The impact of radiation therapy on the TCR Vβ chain repertoire in patients with prostate cancer

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Abstract. Radiation therapy (RT) is an essential component in the therapeutic treatment of patients with localized prostate cancer (LPCa). Besides its local effects, ionizing radiation has been linked to mechanisms leading to systemic immune activation. The present study explored the effect of RT on the T-cell receptor variable β (TCR Vβ) chain repertoire of peripheral blood T cells in patients with LPCa. High-throughput TCR Vβ sequencing was performed on 20 blood samples collected from patients with LPCa at baseline and 3 months post-RT. The diversity index was altered, as were TCR Vβ clonal evenness and convergence before and post-RT; however, these findings were not significant. Notably, marked changes in the frequencies among the top 10 TCR Vβ clonotypes were detected and some patients developed new clonotypes of high abundance. These data provided initial evidence that RT in patients with LPCa may induce systemic immune changes, which could be exploited by future therapies for improved clinical results.

Introduction

Increasing evidence has supported the notion that tumor-targeted local radiation therapy (RT) may induce an *in-situ* tumor vaccine and potentiate immune responses to immunotherapy (1). Thus, in addition to its local antitumor effects, RT may induce systemic immune activation via the release of tumor-associated antigens from dying tumor cells, which are taken up by dendritic cells to cross-present them to naïve $CD8^+$ T cells in the lymph nodes. Following activation, the tumor antigen-specific T cells can recognize and destroy tumor cells in the body at distant, unirradiated anatomical sites, thereby turning tumors into endogenous 'vaccines' through abscopal effects (2-4). Therefore, alterations in the T-cell receptor (TCR) repertoire post-RT may be useful as biomarkers predicting RT-induced systemic T-cell immune activation.

RT is an established treatment option for the management of localized prostate cancer (LPCa) (5), which aims to directly kill tumor cells in the prostate gland and occasionally at distant anatomic sites (6). The immune link for such an abscopal effect in patients with LPCa has been attributed to the generation of autoantibodies (7). Notably, RT has been shown to promote antibody production against tumor antigens in patients with non-small cell lung cancer (NSCLC) (8). The link with cellular antitumor immunity has been demonstrated with the emergence of tumor peptide-specific $CD8^+$ T cells in patients with cancer treated with RT (9), whereas $CD8^+$ T-cell depletion in animals has been shown to significantly attenuate radiation-induced therapeutic effects against tumors (10). Although RT may induce some immune responses, the details are still not clear. High-throughput sequencing of the T-cell receptor variable β (TCR Vβ) chain repertoire is a tool used to examine how therapy changes antigen-specific T-cell immunity. Profiling the TCR Vβ repertoire in serial samples from patients can reveal features such as clonal expansion, persistence and turnover of T-cell clones (11). Previous studies have demonstrated that TCRs clustered by sequence similarity potentially target similar antigens (11,12). By characterizing the TCR Vβ repertoire before and after RT in patients with LPCa, the present study aimed to provide indications as to whether RT affects systemic cellular immunity, resulting in TCR Vβ clonal frequency changes. Evaluation of blood samples at baseline and 3 months post-RT revealed a dynamic remodeling of the circulating T-cell repertoire based on the expansion and contraction of TCR Vβ clonotypes, as well as on the appearance of new ones. To the best of our knowledge, these findings provide the first evidence that RT in LPCa may induce systemic immune changes, which could presumably modulate clinical outcomes. Furthermore, these findings

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support the application of RT in LPCa in combination with other therapeutic treatments.

Materials and methods

Patients and sample collection. A total of 10 patients (age range, 58-83 years; median age, 74 years), treated with external beam RT, were recruited in the present study. Patients received either primary RT (n=8), adjuvant RT post-radical prostatectomy (n=1) or salvage RT post-radical prostatectomy (n=1) at the Department of Radiation Oncology, Saint Savas Cancer Hospital (Athens, Greece). Variable RT regimes with a daily dose/fraction ranging from 1.8 to 2.2 Gy (median, 2 Gy) and total radiation doses between 66 and 72 Gy (median, 70 Gy) were applied for an overall period of 35-38 days (median, 37 days). Detailed clinical and radiation characteristics are presented in Table I. Peripheral blood samples were collected at two distinct time-points: At diagnosis and 3 months after the completion of the therapy. Fig. 1 shows the experimental procedure of the present study.

Ethics approval. The present study was conducted in accordance with the Declaration of Helsinki and all of the participants provided written informed consent. The present study was approved by the Saint Savas Cancer Hospital IRB (approval no. IRB-ID6777/14-06-2017) and the Ethical Committee of the National and Kapodistrian University of Athens as part of a larger project (approval no. ID247/28-01-2020).

RNA isolation. Total RNA was extracted from peripheral blood samples collected in K_2EDTA tubes (BD Vacutainer™; BD Biosciences) using the PureLink™ Total RNA Blood Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The RNA was then quantified using a Qubit Fluorometer 3.0 with the Qubit™ RNA HS Assay Kit (Invitrogen; Thermo Fisher Scientific, Inc.).

TCR Vβ library preparation for next generation sequencing. The Oncomine™ TCR Beta-LR Assay (cat. no. A35386; Thermo Fisher Scientific, Inc.) was adopted for profile analysis of the TCR Vβ repertoire in blood samples from patients with LPCa that were subjected to RT. This is a highly sensitive, RNA-based next generation sequencing assay suitable for the characterization of the TCR Vβ sequences, including all complementarity-determining regions (CDR1, CDR2, CDR3) of the variable domain. The assay accurately determines TCR Vβ diversity and clonal expansion, and allows for identification of allele-specific polymorphisms in peripheral blood samples.

The extracted RNA from the peripheral blood of patients before and after RT was used for TCR Vβ analysis using the Oncomine TCR Beta-LR Assay, according to the manufacturer's instructions. Briefly, following DNase treatment with ezDNase™ (Invitrogen; Thermo Fisher Scientific, Inc.), a minimum of 25 ng RNA was reverse transcribed using the Superscript™ VILO™ cDNA Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Target amplification was performed using the Oncomine TCR Beta-LR Assay followed by library preparation using the Ion AmpliSeq™ Library Kit Plus and the Ion Select™ Barcode Adapters (Ion Torrent; Thermo Fisher Scientific, Inc.). Following purification with the Agencourt™ AMPure™ XP Reagent (Beckman Coulter, Inc.), all individual libraries were quantified using the Ion Library TaqMan™ Quantitation Kit (Ion Torrent; Thermo Fisher Scientific, Inc.), diluted to 25 pM and pooled. Following template preparation and chip loading with the Ion Chef™ System, libraries were sequenced with the Ion GeneStudio™ S5 System using the Ion 510™ & Ion 520™ & Ion 530™ Kit-Chef (cat. no. A34461) and the Ion 530™ Chip Kit (cat. no. A27763) (all from Ion Torrent; Thermo Fisher Scientific, Inc.) (single-end sequencing, 400 bp nucleotide length). Subsequent immune repertoire analysis was performed using the Ion Reporter™ Software 5.16 with the Oncomine TCR Beta-LR Single Sample workflow (both from Ion Torrent; Thermo Fisher Scientific, Inc.).

Graph preparation and statistical analyses. TCR Vβ sequencing results were automatically analyzed using the Ion Reporter Software. Additional data plotting and statistical analyses were performed using GraphPad Prism 6.0 for Windows (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation. Non-parametric Wilcoxon's test

Table I. Clinicopathological characteristics of the patients with localized prostate cancer (n=10) enrolled in the present study and details of RT.

Characteristic	Value
Median age at diagnosis, years (range)	74 (58-83)
PSA	
<10 ng/ml	6 (60%)
10-20 ng/ml	1 (10%)
>20 ng/ml	3 (30%)
Gleason score	
<6	3 (30%)
7 (3+4)	4 (40%)
7 (4+3)	2 (20%)
>8	1 (10%)
T stage	
T1c	4 (40%)
T2a, T2b, T2c	4 (40%)
T3a, T3b	2 (20%)
Type of RT	
Primary	8 (80%)
Adjuvant	1 (10%)
Salvage	1 (10%)
External beam 3D conformal RT characteristics	
Median daily dose, Gy (range)	2 (1.8-2.2)
Median total dose, Gy (range)	70 (66-72)
Median radiation treatment schedule, days (range)	37 (35-38)

PSA, prostate-specific antigen; RT, radiation therapy.

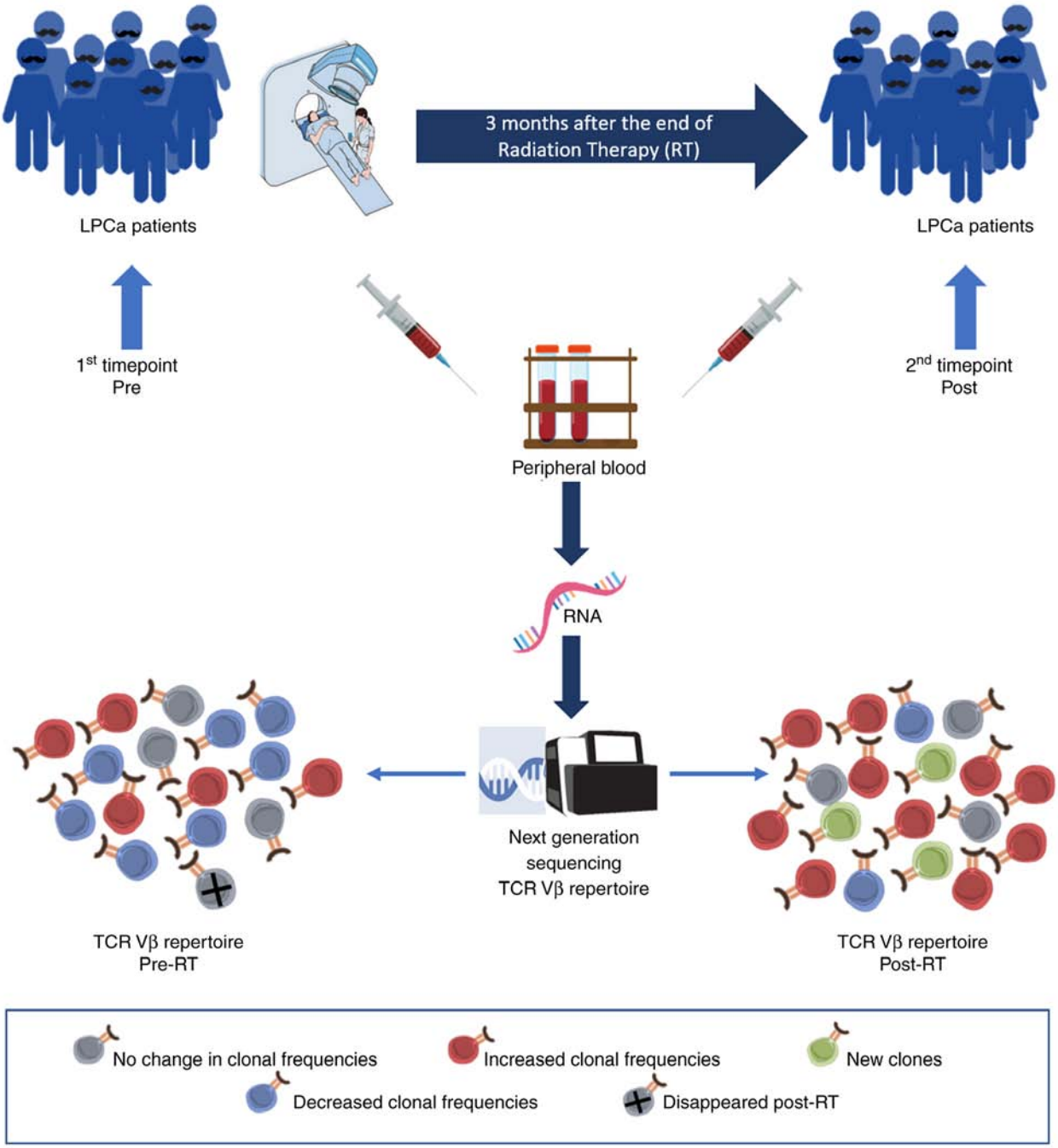


Figure 1. Effect of RT on the TCR Vβ-chain repertoire in peripheral blood samples (n=20) collected from 10 patients with LPCa patients at baseline and 3 months post-RT. RNA was isolated from peripheral blood and the TCR repertoire was analyzed by applying high-throughput TCR Vβ sequencing. Profound changes in the frequencies among the top 10 TCR Vβ clonotypes could be detected, with some patients developing new clonotypes of high abundance. LPCa, localized prostate cancer; RT, radiation therapy; TCR Vβ, T-cell receptor variable β.

was performed for the identification of differences in TCR Vβ sequences among patients before and after RT. For some of the analyses, the patients were divided into two groups, based on the appearance of new TCR Vβ clones (Group II) or not (Group I) following RT. Individual paired t-tests were performed for each patient to analyze V gene segments before and after RT, and unpaired t-tests were performed for each V gene to analyze its frequency in Group I vs. Group II. $P<0.05$ was considered to indicate a statistically significant difference. ClustalX graphical alignment tool was used for clonotype-sequence comparisons (13).

Results

Effect of RT on TCR clonal frequencies in patients with LPCa. A total of 20 samples from 10 patients with LPCa receiving RT, as aforementioned, were included in the present analysis. The median age was 74 years (range, 58-83 years). Six patients had a Gleason Score (GS) of 7 (GS 3+4, n=4; GS 4+3, n=2), three patients had a GS <6 and one patient had a GS >8. Clinical and external beam radiation characteristics are summarized in Table I.

RT-induced changes in the diversity of the TCR Vβ repertoire were determined by TCR Vβ sequencing of RNA isolated

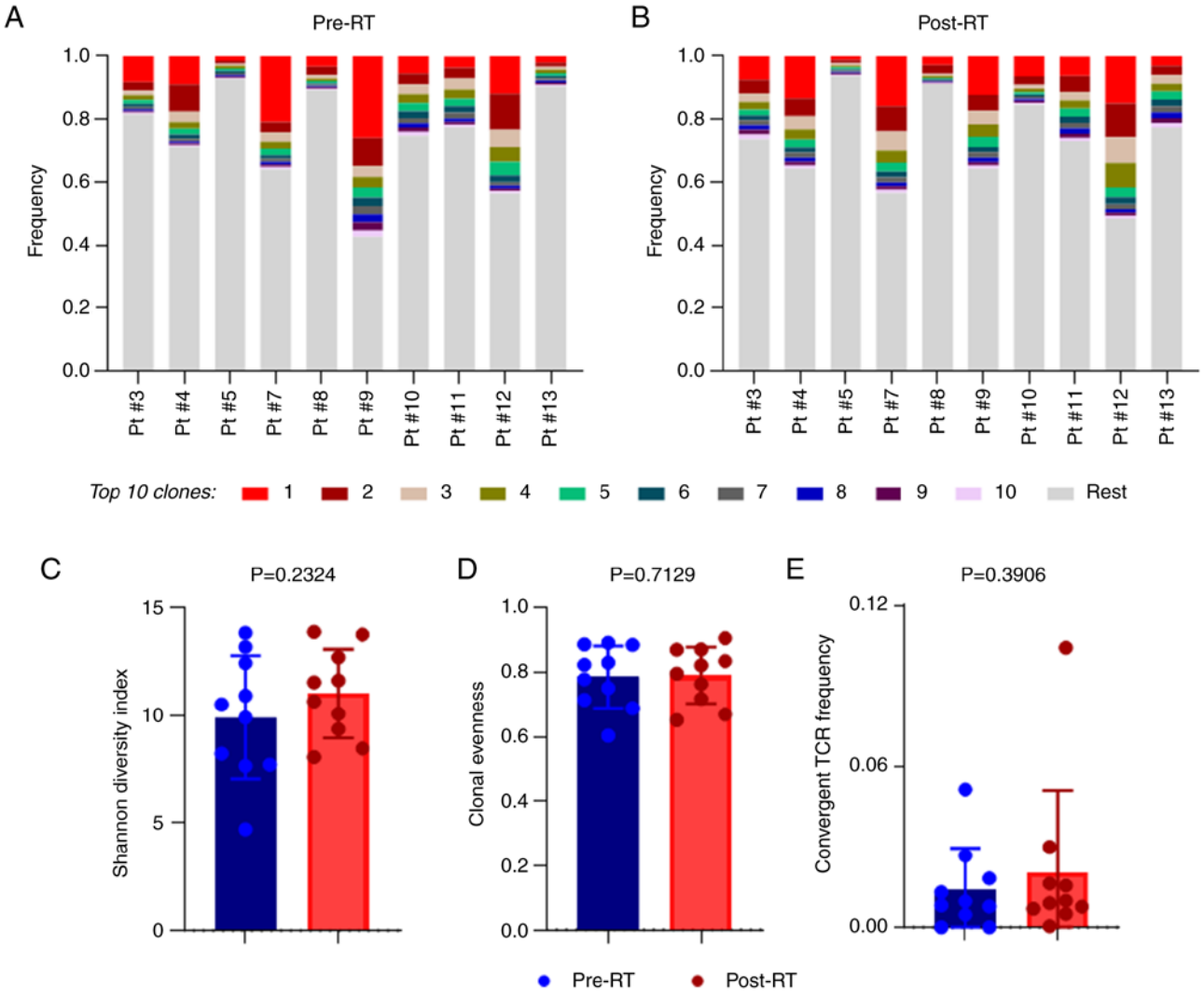


Figure 2. Comparative analysis of TCR Vβ repertoire features. The analysis was performed with blood samples from patients with localized prostate cancer, pre- and post-RT. Graphical representation of the top 10 most frequent clonotypes in each patient (A) before or (B) after RT. Clonal frequency is depicted in descending order, with 1 being the most frequent clonotype and 10 being the least frequent within the top 10 clonotypes. Note that each color does not necessarily correspond to the same TCR Vβ clonotype in all patients. (C) Alterations in T-cell complexity at baseline and post-RT were analyzed for the 10 patients with respect to Shannon diversity (entropy) index, expressing patient clonotype diversity. (D) Clonal evenness, depicting similarities in clonotype sizes. (E) Convergent TCR frequency, estimated as the aggregate frequency of clonotypes sharing an amino acid sequence with at least one other clone. Data are presented as the mean of all 10 patients ± SD. RT, radiation therapy; TCR Vβ, T-cell receptor variable β.

from peripheral blood at baseline and 3 months post-RT. This time point (i.e., 3 months) was chosen based on previous findings, which have demonstrated significant RT-induced immune changes in patients with cancer (14). On average, 534,677.95 sequence reads were obtained, which were mapped to the V and joining segments, and could identify unique TCR Vβ clonotypes. The frequencies of the top 10 clonotypes for all 10 patients analyzed either pre-RT (Fig. 2A) or post-RT (Fig. 2B) ranged from 0.0023 to 0.2591, which suggested a high variability in TCR Vβ diversity between patients.

To explore the effect of RT on the TCR Vβ repertoire, T-cell complexity was measured both at baseline and post-RT. It was revealed that the diversity index was increased post-RT compared with baseline; however, this was not significant (Fig. 2C). The similarity in the TCR Vβ repertoire before and after RT was also reflected by clonal evenness, as shown in Fig. 2D. In addition, the frequency of convergent TCR Vβs was also similar at both time-points (Fig. 2E).

There were changes in clonal frequencies (CFs) among the top 10 TCR Vβ clonotypes following RT. Table SI presents a representative example of how the frequencies of the top 10 clonotypes changed post-RT in one patient. Fig. 3 shows the alterations in the top 10 TCR Vβ clonotype frequencies before and post-treatment in each patient. The CDR3 amino acid (AA) sequences of the clonotypes, which entered the top 10 post-RT in all patients, were mainly occupied by polar (S, T, Y, Q) and negatively charged (D, E) residues (average frequency 58±11%).

RT-induced alterations in V gene segment usage frequencies in patients with LPCa. The highly expressed Vβ gene segments were also analyzed pre-RT as well as post-RT for each of the 10 patients and the results are shown in Fig. 4. Unpaired grouped analysis of each one of the V gene segment usage frequencies for all the patients pre- and post-RT did not give statistically significant differences (data not shown). However,

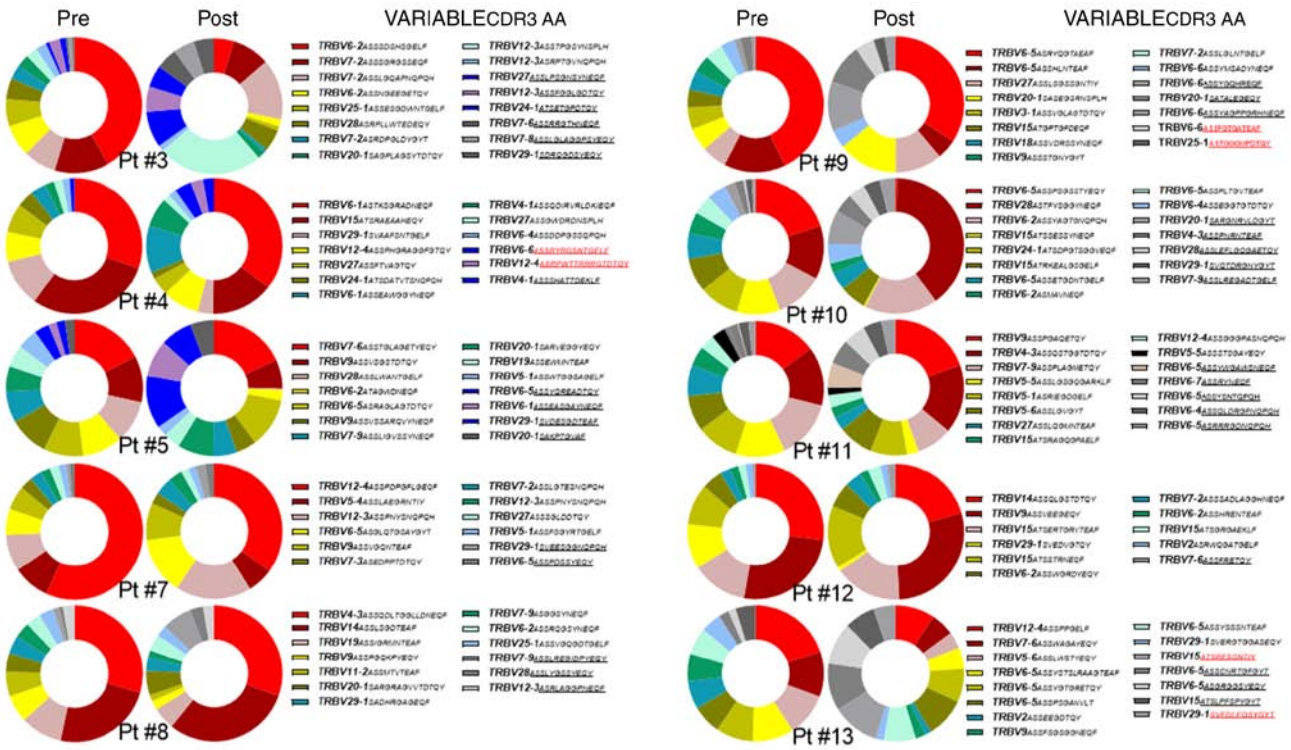


Figure 3. Pie charts illustrating the proportion of the 10 most frequent clonotypes pre- and post-RT per patient. Note that each color does not necessarily correspond to the same TCR clone among patients, nevertheless the same color represents the same clone when examining the same patient pre- and post-RT. The black underlined amino acid sequences represent the clonotypes that were not included among the most frequent clonotypes before RT and appeared in the top 10 most frequent clonotypes post-RT. The red underlined amino acid sequences represent the new clonotypes that appeared in the three patients post-RT. RT, radiation therapy.

by comparing V gene segment usage pre- and post-RT for each patient, statistically significant differences were identified in the frequencies of five patients (Pt #4, Pt #7, Pt #11, Pt #12 and Pt #13). Notably, >2 fold-change increases (ranging from 2.03 to 26.31) and decreases (ranging from 2.01 to 157.59) among the 53 different TCR Vβ clonotypes tested pre- and post-RT, although not statistically significant, were frequently observed for all patients (at various numbers for every patient) and were suggestive of RT-induced alterations in systemic T-cell immunity (Fig. S1). Subsequently, the patients were divided into two groups, based on the appearance of new TCR Vβ clones (Group II) or not (Group I) following RT. Notably, the three patients (Pt #4, Pt #9 and Pt#13) who developed new clonotypes (NCs) (Group II) all had a high GS [8 or 7 (4+3)], in contrast to the other patients who had a GS of 6 or 7 (3+4) (Group I) and did not develop NCs. Statistically significant differences in the usage of specific V gene segments between the two groups of patients were detected, both before and after RT (Fig. 5A). Individual t-tests were performed for each V gene and revealed statistically significant alterations in the usage of certain V gene segments between the two patient groups. Specifically, before RT, there was a statistically significant higher usage frequency of TRBV2 (P=0.0262) and TRBV7-8 (P=0.0036) and lower usage frequency of TRBV6-1 (P=0.0388) and TRBV6-6 (P=0.0037) in the low-risk patient group (Group I) as compared with the high-risk group (Group II) (Fig. 5B). Moreover, there were some notable differences in the CFs between the two groups of patients pre-RT that were not statistically significant. For example, the frequency of TRBV4-3 and TRBV7-7 was lower (P=0.0788 and 0.0557, respectively), and that of TRBV6-5 was higher (P=0.0669) in Group II compared with Group I. Post-RT, there was a statistically significant higher usage frequency of TRBV7-7 (P=0.0239) and TRBV13 (P=0.0493), and lower usage frequency of TRBV6-5 (P=0.0271) and TRBV6-6 (P=0.0226) in the low-risk patient group (Group I) as compared with the high-risk group (Group II) (Fig. 5C). In addition, post-RT there were non-significant trends in CF differences between Groups I and II. For example, the frequency of TRBV4-3 was lower (P=0.0740) and that of TRBV6-1 was higher (P=0.0925) in Group II compared to Group I. Notably, the usage of the TRBV6-6 segment was found to be higher in the high-risk group of patients compared with the low-risk group, both pre- and post-RT. This particular V gene segment was also detected within the NCs of Pt #4 and Pt #9, post-RT (Fig. 3). Intragroup analyses revealed no statistically significant differences between the frequencies of V genes pre- and post-RT among patients belonging to Group I, whereas in Group II, the frequency of TRBV9 was higher post-RT in comparison with the frequency pre-RT (P=0.0336) (data not shown).

RT-induced alterations in the top 10 TCR clonal frequencies and emergence of new clonotypes. Subsequently, the present study aimed to identify TCR Vβ CFs that were not detectable among the top 10 clonotypes before RT, but appeared among the top 10 clonotypes post-RT. By contrast, the present study also searched for TCR Vβ CFs that were present in the top 10 at baseline but were not detectable among the top 10

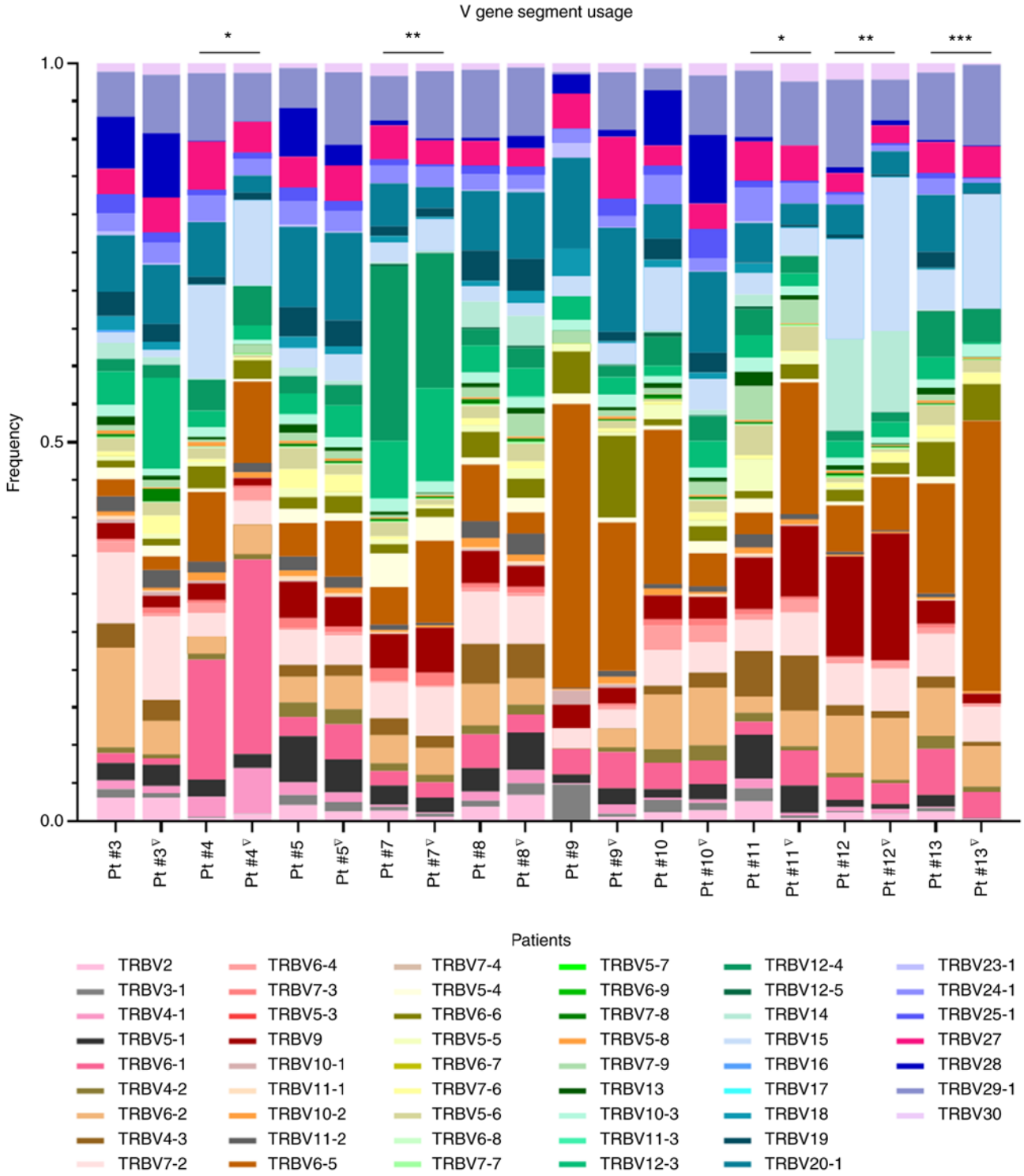
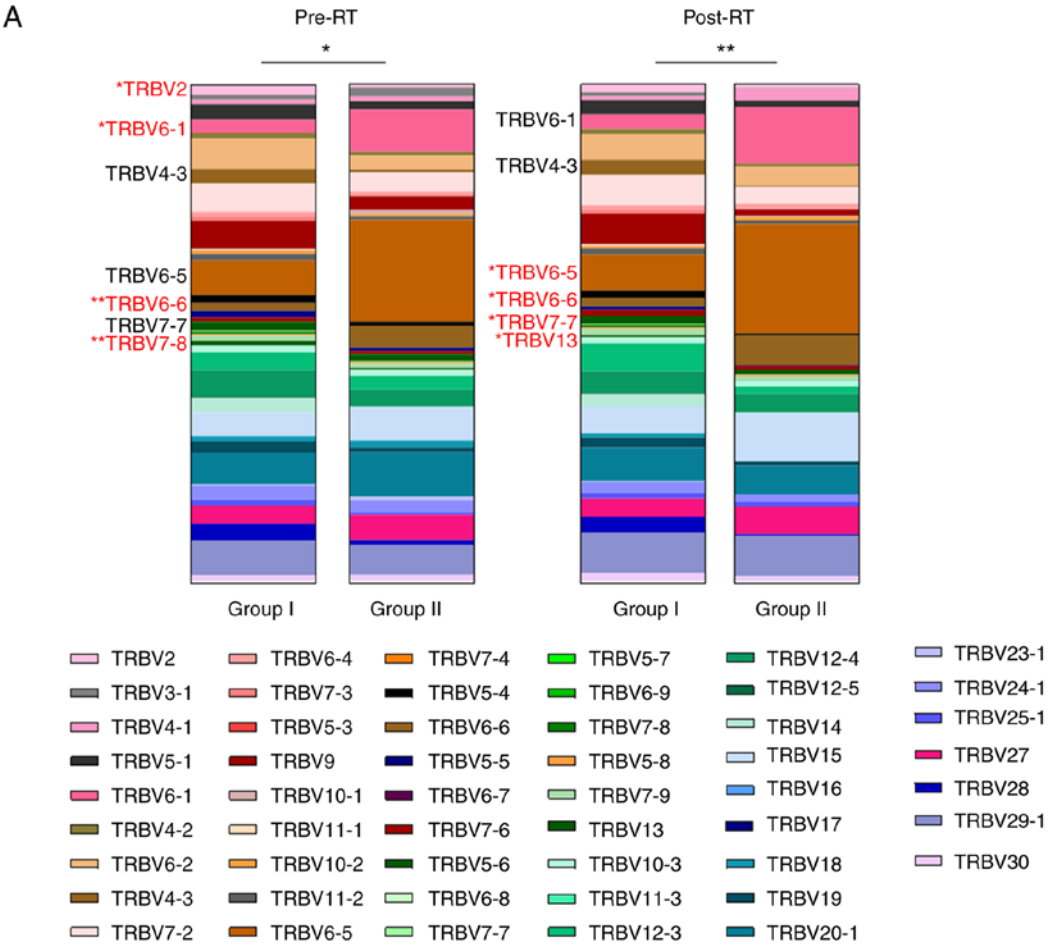


Figure 4. V gene usage analysis in patients with localized prostate cancer pre- and post-RT. Stacked bar chart depicting the frequency of 53 V variant usages. The V variants are color-coded as shown in the figure. In the x-axis, the ▽symbol next to the patient number indicates the post-RT timepoint. *P<0.05, **P<0.01, ***P<0.001. RT, radiation therapy.

clonotypes post-RT. Notably, some clonotypes that had a low CF at baseline, with some being far below the top 10 TCR Vβ CFs, were identified that had expanded post-RT and were in the top 10 TCR Vβ CF. For example, as shown in Fig. 6, Pt #3 had six clonotypes that were below the top 10 TCR Vβ CFs (ranked at positions 11, 24, 34, 46, 80 and 94), with frequencies ranging from 0.0008 to 0.0049, which were increased post-RT, ranging from 0.014 to 0.025, thus entering the top 10 CFs (ranked at positions 4, 5, 6, 8, 9 and 10). In total, for the 10 patients analyzed post-RT, 33 expanded TCR Vβ clonotypes were identified, which entered the top 10 TCR Vβ CFs (Fig. 6). Some exceptional cases included two clonotypes that were ranked at positions 172 (Pt #10) and 109 (Pt #11) at baseline, but post-RT were advanced to position 8 among the top 10 TCR Vβ CF (Fig. 6). Next, the present study searched for new TCR Vβ clonotypes post-RT to suggest for a systemic

A



B

Pre-RT

V gene	P value	Frequency Group II	Frequency Group I	Difference
TRBV2	**0.0262**	0.005929	0.01954	-0.01361
TRBV7-8	**0.0036**	0.0006703	0.003853	-0.003183
TRBV6-1	**0.0388**	0.08431	0.02594	0.05837
TRBV6-6	**0.0037**	0.04344	0.01527	0.02817
TRBV4-3	0.0788	0.005095	0.02973	-0.02464
TRBV7-7	0.0557	0.0002950	0.001363	-0.001068
TRBV6-5	0.0669	0.2039	0.06894	0.1350

C

Post-RT

V gene	P value	Frequency Group II	Frequency Group I	Difference
TRBV7-7	**0.0239**	0.0001343	0.001488	-0.001353
TRBV13	**0.0493**	0.001253	0.004950	-0.003697
TRBV6-5	**0.0271**	0.2196	0.07341	0.1462
TRBV6-6	**0.0226**	0.06036	0.01765	0.04272
TRBV4-3	0.0740	0.001694	0.02960	-0.02791
TRBV6-1	0.0925	0.1127	0.02908	0.08360

Figure 5. V gene usage analysis in patients with localized prostate cancer. (A) Stacked bar chart depicting the frequency of V variant usages in the patients after categorization into two groups, pre- and post-RT. Group I (n=7) consists of patients with GS 6 or 7 (3+4); group II (n=3) consists of patients with GS 8 or 7 (4+3). The V variants are color-coded. *P<0.05, **P<0.01. Red colored V gene segments indicate statistical significance for the particular V gene segment between the two patient groups, pre- and post-RT (*P<0.05, **P<0.01). Frequencies of the V genes that are statistically different (red) or show a strong trend (black) between Group I and Group II (B) pre-RT and (C) post-RT. RT, radiation therapy.

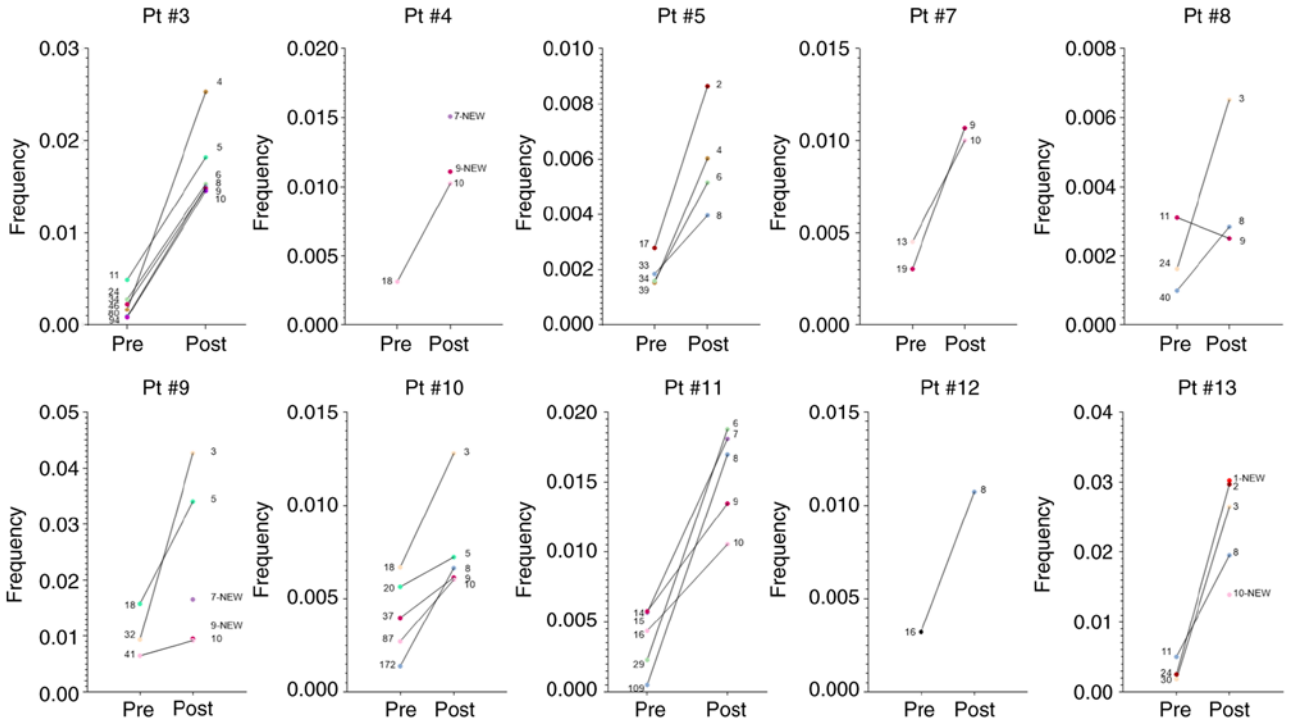


Figure 6. Changes in TCR Vβ clonotypes pre- and post-RT. Identification of TCR Vβ clonotypes with ascending CFs and emergence of new clonotypes with increased frequency in the peripheral blood of patients with localized prostate cancer post-RT. The depicted lines represent conversion of each described clonotype from low ranked frequency to one of the top 10 most frequent clonotypes, with numbers indicating the exact frequency rank. The designation 'NEW' next to a CF number highlights that the specific clonotype was not identified in the referred patient at baseline but entered the top 10 CF post-RT. CF, clonal frequency; RT, radiation therapy; TCR Vβ, T-cell receptor variable β.

		Pt #4	Pt #9	Pt #13
Top 10 Pre-RT	1	ASTKSGRADNEQF	ASRYQGTAEAF	ASSPPGELF
	2	ATSRAEAAHEQY	ASSHLNTEAF	ASSWAGAYEQY
	3	SVAAFSNTGELF	ASSLSGSSGNTIY	ASSLWSTYEQY
	4	ASSPHGRAGGFGTQY	SASEGSRNSPLH	ASSYSTSLRAAGTEAF
	5	ASSFTVAGTQY	ASSVGLAGTDTQY	ASSYGTGRETQY
	6	ATSDATVTSNQPQH	ATGPTGFDEQF	ASSPSGANVLT
	7	ASSEAWGGYNEQF	ASSVDRSSYNEQF	ASSEEGDTQY
	8	ASSQDIRVRLDKIEQF	ASSSTGNYGYT	ASSFSGSGGNEQF
	9	ASSGWDRDNSPLH	ASSLGLNTGELF	ASSYSSSNTEAF
	10	ASSDDPGSSQPQH	ASSYMSADYNEQF	SVERGTGGASEQY
New	NC1	ASSRYRGSNTGELF	ASSFGTGATEAF	ATSRESGNTIY
	NC2	ASRPWTTRRRGTDTQY	ASTGGGHPDTQY	SVEDLEQSYGYT

Figure 7. CDR3 AA sequence changes (highlighted) between the top 10 most frequent clonotypes pre-RT and those of the NCs that emerged post-RT for the indicated patients with localized prostate cancer. AA, amino acid; NC, new clonotype; RT, radiation therapy.

T-cell immune activation more convincingly in these patients. As shown in Fig. 3, the present study identified a total of six NCs ranking in the top 10 TCR Vβ CF at positions 7 and 9 for Pt #4 (two clonotypes; Fig. 6), at positions 7 and 9 for Pt #9 (two clonotypes; Fig. 6) and at positions 1 and 10 for Pt# 13 (two clonotypes; Fig. 6). These identified NCs differed in their TCR Vβ CDR3 AA from the top 10 identified pre-RT. As shown in Fig. 7, NC1 of Pt #4 differed by seven AAs (i.e., Y, R, T, G, E, L and F at positions 5, 6, 10, 11, 12, 13 and 14, respectively). The NC2 of this patient differed by nine AAs (i.e., R, T, R, R, G, T, D, T and Y at positions 3, 7, 8, 10, 11, 12, 13, 14 and 16, respectively). Similarly, Pt #9 had a NC1, which differed by four AAs (i.e. F, T, G and A at positions 4, 6, 7 and 11, respectively) and a NC2 that differed by eight AAs (i.e., T,

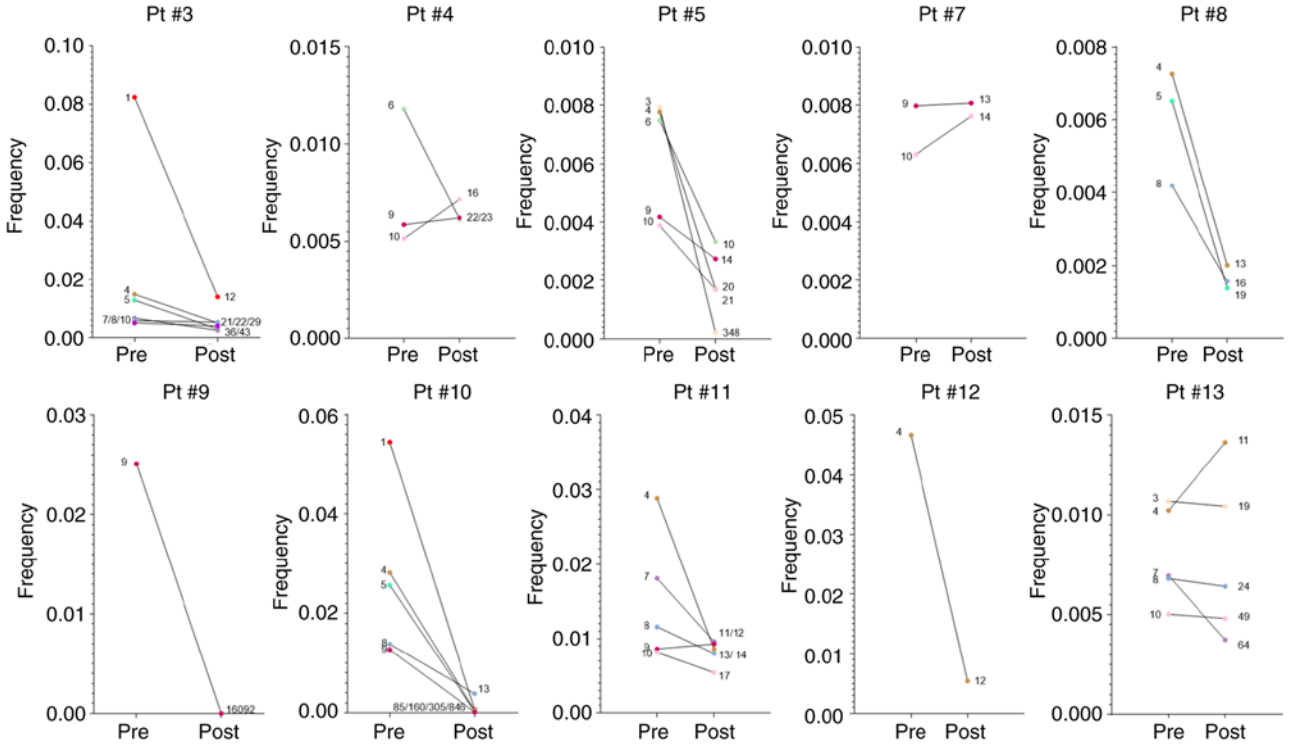


Figure 8. Identification of T-cell receptor variable β clonotypes with descending clonal frequencies in the peripheral blood of patients with localized prostate cancer after radiation therapy. The depicted lines represent the transition of each described clonotype from the top 10 most frequent clonotypes to a lower ranked frequency, with numbers indicating the exact frequency rank.

G, H, P, D, T, Q and Y at positions 3, 4, 7, 8, 9, 10, 11 and 12, respectively), whereas the two NCs of Pt#13 differed by two AAs (NC1; i.e., T and I at positions 2 and 10, respectively) and by eight AAs (NC2; i.e., D, L, E, Q, S, Y, G and T at positions 4, 5, 6, 7, 8, 9, 10 and 12, respectively).

In addition, cases that were characterized by decreased TCR Vβ CF post-RT were identified (Fig. 8). For example, Pt #3 had six TCR Vβ clonotypes, which at baseline were among the top 10 (ranking positions 1, 4, 5, 7, 8 and 10) but post-RT ranked among positions 12-43 (Fig. 8). In total, for the 10 patients analyzed, 35 decreased TCR Vβ clonotypes were identified post-RT, which were relegated from the top 10 TCR Vβ CFs. Some exceptional cases included four clonotypes that were lowered to positions 85, 160, 305 and 846 from positions 1, 4, 5 and 9, respectively (Pt #10) and one clonotype that was lowered from position 9 to position 16,092 (Pt #9) (Fig. 8). Notably, six clonotypes were detected, which although had increased CFs post-RT, they were still relegated from the top 10 TCR Vβ CF; for Pt #4, one clonotype with 0.0058 CF pre-RT and 0.0062 CF post-RT ranked from position 9 to 22, and another one with 0.0051 CF pre-RT and 0.0071 CF post-RT fell from position 10 to 16 (Fig. 8). Furthermore, for Pt #7, there were two clonotypes at positions 10 and 9 with CFs 0.0063 and 0.0079 pre-RT, respectively, which post-RT moved to positions 14 and 13, respectively, despite increased CFs (0.0076 and 0.0080, respectively; Fig. 8). There were also another two cases with clonotypes that were degraded post-RT compared with pre-RT; however, they had higher CFs post-RT compared with pre-RT. Specifically, in Pt #11 the clonotype at position 9 degraded to position 12 (0.0086 to 0.0092) and in Pt #13 the clonotype at position 4 degraded to position 11 (0.010 to 0.014) (Fig. 8). This could be due to the fact that in these patients the frequencies of their TCR Vβ clonotypes were relatively high at the lowest ranking of top 10 post-RT (i.e., position 10). These findings are presented in Table II, where the CFs of the TCR Vβ clonotypes ranking at position 10 (of the top 10 TCR Vβ CFs) post-RT for Pt #3, Pt #4, #7, #11 and #13 ranged from 0.010009 to 0.013911, thus being higher compared with the respective CFs for Pt #5 (0.003327; 3.0 fold-4.2 fold), Pt #8 (0.002364; 4.3 fold-6.0 fold), Pt #9 (0.00917; 11.1 fold-15.4 fold), Pt #10 (0.00632; 1.58 fold-2.2 fold) and Pt #12 (0.008005; 1.25 fold-1.73 fold).

Discussion

RT is a standard treatment for PCa. Clinically, although RT directly induces cancer cell death, an abscopal effect expressed by the regression of distant tumors via systemic immune activation is occasionally also observed (15). To the best of our knowledge, details on TCR CF alterations post-RT linking an abscopal effect with antitumor T-cell immunity in patients with LPCa have not yet been described. In the present study, the dynamics of systemic changes in frequencies among the top 10 TCR Vβ clonotypes before and after RT were investigated, and it was revealed that among the patients analyzed, a total of 33 TCR Vβ clonotypes were expanded in frequencies that ranged from 0.81-fold to 33.18-fold. Taking into consideration the fact that different clonotypes are characterized by marked differences in their CDR3 AA sequence and length, alterations in TCR CFs could indicate alterations in their antigen-targeting and recognition properties. Consequently, the detection of expanded TCR Vβ

Table II. CFs of the T-cell receptor variable β clonotypes ranking in the last position (position 10) of the top 10 clonotypes in each patient post-radiation therapy.

Patient no.	CF of clone at position 10
Pt #3	0.014594
Pt #4	0.010252
Pt #5	0.003327
Pt #7	0.010009
Pt #8	0.002364
Pt #9	0.00917
Pt #10	0.006032
Pt #11	0.010548
Pt #12	0.008005
Pt #13	0.013911

CF, clonal frequency.

clonotypes post-RT was of particular interest since these may signify disease prognosis and/or response to therapy. To this end, significant differences in the usage of specific V gene segments in the two groups of patients stratified by high or low GS were detected, by comparing their CFs before and after RT. Notably, TRBV6-6 segment usage was more frequent in the high-risk group (Group II) compared with the low-risk group, both pre- and post-RT. Similarly, the frequency of TRBV6-5 usage was higher in the high-risk group compared with the low-risk group post-RT. In a recent study, high usage of TRBV6-5 in patients with advanced NSCLC was shown to be associated with disease progression and a poor prognosis, and it was also revealed to be a predictive marker of non-durable clinical benefit of anti-PD-1 treatment (16). Moreover, deep TCR-β sequencing in tissue samples from prostate tumors revealed an abundance of both TRBV6-5 and TRBV6-6 in paracancerous tissue, but not within the tumor (17). By contrast, high usage of certain V segments has been associated with a favorable prognosis in several tumor types. Notably, high TRBV20-1 usage in patients with NSCLC has been reported to be associated with improvements in both progression-free and overall survival, as well as with an increased response to anti-PD-1 treatment (16). Taken together, these data may suggest that the preferential usage of specific V gene segments could have an important role in determining the levels and duration of T-cell-mediated antitumor immunity. Of particular interest was the detection of new TCR Vβ clonotypes post-RT in patients with high GS, presumably recognizing new tumor peptides released by RT-induced tumor cell death. Although PCa is characterized by the expression of unique tumor antigens, which could act as an excellent tool for triggering robust antitumor immune responses (18), its immunogenicity is still hampered by the immunosuppressive tumor microenvironment and the low tumor mutation burden (TMB) (19). However, it has been reported that TMB increases with certain tumor characteristics, such as a higher GS (20,21). Moreover, TMB may be associated with infiltrating immune cells in PCa (22).

In a recent study, the β-chain CDR3 AA sequences of various TCRs were grouped with the aim to sub-group those that recognize the same antigenic epitope (12). By clustering epitope-specific TCR AA sequences it was revealed that differences of at most one AA led to the recognition of the same antigenic peptide. The present study identified marked differences in the AA sequences for the CDR3 of the TCR Vβ chain in the NCs post-RT as compared with the top 10 clonotypes pre-RT (ranging from 2-9 AA for all 10 patients tested), which suggested that these recognize new antigens. The amino acid distribution within the CDR3 has been shown to serve a critical role in TCR assembly and function, and consequently, in the degeneracy of TCR recognition (23). Although characteristics of paired TCR α- and β-chains are more widely used for the determination of T-cell specificity, some deductions can be made by studying alterations in CDR3 AA sequences. It is widely noted that CDR3 mainly consists of 15 AAs (positions 104-118), from which the flanking positions (104-107 and 113-118) are almost exclusively expressed by germline-encoded V or J genes; consequently, these are almost universally conserved. However, AAs in the central region (positions 107-116) of CDR3 are those that directly contact antigens (11). For example, glycine has been found to enhance the flexibility of the CDR3 loop, which in turn serves a role in TCR polyspecificity (24). Notably, in all NCs, except for NC1 of Pt #13, at least one amino acid substitution (or insertion) by glycine was noted. Among the AAs with a higher frequency in all NCs were threonine and tyrosine, which are both polar hydrophilic residues. Apart from NCs, a general trend that was recorded regarding the most frequent clonotypes post-RT was the enrichment of CDR3 with polar and/or acidic AAs, which has been found to contribute to the TCR bonding process and may be related to the restricted localization of TCR on HLA-A2 (25). Notably, it has previously been shown that the nature of AA residues (especially at position 109) within the CDR3 has a crucial role for T-cell autoreactivity, which increases significantly in the presence of hydrophobic residues (26).

TCR sequencing has been used to examine intratumoral T-cell responses in solid types of cancer (27,28); in a previous study, it was shown that changes in pre- and post-treatment TCR repertoires were associated with better outcomes in patients with lung cancer (28). Furthermore, TCR diversity has been shown to be prognostic for overall survival in the absence of any treatment in patients with solid tumors, whereas pre-treatment TCR clonality was revealed to be predictive of response to anti-PD-1 treatment (29). Changes in TCR clonality following stereotactic body RT in patients with NSCLC have also been correlated with disease progression (30), further suggesting that radiation effects on TCR clonality may serve as predictive biomarkers for clinical outcomes.

The present study also detected a number of TCR Vβ clonotypes whose frequencies were either increased or decreased post-RT. Among all 10 patients examined, 33 TCR Vβ clonotypes were identified that at baseline had frequencies not high enough to rank among the top 10 TCR Vβ clonotypes; however, post-RT these were expanded and could be detected at high abundance. Inversely, 35 clonotypes at high baseline frequencies ranking among the top 10 TCR Vβ CFs were found at much lower frequencies post-RT. These findings clearly suggested that RT may induce immune changes,

which differentially influence the expansion of various T-cell clonotypes, either locally or in the blood stream, as a result of systemic immune activation. Notably, reports have indicated that, after irradiation, dying tumor cells can release damage-associated molecular patterns that lead to a variety of immune pathways affecting production of pro-inflammatory cytokines, including IL-1, IL-18 (31,32) and type I and type II interferons (33-37), which in turn activate pathways involved in the antigen-processing machinery and presentation of tumor peptides resulting in the induction of adaptive antitumor immunity (12,15,31,38). Moreover, irradiation-induced IFN-γ has been reported to upregulate the pro-inflammatory chemokines CXCL10 and CXCL16, which in turn activate antitumor $CD8^+$ T-cells (15,39-41). Thus, the present hypothesis to build upon is that the RT-induced release of tumor peptides from the damaged tumor cells in the presence of pro-inflammatory mediators will favor the clonal expansion of T cells specifically recognizing these peptides via their cognate TCRs, which will be then presented at abundant frequencies post-RT. From this perspective, it was proposed that these expanded T-cell clonotypes will be further stimulated to extensive proliferative responses during the continuous release of and stimulation by tumor peptides in the periphery; therefore, their immunodominant outgrowth will suppress the expansion of other T-cell clones having a low average avidity of their involved TCRs for the presented peptide-MHC/HLA class-I complexes. The present results are in line with those recently reported by Chow *et al* (42), which showed that RT in patients with renal cell cancer could induce immune changes in the periphery, which were reflected as dynamic changes in their TCR repertoire. Notably, radiation-induced immunogenicity has been linked to increased type I and type II IFN responses leading to upregulation of the cGAS-STING pathway and enhanced intratumoral infiltration of $CD8^+$ T-cells (40,43).

The present study has some limitations. Firstly, the number of patients examined was low, and therefore a future study with a larger sample size and clinical follow-up is required to confirm the impact of changes in the TCR Vβ repertoires before and after RT on prognostication. Secondly, the present study lacked data regarding the impact of RT on TCR Vβ CF according to T-cell subsets; for example, on $CD8^+$ or $CD4^+$ T cells, or even $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells, which are important for negatively regulating antitumor immunity. Taken together, the results of the present study identified clonal expansion and decreases in response to RT, providing justification for RT as an immune-activating tool in LPCa. The specific observations of T-cell expansion and decrease within a period of 3 months post-RT offer novel therapeutic combination strategies that may leverage RT-activated endogenous systemic T-cell immunity for improved clinical outcomes to future androgen deprivation therapies.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive under BioProject no. PRJNA818160 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA818160).

Authors' contributions

MG, ND, PK and SPF performed the experiments. MG, ND, PB and SPF analyzed the data and performed statistical analysis. MA, ADG and VZ also contributed to data analysis. SS, EM, EV and CZ recruited the patients included in the study, performed the blood sampling and recorded all relevant clinicopathological data. MG, ND, CNB and SPF contributed to the interpretation of the data. MG, ND, PB, VZ, CNB and SPF prepared the original manuscript. CNB and SPF reviewed and edited the final version of the manuscript. CNB conceptualized the study. MG and ND confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Saint Savas Cancer Hospital IRB (approval no. IRB-ID6777/14-06-2017) and the Ethical Committee of the National and Kapodistrian University of Athens as part of a larger study (approval no. ID247/28-01-2020). Written informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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