4.5 h, 9 h, 14 h and 24 h after CIB irradiation. After incubation cells were washed with phosphate buffered saline (PBS) thrice and fixed with 4% paraformaldehyde for 10 min at room temperature. Afterwards, cells were washed twice in PBS. Then cells were made permeable with 0.25% Triton X-100 in PBS for 10 min at room temperature and washed with PBS thrice for 5 min each followed by 30 min incubation with blocking solution having 1% BSA of 0.3 M glycine in PBST (0.1% Triton X-100). Now, cells were incubated with primary antibody rabbit anti-phospho-γH2AX (Ser 139) in PBST having 1% BSA (dilution 1:500) for overnight at 4°C, washed with PBS thrice of 5 min each in rocker at room temperature. Then cells were incubated with rhodamine conjugated donkey anti-rabbit-IgG secondary antibody in PBST having 1% BSA (dilution 1:400) for 1 hr at room temperature in dark, and rinsed with PBS thrice of 5 min each in rocker at room temperature in dark. Finally cells attached in cover slips were mounted with ProLong® Gold antifade reagent with DAPI (Molecular Probes by Life Technologies, USA) in cleaned, grease free glass slides and stored at 4°C in dark condition prior to observation. Images were captured using Multidimensional Acquisition module of AxioVision Release 4.8.2 of Carl Zeiss Fluorescence Microscope or using monochrome camera in AxioScope AI of Carl Zeiss Fluorescence Microscope. Images taken using monochrome system were converted into RGB mode by putting pseudo-colour using Zen software of Carl Zeiss, Germany.

Number of foci was counted manually and the intensity of foci was measured by ImageJ software [7] as described in section 6.2.11. Fluorescence intensities of γH2AX foci of irradiated cells were normalised with the background fluorescence and fluorescence of un-irradiated cells if any found by ImageJ software. Data were analysed in three different ways- number of cells found having at least one foci, number of foci per cell and intensity of foci per cell.

**6.2.6 Cell Cycle analysis by Flow Cytometer**
Cell cycle analysis was done according to the following procedure. After irradiation with different doses (0–4 Gy) of CIB or gamma radiation followed by 24 h of post-irradiation incubation, cells were trypsinized and washed twice with cold PBS. Time dependent study was also carried out using CIB only to check the initial effect of high LET radiation on HeLa cells and the role of PARP-1 in cell cycle regulation. Now cells were fixed in chilled 70% ethanol in PBS for 2 h at 4°C. After fixation, cells were washed thrice with cold PBS and then stained with a solution containing 10 μg/ml propidium iodide (PI), 100 μg/ml of DNase-free RNase A, and 0.1% (v/v) Triton X-100 in the dark for 15 min at room temperature before flow cytometric analysis. Now, the nuclei of each cell were become labelled with PI and the PI fluorescence of individual nuclei was determined by a fluorescence-activated cell sorter (FACS Calibur, BD Bioscience, USA) using the blue laser of 488 nm excitation. Data acquisition was done using CellQuestPro software (Becton Dickinson) and for each sample 20,000 cells were taken. The percentages of cells in each phase of the cell cycle was analyzed using ModFit LT software (Variety Software) supplied by BD Bioscience, USA.

6.2.7 Gene expression studies

Total RNA extraction. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s protocol with slight modification. Both H-vector and HsiI cells were irradiated with CIB either dose-dependently (0–4 Gy) for 24 h or time-dependently (0 h, 0.5 h, 3.5 h and 7 h) at 1 Gy/2 Gy. After proper incubation cells were washed with cold PBS twice. Cells were lysed with 0.5 ml TRIzol reagent (for 35 mm culture dish) and it was kept for 15 min at room temperature. Now, 100 μl chloroform was added with proper mixing and kept for 5 min. After centrifugation at 12,000 x g for 15 min at 4°C clear supernatant (~250 μl) was taken into fresh tube and 250 μl of isopropanol was added to it. After proper mixing it was kept for 10 min at room temperature. Then centrifugation was done at same condition stated earlier for 10 min. Pellet was recovered and 0.5ml of 75% ethanol (in DEPC treated water) was added to it. After mixing well it was centrifuged at 7,500 x g for 5 min at 4°C. After briefly air drying the pellets were dissolved in DEPC (0.1%) treated water and heated at 55°C for 10 min. RNA concentration was checked spectrophotometrically. The purity (A260/A280) was found within 1.70 to 1.90.

cDNA preparation. Complementary DNA (cDNA) was prepared from total RNA according to the protocol described by Ghosh and Bhattacharyya [13] with some modifications. For each reaction of cDNA preparation 1 μg of RNA and 100 ng of oligo-dT were taken in a 200 μl microcentrifuge tube so that the volume of the mixture will be 10 μl. Now it was heated at 70°C for 10 min and kept on ice immediately for 2-5 min. Then 10 μl of RT-mix (containing 5X first strand buffer, MLV reverse transcriptase, RNasin and dNTPs) was added to it and kept at room
temperature for 15 min. After that, the total mixture was incubated at 37°C for 1 h. Now the samples were heated at 90°C for 5 min and immediately kept into ice for 10 min. These RT-products were used directly as template for polymerase chain reaction (PCR) or stored at -20°C for later use.

Reverse transcriptase PCR (RT-PCR). For several gene expression studies at mRNA level, RT-products were used for PCR using different sets of gene specific primers as mentioned in Table 6.2.7. The volume of the reaction was 25 μl and other essential reagents were used as recommended by the supplier of Taq polymerase (HiMedia, India). PCR was carried out in GeneAmp PCR System 2400 (Applied Biosystem, USA) with the annealing temperatures as calculated from the primers of respective genes. Beta actin was used as an internal control. The PCR products were resolved on 1.5-2.0% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide. Gel pictures were photographed using Sony Digital Camera under UV Transilluminator or gel images were taken in Typhoon 9210 – Variable Mode Imager (GE Healthcare, USA). Band intensities were calculated using ImageJ software [7]. Fold change of respective gene expression was obtained after normalising with beta actin.

### Table 6.2.7: List of gene specific primers

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Primer Sequence</th>
<th>PCR Product size (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casp-3</td>
<td>F: 5'-TGCGGCATTGAGACAGAC-3'</td>
<td>159</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CATGGGACAAAGCGACTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP-1</td>
<td>F: 5'-CGTGTGGGTACGGTGA-3'</td>
<td>169</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGCCATAGTCACAATCTCAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-TCCTGTGGCGATCCACGAACT-3'</td>
<td>315</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAAGCATTTGGCGGTGGAC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.2.8 Protein level analysis

**Preparation of whole cell lysate.** Whole cell lysate was prepared using the method as described in [14]. In brief, after washing with PBS twice cells were trypsinised and again washed with 1 ml of wash buffer (Table 6.2.8a).

Then cell lysis was done in ice for 30 min using the lysis buffer having the following composition (Table 6.2.8b). Clear supernatant was obtained after centrifugation at 12,000 X g for 15 min at 4°C. Protein content was estimated by standard Lowry’s method.

### Table 6.2.8a: Wash buffer Composition

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-KOH (pH 7.5)</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>DTT</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

### Table 6.2.8b: Lysis buffer Composition

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl₂ (pH 7.5)</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>β-ME</td>
<td>0.75%</td>
</tr>
<tr>
<td>CHAPS</td>
<td>0.5%</td>
</tr>
<tr>
<td>CHAPS</td>
<td>0.5%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
</tr>
</tbody>
</table>
Protein separation in 10% SDS-PAGE. 35 µg of protein was separated in standard 10% SDS-PAGE in 1X Tris glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at 120 V. After completion of electrophoretic run, the protein gel was processed for immunoblotting.

Immunoblotting. The standard wet transfer method was followed for immunoblotting as described by Sambrook et al [2]. Proteins were transferred into PVDF membrane in transfer buffer (25 mM Tris base, 192 mM glycine, 10% methanol) for 2 h in 4°C chamber with 300 mA current. Then the membrane was blocked with 5% BSA in tris buffered saline (TBS, 25 mM Tris-Cl pH- 7.5, 0.15 M NaCl) for 1 h at room temperature. After that, the blot was incubated with primary antibodies Ku80 (1:2500) & beta-actin (1:6000) separately in TBS-T (TBS containing 0.05% tween-20) for overnight at 4°C on a gel rocker.

Immunodetection. After washing four times with TBS-T, the blots were incubated with host specific secondary antibody conjugated with horse reddish peroxidase, HRP (1:5000) in TBS-T at room temperature for 1 h with constant shaking condition. Again it was washed four times with TBS-T and finally developed using VisGlow plus Chemiluminescent Substrate of HRP (Visual Protein Biotechnology Corp, Taiwan). Images of the protein bands were captured by Typhoon 9210 – Variable Mode Imager (GE Healthcare, USA).

6.2.9 Apoptosis detection

Several key experiments were performed to detect induction of apoptosis like DNA ladder formation, morphological identification of apoptotic body formation, quantification of sub-G1 population, activation of several caspases, measurement of mitochondrial membrane potential (MMP) & apoptosis inducing factor (AIF) translocation study.

Nucleosomal ladder formation. Induction of apoptosis in irradiated cells was determined by formation of nucleosomal ladder at different doses (0- 4 Gy) using conventional 1.5% agarose gel electrophoresis as described elsewhere [15- 17]. In brief, after irradiation with CIB followed by 24 h incubation in complete medium cells were collected by trypsinization, counted in Countess (Invitrogen, USA) and washed with PBS. The equal number of cells was pelleted and lysed in lysis buffer (50 mM Tris, 20 mM EDTA, 1% Nonidet P40) for 10 minutes at room temperature. To collect the supernatants, lysate was centrifuged at 2,800 rpm at room temperature for 10 minutes and it was repeated for 3 times. Now SDS was added to the supernatants at a final concentration of 1% and the mixture was allowed to RNase A treatment (final concentration 0.1 mg/ml) for overnight at 56°C. Then Proteinase K treatment (final concentration 0.1 mg/ml) was done for 6 h at the same temperature. After that, ½ volume of 10 M ammonium acetate was added, followed
by the addition of 2½ volumes of absolute ethanol. Whole solution was mixed well and kept at ~20°C for overnight. After centrifugation at 12,000 rpm at 4°C for 15 min DNA was precipitated out. The pellet was air dried and dissolved in ~15 μl sterile water. Now samples were mixed with gel-loading dye (0.25% bromophenol blue, 30% glycerol) and loaded into 1.5% agarose gel, separated by applying electric field. Then the gel was stained with ethidium bromide solution (0.5 μg/ml) and visualized under UV trans-illuminator. The gel was photographed by Sony Digital Camera. Un-irradiated cells were processed in the same way and used as control. This experiment was repeated three times and a typical gel picture was shown in Chapter 4 (section 4.2.7).

**Nuclear fragmentation or apoptotic body formation.** Nuclear fragmentation or apoptotic body was detected as described by Saha et al [18]. Briefly, both the cells H-vector and HsiI were grown over glass cover slips inside the 35 mm culture plates overnight and then irradiated with CIB and gamma radiation at different doses (varied from 0- 4 Gy) for 24 h. Now the cover slips were washed twice with PBS and the cells were fixed using 1 ml. of fixative solution of Methanol: Acetone (1:1) for 1 h in 4°C condition. Again the cells were washed twice with PBS. Finally, it was stained with Hoechst 33258 dye (1 μM), incubated for 10 min in dark, and washed with PBS. Cells were examined by fluorescence microscope (Carl Zeiss) using the appropriate filter. Apoptotic cells were distinguished by nuclear fragmentation, chromatin condensation followed by apoptotic body formation. The apoptotic cells as well as the normal cells were randomly counted (approximately 250 cells for each dose) and percentage of apoptotic cells was calculated at each dose. The mean percentage of apoptotic cells with standard deviation was calculated from four independent experiments.

**Sub-G1 quantification by Flow Cytometer.** Apoptosis was also detected by quantifying the sub-G1 cell population from the cell cycle profile by fluorescence-activated cell sorter (FACS) as described in this Chapter (section 6.2.6). Both H-vector and HsiI were irradiated with CIB (0- 4 Gy) and incubated for 24 h. Then cells were processed as mentioned earlier. Fluorescence of propidium iodide (PI) was measured by FACS (FACS Calibur, BD Bioscience, USA) using the blue laser of 488 nm excitation. Acquisition of 20,000 cells for each sample was done using CellQuest Pro software (Becton Dickinson). Now, sub-G1 population was marked in the histogram plot of number of counts vs. FL2A (DNA content) using the marker (‘M’) tool from the CellQuest Pro software. For statistical analysis the experiment was repeated three times and the induction of apoptosis by CIB was compared with the un-irradiated control cells.

**Assay of caspase-3 & caspase-8 activity by fluorimetric & colorimetric methods.** Caspase-3 and caspase-8 activity assays were performed according to the protocols recommended by the manufacturer (ApoAlert® Caspase 3 Fluorescence Assay Kit,
ApoAlert® Caspase 3 Colorimetric Assay Kit and ApoAlert® Caspase 8 Fluorescent Assay Kit from Clontech, USA). In short, after irradiation with different doses (0-4 Gy) of carbon ion beam and gamma radiation cells were grown for further 24 h. After trypsinization, cells were counted by Countess (Invitrogen, USA). Two million cells were taken, washed with cold PBS, and pelleted down. Then cells were lysed in 50 μl of chilled lysis buffer (as provided by the respective manufacturer) on ice for 10 min. The lysate was centrifuged at 18,000g for 5 min at 4°C to precipitate cell debris and supernatant was collected. 50 μl of 2X reaction buffer containing 10 mM DTT was added to it. Five μl of caspase-3 substrate (1 mM) - DEVD-AFC (for fluorimetric assay) and DEVD-pNA (for colorimetric assay), was added to each reaction mix and allowed to incubate for 1 h at 37°C water bath whereas, for caspase-8 it was IETD-AFC (1 mM) for fluorimetric assay. The fluorescence of liberated AFC was measured in Spectrofluorimeter with an excitation at 400 nm and an emission at 494 nm whereas the absorbance of liberated free pNA was measured in Spectrophotometer at 405 nm. To check the enzyme-substrate specificity, the inhibitors of the caspase-3 and caspase-8 (supplied by the manufacturer in the assay kits) were used with their respective substrates during the assay and found inhibited by the inhibitors showing the specificity of the assays (data not shown).

**Activation of caspase-8, caspase-9 & caspase-3 by IF studies.** Activation of caspases was also monitored after 24 h of CIB irradiation by IF as described in detail in this Chapter (section 6.2.5). In case of caspase-3, the antibody used in this experiment recognises specifically the cleaved caspase-3, indicating the activation of caspase-3 activity. Rest two antibodies for caspase-8 and caspase-9, recognise both pro and active form of respective enzyme. For caspase-3 and caspase-8 host specific rhodamine conjugated and FITC conjugated secondary antibodies were used for detection purposes respectively. As caspase-9 antibody was conjugated with Alexa Fluor 488, there was no need to use secondary one. Images were captured using Multidimensional Acquisition module of AxioVision Release 4.8.2 of Carl Zeiss Fluorescence Microscope.

Dilutions of primary antibodies were: anti-caspase-3 (1:300), anti-caspase-9 (1:175) and anti-caspase-8 (1:200). All secondary antibodies were used at 1:300 dilutions.

**Measurement of mitochondrial membrane potential (MMP) by IF & flow cytometry.** Rhodamine 123 is a well-known cationic fluorescent probe for mitochondria in living cells to measure MMP and it has been routinely used in cell biology of mitochondria [19, 20]. First, cells were grown over cleaned, sterile glass cover slips of 10 mm diameter inside the 35 mm tissue culture Petri dishes for 14-18 h. After irradiation with 0-4 Gy of CIB cells were incubated for 24 h at 37°C in humidified atmosphere containing 5% CO₂. Then Rhodamine 123 probe was added into the cultured Petri dishes at the final concentration of 0.1 μg/ml for 10 min in dark condition. After that, culture medium was removed and cover slips were washed with PBS twice.
Then cover slips were taken out and placed upside down on glass slides and examined using AxioVision Release 4.8.2 of Carl Zeiss Fluorescence Microscope.

MMP was also analyzed for un-irradiated H-vector, HsiI, HEK-vector and HEKsiI (PARP-1 knocked down HEK 293T) cells by standard flow cytometric method with some modifications [21]. In short, the cells (approximately $10^6$ cells) were trypsinised after 24 h growth condition and washed with PBS thrice. Then Rhodamine 123 probe (at final concentration of 0.1 μg/ml) was added into the cells suspended in 1 ml PBS with 1% FBS and incubated for 10 min at 37°C in dark condition. Finally, cells were analyzed by a fluorescence-activated cell sorter (FACS Calibur, BD Bioscience, USA) using the blue laser of 488 nm excitation and emission at 530±20 nm (FL1-H filter). Data acquisition was done using CellQuestPro software (Becton Dickinson) and for each sample at least 10,000 cells were taken. The overlay histogram plots were generated using the same software.

**AIF translocation by IF.** Translocation of AIF from cytoplasm or specifically mitochondria to nucleus is a characteristic feature of caspase-independent pathway of apoptosis. Here this phenomenon was studied after 24 h of CIB irradiation (0-4 Gy) in both H-vector and HsiI cells following the standard IF technique as described earlier in this Chapter (section 6.2.5). The primary antibody mouse anti-AIF was used at the dilution of 1:300 and goat anti-mouse IgG-FITC secondary antibody was used for the immune-detection purpose. Analysis of the images for AIF translocation study is described in Chapter 4 (section 4.2.6). Analysis from the statistical point of view is mentioned later.

**6.2.10 Cellular NAD$^+$ estimation**

Cellular NAD$^+$ was estimated to measure the PARP-1 activity in HeLa, HsiI (PARP-1 knocked down HeLa) and H-vector (HeLa transfected with only vector plasmid). Whole cell lysate was prepared using the method as described earlier (Section 6.2.8). Protein content was estimated by standard Lowry’s method. Now this whole cell lysate was used for NAD$^+$ assay according to the protocol described by Putt et al [22]. This is a fluorescence based assay measured by spectrofluorimeter. 30 μg of lysate was taken in assay buffer (10 mM Tris pH 8.0, 0.4 mM MgCl$_2$) in 50 μl of volume. Now 20 μl of 2 M KOH was mixed well with it. 20 μl of 20% acetophenon (in ethanol) was then added and kept at 0°C for 10 min. Finally, 90 μl of 88% formic acid was added into the mixture and boiled for 5 min before taking the fluorescence signal at 444 nm with an excitation at 372 nm. Now the content of cellular NAD$^+$ was obtained from the standard curve of known [NAD$^+$] vs fluorescence intensity as measured by the same way.

**6.2.11 Image analysis & Artwork preparation**
The captured photographs were analyzed for relative quantification of protein expression or fluorescence intensities of the fluorophores used in the respective experiments using ImageJ software [7, 12]. Fluorescent areas were manually selected and the intensities were measured. Then the intensities in unit area were calculated. In the same way background intensities were also measured. Then we subtracted background intensities/unit area from the intensities/unit area of fluorescently labelled cells. We considered at least 200 cells (until and unless mentioned in the respective method section) selected randomly for each dose and final results were obtained from three independent experiments. The intensities of PCR product bands and western blot bands were also quantified by this ImageJ software.

All the artworks were prepared using freely available Adobe Photoshop software for the final representation of the data used in this study.

6.2.12 Statistical analysis

Statistical analysis was performed to calculate significance level for the irradiated samples of each cell type with respect to the un-irradiated control of each cell type using one way ANOVA with Dunnett’s test from IBM SPSS Statistics Version 21 software. The significance values were denoted as ‘*’ (0.01<p≤0.05), ‘**’ (0.001<p≤0.01) and ‘***’ (p≤0.001). We did 2-tailed paired-samples T-test using the same software to calculate the statistical significance between H-vector and Hsil cells or CIB and gamma radiation for comparative study at a particular dose and significance values were denoted as ‘#’ (0.01<p≤0.05), ‘##’ (0.001<p≤0.01) and ‘###’ (p≤0.001) as mentioned in the text. We measured Pearson's Coefficient (r) between the green pixels (localization of AIF) and blue pixels (location of nucleus) using JACoP plugin [23] for statistical analysis of AIF translocation from mitochondria to nucleus in ImageJ software [7]. Each experiment was repeated at least 3 times unless otherwise specified.

6.3 REFERENCES

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