Mitochondrial function changes in T cell subsets during radiotherapy for patients with nasopharyngeal carcinoma

QUAN WANG[1*], XIANGZHI YIN[2*], HONGBO LIU[1], QING WANG[3], LU ZHANG[4], YIFAN WANG[5] and HAIJUN LU[1]

[1]Department of Radiation Oncology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266035, P.R. China; [2]Department of Orthopaedics, Affiliated Hospital of Qingdao University, Qingdao, Shandong 266035, P.R. China; [3]Department of Clinical Laboratory, Affiliated Hospital of Qingdao University, Qingdao, Shandong 266035, P.R. China; [4]Department of Radiation Oncology, The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250014, P.R. China; [5]Department of Emergency, Laizhou People's Hospital, Yantai, Shandong 261400, P.R. China

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Abstract. Mitochondrial dysfunction-mediated T cell exhaustion is associated with the efficacy of tumor therapy; however, the effect of radiotherapy (RT) on the mitochondrial function of peripheral blood immune cells remains still unclear. Therefore, the current study aimed to determine mitochondrial function indicators in immune cells, in particular mitochondrial mass (MM) and mitochondrial membrane potential (MMP), to assess the dynamic changes of immune status in patients with nasopharyngeal carcinoma (NPC) during RT. Peripheral venous blood was collected from patients with locally advanced NPC at day 1 pre-RT, at the 10th fraction of RT and within 2 days after RT. Based on a novel immunofluorescence technique, flow cytometry was used to assess the proportion of lymphocytes and their subsets in peripheral blood and the mitochondrial indexes, MM and low MMP (MMP^{low}). Univariate and multivariate logistic regression analyses were performed to evaluate the clinical factors associated with the efficacy of RT. A total of 27 patients were enrolled. After RT, lymphocyte count ($P<0.05$) and the proportion of $CD4^+$ T cells ($P<0.05$) demonstrated a downward trend. In addition, the proportion of $CD4^+$ memory-effector T (Tem; $P<0.05$) cells and $CD8^+$ Tem cells ($P=0.005$) significantly increased during RT. No significant changes were demonstrated for MM in $CD4^+$ effector T (Te) cells, whilst MMP^{low} was significantly reduced ($P=0.047$). However, the mitochondrial function of $CD8^+$ T cells did not significantly change. Multivariate logistic regression analysis revealed that lymphocyte count [odds ratio (OR), 47.317; 95% confidence interval (CI), 1.240-1806.065] and MMP^{low} in $CD4^+$ Te cells (OR, 0.889; 95% CI, 0.792-0.997) were independent factors that could affect clinical efficacy. Receiver operating characteristic curve analysis demonstrated that the area under the curve values for MMP^{low} in $CD4^+$ T cells, lymphocyte count and their combination were 0.72 ($P=0.13$), 0.69 ($P=0.19$) and 0.89 ($P=0.0073$), respectively. These findings suggest that RT could inhibit immune cells in peripheral blood. However, this treatment approach could activate the memory cell subsets of immune cells and enhance the MMP of effector $CD4^+$ T cells. Therefore, the evaluation of mitochondrial function in lymphocytes could be used as a predictor of RT efficacy in patients with locally advanced NPC.

Correspondence to: Professor Haijun Lu, Department of Radiation Oncology, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao, Shandong 266035, P.R. China
E-mail: lhj82920608@163.com

*Contributed equally

Abbreviations: NPC, nasopharyngeal carcinoma; MM, mitochondrial mass; MMP^{low}, low mitochondrial membrane potential; RT, radiotherapy; CXCL, CXC chemokine ligand; PD-L1, programmed cell death 1 ligand 1; TILs, tumor-infiltrating T cells; IL, interleukin; IFN-γ, interferon-γ; RECIST, Response Evaluation Criteria in Solid Tumors; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ROC, receiver operating characteristic

Key words: locally advanced nasopharyngeal carcinoma, radiotherapy, mitochondrial dysfunction, $CD4^+$ T cell, $CD8^+$ T cell

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor that has a high incidence in East and Southeast Asia, with an estimated ~72,000 people dying from NPC in 2018 (1,2). According to the 2022 National Comprehensive Cancer Network guidelines, the standard treatment approach for NPC is concurrent chemoradiotherapy (3). Based on the use of an effective and standardized treatment, the 5-year survival rate of early-stage NPC is ~80%. However, 5-10% of patients experience recurrence or metastasis after treatment, and even in developed countries, the 5-year survival rate of these patients is >40% (4,5). The ongoing phase III clinical study (CONTINUUM), reported at the American Society of Clinical Oncology in 2023, highlighted the promising efficacy of sintilimab combined with concurrent chemoradiotherapy in the treatment of locally advanced NPC (6). Therefore, combination therapy is an inevitable trend, and understanding the effect of conventional treatment on the immune system is of great importance.

Radiotherapy (RT) is a local treatment strategy, which is administered to regulate tumor growth via ionizing radiation, thus promoting double-stranded DNA breaks in cells (7). Similarly, ionizing radiation can also directly damage immune cells. The immunosuppressive effect of RT is associated with the reduction of lymphocyte count, the activation of regulatory T cells and the recruitment of myeloid-derived suppressor cells (8-11). Previous studies have also reported that ionizing radiation could increase the secretion of pro-inflammatory cytokines, including CXC chemokine ligand (CXCL)16, transforming growth factor-β, CXCL10 and CXCL4, and promote the expression of major histocompatibility complex I, programmed cell death 1 ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1) (12-17). In addition, RT exerts its antitumor effects via promoting T cell infiltration and activation through the secretion of pro-inflammatory factors and expression of particular molecules, such as TNF-α, IL-1β and IL-6 (18). Different pathological types and RT plans can affect the efficacy of RT in the immune system (19); however, there is still a lack of qualified markers to dynamically evaluate the changes in the immune microenvironment *in vivo* and elucidate the association between RT and immunity.

The function of mitochondria is primarily, but not exclusively, associated with energy supply (20). Based on their activation status, immune cells can be divided into effector T (Te) cells ($CD62L^-CD45RA^+$) and memory-effector T (Tem) cells ($CD62L^-CD45RA^-$). Te cells are commonly found in peripheral tissues and exert direct antitumor effects, whereas Tem cells exist in peripheral and lymphoid tissues and show antitumor effects after re-exposure to an antigen. $CD4^+$ Te cells, which are involved in tumor immunity, can recruit dendritic cells, activate $CD8^+$ T cells and directly kill tumor cells (21). A previous study reported that the immune status of $CD62L^-CD4^+$ Te cells could predict short- and long-term responses to immunotherapy in patients with non-small cell lung cancer (22).

Furthermore, immune cell activation is characterized by enhanced mitochondrial mass (MM) and mitochondrial membrane potential (MMP). Mitochondrial dysfunction-induced depleted immune cells, and in particular, tumor-infiltrating T cells (TILs), display reduced mitochondrial function (23-25). Notably, a previous study reported that mitochondrial dysfunction was not reversed by the PD-1 blockade of TILs with persistent mitochondrial loss, suggesting that immune checkpoint blockade alone could not yield desirable results in patients with more complex tumors (23,25). The mitochondrial function grouping of peripheral blood immune cells can provide a basis for evaluating the immune status of patients with cancer (26). The maintenance of cellular function requires sufficient MM to maintain oxidative phosphorylation (27). Activated $CD8^+$ T cells and Te cells are characterized by enhanced MM, whilst depleted T cells have low MM (28). MMP is generated by the pumping of protons from the matrix into the membrane space and is associated with adenosine triphosphate production. When T cell receptors are activated, MMP and mitochondrial metabolism are increased, thus promoting the secretion of cytokines, such as interleukin (IL)-17A, IL-17F and interferon-γ (IFN-γ), by several Te cells (29). However, whether MM and MMP can predict the response to tumor therapy remains unknown.

The present study aimed to assess the dynamic changes in immune status in patients with NPC during RT. Using a novel immunofluorescence technique, the mitochondrial function indicators of immune cells, namely MM and MMP, were evaluated. Subsequently, the functional status of immune cells at different time points during RT in patients with NPC and the association between the functional status of immune cells and the efficacy of RT were assessed, thus providing novel insights into the role of MMP of immune cells in predicting the efficacy of RT.

Materials and methods

Study design and patient enrollment. The present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China; approval no. MR-37-23-012058). The study was designed in accordance with the ethical principles laid down in the Declaration of Helsinki. A total of 28 patients with locally advanced NPC were enrolled from the Affiliated Hospital of Qingdao University, between September 2022 and November 2023. Due to the 1 patient contracting coronavirus disease-19 and being excluded from the present study, a total of 27 patients completed the study. The inclusion criteria were as follows: i) New diagnosis of pathologically-confirmed NPC; ii) stage III-IVa NPC according to the 8th edition of the American Joint Committee on Cancer staging system (30); iii) refusal of concurrent chemoradiotherapy due to physical reasons; iv) age of 18-70 years; and v) written informed consent provided. The exclusion criteria were as follows: i) Diagnosis of autoimmune diseases or primary immune dysfunction; ii) diagnosis of other malignant tumors; iii) previous therapy with PD-1, PD-L1 or cytotoxic T lymphocyte-associated antigen-4 inhibitors; iv) presence of inflammatory reactions, such as cold and fever, or infection, including hepatitis B and acquired immune deficiency syndrome; v) diagnosis of metabolic syndrome (diabetes); vi) hemodialysis or organ transplantation performed; and vi) unwillingness to participate in the study. All patients provided written informed consent prior enrollment in the study.

Delineation of the target volume and RT plan design was performed according to International Guidelines for NPC (31). The RT plan was implemented in accordance with the International Commission of Radiation Units reports 50 and 62 (32,33). All patients previously refused concurrent chemotherapy and had received concurrent targeted therapy during RT after induction chemotherapy. Targeted therapy involved the administration of 100 mg nitocilizumab once a week for 8 weeks during RT. Following RT for 4 weeks, the clinical efficacy, which was classified as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD), was evaluated by two attending physicians according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 guidelines (34). The clinical and pathological data were obtained from the electronic medical records of the Affiliated Hospital of Qingdao University.

Peripheral blood samples. At least 3 ml peripheral venous blood was collected in ethylene diaminetetraacetic acid tubes at day 1 pre-RT, at the 10th fraction of RT and within

2 days after RT. The antibodies were as follows: CD3 PE, CD4 FITC, CD45RA PerCP-Cy5.5, CD62L PE-Cyamine7, CD8 APC-Cyanine7 contained in a complete antibody kit (cat. no. KUB32479; Hunan Xiangyi Biotechnology Co. Ltd.) and the mitochondrial staining fluorescent probes, MitoDye (structural formula $C_{34}H_{36}Cl_2N_2$; cat. no. UB32479-8; Hunan Xiangyi Biotechnology Co., Ltd.) and phosphate buffer (DPBS; pH 7.4, 0.12% NaH_2PO_4, 0.88% NaCl, 0.2% gelatin and 0.09% sodium azide), purchased from UB Biotechnology Co. Ltd. A 20 μl volume of antibody detection reagent was placed in a labelled flow tube at room temperature. A 100 μl anticoagulated human whole blood sample turnd upside down at least 7 times, was placed in the flow tube for 3-5 secand incubated in the dark at room temperature for 15 min. A 2 ml hemolysin working solution (BD Biosciences) was added to the flow tube and vortexed at a low speed for 3-5 sec, then incubated in the dark for 15 min at room temperature. Samples were centrifuged at 300 x g for 5 min at room temperature, and the supernatant was discarded. The precipitate was resuspended in 200 μl of DPBS, transferred to an 8-linked tube of mitochondrial staining reagent and incubated at 37˚C in the dark for 30 min. The lymphocyte subtypes in peripheral blood, including $CD3^+$, $CD4^+$ and $CD8^+$ T cells, were assessed using flow cytometry. The $CD62L^-CD45RA^+$ effector and $CD62L^-CD45RA^-$ memory-effector subsets were also detected (35). The mitochondrial indices determined were MM and low MMP (MMP^{low}). Mitochondria were stained with MitoDye-APC as aforementioned and detected using a flow cytometer (DiagCyto 6C2L; Changchun YouBi Biotechnology Co., Ltd.), followed by passing through an antigen-assisted loop gate (36). Using the antibody-assisted ring gate, each target cell gate was read and a binary gate was then drawn to distinguish the low from the high mitochondrial expression groups. The low percentage group represented MMP^{low}, whilst the total fluorescence intensity group indicated MM. Analysis was performed using NovoExpress (version 1.4.1; Agilent Technologies, Inc.). The screening strategy is illustrated in Fig. 1A-D.

Statistical analysis. All statistical analyses were performed using SPSS version 26.0 (IBM Corp.), whilst GraphPad Prism 10.0 software (Dotmatics) was used to draw graphs. Continuous variables are expressed as the mean ± standard deviation and categorical variables as n (%). When the changes in the immune cells during RT were normally distributed, the repeated measures ANOVA test was performed. For non-normally distributed changes, the Friedman's test was performed. For multiple comparisons between two groups, Bonferroni test or Nemenyi test was performed for statistically significant differences. To analyze the potential prognostic factors for clinical response (CR/PR), univariate logistic regression analysis was performed. Prognostic factors with $P<0.2$ were subjected to multivariate logistic regression analysis. Receiver operating characteristic (ROC) curves were utilized to describe the predictive function of mitochondria in immune cells and lymphocytes. To analyze the changes in PD-1 expression in T cell subsets before and after RT, the paired t test was used if the distribution was normal, and the Wilcoxon Rank-Sign test was used if the distribution was not normal. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 27 patients, including 16 men and 11 women, with locally advanced NPC were enrolled from the Affiliated Hospital of Qingdao University (Table I). The median age of patients was 49 years (range, 18-70 years). The majority of the included patients had stage III NPC (63.0%), whilst they all had pathologically confirmed EGFR mutations. Among the 27 patients, 20 were treated with helical tomotherapy, whilst the remaining seven patients were subjected to intensity-modulated RT.

Immune status of peripheral blood cells during RT. All 27 patients underwent pre-RT, mid-RT and post-RT. After RT, the number of peripheral blood lymphocytes ($F=104.554$; $P<0.001$; Fig. 1E) and the proportion of $CD4^+$ T cells were significantly decreased ($F=19.185$; $P<0.001$; Fig. 1G), whilst no significant change was demonstrated for the percentage of $CD8^+$ T cells. In addition, the proportion of $CD4^+$ ($F=30.519$; $P<0.001$; Fig. 1H) and $CD8^+$ ($F=5.827$; $P=0.005$; Fig. 1J) Tem cells was significantly increased, whilst no changes were demonstrated for $CD4^+$ and $CD8^+$ Te cells (Table II). Previous studies have reported that the mitochondrial function of immune cells could be associated with response to cancer therapy (37,38). Therefore, the functional status of mitochondria was used to reflect the changes in immune cell status. A novel immunofluorescence technique was used to detect mitochondrial function indicators, namely MMP and MM. No significant change was revealed for the mitochondrial function of $CD4^+$ T cells after RT (Table II). Additionally, no significant changes in MM were demonstrated in $CD4^+$ Te cells. However, MMP^{low} was significantly reduced in these cells ($F=3.294$; $P=0.047$; Fig. 1I). The aforementioned results indicate that the effector subsets of $CD4^+$ T cells were activated during RT, whilst their mitochondrial function was significantly increased. Notably, the mitochondrial function of $CD8^+$ T cells and their subsets did not change significantly.

Mitochondrial function as a prognostic indicator. At 4 weeks after RT, clinical efficacy was assessed using the RECIST 1.1. guidelines. A total of 22 patients achieved CR or PR, whilst the remaining patients were classified as PD (n=3) or SD (n=2). It was therefore considered that patients with CR and PR had a good response, whilst the remaining patients displayed a poor response to RT. The clinical characteristics of patients, such as age, tumor stage, lymph node stage and pathological features, as well as immunological parameters that changed during RT, were included in the regression analysis. Indicators with $P<0.2$ in the univariate logistic regression analysis were included in the multivariate logistic regression analysis. Therefore, the multivariate logistic regression analysis demonstrated that lymphocyte count and MMP^{low} in CD4+ Te cells were independent factors affecting clinical efficacy. The higher the lymphocyte count, the greater the clinical efficacy [odds ratio (OR), 47.317; 95% confidence interval (CI), 1.240-1806.065]. In addition, the higher the MMP^{low} in $CD4^+$ Te cells, the worse the clinical efficacy (OR, 0.889; 95% CI, 0.792-0.997) (Table III). These results suggest that increased MMP in $CD4^+$ Te cells is associated with improved clinical efficacy. Furthermore, the ROC curve analysis revealed that the combination of

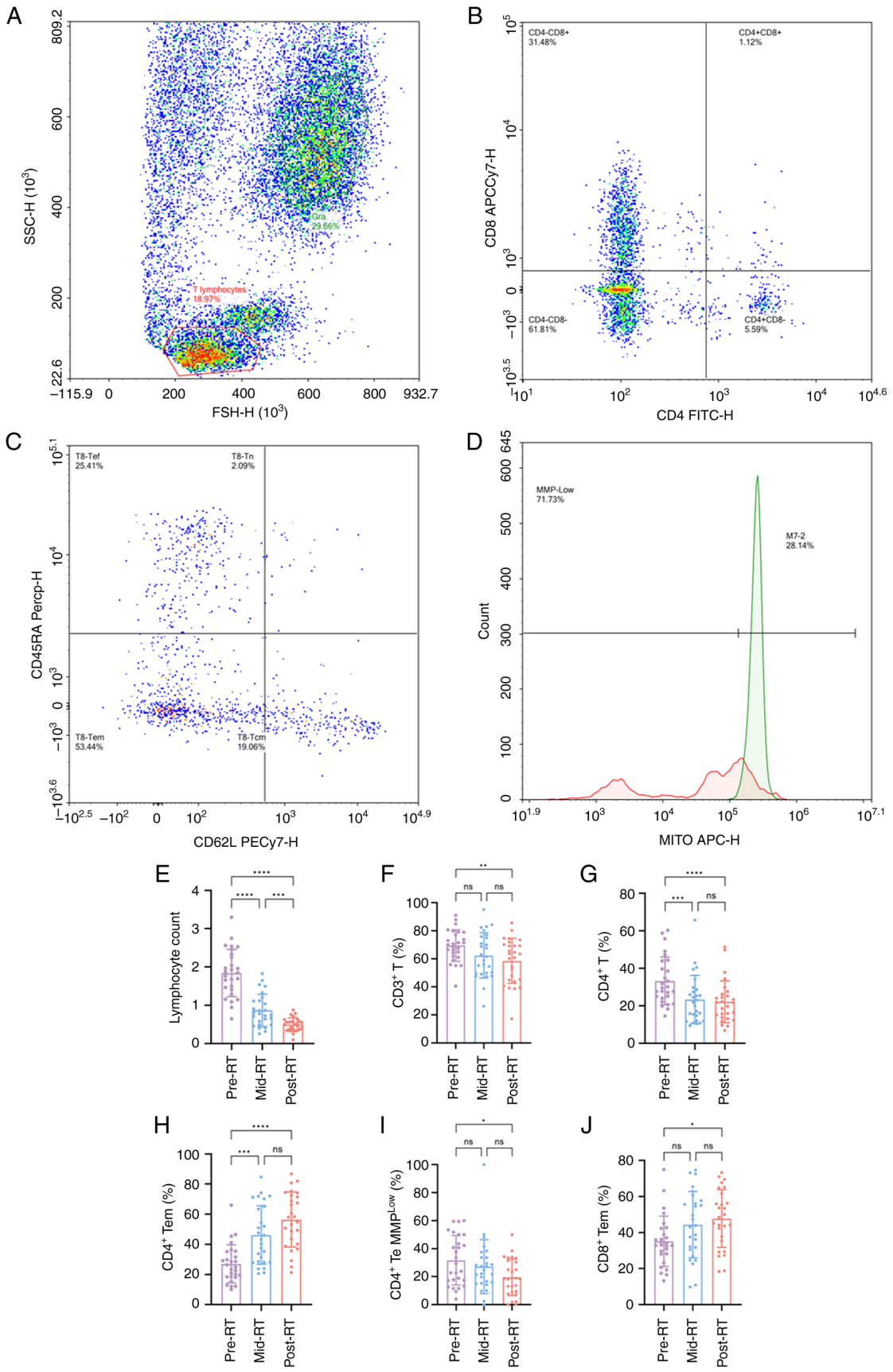

Figure 1. Flow cytometry and changes in immune cells during RT. Gating strategy in flow cytometry. (A) FSC/SSC plot of the lymphocyte populations. (B) CD4/CD8 scatter of the $CD4^+CD8^-$ and $CD4^-CD8^+$ cell populations. (C) CD62L/CD45RA scatter plots of the Tn, Tcm, Tem and Tef cell populations. (D) Count/MITO APC-H of the Tem, Tn, Tcm and Tef cell populations, using a dichotomous gate to calculate the percentage of lymphocytes and fluorescence intensity in each population. Changes in the (E) number of lymphocytes, proportion of (F) $CD3^+$ T cells, (G) $CD4^+$ T cells and (H) $CD4^+$Tem cells, (I) MMP^{low} in $CD4^+$ Te cells and (J) proportion of $CD8^+$ Tem cells during RT. n=27 per group. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. RSC, forward scatter; SSC, side scatter; ns, not significant; RT, radiotherapy; Tem, memory-effector T cells; Te, effector cells T; Tef, effector T cells; Tn, Naïve T cells; Tcm, central memory T cells; MMP, mitochondrial membrane potential.

Table I. Clinical characteristics of patients with nasopharyngeal carcinoma in the present study.

Characteristic	Patients (n=27)
Sex	
Male	16 (59.3)
Female	11 (40.7)
Age, years	49.0±15.2
T stage	
T1	3 (11.1)
T2	16 (59.3)
T3	5 (18.5)
T4	3 (11.1)
N stage	
N0	2 (7.4)
N1	2 (7.4)
N2	16 (59.3)
N3	7 (25.9)
Overall stage	
III	17 (63.0)
IVa	10 (37.0)
World Health Organization pathological type	
Keratinizing carcinoma	2 (7.4)
Differentiated non-keratinizing carcinoma	21 (77.8)
Undifferentiated non-keratinizing carcinoma	4 (14.8)

Data are presented as n (%) or mean ± standard deviation. T, tumor; N, lymph node.

MMP^{low} in $CD4^+$ Te cells with lymphocyte count exerted an improved area under the curve (AUC) value for predicting clinical efficacy, compared with MMP^{low} in $CD4^+$ Te cells and lymphocyte counts. The AUC values for MMP^{low} in $CD4^+$ Te cells, lymphocyte count and their combination were 0.72 (P=0.13), 0.69 (P=0.19) and 0.89 (P=0.0073), respectively (Fig. 2A). These findings suggest that mitochondrial function in lymphocytes could be considered as an indicator to predict the efficacy of RT in patients with locally advanced NPC.

PD-1 expression in peripheral blood T cell subsets. It has been reported that the enhanced expression of PD-1 in peripheral blood immune cells is associated with cell failure and worse clinical efficacy (39,40). Therefore, the present study assessed the expression levels of PD-1 in $CD4^+$ and $CD8^+$ T cell subsets before and after RT. The results revealed that the expression levels of PD-1 in $CD4^+$ Te cells displayed a downward trend and the difference was statistically significant (Z=-2.162; P=0.031; Fig. 2B). By contrast, the expression levels of PD-1 in the other cell subsets were notably increased; however, statistical significance was not reached.

Discussion

The application of immunotherapy in patients with advanced NPC has reached a consensus, whilst the combination of RT with immunotherapy in patients with locally advanced NPC remains in the exploratory stage (41). Currently, the RT-mediated changes in immune status in patients with locally advanced NPC are unclear. Therefore, the timing of combination therapy remains an urgent problem to be solved. Consequently, the present study assessed the mitochondrial changes in immune cells in the peripheral blood of patients with locally advanced NPC before and after RT. The results demonstrated that RT could activate $CD4^+$ Te cells and increase MMP, whilst MMP^{high} was associated with the efficacy of RT. Therefore, we hypothesize that MMP in peripheral blood $CD4^+$ Te cells could be used as an index for evaluating the immune status of patients with locally advanced NPC.

RT can induce the immunogenic death of tumor cells and promote inflammatory and antitumor responses; however, it has been reported that RT can increase the secretion of immunosuppressive antibodies, which in turn deplete immune cells (42). Nevertheless, how to regulate the immunomodulatory effect of RT remains unresolved. RT is the main treatment strategy for NPC; however, ~30% of patients will relapse after systemic treatment (5). In our previous study, it was demonstrated that the compliance of patients with NPC treated with concurrent chemoradiotherapy was poor and patients could not tolerate concurrent chemoradiotherapy (38). In addition, You *et al* (43) and Cao *et al* (44) reported that the clinical effect of synchronous targeted therapy during RT was not markedly different from that of synchronous chemoradiotherapy, and there were fewer adverse effects, which was easier for patients to accept. Moreover, Teng *et al* (45) reported that RT combined with targeted therapy had a small inhibitory effect on the immune system, which enhanced the activation of autoimmune mechanisms in patients with locally advanced NPC and established a foundational framework for combination immunotherapy strategies. Due to patient refusal of concurrent chemotherapy, the inherent limitations of the single-center study design and predefined research objectives, all participants underwent radiotherapy with concurrent targeted therapy instead of the standard chemoradiotherapy regimen.

Single-cell transcriptome analysis of the tumor microenvironment in patients with locally advanced NPC revealed that PD-1 was highly expressed in TILs, accompanied by an increased number of immunosuppressive cells, which may have originated from their migration into peripheral blood (46). Patients with locally advanced NPC have a unique tumor immune microenvironment, which provides the basis for the application of RT combined with immunotherapy. However, the changes in the immune status of patients with locally advanced NPC during RT have not been elucidated (47). Consistent with the study by Xie *et al* (48), in the present study, the number of lymphocytes decreased during RT. Radiation can directly damage the DNA of circulating lymphocytes, and circulating lymphocytes are sensitive to radiation; therefore, DNA fragmentation can be caused by a radiation dose of 2 Gy (49). Previous studies have reported that $CD4^+$ T cells could be more susceptible to radiation compared with $CD8^+$ T cells, thus highlighting the different radiosensitivity of different cells, which could lead to differential lymphocyte subtype distribution (50). Tem cells, as memory effector cells, are located in peripheral lymphoid tissues and

Table II. Analysis of changes in the number of immune cells and mitochondrial function during radiotherapy.

Parameter	Pre-RT (n=27)	Mid-RT (n=27)	Post-RT (n=27)	F	P-value
White blood cell count, 10^9/l	5.86±2.46	5.57±1.68	5.32±2.06	2.889	0.236
Lymphocyte count, 10^9/l	1.85±0.62	0.87±0.42	0.50±0.18	104.554	<0.001
$CD3^+$T, %	69.47±11.26	62.41±16.15	58.54±15.99	6.016	0.009
$CD4^+$T, %	33.31±12.7	23.41±12.89	22.20±11.14	19.185	<0.001
$CD4^+$T MMP^{low}, %	31.57±10.68	35.95±12.82	38.12±13.77	1.855	0.167
$CD4^+$ T MM	3.13±3.13	2.78±3.65	1.70±0.77	2.296	0.317
$CD4^+$Te, %	18.34±13.99	19.98±18.25	17.28±17.31	0.519	0.772
$CD4^+$ Te MMP^{low}, %	31.71±17.67	27.18±19.18	19.62±13.04	3.294	0.047
$CD4^+$ Te MM	2.37±1.54	2.26±0.83	2.48±1.48	0.222	0.895
$CD4^+$ Tem, %	27.04±12.67	46.24±19.39	56.47±18.30	30.519	<0.001
$CD4^+$ Tem MMP^{low}, %	40.41±14.54	43.30±15.75	44.99±17.81	0.621	0.541
$CD4^+$ Tem MM	1.86±1.27	1.46±0.64	1.35±0.71	5.505	0.064
$CD8^+$ T, %	33.16±12.44	33.29±12.52	33.45±14.94	0.963	0.618
$CD8^+$ T MMP^{low}, %	46.66±17.30	38.89±16.00	43.03±15.47	2.005	0.145
$CD8^+$ T MM	1.96±1.73	2.06±2.16	1.39±0.82	3.852	0.146
$CD8^+$ Te, %	27.52±15.29	29.63±20.26	25.79±12.33	0.074	0.964
$CD8^+$ Te MMP^{low}, %	40.75±30.13	40.48±26.69	39.08±16.63	0.222	0.895
$CD8^+$ Te MM	2.14±1.88	1.65±0.93	1.68±1.09	0.296	0.862
$CD8^+$ Tem, %	35.12±13.98	44.38±18.40	47.75±16.01	5.827	0.005
$CD8^+$ Tem MMP^{low}, %	53.08±26.02	53.50±24.62	47.04±18.47	0.802	0.454
$CD8^+$ Tem MM	1.62±1.91	1.17±0.72	1.34±0.86	1.364	0.505
CD4/CD8	1.25±1.13	0.80±0.48	0.85±0.68	15.407	<0.001

RT, radiotherapy; MMP^{low}, low mitochondrial membrane potential; MM, mitochondrial mass; Tem, memory-effector T; Te, effector T. Reference values: White blood cell count, 3.5-9.5x10^9/l; lymphocyte count, 1.1-3.2x10^9/l.

Table III. Univariate and multivariate analysis of radiotherapy efficacy.

	Univariate			Multivariate		
Variable	P-value	OR	95% CI	P-value	OR	95% CI
Age	0.63	1.02	0.95-1.10			
T stage	0.76	-	-			
N stage	0.77	-	-			
Stage	0.88	0.86	0.12-6.26			
Pathological type	0.49	-	-			
Lymphocyte count	0.10	4.73	0.73-30.66	0.038	47.317	1.240-1806.065
$CD3^+$ T	0.24	1.06	0.96-1.17			
$CD4^+$ T	0.40	1.03	0.96-1.11			
$CD4^+$ Te MMP^{low}	0.17	0.95	0.89-1.02	0.045	0.889	0.792-0.997
$CD4^+$ Tem	0.56	0.97	0.89-1.07			
$CD8^+$ Tem	0.86	0.99	0.92-1.07			
CD4/CD8	0.22	2.76	0.55-13.80			

OR, odds ratio; CI, confidence interval; T, tumor; N, lymph node; MMP^{low}, low mitochondrial membrane potential; MM, mitochondrial mass; Tem, memory-effector T; Te, effector T.

respond rapidly when T cell receptors are activated (51). However, Te cells are terminally differentiated effector cells and their proliferation ability is weak (51). Therefore, this explains why the proportion of Tem cells increased during RT in the present study. By contrast, the proportion of Te cells did not change significantly.

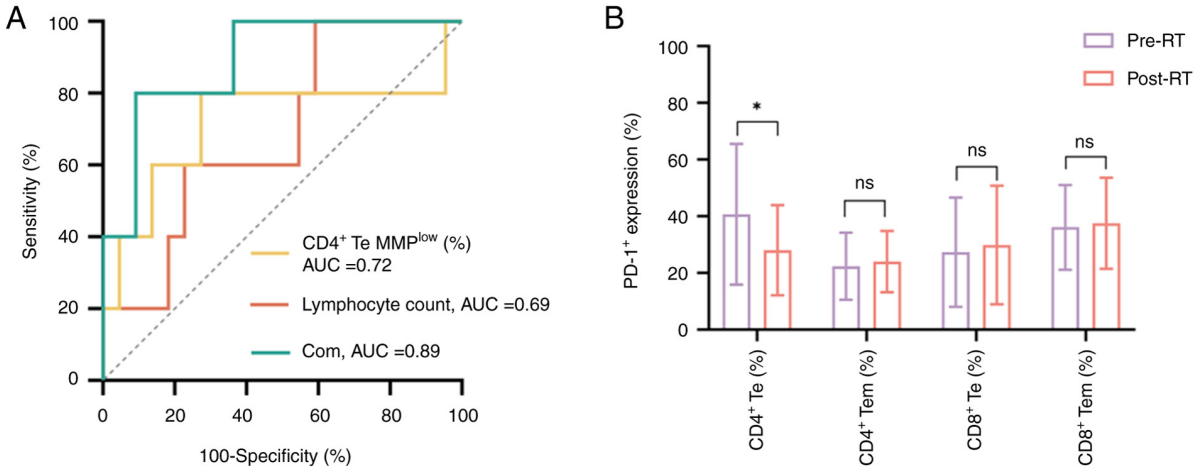

Figure 2. Mitochondrial membrane potential predicts the efficacy of RT. (A) Receiver operator characteristic curves for MMP, lymphocyte number and combined measures. (B) Expression of PD-1 in immune cell subsets before and after RT. n=27 per group. *P<0.05. MMP, mitochondrial membrane potential; PD-1, programmed cell death protein 1; RT, radiotherapy; Te, effector T; AUC, area under curve; Com, combination of MMP^{low} in $CD4^+$ Te cells and lymphocyte count; ns, not significant; Tem, memory-effector T.

The current study demonstrated that RT could activate circulating $CD4^+$ Te cells in patients with locally advanced NPC. Additionally, higher MMP was associated with the efficacy of RT. Previous studies have reported that elevated MMP induced glycolysis and the secretion of different cytokines, such as IL-17A, IL-17F and IFN-γ (29,52). Ma *et al* (53) reported that the MMP and MM of lymphocyte could predict early liver inflammation, indicating that MMP could reflect the inflammatory state of the body. However, the present study is the first to predict MMP and MM during RT in patients with tumor, to the best of our knowledge. Unlike the study by Ma *et al*, the inflammatory state induced by RT in patients with NPC stimulated an antitumor immune response, with the increased metabolism of immune cells promoting their antitumor function. Moreover, it has been reported that T-cell exhaustion is characterized by increased PD-1 expression (54). However, consistent with membrane potential, PD-1 expression decreased in $CD4^+$ Te cells after RT in the present study. These results indicate that RT could activate $CD4^+$ Te cell-mediated immunity. Ogando *et al* (55) reported that PD-1 expression in $CD8^+$ T cells limited the mitochondrial contact site and downregulated cristae organizing system complex proteins, namely coiled-coil-helix-coiled-coil-helix domain containing (CHCHD)3 and CHCHD10, thus promoting the loss of mitochondrial cristae morphology and MMP, eventually resulting in T cell dysfunction. However, the mechanism underlying the effect of PD-1 signaling on MMP in $CD4^+$ Te cells has not been elucidated. Yang *et al* (56) reported that the activation of $CD4^+$ Te cells during chemoimmunotherapy was associated with an improved prognosis in patients with non-small cell lung cancer. Furthermore, it has been reported that $CD4^+$ T cells are associated with the efficacy of RT against malignant tumors, such as ovarian cancer, head and neck squamous cell carcinoma, malignant melanoma, rectal cancer and breast cancer (57,58). This could be due to the RT-mediated activation $CD4^+$ T cells and the release of cytokines, such as IFN-γ and TNF-α, thus enhancing the sensitivity of tumor cells to RT (59). Another study also reported that the production of IFN-γ and that of other cytokines was closely associated with mitochondria-mediated reactive oxygen species generation. The aforementioned process was regulated by the Fas/FasL pathway (60). Herrera *et al* (61) reported that RT could promote the expression of natural killer group 2 member D (NKG2D) by $CD4^+$ Te cells and activate dendritic cells. In turn, the expression of NKG2D was notably associated with glucose metabolism regulated by mitochondria. However, when T cells with stem cell properties are needed, T cells with lower MMP should be screened for CAR-T therapy (62). By contrast, Te cells with high MMP are required to achieve antitumor effects (29). Therefore, the immune system of patients with high MMP in $CD4^+$ Te cells could be activated during RT and therefore, patients who could be more likely to benefit from immunotherapy should be selected. At the same time, as a non-invasive index, MMP could be easier measured.

The current study has certain limitations. Firstly, due to the limitation of time and region, the sample size was relatively small. A strict inclusion and exclusion criteria was adopted to reduce the heterogeneity of patients and improve the universality of the study; however, due to the small number of patients included, heterogeneity was still present. As a result, certain results were not significant, the research data were skewed and the universality of the research requires improvement. Additionally, as long-term follow up was not performed, several P-values could be underestimated. Therefore, further studies with an increased follow-up duration should be performed. Secondly, only the effect of RT on mitochondrial function in peripheral blood immune cells from patients with locally advanced NPC was assessed. However, the underlying mechanism at the cellular level remains unclear. Thus, further *in vivo* and *in vitro* experiments are needed to clarify the underlying mechanism. Furthermore, due to the limitation of clinical practical application, only patients treated with RT concurrent with targeted therapy were included, whilst those treated with RT combined with chemotherapy were excluded. Therefore, the generalizability of the study is lacking and more studies, including more clinical treatment centers, are needed to enhance the clinical data and ensure more comprehensive and reliable experimental results.

In conclusion, the present study was the first to assess the changes in mitochondrial function in peripheral blood immune cells during RT, to the best of our knowledge. The results revealed that RT could increase the MMP of circulating $CD4^+$ Te cells in patients with locally advanced NPC and that MMP^{high} was associated with the efficacy of RT. Therefore, MMP^{low} in $CD4^+$ Te cells could be used as a marker for evaluating the immune status of patients with locally advanced NPC, thus providing a potential treatment approach for NPC.

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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

Conception and design of the research and drafting of the manuscript was conducted by QuW. Acquisition of data and revision of the manuscript for intellectual content was conducted by XY and LZ. Analysis and interpretation of data was conducted by HoL, YW, QiW and XY. Statistical analysis was conducted by HaL. QuW and XY confirm the authenticity of all the raw data. All authors contributed to the manuscript. All author read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (approval no. MR-37-23-012058). All patients provided written informed consent prior to enrollment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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