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Research Letter

Exacerbated Inflammatory Gene Expression After Impaired G2/M-Checkpoint Arrest in Fibroblasts Derived From a Patient Exhibiting Severe Adverse Effects

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Purpose: Recent radiation therapy (RT), such as intensity modulated radiation therapy and particle RT, has improved the concentration of the radiation field targeting tumors. However, severe adverse effects still occur, possibly due to genetic factors in patients. We aimed to investigate the mechanism of exacerbated inflammation during RT.

Methods and Materials: Dermal fibroblasts derived from a patient with severe inflammatory adverse effects during RT were compared with 2 normal human dermal fibroblasts. Micronuclei formation, G2/M-checkpoint arrest, DNA damage signaling and repair, and inflammatory gene expression were comprehensively examined.

Results: We found greater micronuclei formation in radiation-sensitive fibroblasts (RS-Fs) after ionizing radiation (IR). RS-Fs exhibited premature G2/M-checkpoint release after IR, which triggers micronuclei formation because RS-Fs undergo mitosis with unrepaired DNA double-strand breaks (DSBs). Additionally, we found that DSB end-resection and activation of the ATR-Chk1 pathway were impaired in RS-Fs after IR. Consistent with the increase in the formation of micronuclei, which can deliver cytosolic nucleic acids resulting in an innate immune response, the expression of genes associated with inflammatory responses was highly upregulated in RS-Fs after IR.

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All data generated and analyzed during this study will be included after the paper is accepted.

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Conclusions: Although this is a single case of RT-dependent adverse effect, our findings suggest that impaired G2/M-checkpoint arrest due to the lack of DSB end-resection and activation of the ATR-Chk1 pathway causes exacerbated inflammation during RT; therefore, genes involved in G2/M-checkpoint arrest may be a predictive marker for unexpected inflammatory responses in RT.

Introduction

Radiation therapy (RT) can modulate immune and inflammatory responses.[1,2] Cytosolic nucleic acids sensors such as stimulators of interferon genes (STING), cyclic GMP−AMP synthase (cGAS), and retinoic acid-inducible gene I (RIG-I) are involved in the activation of immune and inflammatory genes after ionizing radiation (IR).[3-5] Several mechanisms, such as the release of nucleic acids from micronuclei or mitochondria, instability of telomeres, DNA replication, and R-loop maintenance, are considered.[6-12] Nucleic acid sensor-dependent innate immune response activates the interferon regulatory transcription factor 3 (IRF3) and IRF7 pathways.[13,14] Subsequently, these transcription factors activate downstream molecules, such as type I interferon and interferon-stimulated genes (ISGs).[15,16] The upregulation of these genes is considered to contribute to antitumor activity; however, excessive activation of the inflammatory genes can exacerbate the adverse effects of RT.

DNA double-strand breaks (DSBs) are critical DNA lesions after IR. If cells harboring unrepaired DSBs undergo mitosis due to impaired G2/M-checkpoint arrest, chromosome fragments are released from the primary nucleus during mitosis, resulting in micronuclei formation in the subsequent G1 phase. G2/M arrest is regulated by ataxia-telangiectasia-mutated (ATM)-Chk2 and ataxia telangiectasia and Rad3-related (ATR)-Chk1. ATR-Chk1 and signal mediator proteins, such as 53BP1 and MDC1, contribute to the maintenance of G2/M arrest after IR.[17] Micronuclei formation has also been observed in human tumor cells after RT,[18] highlighting the occurrence of these cellular responses in patients during RT. Here, we comprehensively examined micronuclei formation, G2/M-arrest, DNA damage signaling and repair, and inflammatory gene expression in dermal fibroblasts from a patient with a severe esophageal inflammatory phenotype during RT.

Methods and Materials

Information on tissue-culture, micronuclei analysis, immunofluorescence, immunoblotting, G2/M-checkpoint analysis, RNA-sequencing and its related analysis, and statistical analysis has been provided in the Supplementary Materials.

Results

Fibroblasts derived from a patient with severe adverse effects after RT exhibit the impaired G2/M arrest and enhanced micronuclei formation after IR

As micronuclei is considered a source of innate immune responses after IR,[13] the number of micronuclei per cell was examined after 2 Gy x-rays. Notably, radiation-sensitive fibroblasts (RS-Fs) showed an increased number of micronuclei compared with normal fibroblasts (NHDF1 or NHDF2) after IR (Fig. 1A, B). Micronuclei are formed during mitosis if G2 cells harboring DSBs enter mitosis due to the failure of G2/M arrest. RS-Fs showed a normal reduction in the mitotic index 2 hours after IR (Fig. 1C); however, mitotic index in RS-Fs recovered earlier than that in control fibroblasts, suggesting that the G2/M arrest was not sufficiently maintained in RS-Fs. To investigate the activation of ATM and its downstream signaling, ATM autophosphorylation and its target, Chk2 phosphorylation, were examined. However, RS-Fs showed only a partial reduction in ATM and Chk2 phosphorylation after IR (Fig. 1D), whereas Chk1 phosphorylation, which is a key kinase in ATR signaling that requires the maintenance of G2/M arrest,[17] was substantially reduced in RS-Fs after IR (Fig. 1E).

The recruitment of BRCA1, RPA, and RAD51 is impaired in RS-Fs after IR

To identify the step of impaired DNA damage signaling in the G2 phase, IR-induced foci formation was examined (Fig. A1). γH2AX foci were formed normally in RS-Fs (Fig. 2A); however, MDC1 and 53BP1 foci formation was reduced in RS-Fs, although the reduction was less than ATM inhibitor-treated normal cells (Fig. 2B, C). DSB end-resection, an initial step of HR, was examined by monitoring RPA foci (an RPA-coated single-stranded DNA is a scaffold for ATR-Chk1 activation).[19] RPA foci were modestly reduced in RS-Fs (Fig. 2D). The recruitment of BRCA1, a central regulator of DSB end-resection, was significantly impaired in RS-Fs (Fig. 2E). Furthermore, we found a significant reduction in RAD51 foci, which is a central factor in HR (Fig. 2F).

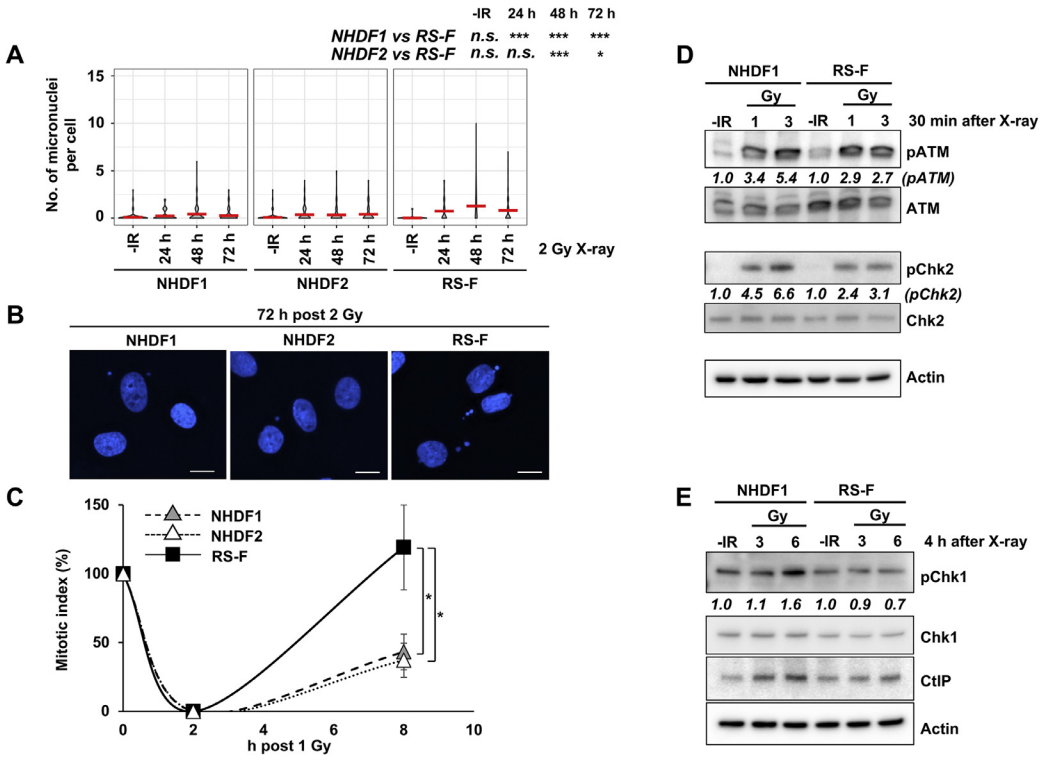

Figure 1 Radiation-sensitive fibroblasts exhibited impaired G2/M arrest and enhanced micronuclei formation after ionizing radiation.

Abbreviations: IR = ionizing radiation; RS-Fs = radiation-sensitive fibroblasts.

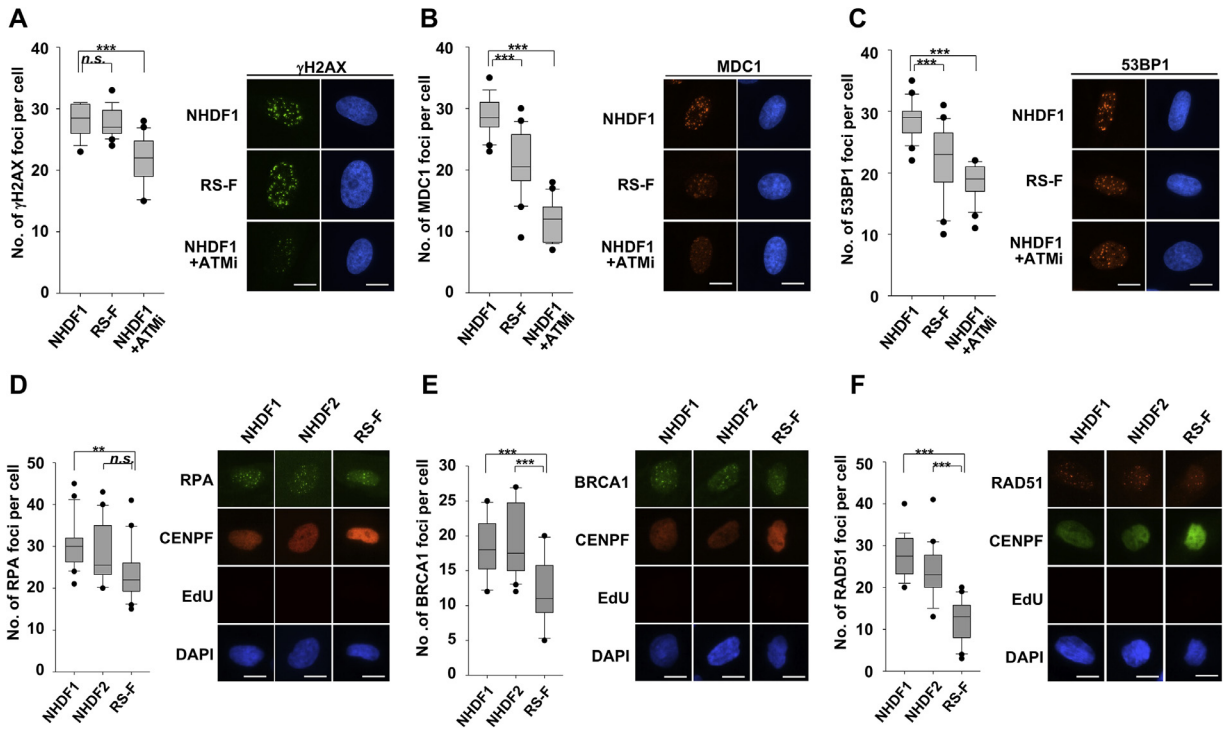

Figure 2 Radiation-sensitive fibroblasts exhibited a reduction of BRCA1, RPA, and RAD51 foci formation in the G2 phase after ionizing radiation.

Abbreviations: RS-Fs = radiation-sensitive fibroblasts.

To investigate DSB repair kinetics, γH2AX foci disappearance was examined (Fig. 3A). We found a slightly faster repair in G2 phase of RS-Fs. Such faster repair might be caused by a repair pathway switch owing to the impairment of resection,[20] although CtIP expression was still observed in RS-Fs (Fig. 1E and Table A3). In contrast, the DSB repair kinetics in the G1 phase of RS-Fs was normal (Fig. 3B-D). Collectively, these data support the notion that the impairment of G2/M-checkpoint maintenance in RS-Fs is caused by the attenuation of ATR-Chk1 signaling, due to the impairment of the HR process in the irradiated G2 phase.

RS-Fs exhibit increased inflammatory gene expression after IR

Using the RNA-seq data set in RS-Fs, we compared the gene expression patterns of ISG selection, inflammatory response, and interferon alfa after IR. We found that RS-Fs exhibited greater upregulation of the genes in ISG selection, inflammatory response, and interferon alfa than normal cells (Fig. 4A-C). These results were confirmed by GSEA (Fig. 4D-F). To assess the expression of cytosolic nucleic acid sensors, the mRNA levels of *cGAS, STING,* and *RIG-I* was examined. *cGAS* expression was consistently low and not significantly different in both normal fibroblasts and RS-Fs, and *STING* expression was slightly lower in RS-Fs than in normal fibroblasts, whereas *RIG-I* expression was comparable (Fig. 5A, B). Furthermore, expression of DSB repair factors related to DSB end-resection during HR was examined; however, there were no significant changes in the mRNA expression (Fig. 5C).

The RIG-I, MDA-5, and IL6 pathways in RS-Fs were upregulated after IR

To investigate the activation of the downstream pathways, signaling cascades were explored using the pathway map included the cGAS-STING, RIG-I, MDA-5, IRF3/IRF7, IL6, and JAK/STATs. The expression of *RIG-I/MDA-5, IRF7, JAK1/2,* and *STAT1/3* was modestly upregulated in normal fibroblasts (Fig. 6A, B); in contrast, the

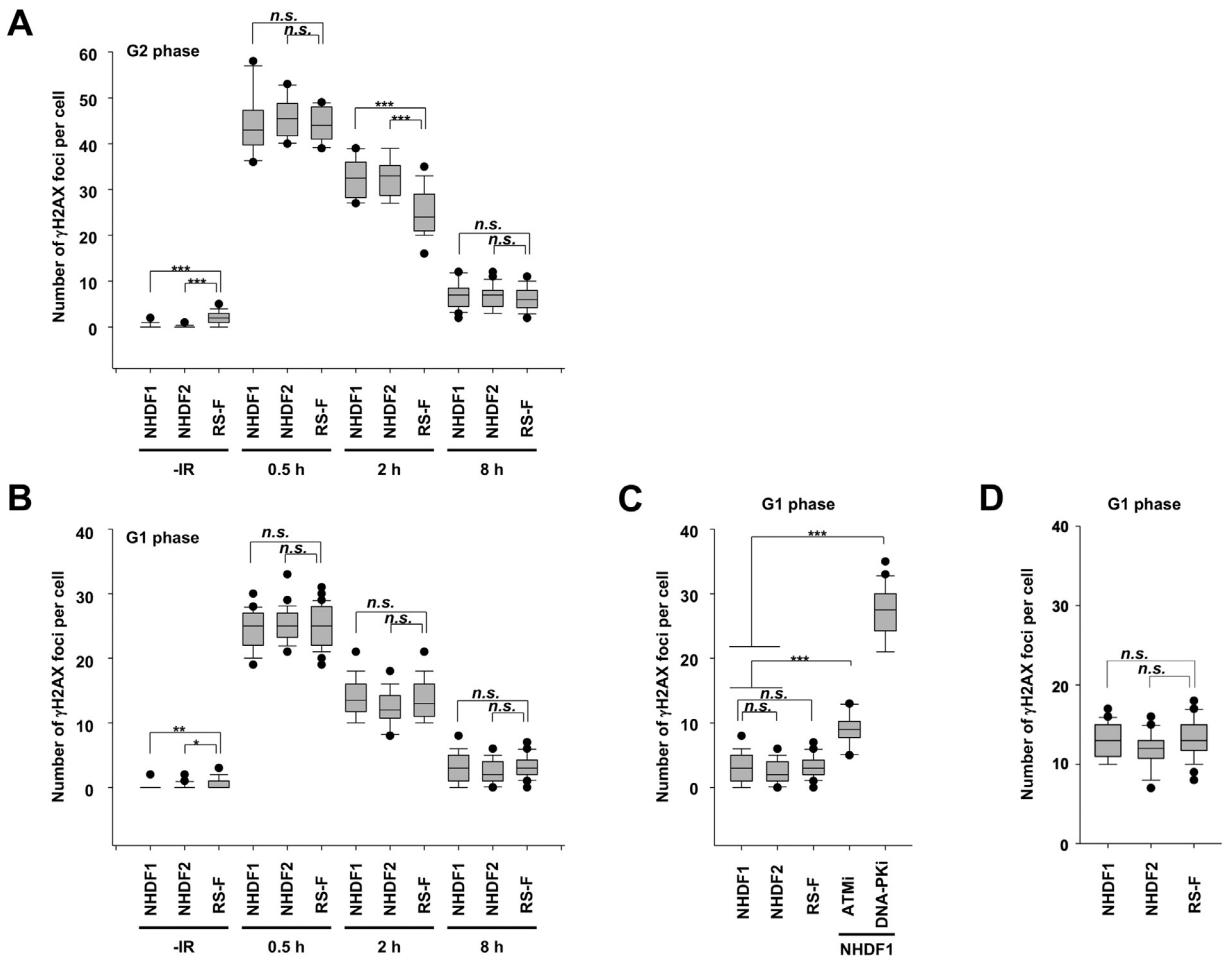

Figure 3 Radiation-sensitive fibroblasts did not show significant change of DSB repair kinetics after ionizing radiation.
Abbreviations: RS-Fs = radiation-sensitive fibroblasts.

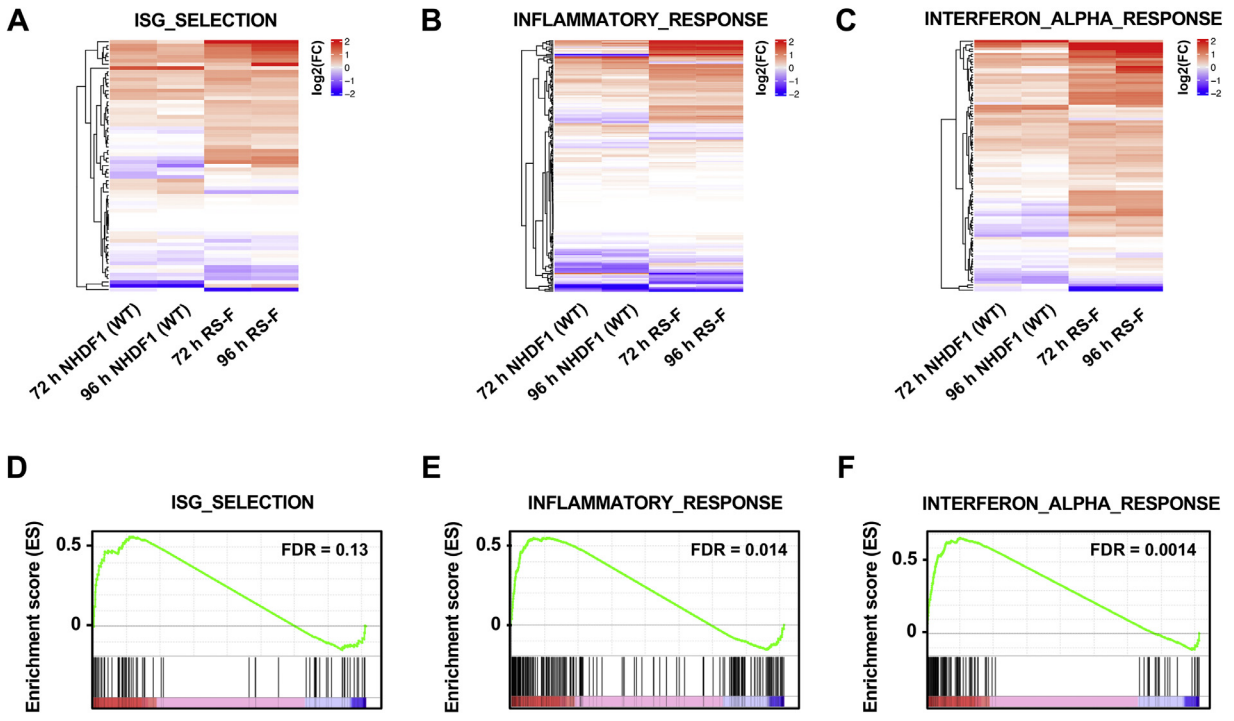

Figure 4 Radiation-sensitive fibroblasts exhibited increased inflammatory gene expression after ionizing radiation.
Abbreviations: RS-Fs = radiation-sensitive fibroblasts.

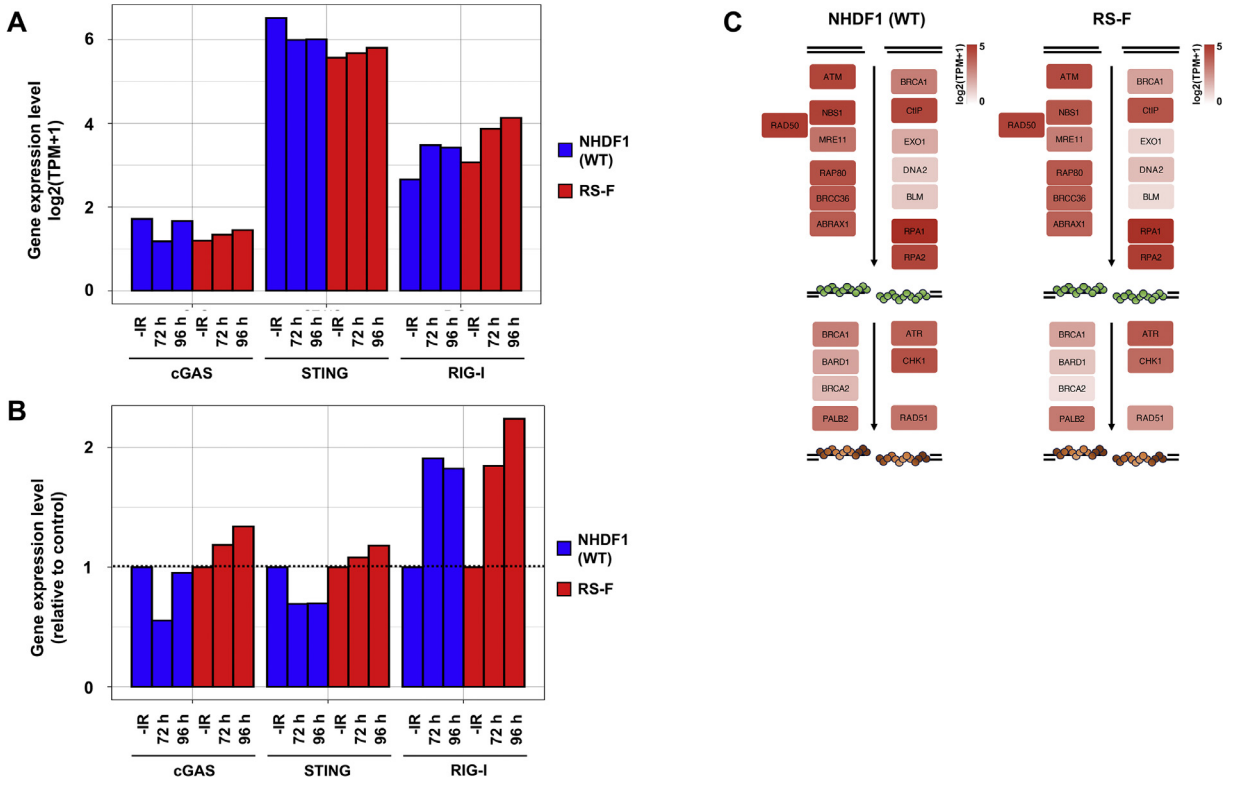

Figure 5 Analysis of cGAS, STING, and RIG-I expression after ionizing radiation.
Abbreviations: RS-Fs = radiation-sensitive fibroblasts.

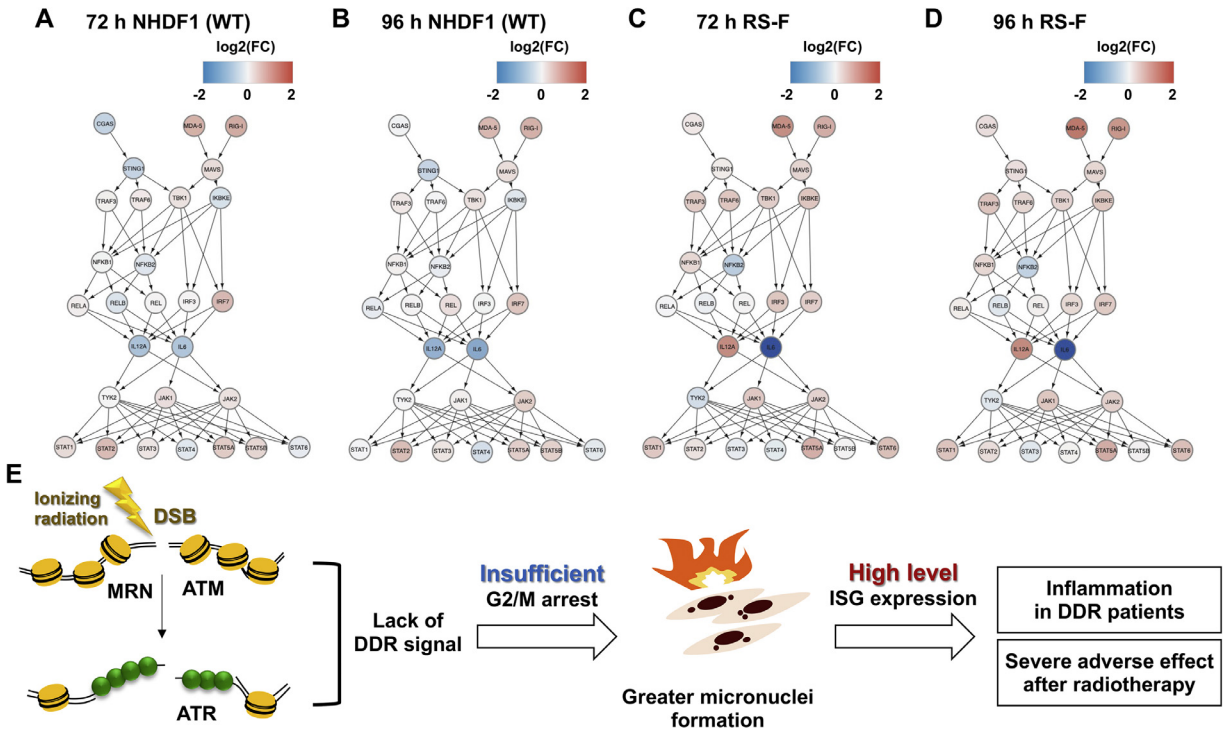

Figure 6 The RIG-I, MDA-5, and IL12A pathways were upregulated in radiation-sensitive fibroblasts after ionizing radiation.
Abbreviations: RS-Fs = radiation-sensitive fibroblasts.

expression of RIG-I/MDA-5, IRF3/7, IL12A, JAK1/2, and STAT1/2 were significantly upregulated in RS-Fs after IR (Fig. 6C, D). Collectively, these data suggest that activation of unusual signal cascades, such as the RIG-I/MDA-5-IL12A and cGAS/STING pathway, may trigger unexpected severe inflammatory responses after RT.

Discussion

We found abnormally high level of IR-induced inflammatory gene expression in RS-Fs derived from a patient with severe esophageal inflammation after RT. From the data obtained in this study, we predicted that a gene is involved in DNA damage signal transduction for G2/M-checkpoint arrest whose expression is downregulated in RS-Fs (Fig. 6E). However, additional evaluation will be required because of the lack of validation by qPCR and proteomics approaches. Although the results are from a single patient, and the responsible gene was not identified, the findings may provide an important information for developing a strategy for the prediction of unexpected inflammatory responses during RT. Additional discussion is provided in the discussion of Supplementary Materials.

Conclusion

Our findings suggest that impaired G2/M-checkpoint arrest due to the lack of DSB end-resection and ATR-Chk1 signaling can exacerbate inflammation during RT. The development of a gene panel related to G2/M-checkpoint arrest based on further investigation with a larger clinical sample cohort may contribute to the prediction of unexpected inflammatory responses during RT.

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.adro.2024.101530.

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