Gamma Radiation Induced Intestinal Proteomic Modulation in Mice: A Two Dimensional Electrophoretic Analysis

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ABSTRACT

Exposure to high doses of radiation causes serious injuries in gastrointestinal tract, by affecting biomolecules of the tissue. To demonstrate the modulation of intestinal proteome by ionising radiations, we analysed changes in protein expression in 9 Gy irradiated C57BL/6 mice at 24 h and 72 h by using two dimensional electrophoresis technique. A total of 19 protein spots with statistical significance (fold change>1.5 and P<0.05) were found to be differentially expressed. Of these 07 spots were identified by MALDI-TOF MS and peptide mass fingerprinting techniques which matched with the known proteins documented in the online database. These proteins belong to biological-functional categories like cytoskeleton system, molecular chaperones, DNA damage response, and stress response. These identified radiation induced proteins can help in understanding the mechanisms behind the intestinal injuries and thus can become potential targets for therapeutics and also aid in drug development.

Keywords: Ionising radiations; GI; Proteomics; Two dimensional electrophoresis; MALDI-TOF MS

1. INTRODUCTION

With technological advancements around the world and increased power consumption, use of nuclear sources in various sectors has tremendously increased. This has led to increased exposure of human beings to high doses of ionising radiations during radiation therapies, nuclear power reactors accidents, casual disposal of radioactive materials or use of radiological nukes in the form of dirty bombs by the terrorist groups etc. It has been reported that post exposure to very high doses of radiations, symptoms like nausea, vomiting, headaches and skin burns appear\(^1\). The main cause of appearance of all the symptoms indicates direct damage or indirect oxidative damage to biomolecules of the cell (DNA, proteins and lipids) which induces complex biological responses, cellular death leading to dysfunction of radiosensitive organs.

Various research studies have proved that proteins, the fundamental entities of the cell, face detrimental effects from radiation exposure such as alteration or/and abolishment of the signalling networks, specific structural and chemical alterations, including oxidation, fragmentation, decarboxylation, aggregation, breakage of disulfide bonds, formation of disulphide radicals, release of aromatic amino acids etc\(^2,3\). These alterations cause specific changes in expression level of the proteins in that particular physiological state with respect to normal conditions. Therefore various experiments have been conducted globally to study the effect of radiations on protein expression in different organs in different physiological conditions with the help of quantitative proteomic analysis methodologies\(^4,5\).

Two-dimensional electrophoresis (2DE) coupled with mass spectrometry (MALDI-TOF MS) is a traditional and the method of choice for conducting quantitative proteomic analysis. This molecular methodology is sensitive to changes in protein expression levels and thus has found its application under identification of low abundance proteins, novel proteins, post translational modifications, quantification of protein expression profiling in different physiological states which further help in organ specific or condition specific or both biomarker development\(^6,7\).

Radiation related functional abnormality in gastrointestinal tract is a major problem after whole body or abdominal exposure\(^8\). Due to a constant phenomenon of cell division and differentiation in the crypt cells of villi, GI is one of most radiosensitive organ of the body\(^9\). With major function of digestion of food and absorption of nutrients, vitamins and water it also harbour’s the bacteria which is required for
maintaining proper homeostasis and good health of the body. But upon radiation exposure not just due to radiation accidents but also during abdominal radiotherapy, patients suffer from problems like gut lining perforation, malabsorption, bacterial infection, fistula, strictures, fibrosis etc. An approved therapeutic measure for radiation injury of GI tract is still not available. Only surgery and supportive care is the help given to these patients. Hence, expression profiling studies of radiosensitive GI proteome may provide a valuable support for injury treatment management not only in case of accidental radiation exposures but also for radiotherapy patients.

The previous differential proteomic studies with 2DE and MS of intestinal tract have identified ERP29, TA1, PGK1, FAS pathway and Glycolysis as most affected critical protein targets and signalling pathways post radiation exposure respectively. But these have been confined to either low dose or localised radiation dose and a single study which reported ERP29 as radiosensitive protein in small intestine was conducted with high dose (9 Gray) of total body irradiation. Thus further extensive study is required for better understanding of molecular mechanisms behind radiation induced gastrointestinal injury. The current study is focused on differentially expressed proteins of lethally irradiated (9 Gy) mice which were analysed at 24 h and 72 h by 2DE methodology. From 2DE gel maps of pH range 4-7, protein spots with expression change over 1.5 fold were identified by MALDI-TOF MS. Further their biological functions were correlated with their expression patterns to understand the cellular and molecular mechanisms that regulate pathophysiology of GI tract of irradiated mice.

2. MATERIALS AND METHODS

2.1 Chemicals

Acrylamide, bisacrylamide, tris base, glycine, sodium dodecyl sulphate, dithiothreitol (DTT), iodoacetamide, urea, thiourea, ethylenediaminetetraacetic acid (EDTA), ethanol, sodium carbonate, sodium acetate, sodium thioglucontate, silver nitrate, glacial acetic acid, formaldehyde, ammonium per sulphate (APS), Tetramethylethylenediamine (TEMED), bromophenol blue, agarose, bradford reagent, protease inhibitor cocktail, coomassie dye, acetonitrile, HPLC grade water, ammonium bicarbonate, trifluoroacetic acid, bis-[3-Cholamidopropyl]-dimethylammonio]-1-propanesulfonate hydrate (CHAPS), potassium ferricyanide were purchased from Sigma-Aldrich Inc. (St. Louis, USA) and Fluka (Buchs, Switzerland). Immobilised pH gradient (IPG) strips, mineral oil, equilibration buffer I, equilibration buffer II, biolytes, were purchased from Bio-Rad laboratories Pvt. Ltd. Sequencing grade trypsin was purchased from Promega corporations (Madison,WI), 2-Cyano-3-(4-hydroxyphenyl) acrylic acid (HCCA Matrix), Peptide calibration standard II were obtained from Bruker Daltonik GmbH and PlusOne Silver Staining Kit, Protein from GE Healthcare Bio-Sciences AB (Sweden).

Tissue lysis buffer (7 M Urea, 2 M thiourea, 4 per cent CHAPS, 60 mM DTT, 0.5 per cent PI cocktail, 0.2 per cent biolytes), rehydration buffer (7 M Urea, 2M thiourea, 2 per cent CHAPS, 50 mM DTT, 0.2 per cent biolytes), 1X phosphate buffer saline (8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium phosphate dibasic, and 0.25 g of potassium phosphate monobasic in 1 l of distilled water at pH 7.4), extraction buffer (1:1 proportion of 0.1 per cent trifluoroacetic acid/100 per cent acetonitrile), resuspension buffer (1:1 proportion of 50 per cent acetonitrile: 0.1 per cent trifluoroacetic acid ) were prepared in laboratory.

2.2 Animals

Male C57BL/6J mice weighing 25 g - 30 g were procured from the in-house animal facility of Institute of Nuclear Medicine and Allied Sciences (INMAS), Delhi. Six animals were housed together in a cage on dust free husk as the bedding material with free access to recommended food pellet and water ad libitum. Ambient temperatures, 25 °C+2 and 12:12 light:dark cycle were maintained.

2.3 Ethics Statement

The maintenance and care of animals during experimental procedures were as per the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of INMAS (INM/IAEC/2013/03, dated 06.06.2013) and conformed to the general national guidelines that were set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

2.4 Irradiation, Treatment and Time Points

Post acclimatisation for a week, four experimental groups with three mice each was formed.
(i) Sham-irradiated control,
(ii) Total body irradiation (9.0 Gy) - 24 h
(iii) Total body irradiation (9.0 Gy) -72 h

For the groups (II) and (III), mice placed in a cage were exposed to total body (TBI) γ- irradiation of 9 Gy in 60Co tele therapy unit (Coabt Tele Therapy, Bhabatron II, Panacea Medical Technologies Pvt. Ltd, India) with source to sample distance set at 120 cm and dose rate of 1.08 ±0.955 Gy/min. The procedure was performed under the supervision of a Radiation Safety Officer. The control mice group was sham irradiated and experiments were repeated twice.

2.5 Sample Preparation

Sham-irradiated control and the treated mice were sacrificed at 24 h and 72 h after total body irradiation. The GI tissue was removed and flushed with 1X ice cold phosphate saline buffer and was immediately harvested in liquid nitrogen. 10 per cent homogenate of the frozen tissues was prepared with the tissue lysis buffer, which was incubated in ice for 15 min and centrifuged at 15000Xg at 4 °C for 20 mins. The supernatant collected was quantified by Bradford method for the protein quantification and was stored in aliquots at -80 °C till further use.

2.6 Two-Dimensional Electrophoresis

17 cm, 4-7 immobilised pH gradient (IPG) strips were rehydrated with 100 µg of GI protein (control, 24 h and 72h irradiated samples) solubilised in 350 µl rehydration buffer overnight at room temperature. Iso-electric focusing of these
rehydrated strips was conducted in PROTEAN IEF cell (Bio-
Rad laboratories Pvt. Ltd) according to optimised program as
follows 250 V for 30 min, 1000 V for 30 min and 8000V for 1
h at linear voltage amplification settings followed by 30000 Vh
with rapid amplification. Focused IPG strips were then reduced
with 3 ml equilibration buffer I containing 135 mM DTT and
alkylated with 3 ml Equilibration buffer II containing 135 mM
iodoacetamide for 15 min each. After rinsing the equilibrated
strip in the tris-glycine electrophoresis buffer, strips were placed
onto 18 cm x 20 cm x 1.5 mm, 12 per cent SDS Polyacrylamide
gels for second dimension which was conducted at 100 V for
first 1 h and then at 180 V till the completion of the run. Then
gels were silver stained using plus one silver staining kit, at
4 °C according to manufactures instructions. The experiments
were performed in the technical replicates of two and biological
replicates of three.

2.7 Image Acquisition and Data Analysis
The silver stained gels were scanned in 16 bit gray Tiff
images with Gel Doc™ XR+ Gel Documentation System and
the images were analysed using PD Quest 2-D analysis
software, version 8.0. Only those three representative gel
images of the biological replicates with correlation coefficient
value of 0.7 and more and relative same number of total spots
in the gel image were selected for match set or master gel
formation. Spot matching between all the normalised images
of different groups were automatically performed and then
added to a master gel image by the software. The fold change
in expression intensities were automatically calculated by
dividing the average intensities of the spot in different treatment
groups with respect to spot in sham irradiated group.

2.8 Spot Processing for Peptide Extraction
The selected differential spots were manually cut out of
the gel with a clean scalpel and washed with ultrapure
water. Washed protein spots were destained and incubated for
30mins with 30 mM of potassium ferricyanide and 100 mM
of sodium thioule at room temperature. The gel pieces
were rinsed several times to remove the destaining solution.
50 mM Ammonium bicarbonate/acetonitrile (1:1) was added
followed by rehydration of gel pieces in 10 mM ammonium bicarbonate
for 5 mins followed by addition of 50 per cent
acetonitrile for 15mins. The gel pieces were dehydrated with
acetonitrile which was removed after 10 mins and then vacuum
dried. Freshly prepared 20 ug/ul of trypsin was added to the
dried gel pieces and incubated at 37 °C overnight. The peptides
were extracted by addition of extraction buffer, followed by
sonication in water bath for 15 mins. The peptide mixture was
vacuum dried to remove the extraction buffer and then were
stored at -20 °C till further use15. These digested protein spots
were analysed by mass spectrometry.

2.9 Mass Spectrometry and Database Analysis
For peptide mass fingerprinting, 50 µl of resuspension
buffer was added to the digested peptides of each spot of
interest. A saturated solution of HCCA matrix in 70 per cent
ACN and 0.03 per cent TFA was prepared to be mixed with
the resuspended peptide extract in equal proportion, which was
manually spotted onto MALDI target plate and dried at room
temperature. The MALDI TOF/TOF (Ultrasflex III, Bruker
Daltonics, Germany) machine was calibrated by the peptide
calibration standards II followed by recording of the peptide
mass spectra in the reflectron mode. The generated mass list was
searched against in-house licensed version of MASCOT using
Uniprot/swissprot and NCBI protein databases using following
parameters: carbamidomethyl modifications of cysteines and
possible oxidation of methionine; one missed cleavage, a mass
accuracy of <100 ppm was requested for PMF. For MS/MS
confirmation of identified protein at least one peptide was
selected and the instrument was used in the lift mode (TOF/
TOF). The mass list was analysed for database search using
mass tolerance error of 0.5 Da -1.0 Da. For ensuring the identity
of the proteins, protein BLAST was performed of the peptide
sequences generated by the MALDI-TOF analysis. Percentage
homology and name of the closest homologue was checked
against the name of identified proteins respectively.

2.10 Statistical Analysis
The data are presented as the mean ±SD of 3 animals
from each group where each experiment was repeated thrice.
Comparisons were made among the untreated and irradiated
groups. And the data were analysed using student’s t-test
and one-way analysis of variance (ANOVA). Identification
of spots as protein were accepted with statistical significance
probability based on MOWSE score (p≤0.05). Overall a p
value of <0.5 were considered significant.

3. RESULTS
3.1 2DE Gel Map Analysis
For the selection of pH range of the immobilised pH
gradients strips the primary experiments with isolated protein
were conducted on 7 cm IPG strips, pH 3 cm - 10 cm and
7 cm IPG strips pH range 4 cm - 7 cm and it was observed that
majority of the protein spots were concentrated in the PI range
of 4-7 and thus IPG strips with 4-7 PI range produced neatly
resolved spots (results not shown). Thereafter the proteins of
respective groups were resolved on 17 cm 4 - 7 IPG strips
only. Each experimental group had three biological and two
technical replicates producing six gel images per group. These
gel maps were analysed with PD Quest software from Bio-Rad
Pvt. Ltd. Hercules, CA, USA. A total of 600±50 protein spots
were detected per gel with 19 differentially expressed protein
spots were found between control and treatment groups with
fold change of 1.5 or more.

3.2 Mass Spectrometry Analysis for Protein
Identification
The selected 19 differential protein spots were cut out of
the gels and subjected to MALDI TOF MS MS analysis, post
which their peptide sequence data was obtained. Only 07 protein
spots were identified with their peptide sequence homology
more than 87 per cent to their closest homologue (Table 2).
Figure 1(a) depicts the representative GI proteome pattern and
the identified differential protein spots has been marked black
rings. Among these proteins identified (Table 1), 03 proteins
were differentially regulated at both the time points. These
three common proteins were Heat shock protein beta-9, Growth arrest and DNA-damage-inducible 45 gamma, isoform CRA_a and RNA helicase DDX6. Protein that expressed significant change after 24 h only was Myosin regulatory light polypeptide 9 and proteins differential expression at 72 h only post radiation exposure were SAAL1, Beta-1,4-galactosyltransferase 3, Disintegrin and metalloproteinase domain-containing protein 28. Figure 1(b) depicts the enlarge images of these identified spots in their respective experimental groups.

4. DISCUSSION

Radiation exposure whether whole body or localised, to the intestinal tissue has been reported as a serious issue\textsuperscript{13}. Various studies have been conducted to understand the mechanism of this pathophysiological condition\textsuperscript{11,12,16}. However, till date no specific protein markers and therapy for radiation related injury has been reported in gastrointestinal tract. As the importance of proteomic analysis tool have been quoted ‘Comparative proteomics analysis provides a promising means to investigate thousands of proteins at the same time to find out proteins that are differentially expressed after radiation exposure’\textsuperscript{17}, we have employed the two dimensional electrophoresis technique to study the modulation in irradiated mice intestinal proteome at 24 h and 72 h when compared with sham irradiated mice.
intestines. Mass spectrometry technique enabled us to identify seven differentially expressed proteins as, ATP-dependent RNA helicase DDX6, SAAL1, Growth arrest and DNA-damage-inducible 45 gamma, isoform CRA_a, Disintegrin and metalloproteinase domain-containing protein 28, Heat shock protein beta-9, Myosin regulatory light polypeptide 9, Beta-1,4-galactosyltransferase 3.

The expression of Myosin regulatory light polypeptide 9 (MYL9) was found significantly upregulated at 24 h post 9 Gy radiation when compared with control. MYL9 is a 19.9 kDa component of nonmuscle myosin II which is a major component of actomyosin cytoskeleton. Phosphorylation is important in controlling myosin II assembly into filaments and pSer19 of myosin regulatory light chain promotes filament assembly in vivo. A recent study has reported phosphorylation of myosin II in direct association with cytoskeletal reorganisation post exposure to 4 Gy X-Ray in human mesenchymal stem cell within 1-3 days. Another study confirms increased expression of myosin II in breast cancer cell line post 6 MW X-ray irradiation and also reported that by inhibiting myosin II expression in breast cancer cell line, ionising radiation induced cell death increased. Thus the upregulation of MYL9 in the present study correlates with previous findings stating increased requirement of myosin components for cytoskeletal reorganisation as an early defense mechanism of the cell.

Heat Shock protein beta-9 is one of the ten member of the small heat shock protein family. Few members of this family have property to prevent poly glutamine aggregates formation, thereby preventing the toxicity which otherwise may lead to Huntington disease and spinocerebellar formation. Binding of HSPB9 to un/misfolded proteins formed due to mutations or stress conditions has been documented. This binding of HSPB9 forms dimers which are then recognised by proteasomes digesting these error proteins and thereby HSPB9 prevent disease formation. Radiation injury is known to cause misfolding of proteins therefore upregulated expression of HSPB9 protein at 24 h and 72 h post irradiation in our study correlates with the fact that HSPB9 helps in clearing defected proteins due to radiation injury.

RNA helicase DDX6 belongs to the DEAD box protein family whose nine common motifs are found to be conserved amongst the studied species. The amino acid sequence of Motif II is D-E-A-D (asp-glu-ala-asp), due to which this family of proteins is named as ‘DEAD box’. These helicases modulate the mRNA function also by promoting post translational modifications (PTMs) under stressful conditions.

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Protein name</th>
<th>Peptide sequences</th>
<th>Homology to close homologue (%)</th>
<th>Name of close homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>RNA helicase DDX6</td>
<td>K.VDHVQMIIVLDEADK.L, R.ELQWLEQR.H</td>
<td>100</td>
<td>probable ATP-dependent RNA helicase DDX6 [Mus musculus]</td>
</tr>
<tr>
<td>02</td>
<td>SAAL1</td>
<td>R.AEQEHLK.I, R.RFLVGR.G, K.VGEVDK.I</td>
<td>88</td>
<td>Saal1 protein, partial [Mus musculus]</td>
</tr>
<tr>
<td>03</td>
<td>Growth arrest and DNA-damage-inducible 45 gamma, isoform CRA_a</td>
<td>R.VTAWQGPDSDRLGDTLAR.C, R.CWLSGPRALECAWR.L, R.TLPVSGEAASTGRPRR.G</td>
<td>100</td>
<td>growth arrest and DNA-damage-inducible 45 gamma, isoform CRA_a [Mus musculus]</td>
</tr>
<tr>
<td>04</td>
<td>Disintegrin and metalloproteinase domain-containing protein 28</td>
<td>K.KPGVVCRA AK.D, K.QHSK.K, K.FSSPVICLR.M</td>
<td>9</td>
<td>a disintegrin and metalloproteinase domain 28, isoform CRA_a [Mus musculus]</td>
</tr>
<tr>
<td>06</td>
<td>Myosin regulatory light polypeptide 9</td>
<td>R.ATSNVFAMFDQ5IQEFK.E, K.EAFNMIDQN.D, R.FTDEEVDEMYR.E, K.GNFRVEFTR.I</td>
<td>88</td>
<td>myosin regulatory light polypeptide 9 [Mus musculus]</td>
</tr>
</tbody>
</table>
environment. Radiation exposures are known to cause PTMs in the cell. Our finding of upregulation of this protein at both the time points post radiation exposure indicates its response to radiation stress also.

Growth arrest and DNA-damage-inducible 45 gamma, isoform CRA_a is protein product of GADD45 or growth arrest and DNA-damage-inducible gene family. These are known to play a crucial role in regulating cellular stress responses and apoptosis. The studies have reported that cancer cells repress this gene to escape cell death thereby indicating that proteins of this family promote cell death during stress conditions. It is also a known fact that this protein expression increases following stressful growth arrest conditions and treatment with DNA-damaging agents. The DNA damage-induced transcription of the gene is mediated by both p53-dependent and independent mechanisms. Its upregulation in our study at 24 h, followed by further enhancement at 72 h supports the interpretation of involvement of GADD45 in the apoptosis due to radiation injury. Its upregulation in our study at 24 h, followed by further enhancement at 72 h supports the interpretation of involvement of GADD45 in the apoptosis due to radiation injury.

Disintegrin and metalloproteinase domain-containing protein 28 is member of ADAM (a disintegrin and metalloprotease) family which is a group of transmembrane proteins containing cell adhesive and proteolytic functional domains. Its members upregulate due to ROS stress and have shown to release heparin-binding epidermal growth factors during oxidative stress. ADAM28 is also involved in cell proliferation. Our findings corroborates with these reports as significant over expression of this protein is noted at 72 h post radiation.

Beta-1,4-galactosyltransferase is the key enzyme known to have important roles in many biological events, including morphogenesis, mammalian fertilisation, brain development, cellular adhesion. B4GALT2 and B4GALT3 gene products catalyze the synthesis of N-linked oligosaccharides in many glycoproteins and specific glycolipids. Its abnormal expression and its mutated forms have been related with a number of diseases and with colon and gastrointestinal cancers too. The under-expression of Beta-1,4-galactosyltransferase 3 in our study indicate this as a radiation responsive protein too.

SAAL1 is a serum amyloid A-like1 protein whose functions are largely unknown. A recent study correlates its expression with abnormal cell proliferation in synovial fibroblasts cell. Our study showed that SAAL1 protein down regulates at 72 h post radiation exposure and may have role in radiation induced damage or repair.

Thus the collective results represent the response of proteins with different biological functions against high dose of ionising radiations which may play important role in cellular defense system, cell proliferation, apoptosis pathway, etc.

However, the current study is limited to only 24 h and 72 h post irradiation. Changes in studied portions are warranted to be studied at other forwarded points for which our work is in progress. Also the validation of the current finding with another technique like ELISA or Immunoblotting will be considered in our further experiments.

5. CONCLUSIONS

We have identified some proteins which otherwise belong to various biological categories like stress responsive, damage indicators, defence related, cell proliferation pathway etc, of gastrointestinal tract which respond to high doses of ionising radiations. We also report differential expression response of SAAL1 protein in intestinal tissue to gamma radiations, whose functions are otherwise largely unknown. As emphasised above, results will be further validated by ELISA or Immunoblotting techniques. Thus, despite the current limitation our data suggests significant expression changes in proteins of various classes like indicating their play major role in radiation injury to intestinal tissue which can contribute to therapeutic targets study for drug development and medical support to affected patients.

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CONFLICT OF INTEREST
The author(s) declare(s) no conflicts of interest.

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