Review

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Single-Cell Sequencing: An Emerging Tool for Biomarker Development in the Event of Nuclear Emergency

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Abstract: Next-generation sequencing (NGS) has been well applied to assess genetic abnormalities in various biological samples to investigate disease mechanisms. With the advent of high-throughput and automatic testing platforms, NGS can identify radiation-sensitive and dose-responsive biomarkers, contributing to triage patients and determining risk groups for treatment in a nuclear emergency. While bulk NGS provides a snapshot of the average gene expression or genomic changes within a group of cells after the radiation, it does not give information on individual cells within the population. On the other hand, single-cell sequencing involves isolating individual cells and sequencing the genetic material from each cell separately. This approach allows for the identification of gene expression and genomic changes in individual cells, providing a high-resolution view of cellular diversity and heterogeneity within a sample. Single-cell sequencing is particularly useful to identify cell-specific features of dose-response and organ-response genes. Although single-cell RNA sequencing (scRNA-seq) technology is still in its infancy in radiation research, it has great potential for identifying biomarkers associated with radiation exposure and for personalized post-radiation medical care. The aim of this review is to focus on current dosimetry methods, recently identified radiation-induced biomarkers, as well as the application of NGS techniques in facing a nuclear accident, specifically the single-cell sequencing technology.

Keywords: next-generation sequencing; single-cell sequencing; radiation dosimetry; radiation biomarkers; cellular heterogeneity

Introduction

Following a major catastrophic event affecting a sizable portion of the populace, such as a pandemic or a natural disaster, a large number of concerned people would rush to hospitals in need of assistance, placing a logistical burden on the medical staff engaged in triage. The response readiness of Canada in the event of a radiological or nuclear emergency is essential, to ensure a prioritization of the triage patients is enacted, given that children and young people are more radiosensitive and susceptible.

The dicentric chromosome assay (DCA) has been recognized as the gold standard for detecting chromosomal rearrangements in peripheral blood lymphocytes. However, the assay is time-consuming, low throughput, and labour-intensive, limiting its usefulness in the event of a large-scale emergency (Macaeva et al. 2019). NGS is a rapidly evolving technology that may evaluate genetic abnormalities in the blood, body fluids, and other non-invasive sample types in an efficient and high-

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throughput manner. By using targeted NGS and processing 1,000 samples in less than 30 hours, a German research team has discovered four genes connected to hematological acute radiation syndrome (HARS) (Port et al. 2019). Following up on the diagnostic time window, HARS-predictive genes can be detected from 2 hours to 3 days after irradiation (Ostheim et al. 2021). The quick workflow in detecting doses or biological markers for adverse effects of radiation exposure facilitates meeting the critical time window for immediate medical interventions. The DCA, on the other hand, requires samples to be collected between 24 hours and 4-6 weeks for optimal results (REMM 2024). Otherwise, the readings might not be stable and representative of the actual radiation exposure. However, in the event of a radiological accident, waiting 24 hours to collect samples for analysis could affect the efficiency of clinical interventions.

Although rapid high-throughput triage might be possible in preparation for a nuclear emergency, we believe NGS will be majorly used for biomarker development instead of fieldwork for biodosimetry assessment. A thorough understanding of radiation responses will provide specific targets to develop an in vitro diagnostics test such as quantitative real-time PCR (RT-qPCR). Specifically, this review will concentrate on the application of NGS technology to radiation accidents and nuclear emergencies, in particular single-cell sequencing technology, which is still in its infancy but rapidly advancing. scRNA-seq can provide detailed characteristics (cellular function and gene expression patterns) of each individual cell, contributing to building up a quick testing method by using a specific group of cells in RT-qPCR. In addition to allowing medical staff to understand the cellular response to radiation exposure at the molecular level, this technology would help in identifying cells that might exhibit resistance or sensitivity to variant radiation doses.

Current Biodosimetry Methods

Biodosimetry is the measurement of the biological effects of exposure to ionizing radiation. It is used to assess radiation-related health risks and determine the appropriate treatment or intervention for individuals exposed to radiation. There are several different approaches to biodosimetry, including physical methods, such as measuring the amount of radioactivity in a sample, and biological methods, which involve analyzing the effects of radiation on living cells or organisms (Wuestermann and Cronkite 1995; Donnelly et al. 2010; Mirrezaei et al. 2020; Meenakshi et al. 2021). One common biological method of biodosimetry is the analysis of chromosome abnormalities in blood or bone marrow cells, which can be induced by radiation exposure. Other methods include measuring DNA damage and analyzing the modulation of gene expression in response to radiation exposure.

The DCA method is the gold standard in biodosimetry, and for a long time was the only available technique (IAEA 2011). It is a technique used to quantify dicentric chromosomes in a sample of cells. Dicentric chromosomes are chromosomes that have two centromeres (the structures that separate the chromosome into two sister chromatids during cell division) and are considered a hallmark of radiation-induced DNA damage. This occurs when both strands of the DNA are damaged (double-strand breaks) and the broken ends of the chromosomes fuse together to form a dicentric chromosome (M'Kacher et al. 2023). In the DCA, cells are exposed to a specific dose of radiation and then allowed to grow and divide. After a certain number of cell divisions, the chromosomes are harvested and analyzed using cytogenetic techniques, such as Giemsa staining or fluorescence in situ hybridization (FISH). The number of dicentric chromosomes present in the sample is then quantified and used to determine the effect of radiation exposure, for which a higher number of dicentric chromosomes is directly proportional to the level of damage caused by ionizing radiation (Brewen and Peacock 1969; Jeong et al. 2022).

Despite its high accuracy, DCA has several drawbacks. One limitation of the DCA is that it is only sensitive to doses of radiation above a certain threshold, typically over 100 – 200 mGy (milligray, a unit of absorbed dose of ionizing radiation) (Manivannan et al. 2018). This implies that the DCA technique might not be able to detect modest to lower levels of radiation exposure, such as those that could happen in the environment or during medical treatments. Another major limitation of the DCA

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is that it can only be used to evaluate acute radiation exposure, making it ineffective for evaluating the effects of chronic or long-term radiation exposure. In addition, the DCA is a cytogenetic technique that requires the analysis of cells under a microscope, which can be time-consuming and may not be practical for high-throughput analyses. In the scenario of a nuclear emergency, not having enough personnel and equipment is known to be a bottleneck for the DCA (Wilkins et al. 2011; Maznyk et al. 2012). To reduce the arduous manual counting time, several DCA with artificial intelligence-powered counting methods are under development, such as the Chromosome ABerration cAlculation Software (CABAS) applied by Ryan's group, the automated Desktop version (ADCI Desktop) for dicentric chromosome counting by Rogan's group, the machined learning method-trained dicentric chromosome score (DCScore) software based on clustering algorithm and watershed algorithm by Shen's group, and the InceptionResnetv2 based deep learning method for dicentric chromosome detection proposed by Wadhwa's group (Rogan et al. 2014; Ryan et al. 2019; Shen X et al. 2019; Angad Singh Wadhwa 2022). A recently developed deep learning-based automatic dose-estimation system (DLADES) in automatic dicentric chromosome counting is introduced with well constructive dose-response curve and dose determination (Jeong et al. 2022). Although this study did not cover exposure doses higher than 4 Gy or lower than 0.5 Gy, its estimation shows a promising high-throughput capability in scoring more cells to estimate doses below 0.1 Gy to meet the higher cell count requirement in conventional methods (Jeong et al. 2022). In another study, Kim et al. used a deep (learning) neural network to automate the DCA by applying the YOLOv5 algorithm, which instead of estimating the dose, focused on automating the approximate number of dicentric chromosomes directly from the metaphase stage (Kim K et al. 2023).

Besides dicentric analysis, some cytogenetic analysis techniques were also introduced to measure radiation-related biodosimetry by applying them to other biological endpoints, including FISH-based translocation analysis, premature chromosome condensation (PCC) analysis, and the cytokinesis-block micronucleus (CBMN) assay (IAEA 2011). FISH-based translocation analysis is a powerful method to assess the absorbed radiation dose retrospectively, by detecting the chromosomal rearrangement biomarker (translocation) and generating a dose-response curve for the genomic translocation frequencies (Bauchinger et al. 1998; Camparoto et al. 2003; Grégoire et al. 2018). Although the miss-scoring of the dicentrics as translocations was questioned in early studies, the problem was fully resolved after applying centromeric probes (Straume and Lucas 1993). PCC analysis was first introduced to visualize X-ray-induced chromosome damage in interphase cells in 1974 exemplified in Chinese hamster ovary (CHO) cells, through which the extent of radiation damage is inversely proportional to the degree of chromosome condensation (Hittelman and Rao 1974). The PCC analysis was then applied in mammalian and human cells showing good sensitivity and dose-effect relationship of radiation-induced micronuclei (Obe and Beek 1975; Witkowski and Anger 1976). In complementary to DCA, the CBMN assay is another one of the best-validated methods to measure chromosome damage in human lymphocytes, especially with its lowest detection limit down to 5 cGy (Fenech and Morley 1986). In addition, CBMN is also sensitive enough to detect ionizing radiation-induced bystander effect and inter-individual variation in DNA damage (Azzam et al. 2001; Fenech 2010).

Scientists are recommending shifting the criteria away from estimates of the dose received by the individual and placing a greater emphasis on the radiosensitive indicators of the patient's biological response in light of the development of the biodosimetry method over the past years and consideration of the initial decision for triage in an emergency scenario (Swartz et al. 2020). The rationale is that even if each person in a radiation disaster receives the same radiation dose, the effects may differ significantly for each individual (common factors are age, sex, individual immunity, and population genetics). Similarly, even if the overall dose amount absorbed by the person is measured, each organ will still exhibit its unique response (Swartz et al. 2020). Due to the heterogeneous absorption, the radiation researchers were prompted to look for particular biological signatures of probable radiation injury, which will benefit not only triage but also detect and analyze risks for subacute and chronic impacts to undertake radiation mitigation (NIH 2019).

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According to Swartz et al., a novel biodosimetry method combining fingernail electron paramagnetic resonance (EPR) with the information provided by organ-specific biomarkers allows the estimation of dose distribution in vivo (Swartz et al. 2020). This new method of physical biodosimetry relies on detecting in vivo radiation-induced free radicals on the nails using EPR, which responds selectively to unpaired electrons that are generated in the keratin of human fingertips and toenails. The intensity of EPR signals is directly proportional to the received dose. A positive aspect of nail dosimetry is that the signal measurement is stable at least for several weeks, and the sample collection method is non-invasive and non-destructive with little training required. Additionally, the reliability of the readings can be checked across multiple limbs on the same individual. More importantly, the resolution has been achieved to at least 1 Gy, which was originally 10 Gy. It is, therefore, expected that in vivo nail EPR together with organ-specific biomarkers would provide a higher resolution and guide effective medical resources for the survival of patients who are the victims of radiation accidents (Swartz et al. 2020). Similarly, an in vivo EPR tooth biodosimetry could be considered due to its potential in triage, however, further testing is required to validate its effectiveness (Swartz and Flood 2023).

Radiation-Induced Biomarkers

Radiation-induced biomarkers and biodosimetry methods are both used to assess radiation exposure, but they are different in their approaches and purposes. Biodosimetry methods are used to directly measure the dose of radiation that an individual has received after exposure to ionizing radiation. On the other hand, a biomarker is a qualitative characteristic that can be quantified and used to identify specific biological, pathological, or therapeutic processes (Singh et al. 2021). The composition of biomarkers includes but is not limited to genetic sequence, receptor expression, radiographic or other imaging-based measurements, blood bioinformation, electrocardiographic parameters, or organ function (Singh et al. 2016). Radiation biomarkers can provide information on the biological effects of radiation exposure at the molecular level, while biodosimetry methods provide a direct measurement of the dose of radiation that an individual has received. Both types of methods can be useful in the management of ionizing radiation exposure, depending on the specific circumstances of the exposure and the information that is needed.

In addition to the traditional cytogenetic biomarkers such as chromosome aberrations, lymphocyte depletion, and γ-H2AX (Rothkamm and Horn 2009), plenty of radio-sensitive molecules have been identified recently (summarized in Table 1). Several “omics” approaches to detecting biomarkers are under development, including genomics, transcriptomics, proteomics, lipidomics, and metabolomics (Satyamitra et al. 2022). Systematic literature research was performed in PubMed, in which the keywords “biomarker”, “radiation” and “accident” were searched, and the 134 articles published within ten years of the interest were collected and reviewed. Just as Olivier Guipaud initiated in 2013, serum and plasma proteomics have been widely applied to radiation biology and biomarker discovery (Guipaud 2013). A recent study by Kwak et al. found that the expression of C-X-C motif chemokine ligand 10 (CXCL10) was altered as a response to ionizing radiation (Kwak et al. 2024). In fact, its expression levels were consistent with those of ferredoxin reductase (FDXR), a known radiation biomarker, in both the non-irradiated and irradiated (1, 3, and 5 Gy) mouse strain (C57BL/6) peripheral blood mononuclear cells (PBMC) samples (Kwak et al. 2024). Therefore, CXCL10 has the potential to serve as a reliable biomarker for assessing ionizing radiation exposure (Kwak et al. 2024). Another in vivo investigation of radio-sensitive mouse strain (C57BL/6J) demonstrated that the serum protein BPI Fold-Containing Family A Member 2 (BPIFA2) can serve as a novel early biomarker for a nuclear accident, as its expression significantly elevated following radiation exposure (He et al. 2022). Additionally, a secondary increase of serum BPIFA2 following a lethal dose (10 Gy) of radiation suggests that BPIFA2 has prognostic significance for predicting fatal radiation injury (He et al. 2022). Similarly, serum amyloid A1 (SAA1) has been identified as a potential early-stage biomarker for radiation damage at both protein and transcript levels, with a

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substantial increase in its expression after irradiation predicting subsequent lethality (Huang J et al. 2019).

Transcriptomics and proteomics approaches have been applied to identify radiation-induced biomarkers to meet the requirements of the large-scale database, providing a comprehensive understanding of radio-sensitive and responsive biomolecules. High-throughput capacity platforms are being developed, among which a few panels of potential biomarkers were chosen for testing. For example, a high-throughput biodosimetry test system (REDI-Dx) has been developed for investigational use in the United States (Jacobs et al. 2020). With a minimum invasive blood collection, a set of 18 dose-response genes plus three normalizer genes and two internal controls are included for RNA Sanger sequencing, and the REDI-Dx shows good sensitivity and specificity on radiation dose measurement on either 2 Gy or 6 Gy (Jacobs et al. 2020). In addition to that, by providing a systematic biology snapshot of the expression of metabolites, metabolomics may be one of the most promising tools for developing biomarkers, particularly when compared to radiation-induced metabolic dysregulation (Singh et al. 2021). Carnitine ($C_7H_{15}NO_3$), citric acid ($C_6H_8O_7$), and many more were identified as frequently altered metabolites in response to variant doses of radiation, which can serve as candidates for radio-sensitive biomarkers (Singh et al. 2021). Maan et al. revealed the dysregulation of several immunological pathways, lipid metabolism, carbohydrate metabolism and amino acid metabolism (histidine, arginine-proline metabolism, arginine biosynthesis) following exposure to 1 Gy and ultra-high (7.5 Gy) dose of radiation highlighting the importance of investigating amino acid responses to varying radiation doses and the extent to which metabolic pathways are affected (Maan et al. 2023). Not only would it enhance our understanding of molecular interactions, but it would also be valuable for identifying potential biomarkers in application of multi-omics based integration of metabolomics and transcriptomics analysis that would otherwise assist in triage management strategies in radiation accidents (Maan et al. 2023).

Single-Cell Sequencing Technology

In contrast to conventional RNA sequencing (RNA-seq), single-cell sequencing (genome, transcriptome, and epigenome) facilitates the identification of gene signatures and differential expression of genes at the individual cell level. Since conventional bulk RNA-seq only reflects the average gene expression within tissues made up of a variety of different cell types and mixed cell populations, it has certain limitations when it comes to analyzing cellular heterogeneity within a tissue or population. Therefore, scRNA-seq technology is a potent and fast-developing technique for determining the variance of gene expression in a single cell and for providing increased insights into intra-tumour or intra-organ heterogeneity (Huang RH et al. 2021). High-throughput scRNA-seq technology, which was first reported in 2009, immediately established itself as one of the most powerful technologies and has since flourished as one of the most expected research tools for scientific groups working on genome, transcriptome, and epigenomics (Tang F et al. 2009). scRNA-seq enables researchers to unveil diverse mechanisms dispersed across a wide range of cell types in tissues, organs, and tumours, revealing the complexities and intricacies in developmental biology, cancer research, and immunology.

A single-cell sequence can be performed using several different approaches. For instance, scRNA-seq can be used to study gene expression, single-cell DNA sequencing can be used to study the genome while single-cell proteomics can deal with global protein expression within thousands of individual cells. In scRNA-seq, all steps associated with bulk RNA sequencing are included, except for the fundamental step of separating the single cells. There are several approaches for the isolation of single cells including conventional manual sorting, dilution, laser microdissection (LCM), fluorescence-activated cell sorting (FACS), and microfluidics/microplate technology (Gross et al. 2015; Potter 2018; Zhou et al. 2021). Specifically, microfluidics technology increases the efficiency of single-cell sorting and the capacity of libraries, thereby making it possible to screen a large number of cells with a high degree of accuracy (Kang et al. 2018; Zhou et al. 2021).

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Cancer involves a range of tumour cell populations with heterogeneous mutations along with a different transcriptional program that contributes to its complexity. Furthermore, there is significant heterogeneity within the tumour and intra-tumour cell populations as well as the tumour microenvironment which also varies at different stages of oncogenesis, making it extremely challenging to develop accurate biomarkers for diagnosis, prognosis, recurrence, and drug resistance. In terms of biomarker study, scRNA-seq offers great promise in cancer research since it allows accurate detection of differential gene expression within individual cells as well as across cell populations (Huang RH et al. 2021; Kim J et al. 2021). scRNA-seq can disentangle the convolution arising from tumour heterogeneity. Gao et al. reported a heterogeneous cellular response to DNA damage induced by ionizing radiation by utilizing single-cell transcriptome sequencing in breast cancer cells, identifying potential radio-sensitive biomarkers (Gao Y et al. 2021). The scRNA-seq method is increasingly being used to predict the prognosis of many cancers. A recent study developed an epithelial cell marker gene-based risk assessment model for predicting the prognosis of colorectal cancer patients based on scRNA-seq analysis (Shen KY et al. 2022). Another scRNA-seq-based study demonstrates considerable cellular and functional heterogeneity of myeloid cells within the tumour microenvironment as well as sex-specific differential gene expression in glioma-activated microglia that may influence presentation and outcomes in patients with gliomas (Ochocka et al. 2021). Additionally, scRNA-seq has been used effectively for the identification of biomarkers within circulating tumour cells from melanoma, hepatocellular carcinoma, and non-small cell lung cancer (Fankhauser et al. 2022; Tang H et al. 2022; Sultana et al. 2023).

Therefore, scRNA-seq can contribute to the discovery of biomarkers for accidental radiation exposure, nuclear incidents, and astronaut exposure to space radiation. Experiencing high radiation doses in a short period can have a variety of effects on individual cell populations in different organs, which can be systematically identified through scRNA-seq. The knowledge gained from scRNA-seq analysis will significantly improve molecular diagnostic screening of individual cells following radiation exposure and the identification of customized countermeasures based on the differential expression of genes across diverse cell populations. Based on the recent experience of early-stage biomarker research on lung cancer, the scRNA-seq can also be exploited for the identification of molecular biomarkers from circulating blood cell populations at different time points following radiation exposure (Kim J et al. 2021). Given the considerable negative impacts on health, space radiation poses a serious risk to astronauts on long-term missions. scRNA-seq will enable us to learn more about how individual cells react to various particle types and energy levels in space. Furthermore, by employing single-cell transcriptomics of human immune cell subpopulations we can identify a cell-specific signature for space radiation sensitivity. For instance, investigators used engineered human tissues (bone marrow and cardiac tissue) and single-cell transcriptomic analysis to investigate the effects of high-energy radiation (neutron sources and photon radiation) to simulate conditions experienced during long space missions. They observed several molecular changes, including decreased proliferation of CD45+ cells, increased inflammatory signatures, and the presence of myeloid cells (Tavakol et al. 2023). Additionally, the study identified genes, such as oxidative stress-related MIR22HG and HMOX1, pro-fibrotic and senescence-related COL24A1, p53-related MIR34AHG and PHLDA3 and many more that could serve as biomarkers for secondary radiation damage during space missions (Tavakol et al. 2023). Furthermore, scRNA-seq can reconstruct our existing understanding of biodosimetry approaches since the heterogeneous gene expression in different cells provides a more precise landscape of molecular markers after radiation exposure. A recent scRNA-seq study on the human T lymphocyte subpopulation following ex vivo radiation exposure demonstrates differential gene expression in different subpopulations (Moreno-Villanueva et al. 2019). Though the application of scRNA-seq technology in radiation research has just emerged, it has a huge potential for radiation exposure-related biomarker identification and personalized medical counter-measurement following radiation exposure.

While scRNA-seq may be a new concept for radiation-induced biomarker study, radiation therapy, as the most cost-effective treatment of cancer, has been applied to more than half of cancer

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patients alone or in combination with surgery or chemotherapy. Radiation-induced biomarkers identified can be used in personalized radiation therapy for cancer to help determine the best radiation dose and treatment plan for individual patients. For example, by comparing the radiation-induced biomarkers before and after radiation, researchers can identify the radiation sensitivity of each patient. This information can be used to develop personalized and customized treatment plans that are tailored to each patient's unique genetic profile. Therefore, the characterization of cell response after irradiation treatment by single-cell sequencing may capture the radiation-induced changes not only in tumour cells, but also surrounding environment, such as immune cells, blood cells, and mesenchymal cells. It also can provide a comprehensive understanding of in vivo studies and patient studies systematically. A recent conference abstract showed evidence that radiotherapy can stimulate immune cells infiltrating the irradiated colorectal carcinoma cells, enhancing the CD8+ T cells trafficking to locally treated areas (Ponthan et al. 2022). By using 10x genomics single-cell sequencing, Ponthan et al. revealed that radiotherapy increased natural killer cell granule protein 7 (NKG7)-expressing cytotoxic T cells, indicating a target for immune modulating medication development (Ponthan et al. 2022). Meanwhile, NFKB1 has been identified by single-cell whole genome sequencing to increase the radiation sensitivity of cervical cancer cells in response to radiotherapy, suggesting to be a potential target for personalized cancer treatment (Yang et al. 2017). By identifying potential biomarkers, single-cell sequencing may direct the following treatment, comprehensively guiding personalized health management.

Discussion

Radiation exposure is a double-edged sword to the living system. It can damage cells causing unpleasant endpoints such as tumorigenesis, cataracts, cardiovascular diseases, and neurological degeneration; while it can benefit humans in radiation therapy for cancer treatment to save lives. Moreover, by summarizing the recent clinical trials, radiotherapy can also serve as a promising treatment option for severe patients with COVID-19-induced pneumonia (Yu et al. 2021). In the event of a nuclear emergency, the doses and types of ionizing radiation are complicated, making it essential to quickly identify and evaluate potential biomarkers that could indicate radiation-induced biological effects and that could guide the specific treatment to mitigate the symptoms. Single-cell sequencing technology is an emerging tool in biomarker development, especially in the identification of gene expression and DNA damage in different clusters of cells. With its fast development, it has been applied in many studies, including cancer biology, microbiology, neurology, immunology, etc.

Although an automatic platform has been introduced in single-cell sequencing technology, the minimum conducting time of the sequencing library preparation is still 10 hours, without accounting for the extra sequencing and data analysis time (Gao C et al. 2020). The necessary devices and equipment need stable lab space, which poses another challenge for quick response in a dynamic fieldwork environment. However, the advantage of single-cell sequencing in discovering unidentified cell clusters and specific gene expressions in multiple cell types makes it a valuable tool for the development of radiation-induced biomarkers, not only in paving for quick ex vivo triage methods but also in analyzing and understanding long-term biological effects. With the refinement of technology, more information can be revealed by the single-cell multi-omics approach to explore specialized mitigation. Therefore, we propose that candidate biomarkers sensitive to radiation exposure could form a comprehensive biomarker matrix panel (see Figure 1). In conclusion, single-cell sequencing is a promising future methodology for biomarker development in response to nuclear emergencies.

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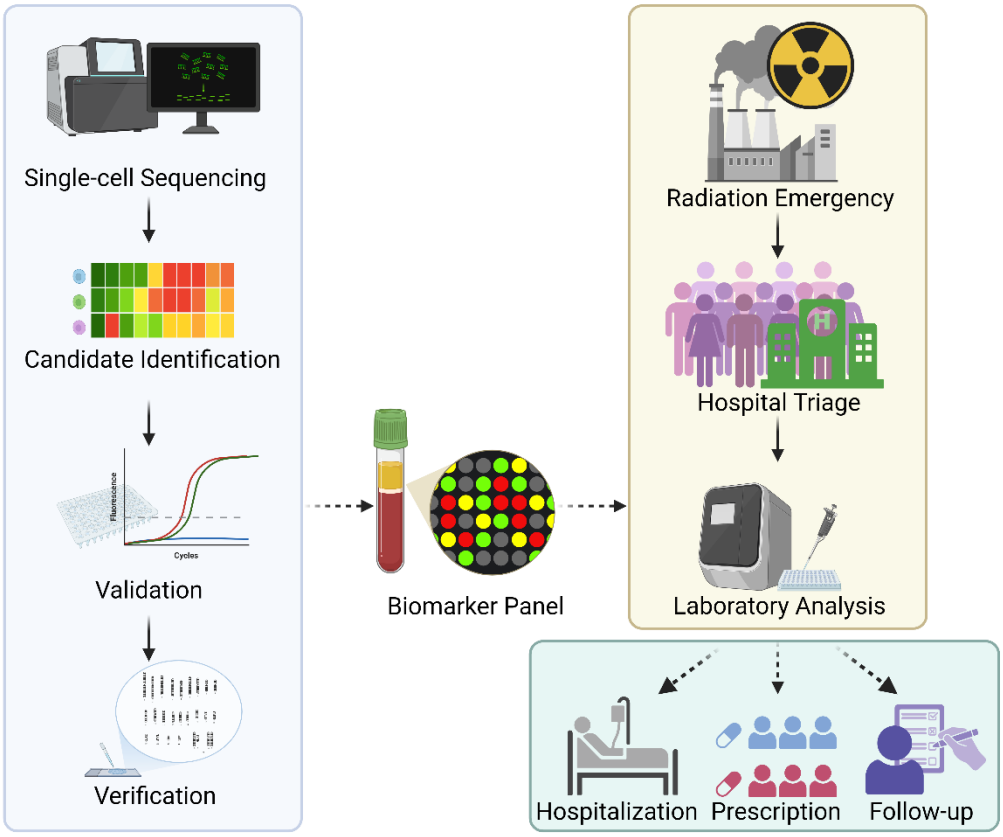

Figure 1. The development of the radiation-sensitive biomarker panel. Single-cell sequencing would provide biomarker candidates to be selected and validated with faster methods such as qPCR. Once the validation is completed, the candidates would be measured against the golden standard methods such as DCA for verification. The biomarker panel would benefit patients for triage and laboratory analysis in the event of a radiation emergency, which would serve not only for dosimetry and radiation protection, but also for guiding treatment in hospital settings, managing prescriptions, and facilitating follow-up healthcare.

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