Analysis of the TCRβ repertoire characteristics when combining radiotherapy and immunotherapy in cancer

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Abstract. Cancer is a global public health concern. Radiotherapy (RT) and immunotherapy are increasingly used in cancer treatment, but the specific immune mechanisms underlying their effects remain to be elucidated. The present study analyzed publicly available datasets, in which the T-cell receptor β chain (TCRβ) repertoire in tumor-bearing mice treated with RT and immunotherapy, as well as the TCR repertoire of T cells derived from hematopoietic stem cells (HSCs) of mice exposed to radiation doses of 0, 10 and 100 mGy were comprehensively evaluated. The results of the present study indicated that the diversity of the TCRβ complementarity-determining region 3 (CDR3) in tumor-bearing mice decreased after RT, anti-cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and anti-programmed cell death protein-1 (PD-1) treatment, especially in the RT and RT + anti-CTLA-4 or anti-PD-1 treatment groups. Furthermore, the CDR3 length markedly decreased in the RT group and markedly increased in the anti-CTLA-4 and anti-PD-1 groups. Next, the TCR profile of T cells differentiated from HSCs exposed to different radiation doses was examined. As the radiation dose increased, the TCRβ diversity in mice gradually decreased and the degree of clonal expansion markedly increased, the percentage of private sequences (unique sequences detected in only a single mouse) was markedly increased and the degree of overlap was markedly decreased. Furthermore, there was a notable difference in the usage frequency of TCRβ chain variable/joining gene segments among the different radiation dose groups. The present study demonstrated that RT and immunotherapy may alter the TCR repertoire owing to their effects on HSCs.

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Abbreviations: RT, radiotherapy; TCRβ, T-cell receptor β chain; TME, tumor microenvironment; CDR3, complementarity-determining region 3; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; PD-1, programmed cell death-1; HSC, hematopoietic stem cell; NSG, NOD scid γ; TRBV, TCR β chain variable gene; TRBJ, TCR β chain joining gene

Key words: cancer, TCRβ, high-throughput sequencing, radiotherapy, anti-CTLA-4 therapy, anti-PD-1 therapy

Introduction

Improvements in socioeconomic conditions have enhanced the average life expectancy of humans (1). However, an aging population has been associated with some public health concerns (2). In particular, the cancer incidence and mortality rates have been increasing (3). According to published epidemiological studies, global cancer incidence in 2012 reached 14.1 million new cases with 8.2 million associated deaths (4). By 2022, however, these figures had risen to ~19.98 million newly diagnosed cases and 9.74 million cancer-attributable deaths worldwide (3). Globally, cancer, a chronic disease, is a major cause of death (5,6).

Chemotherapy, radiotherapy (RT) and surgical resection were the main therapeutic strategies for cancer until the 21st century. RT was traditionally assumed to function by

irreversibly damaging tumor cell DNA, which led to cell death or loss of replicative ability (7). Preclinical and clinical studies have reported that the immune system also determines the response to RT (8,9). More than a century ago, clinicians observed complete remission in advanced cancer cases after acute bacterial infections (10). This observation established the concept of immunological approaches for cancer treatment. Immunotherapy has since transformed cancer treatment and improved the current understanding of tumor biology (11). It has been highlighted that cancer treatment should not only target the cancer cells but also consider the entire tumor microenvironment (TME). The cellular mediators of the anticancer effects of immunotherapy are immune cells (11). Previous studies have reported that the combination of RT and immunotherapy significantly improves cancer treatment outcomes (12,13). However, the effect of the combination of RT and immunotherapy on the T-cell receptor β chain (TCRβ) repertoire is unclear.

The immune system determines the anticancer efficacy of both RT and immunotherapy. T cells are involved in adaptive immune responses and complementarity-determining region 3 (CDR3) determines the specificity of T cells (14). CDR3 is generated through the recombination of germline V, D and J genes, as well as the deletion and insertion of nucleotides at the V(D)J junctions (15-17). As CDR3 closely interacts with antigen peptides, its sequence diversity is a key indicator of T cell diversity. Recombination events at the TCR loci can lead to the production of non-functional (out-of-frame) TCRs with frameshift mutations or premature stop codons (18,19). In this situation, the T cells attempt to rearrange the second allele. If a functional (in-frame) TCR is successfully formed, the T cells carry both functional and non-functional TCR genes (18). There is a certain level of mRNA in the T cells, although non-functional TCR genes cannot be translated into functional TCRβ chains. Non-functional TCRs can represent the pre-selection TCR repertoire as they are not influenced by the functional selection processes (both positive and negative selection) (19-21). Functional TCRs can be used to investigate the TCR repertoire after selection. Certain studies have demonstrated that deep sequencing analysis of TCRβ CDR3 can provide useful insights into the effect of treatment on the T cell response (22,23).

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) are immunosuppressive molecules expressed on the immune cell surface and can regulate immune activation (24). Furthermore, CTLA-4 and PD-1 prevent the occurrence of autoimmune effects. The present study analyzed a publicly available sequencing dataset of the immune repertoire of a mouse cancer model treated with a combination of RT and anti-CTLA-4/anti-PD-1 therapy. Furthermore, a public dataset of T cell repertoire sequencing derived from the hematopoietic stem cells (HSCs) of mice exposed to various radiation doses was also analyzed. The present study focused on examining the effects of RT and immunotherapy on the TCRβ repertoire in cancer treatment based on various parameters, such as TCRβ CDR3 diversity, frequency distribution of CDR3, length distribution of CDR3, V/J gene usage, V-J pairing and overlap indices. The findings from the present study will enhance the current understanding of radiation-induced adaptive immune responses and the TCRβ repertoire after RT and anti-CTLA-4/anti-PD-1 therapy, which can potentially provide valuable insights into the complex dynamics driving global cancer patterns.

Materials and methods

TCR sequencing data from public databases. The TCR sequencing data of T cells derived from CBA/H^{mCherry} mouse HSCs exposed to radiation doses of 0, 10 and 100 mGy were obtained online from the Adaptive Biotechnologies immuneACCESS database (https://clients.adaptivebiotech.com/pub/candeias-2019-mouse; immuneACCESS DOI: https://doi.org/10.21417/SC012019) (25). Meanwhile, the TCR data of tumor-bearing mice treated with the combination of RT and anti-CTLA-4/anti-PD-1 therapy were downloaded from different sources [https://clients.adaptivebiotech.com/pub/rudqvist-2017cancerimmunology-research, immuneACCESS DOI: https://doi.org/10.21417/B7H34S (26) and https://clients.adaptivebiotech.com/pub/dovedi-2017-clincancerres, immuneACCESS DOI: https://doi.org/10.21417/B7TS67 (27)]. The T cell repertoires in 45 samples (9, 16 and 20 samples from mice exposed to different radiation doses, mice treated with RT and anti-CTLA-4 therapy for breast cancer and mice treated with RT and anti-PD-1 therapy for colon cancer, respectively) were analyzed. TCRβ sequencing was performed on peripheral blood samples exhibiting the highest levels of donor-derived T lymphocyte reconstitution. Based on the percentage of T lymphocytes, the top samples were chosen from the following groups: 11-15% (HSCs exposed to 0 mGy), 4-13% (HSCs exposed to 10 mGy) and 3.5-10% (HSCs exposed to 100 mGy). The yield of unique TCRβ rearrangements was extremely low in 1 NSG mouse reconstituted with 100 mGy-irradiated HSCs, which exhibited a low T-cell frequency of merely 3.5% among leukocytes. Thus, these mice were classified as outliers based on the Hubert and Vandervieren test and excluded from subsequent analyses (25). The original research had obtained ethical approval.

DNA extraction, TCRβ immunosequencing and data analysis. The experimental procedures, sample collection, DNA extraction and TCRβ immunosequencing were previously described in the original study (26), while data analysis based on process data was conducted as part of the present study. Briefly, blood samples were collected from mice 6 months after receiving HSC transplants with radiation doses of 0, 10 and 100 mGy. Additionally, breast cancer and colon cancer tissues were obtained from mice treated with RT combined with anti CTLA-4, RT combined with anti PD-1 and untreated mice. The mouse blood samples and the untreated and treated tumor samples had been subjected to TCRβ CDR3 immunosequencing using the ImmunoSEQ™ assay (28,29). A synthetic immune receptor library was used to identify and reduce PCR biases and computational techniques were applied to remove any remaining biases after sequencing and ensure the quantitative accuracy of the ImmunoSEQ™ detection kit (29). PCR bias assessed the total amplification bias by computing the following two metrics for the amplification bias of each template compared with the mean: The dynamic range (maximum bias divided by minimum bias) and the sum of squared logarithmic bias values. The process of altering primer concentrations was repeated until improvements ceased (29). In the biased-controlled multiplex PCR experiment, the extracted DNA was amplified using 54 forward primers targeting V genes and 13 reverse primers targeting J

genes. High-throughput sequencing was then performed. The raw data were processed and analyzed with an ImmunoSEQ™ analyzer (http://www.adaptivebiotech.com/immunoseq). Then, a reanalysis of the data from the original study was performed in the present study. Subsequently, correction of sequencing errors and PCR amplification bias was performed with MiTCR (http://mitcr.milaboratory.com/) (30). The international ImMunoGeneTics database (www.imgt.org) provided gene definitions for the TCRβ V, D and J genes. As the CDR3 sequence can be generated by various pathways, it cannot directly provide the probability distribution of hidden recombination events (31,32). Consequently, TCR nucleotide sequence probabilities were calculated using the existing recombination model (33). According to the probability model, specific sequences of CDR3 may be generated based on the original recombination process. This enables the annotation of the V(N)D(N)J genes for each distinctive CDR3 and the determination of the amino acid sequence for each gene. Furthermore, the combat function in the sva package (R version 4.4.1) was used to address batch effects across different datasets. Next, TCR data were subjected to various bioinformatics analyses based on the R statistical programming language (R version 4.4.1), which encompassed the diversity of TCRβ repertoire, usage of V/J gene, V-J gene pairing, length distribution of CDR3 and vDeletion, d3Deletion, d5Deletion, jDeletion, n1Insertion and n2Insertion distribution (14,34-37). These analyses were evaluated using previously published research (14,34,37). The D50 index, Gini index, inverse Simpson index, Shannon index and Simpson index were used to calculate the TCR repertoire diversity (38,39). Shared TCR repertoires between species were quantified using overlap coefficients of |X and Y|/min (|X|, |Y|). The TCRβ overlap was calculated based on the number of common nucleotide sequences in each sharing category (summed across all subjects in which this sequence was found)-[Percentage (n)=number of common nucleotide sequences appeared in ≥n subjects/total number of nucleotide sequences in sample]. In addition, principal coordinate analysis (PCA) was used to perform cluster analysis on the V/J genes of mice in the radiation exposure group and healthy control (HC) group.

Statistical analysis. Means between two groups were compared using the unpaired t-test or the Mann-Whitney U test. One-way ANOVA or Kruskal-Wallis test was used to compare group means across three or more groups and Bonferroni was used to correct for multiple comparisons. Additionally, two-way ANOVA followed by Tukey's test was used. Data are represented as mean ± SD or percentages (%). All statistical analyses were performed using SPSS (version 20; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

RT and anti-CTLA-4 therapy alters the TCR repertoire

Alterations in TCRβ repertoire diversity. The TCRβ repertoires of T cells sorted from tumors of the untreated (control), RT, anti-CTLA-4 and anti-CTLA-4 + RT groups were analyzed. The diversity indices were determined based on D50, Gini, inverse Simpson, Shannon and Simpson indices (Fig. 1). When considering the Gini or Simpson indexes, a smaller value is associated with a higher CDR3 diversity. The greater the index, the more diverse the CDR3, when it comes to the D50, Shannon and inverse Simpson diversity indices. In the post-selection repertoire (in-frame sequences; Fig. 1A-E), the D50 index in the anti-CTLA-4 + RT group was lower than that in the control ($P<0.01$; Fig. 1A). The Gini index in the anti-CTLA-4 + RT group was higher than that in the control ($P<0.01$), RT ($P<0.05$) and anti-CTLA-4 ($P<0.01$) groups (Fig. 1B). The inverse Simpson index in the RT and the anti-CTLA-4 + RT groups was lower than that in the control group (both $P<0.01$; Fig. 1C). The Shannon index in the RT group ($P<0.05$) and anti-CTLA-4 + RT ($P<0.001$) groups was lower than that in the control group, furthermore, it was also significantly lower in the anti-CTLA-4 + RT group compared with that in the anti-CTLA-4 group (Fig. 1D). Other parameters were not significantly different between the control and treatment groups. Furthermore, similar trends were observed in the pre-selection repertoire (out-of-frame TCRβ sequences; Fig. 1F-J). In summary, the diversity of the TCRβ CDR3 amino acid sequence in the treatment group (especially in the RT + anti-CTLA-4 group) was lower than that in the control group.

Alterations in CDR3 length distribution. To further understand the changes in the TCRβ repertoire induced by radiation and anti-CTLA-4 treatment, the length distribution of the CDR3 nucleotide sequences was examined. The overall difference in CDR3 length between the anti-CTLA-4 group and the control group did not reach statistical significance ($P>0.05$), but the CDR3 length was longer at 45-nucleotides compared with the control group ($P<0.01$) (Fig. 1K). The CDR3 length in the RT group was lower than that in the control group ($P<0.001$; Fig. 1L). Additionally, the CDR3 length distribution was significantly different between the RT + anti-CTLA-4 and control groups ($P<0.05$, Fig. 1M). Furthermore, the CDR3 length in the anti-CTLA-4 group was higher than that in the RT group ($P<0.05$; Fig. 1N).

RT and anti-PD-1 therapy alters the TCR repertoire

Alterations in TCRβ repertoire diversity. Next, the present study examined the TCRβ diversity and complexity after RT and/or anti-PD-1 treatment. The D50, Gini, inverse Simpson, Shannon and Simpson indices were used to evaluate the TCRβ amino acid sequence diversity in the control, RT, anti-PD-1 and RT + anti-PD-1 groups. The diversity of the in-frame TCRβ sequences was first examined (Fig. 2). As shown in Fig. 2B, within the radiation field (radiation irradiated tumors), the Gini index in the RT and RT + anti-PD-1 groups was higher than that in the control group (both $P<0.01$). Additionally, the Gini index in the RT and RT + anti-PD-1 groups was higher than that in the anti-PD-1 group (both $P<0.01$). Additionally, although changes in other parameters were observed between the treatment group and the control group, these differences did not reach statistical significance ($P>0.05$; Fig. 2A and C-E). Next, the diversity of TCRβ outside the radiation field (tumors that have not been irradiated with radiation) was examined. The Gini index in the RT + anti-PD-1 group was higher than that in the RT group ($P<0.05$; Fig. 2G). Similar to within the radiation field, although changes in other parameters between the treatment group and the control group could be observed outside the radiation field, these differences did not reach statistical significance ($P>0.05$; Fig. 2F and H-J). The differences in

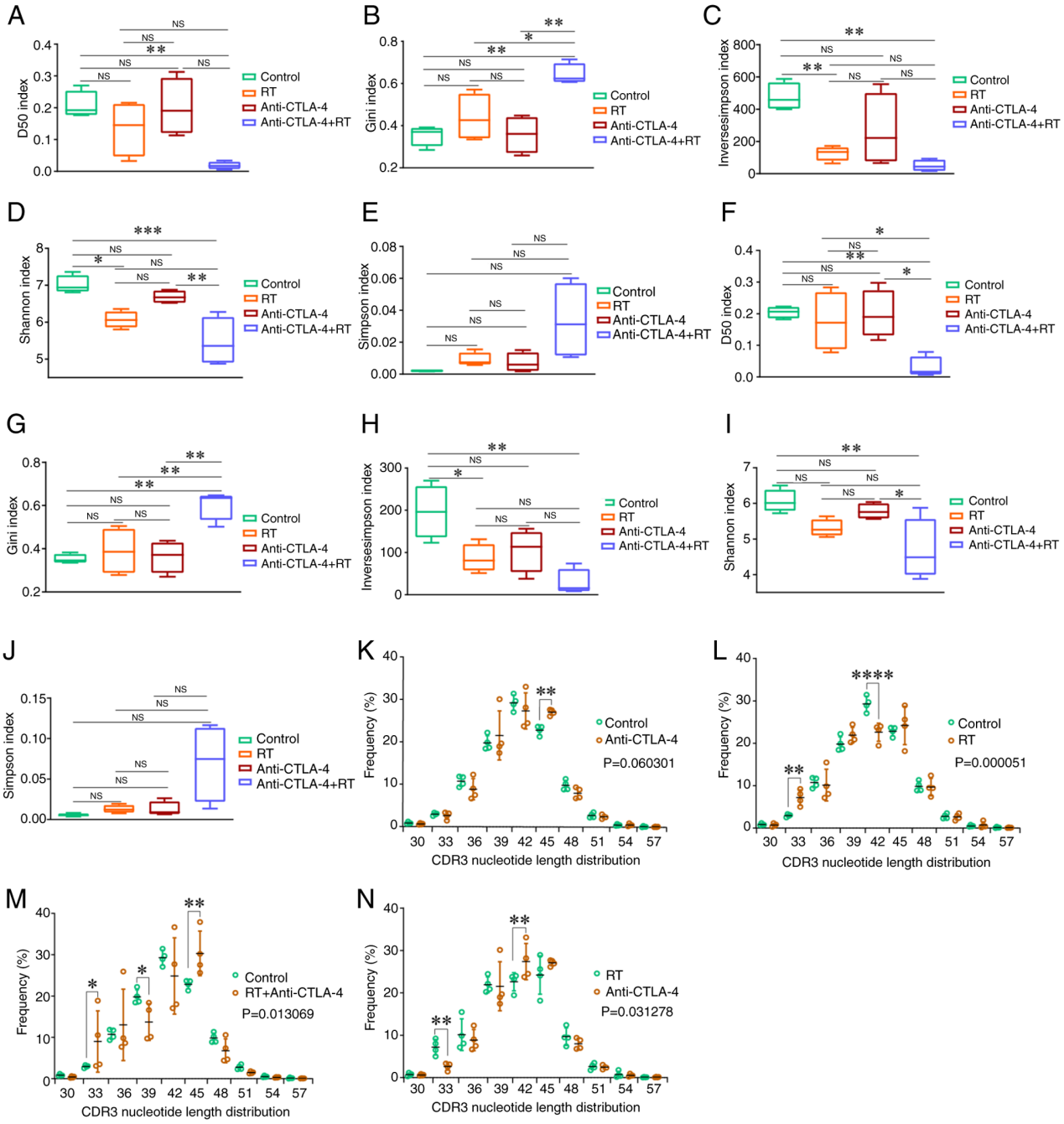

Figure 1. CDR3 nucleotide length distribution and diversity analysis of the TCRβ repertoire in mice with RT and anti-CTLA-4 therapy, and the control group. The TCRβ CDR3 diversity in the post-selection repertoire (in-frame sequences) was estimated by (A) the D50 index, (B) Gini index, (C) Inverse Simpson index, (D) Shannon index and (E) Simpson index. (F-J) The TCRβ CDR3 diversity in the pre-selection repertoire (out-of-frame sequences) was estimated by the aforementioned five diversity indices. Data are presented as the mean ± SD; comparisons were made using one-way ANOVA or Kruskal-Wallis test and corrected for multiple comparisons using Bonferroni. *P<0.05, **P<0.01 and ***P<0.001. (K-N) The comparison of CDR3 length between the different groups. All of the CDR3 lengths that differed significantly (P<0.05) between treatment group mice and controls are presented. Data are presented as the mean ± SD; the overall differences of the CDR3 length distribution (ten length categories) between groups were compared using two-way ANOVA, and Tukey's test was performed after the two-way ANOVA to compare the differences in each CDR3 length between groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CDR3, complementarity-determining region 3; TCRβ, T-cell receptor β-chain; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; RT, radiotherapy; NS, not significant.

TCRβ diversity in the post-selection repertoire were similar to those in the pre-selection repertoire (Fig. S1).

Alterations in CDR3 length distribution. The CDR3 length distribution within and outside the radiation area was examined. The TCRβ CDR3 length within the radiation area in the anti-PD-1 and RT + anti-PD-1 groups was significantly longer than that in the control group (P<0.05; Fig. 3A and C). Additionally, the CDR3 length of the anti-PD-1 group was shorter than that of the RT group (P<0.01; Fig. 3D). The CDR3 length outside the radiation area in the anti-PD-1 group was longer than that in the control group (P<0.001; Fig. 3E). In addition, whether within or outside the radiation field, the difference in CDR3 length between the RT group and the control group did not reach statistical significance (P>0.05; Fig. 3 and F). Furthermore, it was also observed that the CDR3 length was similar outside the radiation field

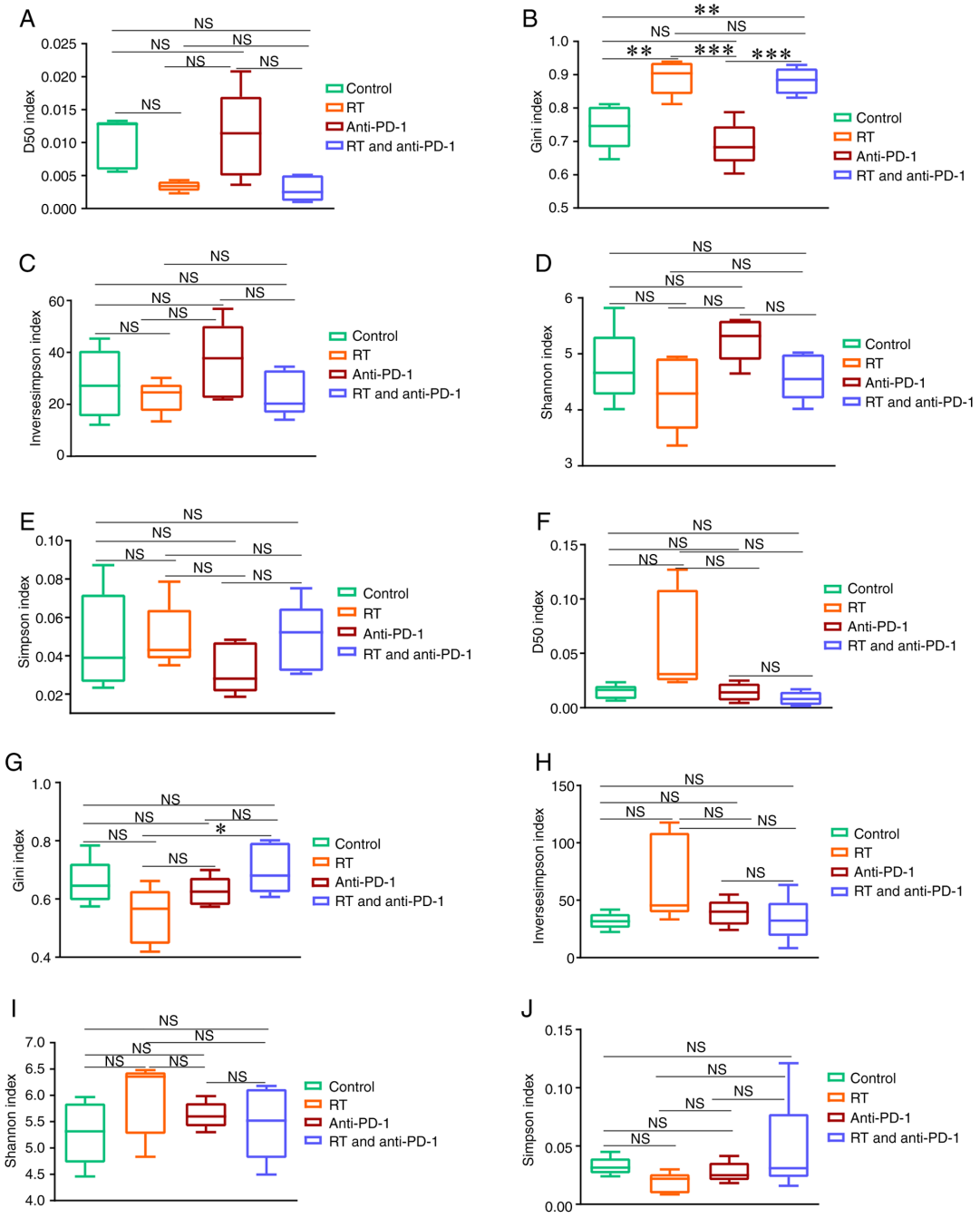

Figure 2. Diversity indexes of the post-selection repertoire in mice with RT and anti-PD-1 therapy, and the control group. The diversity of the treatment area was measured by (A) the D50 index, (B) Gini index, (C) Inverse Simpson index, (D) Shannon index and (E) Simpson index. (F) The D50 index, (G) Gini index, (H) Inverse Simpson index, (I) Shannon index and (J) Simpson index were all used to evaluate the diversity of the outside the RT field. Data are presented as the mean ± SD; comparisons were made using one-way ANOVA corrected for multiple comparisons using Bonferroni. *P<0.05, **P<0.01 and ***P<0.001 (two-tailed). PD-1, programmed cell death-1; NS, not significant; RT, radiotherapy.

between the RT + anti-PD-1 group and the control group, as well as between the RT group and the anti-PD-1 group (P>0.05; Fig. 3G and H).

Analysis of the TCR profile of T cells differentiated from the HSCs of mice exposed to various radiation doses. The findings of the present study indicated that the RT-immunotherapy combination decreased CDR3 diversity and altered the CDR3 length. However, the potential reasons for these alterations were not elucidated. Hence, the changes in the TCR repertoire in the peripheral blood samples of NSG mice 6 months after the transplantation of HSCs derived from CBA/H^{mCherry} mice exposed to different radiation doses for 7 days were next investigated.

Diversity of the TCRβ repertoire varies under different radiation doses. The D50 index, Gini index, inverse Simpson index, Shannon index and Simpson index were examined to assess the impact of different radiation doses on TCRβ diversity and nucleotide sequence clonal expansion in the peripheral blood of NSG mice following the aforementioned treatment. As shown in Fig. 4, the highest diversity was observed in the HC group. The TCR diversity in the

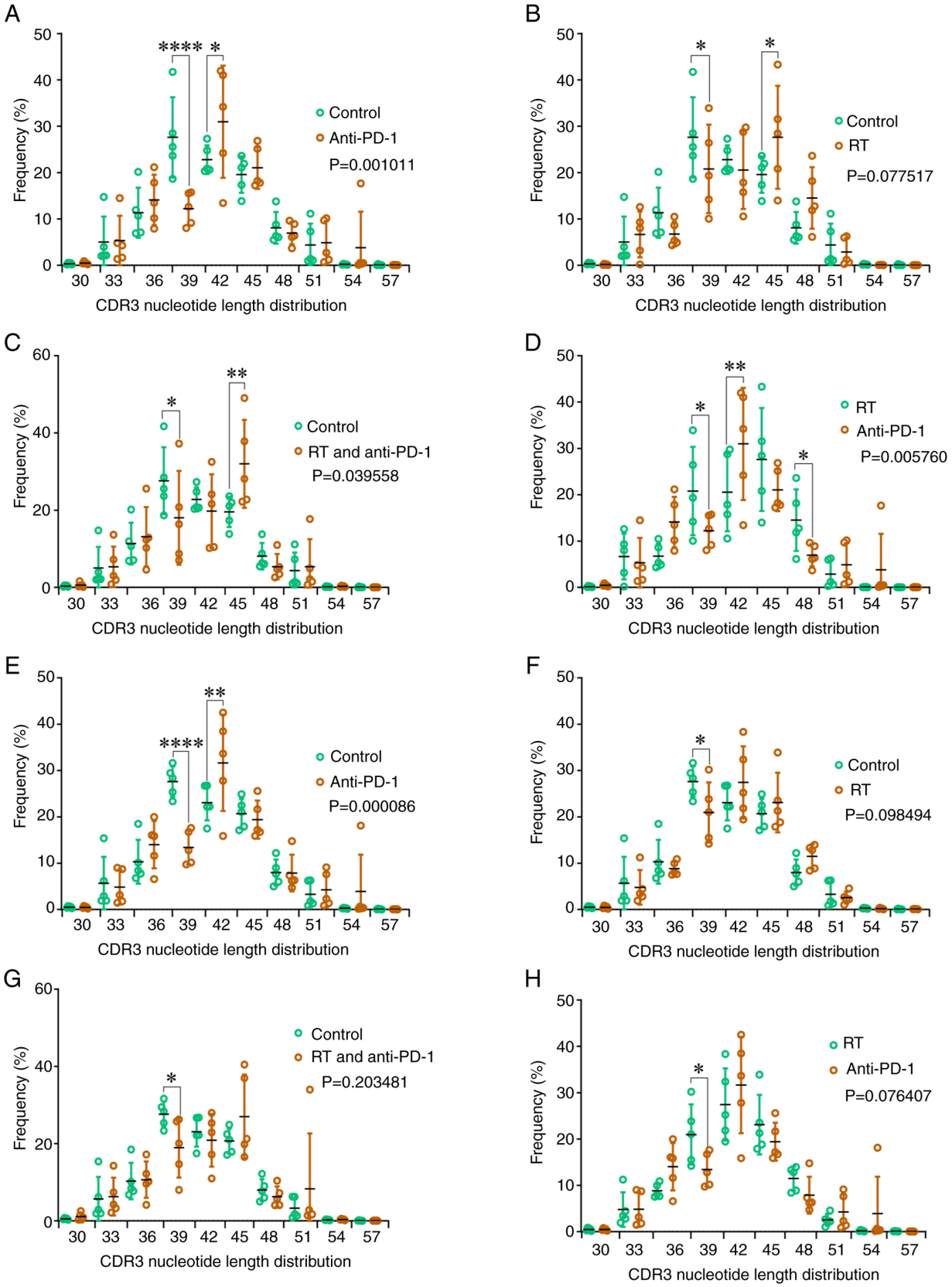

Figure 3. Distribution of TCRβ repertoire CDR3 length within and outside the treatment area in mice receiving RT and anti-PD-1 therapy, as well as in the control group. (A) The CDR3 length distribution map compared between the control and anti-PD-1 groups. (B) The CDR3 length distribution map compared between the control and RT groups. (C) The CDR3 length distribution map compared between the control and RT + anti-PD-1 groups. (D) The CDR3 length distribution map compared between the RT and the anti-PD-1 groups. (E) The CDR3 length distribution map compared between the control and anti-PD-1 groups. (F) The CDR3 length distribution map compared between the control and RT groups. (G) The CDR3 length distribution map compared between the control and RT + anti-PD-1 groups. (H) The CDR3 length distribution map compared between the RT and the anti-PD-1 groups. Data are presented as the mean ± SD; the overall differences of the CDR3 length distribution (ten length categories) between groups were compared using two-way ANOVA, and Tukey's test was performed after the two-way ANOVA to compare the differences in each CDR3 length between groups. *P<0.05, **P<0.01 and ****P<0.0001. TCRβ, T-cell receptor β-chain; CDR3, complementarity-determining region 3; PD-1, programmed cell death-1; RT, radiotherapy.

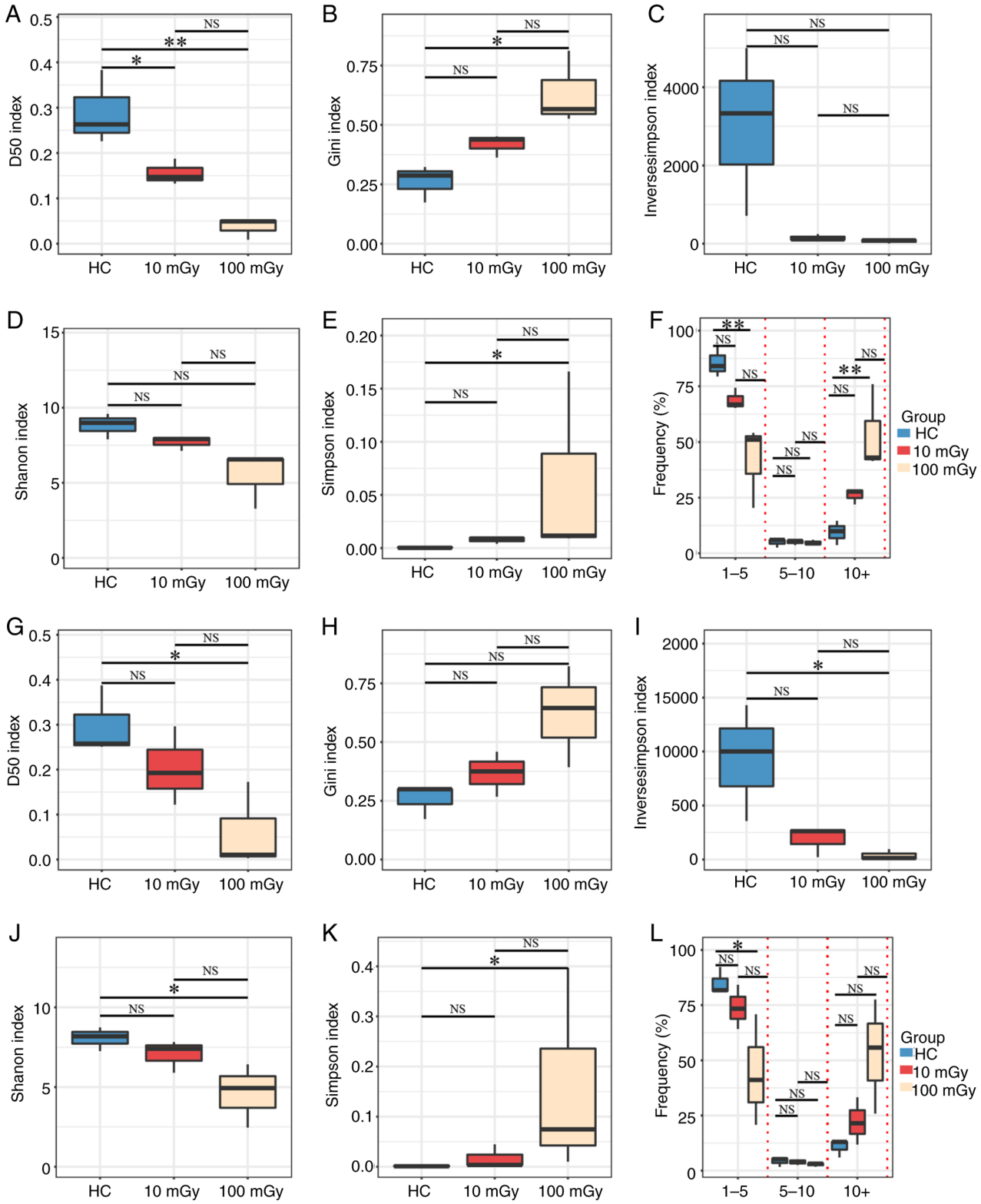

Figure 4. Degree of expansion and diversity indices of the TCRβ repertoire in the HC and RT groups. The diversity of the post-selection repertoire was measured by (A) the D50 index, (B) Gini index, (C) Inverse Simpson index, (D) Shannon index and (E) Simpson index. (F) Distribution in the clonal abundance of the post-selection repertoire. Five diversity indices were used to assess the diversity of pre-selection repertoire: (G) D50 index, (H) Gini index, (I) Inverse Simpson index, (J) Shannon index and (K) Simpson index. (L) Distribution in the clonal abundance of the pre-selection repertoire. Data are presented as the mean ± SD; comparisons were made using the one-way ANOVA or Kruskal-Wallis test and corrected for multiple comparisons using Bonferroni. *P<0.05 and **P<0.01. TCRβ, T-cell receptor β-chain; HC, healthy control; ns, not significant.

radiation (10 or 100 mGy)-exposed groups was lower than that in the HC group in both post-selection (Fig. 4A-E) and pre-selection repertoires (Fig. 4G-K). Additionally, a trend towards decreased TCR diversity with increasing radiation dose was observed, although this trend did not reach statistical significance.

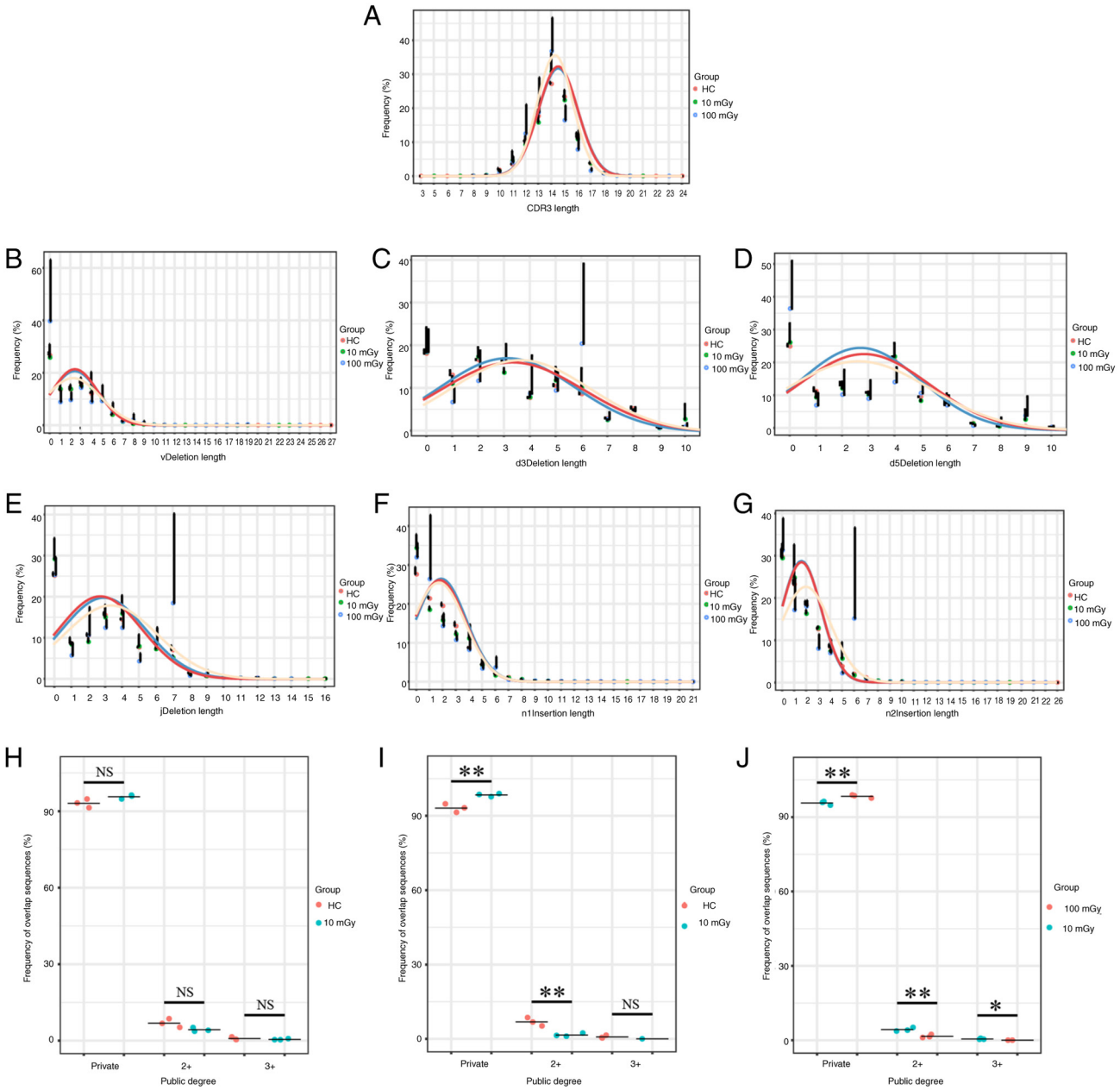

Figure 5. Altered CDR3 length distribution, InDel patterns and public degree in the RT group. (A) The changes in the CDR3 length in the radiation group mice from the 10 and 100 mGy groups were compared with those of the HC group. (B-G) TCRβ CDR3 sequences from the radiotherapy and HC groups in the post-selection repertoire were analyzed for the frequency of clonotypes with a specific number of InDels at each of the six rearrangement sites: (B) vDdel, (C) d3Del, (D) d5Del, (E) jDel, (F) n1Ins and (G) n2Ins. (H-J) Overlap indices were calculated from 9 HC, 9 mice with 10 mGy and 9 mice with 100 mGy, where (H) is the comparison between HC group mice and mice receiving 10 mGy radiation, (I) is the comparison between HC group mice and mice receiving 100 mGy radiation and (J) is the comparison between mice receiving 10 mGy radiation and mice receiving 100 mGy radiation. Data are presented as the mean ± SD and the means between the two groups were compared using the unpaired t-test or the Mann-Whitney U test. One-way ANOVA or Kruskal-Wallis test was used to compare group means across three or more groups and corrected for multiple comparisons using Bonferroni. *P<0.05 and **P<0.01. TCRβ, T-cell receptor β-chain; CDR3, complementarity-determining region 3; InDel, insertions and deletions; NS, not significant; HC, healthy control.

Based on the read frequencies of unique TCRβ nucleotide sequences (after correction), the TCRβ sequences were categorized into the following three groups: Low-abundance (1-5 reads), medium-abundance (5-10 reads) and high-abundance (>10 reads) sequences, represented as percentages of the total sequences for each TCRβ [Fig. 4F (in-frame) and Fig. 4L (out-of-frame)]. The average percentages of low-abundance and medium-abundance TCRβ sequences were the highest in the HC group, followed by the 10 mGy-exposed and 100 mGy-exposed groups. Meanwhile, the average percentage of high-abundance TCRβ sequences was the highest in the 100 mGy-exposed group, followed by the 10 mGy-exposed and HC groups. However, the differences were not always significant (P>0.05), particularly between the HC group and the 10 mGy-exposed group, and between the 10 mGy-exposed and 100 mGy-exposed groups. These results indicate that the HC group exhibits the highest TCRβ diversity and the lowest clonality, whereas the 100 mGy-exposed group exhibits the lowest TCRβ diversity and the highest clonality.

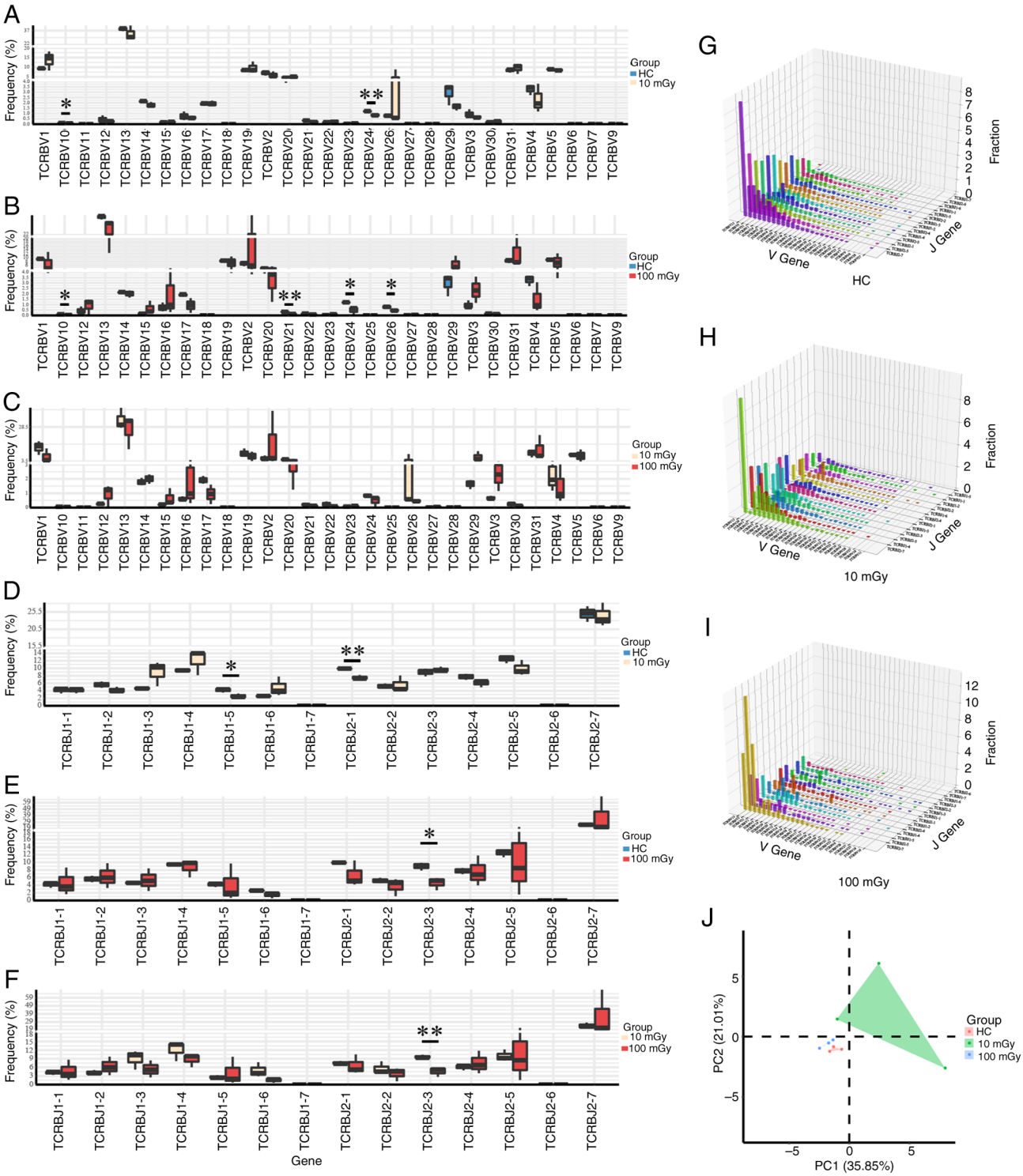

Figure 6. Usage frequency of TRBV/J segments is different following three different radiation doses in the post-selection repertoire. Significant differences in TRBV segment usage in the (A) 10 mGy and (B) 100 mGy groups, compared with the HC group. Differential usage of the TRBJ gene segment in the (D) 10 mGy and (E) 100 mGy groups, compared with the HC group. Use of TRBV (C) and TRBJ (F) gene fragments in the 100 mGy group compared with the 10 mGy group. Significant differences were analyzed by unpaired t-test and false discovery rate correction. All of the TRBVs and TRBJs that were found to differ significantly ($P<0.05$) between radiation group mice and HC are presented. Data are presented as the mean ± SD. Three-dimensional landscapes depicting the mean utilization frequency of specific Vβ-Jβ gene segment combinations in the in-frame TCRβ chains expressed in the (G) HC group and mice with radiation from the (H) 10 mGy and (I) 100 mGy groups. (J) PCA based on the TRBV segment out-of-frame usage demonstrated notable differences between the radiation and HC groups. $^{*}P<0.05$, $^{**}P<0.01$. TCRβ, T-cell receptor β-chain; TRBV, TCR β chain variable gene; TRBJ, TCR β chain joining gene; PCA, principal coordinate analysis; HC, healthy control.

Length distribution and overlap degree of TCRβ CDR3. In the present study, the CDR3 length distribution in the 10 mGy-exposed and 100 mGy-exposed groups was not significantly different from that in the HC group, although some changes were observed (Fig. 5A). To understand the molecular mechanisms underlying the effect of radiation on

TCRs, six recombination positions (vDeletion, d3Deletion, d5Deletion, jDeletion, n1Insertion and n2Insertion) for both in-frame and out-of-frame insertion/deletion (InDels) events were analyzed. The length distributions for the six recombination events (indels) were similar between the 100 mGy-exposed and HC/10 mGy-exposed groups in the post-selection (Fig. 5B-G) and pre-selection repertoires (Fig. S2). A previous study has demonstrated that the TCRβ CDR3 length has been associated with the degree of sequence sharing (34). Thus, the overlap indices of TCRβ nucleotide sequences were calculated for each group. Based on the shared degree of amino acid sequences between samples, the samples were divided into the following three groups: Private, co-owned by ≥2 and co-owned by ≥3 samples. The results indicated that, as the radiation dose increased, the percentage of private sequences was significantly increased and the degree of overlap was significantly decreased (Fig. 5H-J).

Radiation alters the usage frequency of the TCRβ chain variable (TRBV) and TCRβ chain joining (TRBJ) gene segments. To identify whether there was a skewed and restricted VDJ segment usage following radiation, the usage frequency of the TRBV and TRBJ genes in the radiation-exposed group was compared with that in the HC group in both the post-selection (Fig. 6A-F) and pre-selection (Fig. S3A-F) repertoires. For the post-selection repertoire, the usage frequency of TRBV10 and TRBV24 in the radiation-exposed groups was significantly lower than that in the HC group. For the pre-selection repertoires, the usage frequencies of TRBJ1-2, TRBJ1-5 and TRBJ1-6 in the radiation-exposed groups were lower than that in the HC group, but not all showed statistical differences. Furthermore, through PCA, the radiation-exposed mice can be distinguished from the HC mice based on the usage of TRBV genes, in the post-selection (Fig. 6J) and pre-selection (Fig. S3J) repertoires. Furthermore, the three-dimensional landscapes of TRBV and TRBJ gene usage were plotted. In the post-selection (Fig. 6G-I) repertoires, the top three V-J combinations in the 10 mGy-exposed group were TRBV13-TRBJ2-7, TRBV13-TRBJ1-4 and TRBV13-TRBJ2-5, while those in the 100 mGy-exposed group were TRBV13-TRBJ2-7, TRBV2-TRBJ2-7 and TRBV31-TRBJ2-7. The top three V-J combinations in the HC group were TRBV13-TRBJ2-7, TRBV13-TRBJ2-5 and TRBV13-TRBJ2-1 (Fig. 6G). Additionally, in the pre-selection (Fig. S3G-I) repertoires, the top three V-J combinations in the 10 mGy-exposed group were TRBV31-TRBJ2-4, TRBV13-TRBJ2-7 and TRBV13-TRBJ2-1, while those in the 100 mGy-exposed group were TRBV13-TRBJ2-7, TRBV5-TRBJ1-4 and TRBV13-TRBJ2-4. The top three V-J combinations in the HC group were TRBV13-TRBJ2-7, TRBV4-TRBJ2-7 and TRBV13-TRBJ2-5 (Fig. S3G).

Discussion

At present, cancer remains a primary public health problem. According to the International Agency for Research on Cancer, 19.88 million new global cancer cases and 9.74 million cancer-related deaths were reported in 2022 (3). RT is one of the most important treatment strategies for cancer (40). The clinical application of various immunomodulatory drugs has contributed to the field of cancer immunotherapy, achieving notable progress in the design of effective immunotherapy regimens. According to relevant research reports, there is a correlation between neoantigen burden and clinical benefits when using CTLA-4 blocking monoclonal antibodies to treat patients with melanoma (41,42), and similar associations have also been reported in patients with lung cancer receiving anti-PD-1 treatment (43). However, to the best of our knowledge, there is currently no systematic understanding of the heterogeneity in immune cells following the use of these treatment methods for cancer. The present study analyzed the TCRβ repertoire from a public dataset of tumor tissues from mice treated with RT and/or anti-PD-1/anti-CTLA-4 therapies, as well as in peripheral blood mononuclear cells of NSG mice 6 months after transplantation with HSCs isolated from CBA/H^{mCherry} mice exposed to radiation doses of 0, 10 and 100 mGy for 7 days.

Analyses of the TCRβ repertoire of the RT and anti-CTLA-4 treated cancer samples revealed that the diversity of the TCRβ repertoire in the RT and RT + anti-CTLA-4 groups was significantly lower than that in the control group, especially in the RT + anti-CTLA-4 group. Although not all differences were significant, some changes were observed. This may be due to the synergistic inhibitory effects of RT and anti-CTLA-4 therapy on T cells. The arrest of T cells in contact with tumor cells is dependent on major histocompatibility complex class I (44). A previous study indicated that the TCRβ repertoire diversity is positively associated with the polymorphism of human leukocyte antigen class I loci (45) and the present study findings further confirmed these conclusions. Furthermore, a short biased TCRβ CDR3 length may lead to an increased degree of sequence sharing as short sequences (with limited variability in sequence combinations) increase the probability of two TCRβ CDR3 sequences being coincidentally identical. The present study results are contrary to those presented by Rudqvist *et al* (26), who reported that the overlap between mice receiving RT combined with anti-CTLA-4 therapy was significantly reduced compared with mice receiving RT alone.

TCR diversity is key for neoantigen recognition (46,47). In the present study, compared with the control group, the TCRβ repertoire diversity of the in-field tumors was lower in the RT and RT + anti-PD-1 groups, although not all differences were statistically significant. However, the TCRβ repertoire diversity in the anti-PD-1 group was comparable with that in the control group. Additionally, analysis of the diversity of the TCRβ repertoire in the out-of-field tumors revealed that the TCRβ repertoire diversity in the RT group was higher than that in the control and RT + anti-PD-1 groups. RT can enhance the mobility of T cells toward the localized treatment site of tumors and augment existing anticancer T cell responses. The combination of RT and anti-PD-1 therapy facilitates the regression of both local and distant tumor lesions (27). A recent study reported that the combination of RT and anti-PD-1 therapy can promote the circulation of T cell clones between the peripheral blood and tumor tissues (48). Peripheral blood serves as a reservoir for T cell clones, which contributes to the diversity of the TCR repertoire of tumor tissue. We hypothesize that the diversity of the TCR in the in-field tumors of the RT and RT + anti-PD-1 groups was lower than that in the control group due to the RT-induced potent local inflammatory response against tumor tissue. This leads to an enhanced antitumor immune response and increased utilization of the TCR. Additionally,

the antitumor immune response of off-site tumors may be slightly delayed compared with on-site tumors. A key feature of the TCRβ repertoire is the distribution of the CDR3 length. Epitope-specific T cell repertoires often have a bias in CDR3 length (37,49,50). Compared with the control group, the clonotypes in the in-field tumors of the anti-PD-1 and the RT + anti-PD-1 groups shifted toward longer clonotypes. However, the CDR3 length in the RT group was comparable with that in the control group. Consistent with the CDR3 length in some in-field tumors, the CDR3 length in the anti-PD-1 group was longer than that in the control group in the out-of-field tumors. These results may be due to the random insertion of a large number of nucleotides into the CDR3 region in the presence of terminal deoxynucleotidase after cancer treatment, which increases CDR3 length (16).

The diverse TCRs provide protection to the human body, shielding it from various external pathogens and internal cancer cells (51). However, in the present study, further investigation of the TCRβ repertoire characteristics in mice exposed to different radiation doses revealed that, as the radiation dose increased, the TCRβ diversity in mice decreased while the degree of clonal expansion increased. This may be associated with immune system damage caused by reduced TCRβ diversity after radiation exposure, further confirming the findings by Hollingsworth *et al* (52), in which it was reported that radiation damage leads to impaired immune response and a decrease in T cell repertoire diversity. Additionally, in the present study, the CDR3 length and InDel length distribution were not significantly different between the radiation-exposed and HC groups. Furthermore, as the radiation dose increased, the percentage of private sequences was significantly increased and the degree of overlapping sequences was significantly decreased. The shared TCRβ clonotypes among individuals is associated with enhanced cross-reactivity toward potentially related antigens, which is considered key for pathogen-specific responses and infection control (53-56). Furthermore, in the present study, the usage frequencies of TRBV/J segments significantly varied between the radiation-exposed and HC groups in both the pre-selection and post-selection repertoires. The biased usage of TRBV/J segments may result from radiation-induced DNA rearrangement (57). Upon radiation exposure, activated T cells undergo clonal expansion to maintain physiological functions. However, immune cell composition remains stable. An increase in the number of dominant T cells can suppress the proliferation of other T cells (58). Therefore, radiation exposure may have increased the proportions of some TRBV/J genes but decreased the proportion of other genes.

The results of the present study indicated that different treatments decreased the TCRβ repertoire diversity of mice. RT may be the main factor contributing to this decreased diversity of the TCRβ repertoire. The decreased TCRβ diversity may weaken the ability of the immune system to recognize and clear tumor cells, affecting treatment efficacy. RT may affect the diversity of TCRβ repertoire through different mechanisms. First, radiation may directly damage and kill the lymphocytes in the TME, reduce the number of T cells and consequently decrease TCRβ diversity (59). Second, radiation may affect thymic function. The thymus is the site for T cell development and maturation. RT-induced thymic tissue damage can adversely affect T cell production and TCRβ rearrangement, which decreases TCRβ diversity (60). Third, RT may induce alterations in the TME, such as eliciting inflammatory responses and increasing the number of immunosuppressive cells. These changes may adversely affect the infiltration and function of T cells, indirectly decreasing the diversity of TCRβ (61,62). Future studies should consider reducing the negative impact of RT on TCRβ diversity through the optimization of RT. Radiation dose may be decreased to a level that minimizes direct damage to lymphocytes without compromising treatment efficacy (63). A previous study has demonstrated that fractionated low-dose RT is efficacious for the activation of the immune system, mitigating the negative impact on TCRβ diversity (63). Furthermore, adoptive T cell therapy can be used after RT. Adoptive T cell therapy involves re-infusing tumor-specific T cells expanded *in vitro* into the body of the patient to enhance TCRβ diversity and antitumor activity (64). The management of TCRβ diversity is dependent on multiple factors, such as tumor type, staging and patient immune status. Thus, different strategies can be employed to overcome these issues. For example, thymus function can be enhanced. The thymus is a key organ for T cell development and diversification. The improvement of the thymic microenvironment (such as supplementing IL-7, growth hormone and thymosin) or the suppression of age-related thymic atrophy will promote the generation of initial T cells and the maintenance of TCR repertoire diversity (65-67). Furthermore, strategies can be adopted to regulate gut microbiota. The gut microbiota affects T cell differentiation by modulating the immune microenvironment (68). For example, probiotics or fecal transplantation may indirectly enrich the TCR repertoire through antigen cross-reactivity (69). Treatment effectiveness may vary depending on the type of tumor, individual patient and treatment plan, highlighting the importance of developing personalized treatment plans. Treatment plans for patients with cancer can be personalized to achieve optimal therapeutic effects using two strategies: i) TCR sequencing technology can be used to analyze the TCRβ diversity of the patient and evaluate their immune status (70,71); and ii) the identification of biomarkers associated with TCRβ diversity can be used to predict the efficacy of RT and immunotherapy (72,73).

The present study has several limitations. The sample size was small; hence, the conclusions of the present study need to be validated using a larger sample size. Additionally, the types of cancer involved in the present study are limited and future research needs to be conducted on more categories of cancer samples to confirm the conclusions. Furthermore, this was a cross-sectional study that evaluated the TCRβ repertoire at a specific time point. The dynamic changes of the TCRβ repertoire during treatment should be evaluated in the future, which will aid in the identification of the optimal treatment window. Finally, TCR sequencing, which was used to determine TCR rearrangement, does not fully represent the functionality of T cells. Therefore, future research needs to combine more functional experiments, such as i) Cytokine release experiments: detecting the types and quantities of cytokines released by T cells after stimulation to evaluate the activation status of T cells; and ii) cytotoxicity assay: Evaluate the ability of T cells to kill tumor cells.

In conclusion, the present study demonstrated that the TCRβ repertoire may serve as an ideal predictive biomarker

for the efficacy of combination therapies. In the future, further research should be conducted on: i) The molecular mechanisms by which the CDR3 length is significantly shorter under RT and significantly longer under immunotherapy, as well as the role of changes in CDR3 length in tumor treatment and prognosis; and ii) identifying methods that can be used to maintain and improve the diversity of TCR profiles in RT and immunotherapy. Future studies may explore and evaluate the potential mechanisms underlying antitumor immune responses to address key challenges in clinical treatment.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XY and WW curated the data, performed the formal analysis, prepared and wrote the original draft and analyzed and interpreted the data. XY and WW confirm the authenticity of all the raw data. ZF, BZ, YL and FY participated in data analysis and reviewed and edited the manuscript. CZ and MO reviewed and edited the manuscript as well as supervised and conceptualized the present study. SL conceptualized the present study, performed the formal analysis, designed the present study methodology and supervised and aided with the visualization of data. XH conceptualized the present study, performed the formal analysis, obtained funding, designed methodology and supervised and conceived the present study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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