Volume 23, No 4 | International Journal of Radiation Research, October 2025

Integrative analysis of immune gene features and radiotherapy sensitivity in rectal cancer

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► ***Original article***

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Received: October 2024
Final revised: December 2024
Accepted: December 2024

Int. J. Radiat. Res., October 2025; 23(4): 945-951

DOI: 10.61186/ijrr.23.4.16

Keywords: *Immunotherapy, PLA2G2A, PLA2G4A, rectal cancer, radiotherapy.*

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ABSTRACT

Background: To investigate the correlation between immune gene expression patterns and radiotherapy (RT) response, aiming to identify novel biomarkers for personalized rectal cancer treatment. ***Materials and Methods:*** Data from rectal cancer patients were clustered into two subgroups based on immune gene expression. Differentially expressed genes (DEGs) between subgroups were identified by using the limma package, and survival analysis was conducted with the Kaplan-Meier (KM) method. ClusterProfiler was used to conduct GO and KEGG pathway enrichment analyses on the DEGs. Protein-protein interaction (PPI) networks were utilized to pinpoint key modules or hub genes through interaction scores. In the TCGA rectal cancer dataset, 165 samples were divided into high (n=39) and low (n=126) immune gene expression groups based on the expression of 1,959 immune-related genes. ***Results:*** Between the two groups, 775 DEGs were up-regulated and 35 were down-regulated. Analysis of the GSE35452 dataset revealed 308 up-regulated and 209 down-regulated DEGs between the RT-responsive group (n=24) and the non-responsive group (n=22). PPI analysis showed that PLA2G2A and PLA2G4A exhibited the highest interaction score (value = 0.918). ***Conclusion:*** Through gene enrichment and PPI network analysis, potential core targets PLA2G2A and PLA2G4A were identified, providing new biomarkers for personalized treatment in rectal cancer.

INTRODUCTION

Colorectal cancer (CRC) ranks among the leading malignancies in terms of incidence and mortality, imposing a significant economic and public health burden [1]. A study projected that in 2024, rectal cancer would constitute 30.25% (n=46,220) of the 152,810 new invasive colorectal cancer (CRC) cases in the United States [2]. Approximately 70%-75% of rectal cancer patients are diagnosed with mid-low rectal cancer, which predominantly affect the rectal wall and mesentery below the peritoneal reflection [3]. Early clinical manifestations of rectal cancer are often atypical. As the disease progresses, patients may experience changes in bowel habits, rectal bleeding, mucus discharge, intestinal obstruction, and weight loss, all of which are associated with a poor prognosis [4]. Identifying effective treatment strategies for rectal cancer is crucial for improving patients' quality of life, and reducing the individual and societal burden of the disease.

Current treatment modalities for rectal cancer mainly include surgery, chemotherapy, radiotherapy (RT), immunotherapy, and targeted therapy [5]. Patients with rectal cancer who face challenges in preserving the anus or strongly wish to do so are advised to consider local excision (LE) followed by a wait-and-see approach [6]. With total mesorectal excision (TME) becoming the standard procedure for low and intermediate rectal cancer, surgical radicality has been effectively improved, and patients with rectal cancer have achieved significant improvement in survival and local recurrence rates [7, 8]. Challenges like limited visual field during low rectal cancer surgeries and instrument interference due to restricted operating space elevate the risk of surgical complications [9, 10]. Meanwhile, the colostomy performed by the patient after surgery will change the patient's defecation pathway, making it difficult for the patients to control the frequency and duration of defecation, which seriously affects the quality of life [11]. Chemotherapy is the primary treatment for

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[DOI: 10.66224/ijrr.23.4.16]

intermediate and advanced rectal cancer. However, drug resistance and low sensitivity in most patients elevate the risk of treatment failure and tumor progression [12]. RT can accurately locate the lesions through three-dimensional conformal therapy, which is more suitable for patients with rectal cancer with complex anatomical locations [13]. However, some groups of rectal cancer may modulate resistance to RT through mechanisms of apoptosis, autophagy, cell cycle and DNA damage repair [14]. Targeted therapy against specific molecular markers and immunotherapy using the immune system to attack cancer cells provide more treatment options for patients with rectal cancer [15]. Research has focused on identifying populations suitable for immunotherapy, as programmed death receptor 1 (PD-1) / programmed death ligand 1 (PD-L1) inhibitors have demonstrated promising responses in patients with different mismatch repair (dMMR) / microsatellite instability-high (MSI-H) genotype rectal cancer [16].

Combined RT and immunotherapy have demonstrated clinical benefits in the treatment of rectal cancer patients [17-19]. As our understanding of the tumor immune microenvironment deepens, research indicates that the expression patterns of immune genes may significantly influence the efficacy of both RT and immunotherapy. The novelty of this study lies in the first integration of immune gene expression patterns and RT response data to systematically explore their interrelationships and reveal the potential roles of immune genes in rectal cancer RT sensitivity. This study analyzed The Cancer Genome Atlas (TCGA) rectal cancer dataset and the GSE35452 dataset, identifying key genes linked to immune response and radiotherapy sensitivity, and discovering new core target factors, PLA2G2A and PLA2G4A, through gene enrichment and PPI network analysis. These findings introduce a novel biomarker for personalized rectal cancer treatment, particularly in the context of combining immunotherapy with radiotherapy.

MATERIALS AND METHODS

TCGA rectal cancer clustering analysis

Rectal cancer tumor sample data and associated clinical information were obtained from the TCGA database (https://portal.gdc.cancer.gov). We extracted gene expression data in Transcripts Per Million (TPM) format and performed log2(TPM + 1) normalization [20]. Samples with complete RNAseq data and clinical information were included in subsequent analyses. A consensus clustering analysis was performed on rectal cancer samples using the expression data of 1,959 immune genes. The analysis involved a maximum of six clusters, with 100 iterations and random sampling of 80% of the samples for each iteration. The clustering analysis was performed using the ConsensusClusterPlus package [21]. The clustering algorithm selected was clustering = "hc," with innerlinkage = "ward.D2" to optimize the clustering results.

TCGA rectal cancer differential gene analysis

We utilized the limma package in R to analyze differential mRNA expression [22]. We analyzed adjusted P-values in the TCGA and GTEx datasets to correct for false positives. Differentially expressed mRNAs were identified using thresholds of an adjusted P-value <0.05 and log2(fold change) > 2 or < -2. Clustering heat maps of gene expression were created with the pheatmap package (https://CRAN.R-project.org/package=pheatmap) [23]. In the heatmap analysis, we first filtered for genes with a variance greater than 0.1. If the number of target genes exceeded 1,000, we ranked them by expression variance and selected only the top 25% of genes with the highest variance for further display. All statistical analyses were conducted using R (R software, version 4.0.3, USA). A P-value below 0.05 was deemed statistically significant.

TCGA rectal cancer subgroup survival analysis

We conducted a Kaplan-Meier survival curve analysis to evaluate the association between immune gene expression patterns and both overall survival (OS) and progression-free survival (PFS) in rectal cancer patients [24]. Survival differences between groups were compared using the log-rank test, with statistical significance determined by P-values <0.05. Hazard ratios (HR) and 95% confidence intervals (CI) were used in the survival curve analysis to assess the relative survival risk differences between groups. The median survival time (median time) was used to describe the survival time when the survival rate was 50%, expressed in years. Analyses were performed using the survival and survminer packages in R (25), considering P-values below 0.05 as statistically significant.

Gene expression Omnibus databas (GEO) rectal cancer dataset selection

We downloaded data in MINiML format from the GEO database. For datasets that had not undergone normalization, we applied log2 transformation. For datasets lacking standardization, we applied the normalize.quantiles function from the preprocessCore package (http://www.bioconductor.org/packages/3.0/bioc/html/preprocessCore.html) to achieve data normalization. We utilized platform annotation data to convert probe IDs into gene symbols, excluded probes linked to multiple genes, and averaged values for genes represented by multiple probes. We removed batch effects using the removeBatchEffect function from the limma package in R (R software,version 4.0.3, USA) .

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All patients underwent standardized curative resection after a 4-week interval following chemoradiotherapy, which included a total radiation dose of 50.4 Gy, UFT (300-500 mg/day), and LV (75 mg/day).

GEO rectal cancer differential genes in RT response/non-response

We utilized the limma package in R (R software,version 4.0.3, USA) to explore differential mRNA expression. We analyzed adjusted P-values to correct for false positives. Differentially expressed mRNAs were identified using thresholds of an adjusted P-value less than 0.05 and a log2(fold change) greater than 1.3 or less than -1.3.

Key and Hub gene identification

We utilized the Venn online tool (https://www.anychart.com/zh/products/anychart/gallery/Venn_Diagram/) for intersection analysis of differentially expressed genes and identified key genes via the STRING database (https://cn.string-db.org/).

RESULTS

Dataset

The final TCGA colorectal cancer dataset included 165 samples that met our analytical criteria. The rectal cancer patient RT dataset, GSE35452, was incorporated into the analysis, which was based on the GPL570 platform and comprised 46 samples.

TCGA subgroup clustering

The disparity between groups was more pronounced when the data were clustered into 2 categories according to the 1959 immunity genes. Figure 1A presented the cumulative distribution function (CDF) curve alongside the CDF Delta area curve. In consistent clustering, the Delta area curve depicted the variation in the area under the CDF curve for each category number k compared to k-1. The x-axis represents the category number k, and the y-axis shows the relative change in the area under the CDF curve. At K=2, we observed the heatmap of the ConsensusClusterPlus clustering results (figure 1B) and the principal component analysis (PCA) plot (figure 1C), where both rows and columns represented samples, and different colors signified different categories, showcasing significant gene expression differences between the two groups. We also included a heatmap illustrating immune-related gene expression across various subgroups (figure 1D), with red representing high expression levels and blue indicating low expression levels.

Survival analysis and differential gene expression in TCGA subgroups

Survival analysis indicated no significant difference in OS between high and low immune gene expression groups in rectal cancer (HR=1.283, 95% CI=0.501-3.289, P=0.604) (figure 2A). The median OS for the low expression group was 4.6 years, with a 50% survival rate. PFS showed no significant difference between the groups (HR=0.747, 95% CI=0.327-1.706, P=0.488), with the low expression group having a median PFS of 6.5 years (figure 2B). The numbers listed in the table below the figures represent the number of patients still under observation at specific time points. The cumulative probability curve in the upper right corner typically displays time on the x-axis and the cumulative risk of adverse prognostic events on the y-axis. The shape and position of the curve indicate the magnitude and distribution of disease progression risk. Differential gene expression analysis between the high (n=39) and low (n=126) expression groups revealed 775 upregulated and 35 downregulated genes (figure 2B).

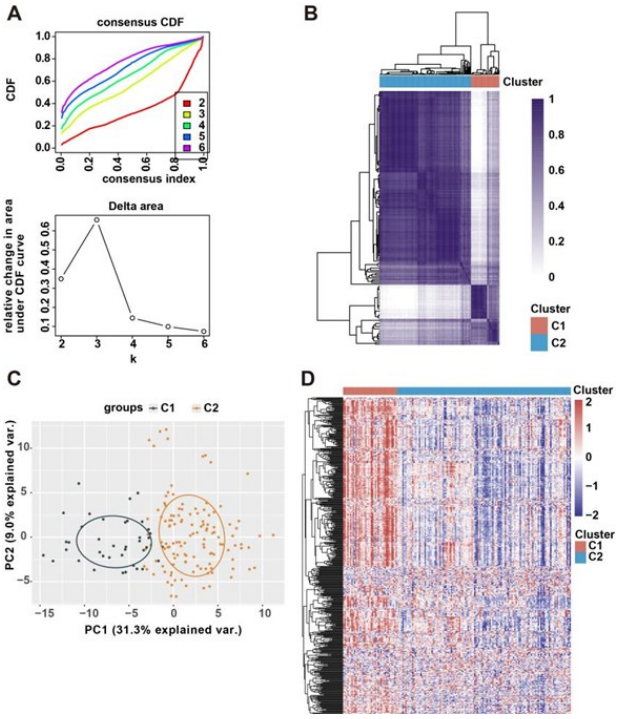


Figure 1. Immune gene clustering and expression analysis. **(A)** Cumulative Distribution Function (CDF) and CDF Delta area curves; **(B)** Clustering outcomes for K=2; **(C)** PCA plot showing clustering results; **(D)** Heatmap of immune-related gene expression among subgroups.

Differential genes in response/non-response to RT in GEO

The GSE35452 dataset included RT responders (n=24) and non-responders (n=22). The analysis of DEGs revealed 308 upregulated and 209 downregulated genes between the two groups (figure 3A). A heatmap of the DEGs between the groups was presented in figure 3B. The GO analysis of molecular function (MF) identified 1,094 enriched pathways, with key pathways including gated channels, neurotransmitter receptors, and actin binding. The biological process (BP) analysis revealed 4,932

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[DOI: 10.66224/ijrr.23.4.16]

enriched pathways, highlighting major pathways such as regulation of hormone levels, chemical synaptic transmission, and cellular responses to organonitrogen compounds. The cellular component (CC) analysis identified 580 enriched pathways, focusing on presynapses, monoatomic ion channel complexes, and the apical part of the cell. KEGG pathway enrichment analysis identified 272 pathways, highlighting neuroactive ligand-receptor interactions, steroid hormone biosynthesis, and GABAergic synapses as key pathways.

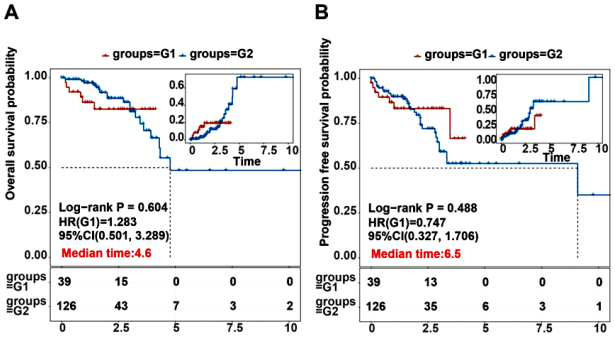


Figure 2. Survival analysis and differential gene expression of immune genes in colorectal cancer. **(A)** KM survival curve for OS between the high and low expression groups of immune genes; **(B)** KM survival curve for PFS between the high and low expression groups.

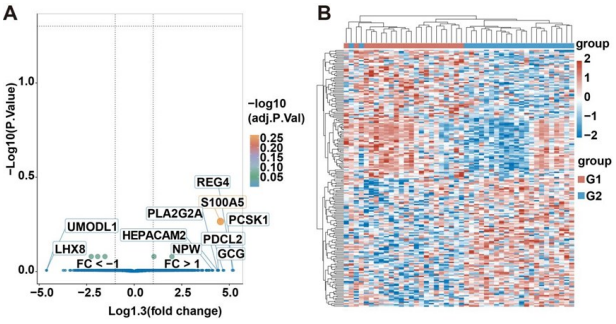


Figure 3. Differential gene expression and pathway enrichment analysis in RT responders and non-responders. **(A)** Analysis of differential gene expression between RT responders (n=24) and non-responders (n=22); **(B)** Heatmap illustrating gene expression differences between these groups.

Identification of intersection and Hub genes

The Venn online database indicated that there were 15 intersecting genes between TCGA and GEO (figure 4), including CD163L1, FRMD3, PLA2G4A, MYOF, PLA2G2A, RARRES1, PTGER2, SPON1, SCG2, PDGFRA, CXCL11, OGFRL1, ADAMTS12, FLNA, and LRRC15. The string interaction results showed that PLA2G2A and PLA2G4A exhibited the highest interaction score (value=0.918).

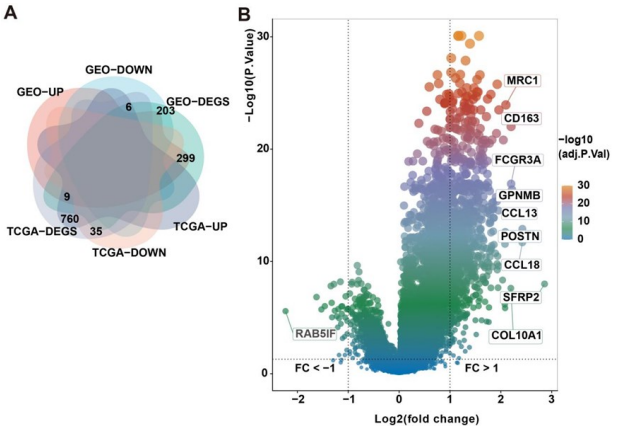


Figure 4. Intersecting genes between TCGA and GEO and their protein-protein interactions. **(A)** Venn results; **(B)** Differential gene expression analysis between the high (n=39) and low

DISCUSSION

In recent years, the combination of RT and immunotherapy has shown promising prospects in the treatment of various cancers, especially in rectal cancer [26]. Apart from directly killing tumor cells, previous studies have demonstrated that RT can effectively modulate and reprogram the tumor microenvironment, promoting the transition from an immunosuppressive phenotype to an immune-stimulatory phenotype. This, in turn, triggers the recruitment and activation of immune cells, leading to immunogenic cell death and enhancing the efficacy of immunotherapy [27]. Our results suggest that PLA2G2A and PLA2G4A could serve as core targets for RT combined with immunotherapy in rectal cancer patients, offering new insights for RT-immunotherapy strategies.

In recent years, more researchers have been exploring the efficacy of RT combined with immunotherapy, aiming to enhance the sensitivity of radiotherapy and provide a solid foundation for the application of immune checkpoint inhibitors [28]. This could be related to the following mechanisms. RT enhances T cell proliferation and activation, and induces dendritic cell maturation, improving their function as antigen-presenting cells [29, 30]. Conversely, RT promotes antigen release, aiding the targeted migration and infiltration of T cells into tumors [31]. RT enhances tumor cell sensitivity to CD8+ T cell-mediated cytotoxicity. RT activates the cGAS-STING pathway, promoting the expression of type I interferons, which further initiates and sustains T cell

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[DOI: 10.66224/ijrr.23.4.16]

-mediated adaptive immune responses, leading to tumor cell cycle arrest and immunogenic cell death, thus enhancing the effect of RT [32]. Therefore, RT combined with immunotherapy significantly strengthens immune responses, and the synergistic effect of both can greatly improve tumor treatment outcomes.

PLA2G2A, a marker of tumor-associated fibroblasts (TAFs), has been shown to promote tumor immune escape by inhibiting the antitumor immune response of CD8+ cytotoxic T cells [33]. Research by Li *et al*. demonstrated that PLA2G2A is associated with shorter OS in rectal cancer patients [34], a finding confirmed by other studies [35, 36]. He *et al*. Immunohistochemical analysis of pathological specimens from 172 rectal cancer patients revealed that PLA2G2A is linked to both survival outcomes and poor treatment response in those undergoing chemoradiotherapy [37]. However, no study has directly investigated the impact of PLA2G2A on survival and efficacy in rectal cancer patients undergoing RT combined with immunotherapy. Our findings indicate that PLA2G2A is one of the core targets for RT combined with immunotherapy in rectal cancer patients, which may provide new therapeutic insights for clinical treatment. We believe this could be related to the high expression of PLA2G2A during RT, which interferes with the activation of the cGAS-STING immune pathway, thereby weakening the radiotherapy effect [38]. The mechanism of this combination therapy may involve immunogenic cell death and remodeling of the tumor microenvironment induced by RT [39]. Targeting PLA2G2A could enhance immune cell recognition and attack of tumor cells, thereby improving patient survival [40].

PLA2G4A is a crucial enzyme in lipid metabolism, significantly influencing cell signaling, immune responses, and inflammation [41]. PLA2G4A is a key enzyme in the synthesis of various cytokines and prostaglandins, converting them into bioactive molecules such as prostaglandins and leukotrienes through the cyclooxygenase (COX) or 5-lipoxygenase (5-LOX) pathways, thereby regulating immune and inflammatory responses [42]. In the tumor microenvironment, PLA2G4A regulates inflammatory responses, possibly by altering the function of immune cells, promoting immune escape, and consequently enhancing tumor growth and metastasis [43]. Several studies have shown that the expression of PLA2G4A is significantly elevated in rectal cancer tumor tissues compared to normal tissues, and high expression of PLA2G4A is associated with tumor invasiveness and poor prognosis [44, 45]. Suppressing PLA2G4A expression using siRNA or specific inhibitors significantly reduces rectal cancer cell proliferation, migration, and invasion. Some studies have also suggested that inhibiting PLA2G4A activity may enhance the effects of RT [46- 48]. No research has examined the link between PLA2G4A expression and prognosis in rectal cancer patients receiving combined radiotherapy and immunotherapy. The study indicates that targeting PLA2G4A and PLA2G2A could be crucial for enhancing the efficacy of radiotherapy combined with immunotherapy in rectal cancer patients, offering significant insights for clinical drug and treatment selection.

This study has some limitations. First, although the sample size in the dataset is relatively large, the sample size in specific subgroups of patients may be small, which could affect the statistical power of the analysis. Second, although we identified potential associations between immune genes and RT response through intersection gene analysis, the specific biological functions and clinical applications of these genes still require further experimental validation. Despite providing new insights into the potential role of immune genes in RT response in rectal cancer, questions regarding RT dose, fractionation, and the duration of combination with immune checkpoint inhibitors (ICIs) remain to be addressed in future multicenter, large-sample, randomized controlled studies.

CONCLUSION

We identified that PLA2G2A and PLA2G4A may play significant roles in the immune microenvironment and RT responses in rectal cancer, offering potential biomarkers for exploring precise treatments. As the mechanisms underlying the improved prognosis of rectal cancer patients undergoing RT combined with ICIs are further investigated, and as new biological markers beyond MSI-H/dMMR emerge, the prospects for the application of RT combined with immunotherapy in rectal cancer appear promising.

***Acknowledgment*:** We sincerely thank all contributors and supporters of this research. First and foremost, we would like to thank KeJie Chen and ChengLong Liao for their guidance, insightful feedback, and constant encouragement throughout the study. The expertise and support were invaluable in shaping this project. We also extend our appreciation to TCGA and GSE35452 dataset providers (Watanabe T, Kobunai T, Hashimoto E) for making their datasets available to us. Without these resources, this study would not have been possible .resources, this study would not have been possible.

***Conflicts of Interest*:** All authors unanimously declare that there is no conflict of interest in this study.

***Funding*:** This study was supported by no funding.

***Ethical consideration*:** Not applicable.

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[DOI: 10.66224/ijrr.23.4.16]

***Author contribution*:** J.G. and H-C.L. were the co-first authors of this study, and both participated in the design of the study, data analysis, interpretation of the results, and writing of the paper. Z-J.R. was responsible for data collection and preliminary analysis, and assisted in the literature review. J.L. provided technical support for the data analysis part and provided important inputs on the presentation of the results. T-T.Y. and K-L.L. assisted in data organization and graph production. K-J.C. provided guidance during the study design and data analysis, and participated in the discussion of the results. C-L.L., as the corresponding author, was responsible for planning the overall research direction, supervising the data analysis, and guiding the writing and revision of the paper. All authors made significant contributions to the final content of this paper and agreed to its publication.

***Possible AI usage for manuscript preparation*:** No AI tools were used in the preparation of this manuscript.

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