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Radiation-Induced Gene Expression Signatures for Triage Biodosimetry

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ABSTRACT

Medical management of radiation emergencies will require quick and reliable biodosimetric tools for assessment of absorbed dose. Dicentric chromosomal assay (Gold standard) has a limitation of being time intensive, requires specialised human skill and cannot be used for triage and mass screening. Dose assessments of suspected individuals are critical for the medical management of radiation emergencies. For effectively utilizing the available resources, there is an urgent need for developing triage biodosimetry tools for determining the exposure status of suspected individuals. High-throughput methods, utilising the novel "omics" science approaches are emerging as new technologies and gene expression-based biodosimetry is considered a promising technique for radiation dose assessment. Gene expression signatures of radiation have demonstrated the potential for triage biodosimetry. It is a minimally invasive, rapid and reliable approach that has the ability to be a robust field-deployable point-of-care high throughput technique. In addition gene expression based biodosimetry can be useful for long-term epidemiological assessment, clinical radiation oncology and radiodiagnosis.

Keywords: Radiation exposure; Biodosimetry; High throughput; Genomics; Biomarker; Microarray

1. INTRODUCTION

Ionizing radiation (IR) is omnipresent and exposure to low background radiation is inevitable. The natural sources of exposure range from the Earth's crust generated radon exposure to cosmic radiation. Artificial sources of IR mainly comprise medical, industrial and research applications but the exposure level is very low. On average, we are exposed to a few mSv/year of background radiation and depending on the geographical location, it may range from 1 to 10 mSv/year and can reach up to 50 mSv/year. Kerala in India and places in Brazil, Sudan and Iran have higher background radiation ranging from 15- 40 mSv/year[1].

In today's times, there is always a threat of terrorist activities involving radiation exposure devices (RED), radiation dispersal devices (RDD) or improvised nuclear devices (IND) and possible radiation exposure from nuclear reactor accidents or lost radioactive sources. Highly penetrating IR deposits energy to biomolecules (DNA, proteins and lipids) and cause a wide range of structural changes. Damages to genetic material, in turn, cause dysfunction in cells, tissues and organs. External contamination by radioactive dust can damage skin and if ingested, these radioactive materials deposit internally in bone and other tissues causing irreversible local injuries. Possibilities of such radiation exposure exist at accident sites and work environment with radiation sources. Clinical signs and symptoms do not occur at the low dose range (<0.1 Gy) however, possibilities of health problems can increase in subsequent years of exposure. Since the discovery of radiation in 1895 by Roentgen, radiation accidents of different scales have occurred with nuclear accidents of Chernobyl, now in Ukraine (1986) and Fukushima, Japan (2011) being of the highest scale. These accidents caused radiation emergencies involving few hundreds to lakhs of individuals. Health monitoring of such a large population involved screening using radiation detection equipment like handheld dosimeters, whole-body counters, thyroid scanners and even high-end isotope detection systems for body fluids. Individuals suspected of exposure were further assessed using various cytogenetic assays including dicentric chromosomal assay (DCA)[2]. In such scenarios, biodosimetry plays a central role along with physical and clinical dosimetry for medical management. Radiation emergency is manifested in two ways:

- Amount of activity released in the environment and
- Doses received by individuals.

In practice, area monitoring by remote-controlled systems including gamma-ray spectrometers is used to assess by following the guidelines published in the 'users manual' of IAEA[3] and related documents. The primary purpose of radiation emergency assessment is to predict deterministic health effects on the size of the population in the vicinity of accident or incident sites. The scale of nuclear radiation emergency is decided by IAEA, Vienna. According to Swartz et al[4] during any radiological emergency following types of exposure scenarios are possible:

- Large-scale radiation event: Regulatory agencies of countries use varying scales for considering large-scale

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events that may range from exposure of 100 and more people[3]. As the medical machinery is expected to be overwhelmed in such a scenario, the most imperative requirement is to segregate suspected individuals based on triage for assisting medical management. Accidents of Chernobyl, now in Ukraine, and Fukushima, Japan required the evacuation of a few lakhs of the population from the sites of the accident. Historically, these two accidents were of the highest scale according to the guideline of the International Nuclear Event Scale. Medical management comprised of various investigations related to radiation contaminations including internally deposited radioisotopes and clinical signs and symptoms. In a radiological accident at Goiania, Brazil more than one lakh people were screened for radioactive contamination[2]. These processes continued for months to years after the accident.

- Small-scale radiation event: It may involve exposure of very few to < 100 individuals. These are mainly the radiation accidents that have formed the basis of biodosimetry in the past[4]. In such a scenario it is convenient to medically assist all the potentially exposed individuals.
- Exposure concentrating on long-term effects: Biodosimetry is an effective tool to assess low dose exposures and segregate individuals that do not require immediate medical attention (radiation dose ≤ 1Gy, whole body). However, in this case, the dose received could be possibly sufficient to warrant a long-term follow-up. Individuals requiring urgent medical care (radiation dose ≥1Gy) would also be observed for long term effects. Physiological perturbations are likely to manifest at 48 - 72 hours in individuals exposed to doses > 0.7Gy.

2. NECESSITY OF BIODOSIMETRY

In the event of radiation exposure, there are three possible ways to determine the exposure status of an individual:

- Physical dose reconstruction using physical dosimeters at sites,
- Clinical assessment using blood cell counts and other physiological symptoms like nausea, vomiting and diarrhea
- Biodosimetry for confirmation of radiation exposure and dose assessment.

Biodosimetry relies on the quantifiable biological endpoints occurring due to the interaction of ionizing radiation with the biological matter and can be utilised to predict clinically relevant doses. Among various biological indicators formation of dicentric chromosomes is specific to radiation and sensitive to doses and therefore recommended for biodosimetry[2].

In any accident, it is not expected that the affected public would be wearing physical dosimeters or their prior exposure status would be available. Moreover, the clinical signs and symptoms of radiation exposure (primarily nausea, vomiting and diarrhea) are not exclusive to IR. In such a situation, biodosimetry becomes the only reliable medical management tool to estimate the exposure status of individuals. During any radiological calamity, a combination of physical dosimetry, medical signs and symptoms, a record of the individual's location, hematology and evaluation with established biodosimetric methods are used for long term risk assessment[5].

2.1 Conventional Biodosimetry

Biodosimetric dose assessments have been utilised for decades in various major accidents from Chernobyl (1986) to Fukushima Daiichi (2011). International Atomic Energy Agency (IAEA) manual 2011[2] has enlisted cytogenetic assays like DCA, Cytokinesis Blocked Micronucleus Assay (CBMNA), Premature Chromosome Condensation (PCC) and Fluorescence *in situ* hybridisation (FISH) as key techniques for biodosimetry.

With increasing evidence of radiation overexposures amongst patients, occupational workers and rising nuclear threats after the 2001 incident, US FDA has issued guidance for the development of biodosimetry devices. Accordingly, the clinically relevant radiation levels could be 2- 10 Gy and DCA has an upper limit of 5 Gy for dose detection. DCA is considered as the reigning "gold-standard" by IAEA and WHO. The low background frequency of the dicentric chromosome and the range of dose detection (0.1- 5 Gy) make it a method of choice for global biodosimetry laboratories. However, like other cytogenetics-based assays, DCA is completely manual and time-intensive where the dose estimation can only be made after a minimum of three days. Moreover, DCA requires skilled and qualified scorers, which poses major limitations for radiation dose assessment in a crisis.

Other important techniques like the gamma-H2AX foci assay, electron paramagnetic resonance, or mechanisation of existing techniques are rapid to use but necessitate cost-intensive equipment and large facilities[6-7].

2.2 Advances in Biodosimetry

With the possible threat of a probable radiological or nuclear event, there is an urgent need to develop new technologies and countermeasures for managing any large scale population exposure possibilities. Post 11th Sept 2011 attack on World Trade Centre, USA, various programs are directed towards strengthening the capability and capacity in dealing with radiological terrorism. Biodosimetry laboratories started expanding in terms of networking and automation. After a decade of research, DCA remains the gold standard. However, conventional DCA requires extensive manual microscopy for detection and quantitation of dicentric frequency. With the availability of automatic image acquisition using Metafar microscope and similar systems, capturing of metaphase spreads of a few hundred in one slide to few thousand metaphases in multiple slides is possible. Romm et al[8,9] have established and validated semi-automated scoring of dicentric chromosomes under the MULTIBIODOSE EU FP7 project with six participating laboratories establishing semi-automated dose-response calibration curves. Machine learning and artificial intelligence approaches have substantially increased the efficiency in terms of speed and accuracy. For example, the time taken for analyzing 150 metaphases using this approach was effectively reduced to few minutes as compared to taking 60 minutes by manual scoring of 50 metaphases[8,9]. Different

approaches for metaphase image analysis are being considered at other laboratories including ours.

David Brenner's group at Columbia University has developed Rapid automated tools based on CBMNA and phosphorylation of Histone (γ H2AX).[6,10,11] Robya et al., recently developed Rapid Automated Biodosimetry Tool II (RABiT-II) robotic system that utilises an improvised centromere FISH protocol and the entire assay is performed exclusively on an automated system[11]. RABiT-II can process multiple samples utilizing multiwell plates, thus making it useful for triage applications. In an attempt to increase the sample analysis capacity, a miniaturised adaptation using a 96-microtube template, a "mini-DCA" was established with semi-automated DCA scoring that reduced the sample handling and analysis time by a factor of 4, along with analyzing a large number of samples together[12].

Despite advances in semi-automatic image-based dicentric analysis and the possibility of complete automation DCA will not be able to serve the purpose of triage, as it cannot negate the requirement of stimulation of lymphocytes and culturing, thus requiring at least 2-3 days before the results could be available. DCA also requires an off-site laboratory setting and thus cannot be utilised as a point-of-care diagnostic.

Radiation biodosimetry is emerging beyond its preliminary objectives and identifying strategies that may be employed for mass screening. Novel biological markers of radiation injury, utilizing the "omics" approach in the field of genomics, proteomics, transcriptomics and metabolomics are taking center stage for giving a new dimension to conventional biodosimetry. Sullivan et al[5] critically reviewed the different biodosimetry approaches including conventional DCA, CBMNA, gamma-H2AX foci assay and "-omic" assays. These novel "omics" based dosimeters utilise a varied class of molecules like DNA, mRNA, miRNA, protein expression and metabolomic profiles, exploiting different technology platforms. All these prospective markers are minimally invasive and are mostly investigated in blood, serum, plasma, saliva, or urine; which can be readily acquired in field settings, making them a good candidate for point-of-care diagnostic possibility. The current mini-review focuses on "gene expression-based biodosimetry" as a promising new approach that can overcome the limitations of existing cytogenetic assays.

3. GENE EXPRESSION BASED BIODOSIMETRY

Environmental stresses including IR triggers numerous signal transduction pathways, consequentially generating multiple gene expression changes, which could be utilised as one of the most potential assays to analyse the effect of radiation on the cell. Studies have developed gene expression-based radiation exposure signatures in *ex vivo* irradiated human peripheral blood[13-15], *in vivo* using blood from total body irradiated (TBI) patients[14,16-17], isolated human monocytes[18], CD4+ lymphocytes[19], biopsy samples[20-21] and human cell lines[22-23]. The method relies on identifying the radiation responsive genes that preferentially transcribe in response to ionizing radiation.

3.1 Approaches used in Genomics-based Biodosimetry

Based upon the technologies involved a majority of the gene expression-based biodosimetry studies have utilised two major platforms:

3.1.1 Whole-genome Microarray-based Method

A large number of studies utilised whole-genome microarray studies to identify radiation responsive panel of genes[13-14,24-25]. Macaeva and co-workers analysed the response of 0, 0.1, 1 Gy radiation doses on *ex vivo* irradiated human peripheral blood mononuclear cells (PBMCs), where the radiation responsive differential gene signatures were transcribed and dose predicting genes were identified[25]. They also utilised exon-specific qRT-PCR approach to demonstrate the alternate splicing of many candidate biomarker genes upon radiation exposure and demonstrated that expression of highly responsive exons might have a better predictive significance especially at higher doses of about 1 Gy as compared to genes, where expression signals of the exons of these genes become averaged[25].

Using a similar approach, a 74-gene signature discriminating radiation doses from a low dose range of 0.5 Gy to a very high dose range of 8 Gy was identified, where a majority of these genes were regulated by transcription factor p53. Nearest Centroid classifier was used to accurately distinguish 98 per cent of samples at six or twenty-four hour time points as exposed or non-exposed[13]. A meta-analysis comparing eleven microarray studies identified a 29-gene signature forecasting high (>8Gy) and low (<2Gy) radiation exposure[26]. Lacombe and co-workers compared 24 microarray studies in a meta-analysis to quantify gene expression levels in human blood exposed to ionizing radiation *ex-vivo* or *in-vivo* with 10,170 unique genes and the twenty-seven genes were common in half of the studies and *TNFSF4, FDXR, MYC, ZMAT3* and *GADD45A* specifically had the greatest diagnostic potential of discriminating radiation dose < 2Gy and dose ≥ 2Gy[27].

FDXR (Ferredoxin Reductase) especially scores as a biodosimetry marker of radiation-induced transcriptional changes both as a candidate gene and as part of the validation panel of genes[17,27-28]. Using the candidate gene approach, O'Brien et al., systematically analysed *FDXR* and presented its *in vivo* dose-response at very low doses and found a strong correlation between physical and biological dose estimates, thus concluding it as a strong gene radiation marker[29]. Participating laboratories of the European Union's Realizing the European Network of Biodosimetry project (EU-RENEB) analysed gene expression profiles both *in vivo* and *ex vivo* in blood samples of prostate cancer patients and healthy volunteers respectively and demonstrated that dose estimates of *FXDR* were similar, regardless of the methodology adopted by the laboratories, implying that the approach adopted for blood incubation did not affect results[17].

3.1.2 Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Based Method

qRT-PCR candidate gene biodosimetry is the most

sensitive technique for the detection and quantification of gene expression levels and is a reliable biodosimetry tool. Majority of the studies focused on genes related to DNA repair, apoptosis and cell cycle checkpoints in different models like mice, radiotherapy patients and *ex vivo* human blood[30,31,32,33].

3.2 Radiation Responsive Genes

Different studies have utilised various bioinformatics approaches to identify a set of genes that are differentially modulated upon radiation exposure. Literature survey revealed more than 10,000 differentially altered genes in response to radiation where different dose rates, time points, doses, *ex vivo, in vivo* irradiation and other variables are evaluated[13-15,17,24-25,27,34]. A common aspect in the majority of these studies is the activation of tumor suppressor *p53* target genes across a cross-section of parameters. *p53* is a DNA-binding transcription factor that plays a key role in cellular adaptation to stress like ionizing radiation exposure. It regulates the transcription of genes in various pathways including DNA repair, cell cycle checkpoint, or apoptosis, thus maintaining genome integrity.

An account of the key genes projected as radiation biomarkers in some of the studies that focussed primarily on human blood irradiated with X-rays or γ rays and genes validated by qRT-PCR are summarised in Annexure I. This mini-review comprehends a limited number of studies and for an extensive list of genes, other detailed reviews are suggested.[27,35]

3.3 Merits of Genomics Based Biodosimetry

Many efforts have been made to advance biodosimetry using end-points that can offer dose assessments rapidly with better sample throughput. Studies have recommended the advancement of gene-expression based radiation biodosimetry as a promising alternative approach to conventional methodologies[15,25,35,38,40-42]. This approach is minimally invasive where whole blood can be used for biomarker discovery. Analyzing gene expression does not require cell division, which is the main time-consuming process in performing assays like DCA and CBMNA, thus making it a rapid technique. In case of any radiological emergency, gene expression assays can be performed easily utilizing qRT-PCR which is available in most biomedical research laboratories. Gene signatures in peripheral blood can differentiate irradiated and nonirradiated samples without having the prerequisite of having a matching pre-exposure sample and thus is considered a potent tool in biodosimetry assay advancement[13]. With current advancements in high throughput gene expression screening, it is promising to develop gene expression signatures that correlate with the timing and dose of radiation exposures. Another recent study addressed the temporal gene expression responses and RNA extraction methodologies to be utilised in emergency situations[39]. More such studies are needed to correlate these responses *in vivo* and generate sufficient data for this methodology to be useful in the triage of potentially exposed populations.

Despite the non-homogeneous nature of the transcriptional response in 24 studies analysed as a systematic review by Lacombe et al., an evident p53-regulated response was observed, suggesting the involvement of p53-mediated pathways such as DNA damage repair, cell cycle regulation and apoptosis[27]. Similar patterns can be observed in studies listed in Annexure I.

Radiation responsive gene signatures can be practically helpful for mass screening using microfluidics and "lab-on-chip" technology[43,44,45] as a robust field-deployable point-of-care high throughput device that can analyse large numbers of samples quickly.

3.4 Limitations of Genomics Based Biodosimetry

Several large-scale studies have utilised gene expression-based biodosimetry for dose prediction. However, there is inconsistency in reproducing results for identified biomarkers[27]. The possible explanation for the encountered variation could be the usage of non-homogenous microarray platforms, different experimental practices like radiation sources varying doses, time points, dose rates and divergent bioinformatics and statistical approaches. A limited sample size of patients studied also restricts the parameters for direct comparisons. Inter-individual variations, age and gender-based variations and other stresses like disease state and smoking status[16] also play a vital role in gene responses to radiation. To counter these limitations, always a panel of radiation responsive gene biomarkers should be used that cater to wider variables to improve their performance.

The extremely dynamic and transitory nature of gene expression signals is one of the major limitations of using this assay as a radiation exposure biomarker. To understand the kinetics of expression of the panel of candidate genes, it is pivotal to have prior knowledge of their baseline expression and accurate time of exposure while assessing the dose. Direct validation in radiotherapy patients may cause limitation with pre-existing diseases like cancer and could be a source of interference while analyzing gene expression. However, inter-lab comparisons with harmonised protocols are necessary for biodosimetry laboratories.

4. FUTURE AREAS OF ADVANCEMENT

To circumvent the limited accessibility of human samples to study radiation stress responses across a range of doses, a humanised mouse model was used as *in vivo* model for gene expression biodosimetry using engrafted human cells[45]. A recent study proposed IR induced extended presence of stable circular RNAs (circRNAs) in p53-dependent genes (*Pvt1*, *Ano3*, *Sec14l5,* and *Rnf169*) in embryonic mouse brain, primary cortical neurons and blood. These circRNAs were independently regulated as compared to linear mRNAs and since the radiation-induced expression of mRNAs rapidly reduces, some circRNAs remain stable or persistently rise for a longer window of time, making them durable transcription exploratory markers[46]. The single-cell RNA sequencing (scRNA-seq) is used in cancer cell biology[48] and to determine intricate regulatory interactions between genes[49] and warrants further exploration for radiation response pathways in diverse cell subsets.

Further studies on larger population sizes that include a wide range of ages, ethnicities and lifestyles, along with information of the physical dose received will assess inter-

individual variations coherently, giving insights into individual radiation sensitivity. These insights could be useful in long-term epidemiological assessment after a large-scale radiological event. Similarly, this information could be useful for clinical radiation oncology in assessing long-term outcomes like carcinogenesis.

Other important areas for exploration could be the gene expression responses to partial body irradiation[50], internal contamination and combined injuries, different dose-rates and qualities of radiation. Correlation with established assays in an accredited lab will also add more reliability to this assay, thus making gene expression-based biodosimetry an indispensable tool for future applications.

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Annexure 1

Radiation responsive gene markers identified in different studies using Microarray and qRT-PCR

Validated Gene Biomarkers	Analysis System	Biological Function	Radiation source and type	Radiation Dose	References
CDKN1A, SESN1, PHPT1, BBC3(PUMA), FDXR	*Ex vivo* healthy human peripheral blood	Cell cycle regulation, DNA repair, Apoptosis	Cs-137 (gamma)	0.5, 2,5,8 Gy	[13]
PRKCH	Human Peripheral Blood Mononuclear Cells (PBMC) From TBI pre-transplantation patients	DNA repair, hematopoietic cell activation	X-rays	200 cGy	[14]
PRKCH, Rag1, Ms4a1, Ei24, Ppp1r2	C57BL6 female TBI mice	Anti-apoptotic factors	Cs-137 (gamma)	50, 200, 1,000 cGy	
DDB2, CDKN1A, XPC, cyclin G1 (CCNG1), PCNA and IL1B	*Ex vivo* healthy human peripheral blood	Nucleotide Excision Repair Pathway, Human Tumor Cell Lines	Cs-137 (gamma)	20 cGy to 2 Gy	[15]
CDKN1A, SESN1 PCNA, DDB, PHPT1, BBC3(PUMA), FDXR	*In vivo* human peripheral blood	Cell cycle regulation, DNA repair, Apoptosis	Linear Accelerator (LINAC), (high energy X-rays)	1.25, 3.75 Gy	[16]
FDXR, PHPT1, DDB2, TNFSF8, GADD45A, MDM2, PRKAB1	*Ex vivo* healthy human peripheral blood	Cell cycle regulation, DNA Repair, Apoptosis	X-rays	0, 0.25, 0.5, 1, 2, 3 and 4 Gy	[17]
FDXR, GADD45, PCNA	*In vivo* human peripheral blood		LINAC	2 Gy local exposure	
ASTN2, FDXR, PCNA, MDM2, NDUFAF6, POLH, TNFRSF10B	Human PBMC	Cell cycle regulation, DNA damage repair and apoptosis	X-rays	0.1, 1.0 Gy	[25]
CDKN1A, PCNA and MYC	*Ex vivo* healthy human peripheral blood	DNA damage response, DNA repair, cell cycle regulation, ROS (reactive oxygen species) associated apoptosis	X-rays	Low doses: 5,10,20, 50, 75 and 100 mGy	[28]
XPC, DDB2, POLH, GADD45A, PCNA	*Ex vivo* healthy human peripheral blood	Cell cycle regulation, DNA damage repair	X-rays	0.56, 2.23, 4.45 Gy	[34]

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Validated Gene Biomarkers	Analysis System	Biological Function	Radiation source and type	Radiation Dose	References
BAX, TNFRSF10B, ITLN2, AEN	*Ex vivo* healthy human peripheral blood	DNA damage response	Neutrons X- rays	0.1, 0.3, 0.5, 1 Gy 0.1, 0.3, 0.5, 1, 2 or 4 Gy	[36]
GADD45A,DDB2(XPE),BAX	*Ex vivo healthy human peripheral blood*	Cell cycle regulation, DNA repair, Apoptosis	Co-60 (gamma)	1,2,3 Gy	[37]
CDKN1A, BAX, MDM2, XPC, PCNA, FDXR, GDF-15, DDB2, TNFRSF10B, PHPT1, ASTN2, RPS27L, BBC3, TNFSF4, POLH, CCNG1, PPM1D, GADD45A	*Ex vivo* healthy human peripheral blood	DNA damage response, DNA Repair, Cell proliferation, Apoptosis	Co-60 (gamma)	0, 0.5, 1, 2, 3, 4, 6, 8 Gy	[38]
FDXR,DDB2,TNFSF10B,AEN,XPC,BAX,ASTN2,NDUFAF6,MAMDC4,PHPT1,ASCC3,TRIAP1,LR5,AEN,ASCC3,CDKN1A,GNG11, CCR4	*Ex vivo* healthy human peripheral blood	Apoptosis, DNA repair	X-rays	0,25,50,100,500, 1000,2000 mGy	[39]