Radiation-Induced Intestinal Normal Tissue Toxicity Protein Signatures

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Radiation-induced toxicity to healthy/normal intestinal tissues, especially during radiotherapy, limits the radiation dose necessary to effectively eradicate tumors of the abdomen and pelvis. Although the pathogenesis of intestinal radiation toxicity is highly complex, understanding post-irradiation alterations in protein profiles can provide crucial insights that make radiotherapy safer and more efficient and allow for increasing the radiation dose during cancer treatment.

radiation enteropathy proteomics intestine biomarkers

1. Introduction

The intestine is one of the most radiation-sensitive organs, and the risk of damage to healthy/normal tissues surrounding a tumor that is targeted by radiotherapy is very high. Intestinal toxicity caused by radiotherapy, commonly known as radiation enteropathy [1][2][3], is a major but largely unaddressed problem for ~3.4 million radiation-treated cancer survivors in the United States (US). Despite recent progress in radiation delivery techniques, radiation enteropathy is a major dose-limiting factor during abdominal and pelvic radiation therapy [1][4][5]. This off-target damage is unavoidable due to the highly penetrating nature and uniform dose deposition per unit distance of X-rays and γ-rays, largely used in external beam radiation therapy, which is delivered in multiple fractions in cancer clinics.

Radiation enteropathy symptoms can appear within hours (early), weeks (acute), or even months or years (delayed or chronic) after radiotherapy [1]. Following radiotherapy, 60% to 80% of patients commonly develop acute clinical and histopathologic symptoms (e.g., diarrhea, abdominal pain, bloody stool, nausea, inflammatory cell infiltration, reduced crypt cell mitosis, and mucosal epithelial denudation and ulceration) [1][5]. While most acute symptoms resolve within 1 to 3 months after radiotherapy, about 90% of these patients suffer from permanent changes in bowel habits (particularly dysmotility and malabsorption). Of these, 50% experience substantial decreases in quality of life, and 10% to 15% develop life-threatening complications within 10 to 20 years after radiotherapy (e.g., submucosal fibrosis, vascular sclerosis, secondary cancer, bowel obstruction, and tissue necrosis) [1][5]. The chronic symptoms tend to be irreversible and progressive and generally have a poor prognosis [6]. Surgery is the preferred treatment option, but the mortality rate is high. Although chronic radiation enteropathy is more prevalent than inflammatory bowel disease [1], radiation enteropathy draws less attention because of false perceptions that it is not prevalent, the lack of multidisciplinary expertise required to treat these patients, and the absence of consensus on the treatment success rate [1].

The pathogenesis of radiation enteropathy is not fully understood. It involves a complex network of cellular death, proliferation arrest, and/or activation. Acute radiation enteropathy involves apoptotic or mitotic loss of intestinal epithelial stem and transiently amplifying cells in the crypt region [7]. Crypt damage impairs the replacement of the mucosal epithelial surface, blunting villus height and diminishing barrier integrity. A compromised mucosal barrier impairs absorption and facilitates the invasion of luminal intestinal bacteria into the systemic circulation, increasing the risk of inflammation and sepsis. In contrast, chronic radiation enteropathy is thought to result from the proliferative arrest (senescence) of different cell types in the irradiated microenvironment as well as from changes in their function [1][7]. Functional changes may affect the secretion of soluble mediators (e.g., cytokines, chemokines, and growth factors), cell-cell interactions, profiles of cell-surface adhesion molecules, and cell trafficking, all of which are the consequences of altered protein expression [1][6][7].

2. Proteomics Methods Used to Separate Proteins

Gel- and MS-based methods are the 2 most popular proteomics methods used to separate proteins for protein identification, quantitation, localization, and assessing post-translational modification and functional and structural changes in proteins.

2.1. Gel-Based Proteomics Method

2-dimensional gel electrophoresis (2-DE), which uses an electric current to separate proteins in a gel based on their charge (1st dimension) and mass (2nd dimension), was the first proteomic technique developed [8]. 2-DE is also known as differential display proteomics or expression proteomics because it permits the analysis of differentially expressed proteins under specific conditions in a targeted manner. However, with 2-DE, only one sample can be run per gel. 2-dimensional difference gel electrophoresis (2D-DIGE) was developed to overcome this limitation. 2D-DIGE uses different covalently tagged fluorescent dyes that allow the simultaneous comparison of 2 to 3 protein samples on the same gel without compromising the migration of proteins [9].

2.2. Mass Spectrometry-Based Proteomics

There are several “gel-free” methods for separating proteins, such as MS-based methods. MS was invented more than a century ago; however, the technique has experienced a recent surge in usage in proteomics [10]. The application of MS in protein studies involving matrix-assisted laser desorption ionization and time-of-flight MS in early 1990s advanced the field to a great extent [11][12][13]. Since then, efforts have been made to enhance its accuracy, sensitivity, and dynamic range [14][15]. Today, a single MS-based proteomics operation can be used to estimate the absolute and relative abundance of all proteins in a given cell or tissue with high sensitivity and throughput compared to other methods. Moreover, the same detection method is applicable to different samples (blood, serum, or tissue) with no limitation in sensitivity or specificity [16]. A classical bottom-up workflow in MS-based proteomics involves protein digestion into peptides, liquid chromatography separation, measurement of peptides using tandem MS, and subsequent database searching to use the information on all known peptides in the database for the assignment of peptides to proteins. This strategy makes it possible to identify proteins in a

complex mixture [17] and is now an integral part of MS-based proteomics due to its robustness and accuracy [14]. There are multiple variations of the MS method with modifications made to the various steps in the typical bottom-up proteomics workflow to accomplish specific goals.

3. Radiation-Induced Change in Protein Profile in Intestinal Cells or Tissues

3.1. Radiation Alters Protein Profiles in Intestinal Cells in Culture

An in vitro proteomics study with rat small intestinal epithelial cells (IEC-6) revealed that a single exposure to 25 Gy ^{60}Co-γ-rays differentially altered 16 proteins compared to sham-irradiated cells at 24 h [18]. The proteomics data also indicated that radiation-induced differentially expressed proteins in IEC-6 cells are involved in the cellular processes of anti-oxidation, structural development, metabolism, and protein post-translational modifications [18]. Further confirmation of proteomics data with immunoblot analysis demonstrated a significant reduction in the expression of stress-70 protein (also known as GRP75) in IEC-6 cells after radiation [18]. This proteome analysis may contribute to the elucidation of a molecular mechanism of radiation damage in intestinal cells.

3.2. Radiation Alters Protein Profile in the Intestinal Tissue of Rodents

A proteomics study of intestinal tissues of 6- to 8-week-old male C57BL/6 mice at 1 h after exposure to 9 Gy γ-rays found 17 proteins were expressed only in the irradiated group compared to the unirradiated control group [19]. The dysregulated proteins were involved in biological roles, including post-translational modifications, protein turnover and chaperones, bimolecular transportation and metabolism, cytoskeletal structure, energy production and conversion, and signal transduction mechanisms [19]. Significantly, MYC transcription factor was identified as the only upstream regulator affected by radiation exposure [19]. With the help of commercially available antibody kits, the abnormal expression of ATP synthase subunit D, aldehyde dehydrogenase, Cox5a, CRP, multifaceted C1qbp, Oat, and Pcna was confirmed after irradiation [19].

3.3. Radiation Alters Protein Profile in the Intestinal Tissue of Nonhuman Primates

PBI with minimal bone marrow sparing (2.5% to 5% sparing) causes a change in the intestinal proteome landscape of nonhuman primates. Huang et al. exposed male rhesus macaques (*Macaca mulatta*) to 12 Gy PBI with 2.5% bone marrow sparing (PBI/BM2.5) using a 6 MV linear accelerator and harvested intestinal tissues on days 4, 8/9, 11/12, 15, and 21/22 [20]. Out of the 3700 proteins that were identified, 3245 were quantified [20]. Notably, PBI/BM2.5 altered the expression of 289 proteins significantly and consistently across at least 3 time points, of which 263 proteins were upregulated while 26 proteins were downregulated [20]. Further analysis revealed that 18 upstream regulators were significantly dysregulated, out of which 15 were upregulated and 3 were downregulated [20]. In addition, the scholars observed a strong positive correlation between downregulated proteins and reduction in crypt number [20]. Finally, the scholars found that inflammatory proteins such as ACTA1, DUOX2, DNM1, COL6A3, GAL, HP, and S100A8 were significantly and consistently upregulated, while many proteins related to

retinoic acid activity, such as retinal dehydrogenase and retinal reductase, were downregulated following PBI/BM2.5 [20].

4. Therapeutic Radiation Alters the Plasma Protein Profile of Rectal Cancer Patients

Radiation is an integral part of cancer treatment. Approximately 50% of the patients with cancer in the US, including those suffering from abdominopelvic cancer, receive radiation therapy at a certain stage of their cancer treatment. One of the major detrimental side effects of abdominopelvic radiation therapy is intestinal healthy tissue toxicity, which may alter plasma protein profile. A study by Holm et al. of patients with stage II and III rectal cancer who were rectally exposed to 5 fractions of 5 Gy radiation for 5 consecutive days demonstrated significant alteration of 14 plasma proteins in stage II patients and 28 in stage III patients compared to rectal cancer patients who did not receive radiation therapy [21]. Interestingly, in stage II patients, all 14 altered plasma proteins were downregulated, while in stage III patients, all 28 altered proteins were upregulated following radiation therapy compared to rectal cancer patients who did not receive radiation therapy [21]. The significantly downregulated proteins in stage II patients include hemoglobin subunit beta, hemoglobin subunit alpha, and lysozyme C, while the highly upregulated proteins in stage III patients were serum amyloid A1 and synapsin 2 [21]. It indicates that cancer stage modulates plasma protein levels in radiation-induced intestinal damage following radiotherapy.

5. Signaling Pathways Altered as a Result of Intestinal Radiation Toxicity

Systematic studies of altered signaling pathways in the intestines following radiation damage may provide crucial information to develop countermeasure strategies. A number of preclinical studies with murine models of intestinal radiation toxicity exhibit dysregulation of various pathways in the intestinal tissue. For example, the proteasome and protein processing pathways were significantly altered in the intestinal tissue samples of 6- to 8-week-old male C57BL/6J mice exposed to 9 Gy γ-rays at 1 h post-exposure [19]. Another study demonstrated alterations in 5 canonical signaling pathways such as Rho family GTPases, glycolysis I, xenobiotic metabolism, 14-3-3-mediated signaling, and retinol biosynthesis in the intestinal tissues of male CD2F1 mice 24 h after exposure to 11 Gy TBI [22].

Preclinical studies with rat models of intestinal radiation toxicity also exhibit that radiation dysregulates a number of intestinal signaling pathways. FAS and glycolysis signaling pathways were dysregulated in the intestinal tissues of Sprague–Dawley rats following 10 Gy abdominal γ-ray irradiation on day 4 after exposure [23]. A study of 6-week-old male Sprague–Dawley rats exposed to 20 Gy abdominal X-ray irradiation exhibited dysregulation in complement and coagulation cascades, amoebiasis, phagosome, lysosome, focal adhesion, oxytocin signaling in the intestinal tissue 10 weeks after exposure [24].

In addition, radiation was shown to affect various signaling pathways in nonhuman primates. For example, 4 canonical pathways—GP6 signaling pathway, acute phase response signaling, LXR/RXR activation, and intrinsic prothrombin activation pathway—were dysregulated in the intestinal tissue of nonhuman primates following 12 Gy PBI/2.5 BM at various time points ranging from 4 to 22 days [41].

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