Heterogeneity of IFN-Mediated Responses and Tumor Immunogenicity in Patients with Cervical Cancer Receiving Concurrent Chemoradiotherapy

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ABSTRACT

Purpose: To ask whether the expression of immune markers and IFN signaling in tumor biopsies changes during concurrent chemoradiotherapy (CCRT).

Experimental Design: Tumor biopsies and peripheral mononuclear blood cells (PMBC) before and immediately after 20 Gy/10 fractions (F) of radiation treatment (RT) from 30 patients with cervical cancer receiving CCRT were evaluated by IHC and qRT-PCR for immune markers and correlated with the short-term response.

Results: Tumor immune response to radiation before and after 10F RT as reflected by CD8+ T-cell infiltration had substantial heterogeneity with increases, decreases, and no change all evident. Increases in CD8+ T cells during CCRT correlated with the presence of nuclear IRF1 in tumor cells (r = 0.68, P < 0.0001) and the patient short-term response (P < 0.01). Similarly, in a subset of patients (~40%) PD-L1 positivity in tumor cells increased, which also correlated with nuclear IRF1 staining (r = 0.48, P < 0.01). Patients with augmented PMBC IFN signature expression after 10F had a significantly higher probability of PD-L1 induction (83% vs. 7%, P < 0.0001). Most patients exhibited abundant expression of SERPINB9 and CD47 in tumor cells, and tumor infiltration by CD68+ cells. SERPINB9 expression correlated with STAT1 signaling in tumor cells.

Conclusions: CCRT leads to differential tumor immunogenicity and IFN signaling in patients with cervical cancer, suggesting radiation induction of immunity is limited to a subset of patients and may reflect the heterogeneity of intratumoral induction of IFNs.

Introduction

Immunotherapy using immune checkpoint blockade (ICB) has emerged as the fourth main therapeutic modality for cancer. ICB depends on preexisting antitumor immunity so that inhibition of immune checkpoints can release an effective immune response against the tumor. The immunogenicity of a tumor is thought to be determined by a number of factors, including the number of somatic mutations that might generate neoantigens, the presentation of these neoantigens, the expression of immune checkpoints in the tumor, and the infiltration and status of tumor-specific CD8+ T cells. The key etiologic factor for cervical cancer is persistent human papillomavirus (HPV) infection, present in 85% of invasive cervical cancer (1). Despite HPV vaccination, cervical cancer is still the fourth leading cause of cancer-related deaths in women. HPV-positive tumors actively transcribe HPV-related genes associated with their neoantigen landscape (2). Cervical cancer is one of the cancer types carrying a high genomic mutation burden (3). These data suggest that cervical cancer could often be immunogenic.

Radiotherapy (RT) is one of the main methods of cancer treatment. In addition to directly killing cancer cells, ionizing radiation has the potential to trigger an antitumor immune response. Many mechanisms including the generation of damage-associated molecular patterns and neoantigens, activation of dendritic cells, and production of IFN have been identified as potential contributors to a RT-stimulated immune response (4–7). Patients with locoregionally advanced cervical cancer are routinely treated with conventionally fractionated external beam RT (EBRT) in combination with concurrent chemotherapy followed by brachytherapy with the 5-year survival very dependent on stage; the survival of the local, regional, and distant stages is approximately 87%, 49%, and 11%, respectively (8). More recently, clinical evidence suggests that cervical cancer also responds to ICB further supporting the presence of tumor immunogenicity. Programmed cell death-1 (PD-1) blockade (pembrolizumab) has now been approved by the FDA for treatment of recurrent or metastasized cervical cancer with positive programmed death-ligand 1 (PD-L1) expression in tumors (9). Furthermore, CTL-associated protein 4 (CTLA-4) blockade after chemoradiotherapy in curative-intent treatment is effective in cervical cancer (10). Combining qPD-L1/PD-1 with chemoradiotherapy in treatment of cervical cancer is under evaluation in a number of clinical trials in various setting (11). One question that arises is whether treatment with RT and chemotherapy alters the immune response in cervical cancer.

If RT augments tumor immunogenicity and alters the microenvironment in favor of an inflammatory immune response, ICB would be more effective when used in conjunction with RT (12). In preclinical models, combining RT and ICB has a synergistic effect, which is
Translational Relevance

The combination of immune checkpoint blockade (ICB) with radiotherapy for cancer is of great promise, although the extent of antitumor immunity that develops in response to irradiation is uncertain. Here we show in patients with cervical cancer that increased CD8 T-cell infiltration during concurrent chemoradiotherapy (CCRT) is limited to a subset of patients. Similarly, only a subset of patients showed tumoral IFN induction during CCRT, which correlated with CD8 T-cell infiltration, and only 40% of patients displayed PD-L1 induction in tumor cells during chemoradiotherapy, also correlating with IFN signaling. The limitation of induction of antitumor immunity and interferon to a subset of patients suggests that the combination of CCRT and ICB will only be effective selectively dependent upon IFN signaling in response to CCRT. In addition, our results suggest the interferon signature in peripheral mononuclear blood cells could be an easily accessible biomarker to select patients for these combinational therapeutics.

dependent on the proinflammatory effect of RT (13–15). Promising results have been demonstrated in a few clinical trials examining ICB in combination with stereotactic body RT (SBRT) in metastatic tumors, or with conventional chemoradiotherapy in locally advanced diseases (16–18). Intrigued by these positive findings, clinical investigators have launched hundreds of trials to examine the efficacy of various combination between ICB and RT in a number of cancer diseases.

Despite this enthusiasm, only a small proportion of patients have been shown to benefit from adding ICB to RT. In the PACIFIC-1 trial, administration of antibodies against PD-L1 (aPD-L1, durvalumab) after definitive chemoradiotherapy resulted in significant improvement of both objective responses and 2-year overall survival (OS) rates in patients with stage III non–small cell lung cancer (NSCLC; ref. 18). However, the majority (70%) of patients did not display improved response from the PD-L1 blockade. The mechanisms underlying lack of response in these patients are still not fully understood.

The proinflammatory effect of RT and its synergistic effect in combination with ICB depends on generation of an IFN response in tumors (4, 17). Ionizing radiation and other means of generating DNA damage result in cellular synthesis of IFNs through the cGAS-STING-STAT1- or IRF1-mediated IFN responses and a variety of IFN-responsive molecules in tumor cells that are critical for the antitumor immune response. We found wide variation in the extent of infiltration by CD8+ T cells and in their response during therapy with increases, no change, and decreases all evident. However, increases of CD8+ T cells during therapy roughly correlated with IFN signaling and tumor response at the completion of therapy. We also examined an IFN gene signature, which has shown excellent predictive value for both local and systemic IFN response in autoimmune diseases (28), in PMBCs as a surrogate for evaluation of IFN response in local tumors. These data suggest that the immune and IFN response of cervical cancer to therapy is highly variable and that IFN signaling in PMBCs could be a surrogate biomarker.

Materials and Methods

Study design

A prospective observation study was launched in 2018 to explore potential biomarkers to predict cervical cancer response to CCRT. The study design is shown in Fig. 1. Briefly, patients diagnosed with primary squamous cell carcinoma (SCC) of the cervix were subjected to CCRT. Tumor biopsies at primary sites and PMBCs collection were performed at baseline before treatment and within 12 hours after 10F RT. The expression of biomarkers in these samples was analyzed as described below. This study is in compliance with the declaration of Helsinki. It was approved by the clinical study ethic committee in Cancer Hospital of Shantou University Medical College (Shantou, P.R. China) and registered on Clinicaltrial.gov (NCT03744819). Written informed consent was obtained from all enrolled patients.

Patient eligibility criteria

The inclusion criteria were as follows: (i) Pathologic proven diagnosis of primary cervical SCC; (ii) Patient will receive CCRT; (iii) The primary tumor was accessible for biopsy during the course of CCRT. The exclusion criteria included: (i) History of autoimmune diseases; (ii) History of antitumor immunotherapy; (iii) Prior RT that would result in overlapping of planned RT fields; (iv) Concomitant immunotherapy during the course of RT; (v) Contraindications for biopsy.

Treatment

Enrolled patients were subjected to CCRT. RT consisted of EBRT (median dose 46 Gy/23F) to the whole pelvis followed by EBRT boost (median dose 14 Gy/7F) to positive lymph nodes and brachytherapy boost (24–32 Gy/4–5 fraction) to the primary tumor and surrounding subclinical disease. During EBRT, patients also received platinum-based concurrent chemotherapy. The chemotherapy regimen consisted of either weekly cisplatin (30–40 mg/m² body surface area) or a combination of cisplatin (25 mg/m², day 1–3) and fluorouracil (0.5 g/m², day 1–4) on week 1 and week 4.

Clinical evaluation

Patients received the following examination prior to treatment: (i) tumor biopsy; (ii) PMBC collection; (iii) other examinations for tumor staging and pretreatment evaluation. Bone marrow, liver, and renal...
functions were monitored during treatment. Immediately after 10F of RT, another tumor biopsy and PMBC collection was obtained. The short-term tumor response to CCRT in patients was assessed after the whole course of pelvic EBRT according to the RECIST 1.0 version. Complete regression (CR) is defined as disappearance of cervical lesion as evaluated by the bimanual examination. Partial regression (PR) is defined as at least 30% decrease in the lesion maximum diameter compared with baseline. The evaluation of tumor response was blind to the biomarker assessment.

Sample collection
Each tumor biopsy was divided into two parts; one fixed in 10% formalin for at least 24 hours, paraffin embedded, and stored at room temperature. These blocks were later processed into 4-μm consecutive sections and stored at −80°C. The other part was submerged in RNAlater solution (Thermo Fisher Scientific) and stored at −80°C. Whole blood samples were collected in EDTA tubes. PMBCs were isolated using Ficoll Paque Plus media (GE Healthcare Life Sciences) according to the manufacturer’s protocol. Isolated PMBCs were then submerged in RNAlater solution and stored at −80°C.

IHC staining and evaluation
The antibodies used for IHC staining in this study are listed in Supplementary Table S1. Tumor sections were deparaffinized using a series of xylene and ethanol and rehydrated with dH2O. Microwave-stimulated antigen retrieval in buffers was performed as recommended by the manufacturers. Staining was as described previously (22). Slides were scanned (Magascanner, KFBIO) at 40× magnification. Images were processed using K-Viewer (KFBIO). These IHC images were evaluated by two independent researchers. Up to 10 representative areas of tumor islands were selected. PD-L1, SERPINB9, CD47, nuclear IRF1, nuclear STAT1, HLA-A, HLA-B/C, β2M, TAP1, LMP2, LMP7, and CD47 stainings were assessed. For each area, the percentage of cancer cells with positive staining (positivity) was measured. The expression intensity was scored as follows: negative, score = 0; weak, score = 1; moderate, score = 2; strong, score = 3 (representative images in Supplementary Fig. S1). The H score is the product of positivity multiplied by the score. For infiltrating CD8⁺ T cells or CD68⁺ cells, the number of immune cells per mm² was quantified. The mean expression (positivity, H score, or density) over multiple areas was used in subsequent analyses. Representative images were selected from various patients or the same patient for illustration in the figures as indicated in the legends. The image of IRF1 staining is the same in Figs. 2G and 5C and the images of PD-L1, STAT1, and HLA-A staining are the same in Supplementary Figs. S1 and S8 allowing direct comparison.

qRT-PCR
After removal of RNAlater, tumor samples and PMBCs were homogenized in TRIzol (Thermo Fisher Scientific) at 4°C using a magnetic bead homogenizer. The procedures for RNA isolation and qRT-PCR have been described previously (22). The IFN gene signature used was as described in Rice and colleagues and consists of IFI27, IFI44L, IFIT1, ISG15, RSAD2, SIGLEC1 except that the expression
Figure 2.
Changes in tumor-infiltrating CD8^+ T-cell number correlate with nuclear IRF1 staining and tumor response to CCRT in patients with cervical cancer. Tumor-infiltrating CD8^+ T-cell number before and after 10F RT in aggregate or in subgroups with different dynamic change patterns (decrease, no change, and increase) are shown in A and B, respectively. The percentage of various change patterns is summarized in C. Definition of change patterns for CD8^+ T cells: Increase (in red), cell number increased by > 30 per mm^2; Decrease (in blue), cell number decreased by > 30 per mm^2; No change (in gray), the difference of cell number ≤ 30 per mm^2. The number of tumor-infiltrating CD8^+ T cells and the expression positivity of nuclear IRF1 in tumor cells before and after 10F RT, and their dynamic changes are plotted in D–F. (Continued on the following page.)
of SIGLEC1 was not robust in these samples and was excluded from analysis (28). Genes of the DNA damage response (DDR) signature include BBC3, DDB2, E2F4, FBXO22, FDXR, GRAD45A, PCNA, SESH1, and TRIAP1. These genes were selected from a previous report excluding genes that could be upregulated by IFNs (http://www.interferome.org/interferome/home.jsp; ref. 29). Of a macrophage signature consisting of ARGI1, TNF, CXCL9, IFNG, IL1B, IL10, IL4, IL12B, NOS2, and TGFβ1, only IL1B, TNF, CXCL9, and TGFβ showed sufficient robustness in all samples (30). Housekeeping genes were 18sRNA, HPRT1, and ACTB. The primer pairs used are in Supplementary Table S2. All genes displayed single peak on melting curve analysis after the amplification of qPCR (Supplementary Fig. S2).

Knockout of IRF1 in tumor cell line by using CRISPR/Cas9

IRF1 knockout in HT29 cells (a human colorectal cancer cell line, RRID: CVCL_0320) using CRISPR/Cas9 was as described previously (19).

The Cancer Genome Atlas data analysis

The Cancer Genome Atlas (TCGA) gene expression data (RNA-sequencing) of CD8A, IRF1, HPRT1, and ACTB and clinical information in cervical SCC (n = 294) were downloaded via cBioPortal (http://www.cbioportal.org/; RRID: SCR_014555). The expression of CD8A and IRF1 was normalized to HPRT1 and ACTB. Pearson correlation coefficients (r) are indicated. The predictive capacity of tumor expression of CD8A or IRF1 in patient OS was evaluated by using ROC analysis. Their optimal cutoff was determined using the Youden index. Patients who received RT were divided into subgroups (high vs. low) according to the expression level of CD8A or IRF1. The survival curves for subgroups of patients were generated using the Kaplan–Meier method. The log-rank test was used to compare the median survival time.

Statistical methods

This study aimed to enroll at least 30 patients with evaluable paired samples. All values represent means (M) and SD. Mean comparisons were performed using the paired Student’s t test in paired groups with normal distribution (evaluated using D’Agostino and Pearson omnibus normality test), the Wilcoxon matched-pairs signed rank test in paired groups with uncertain distribution normality, the Mann–Whitney test in two independent groups with uncertain distribution normality and the one-way ANOVA with Tukey multiple comparisons test in three groups. Ratio comparison in two groups was performed by using the χ² test. The correlation between the expression of markers was evaluated by Pearson correlation. P value: NS > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. All graphs were plotted using Graphpad Prism 8 (RRID: SCR_02798). ROC analysis was performed using IBM SPSS Statistics 23 (RRID: SCR_002865).

(Continued.) G, Representative IHC staining of CD8 (top row) and IRF1 (bottom row) before and after 10F RT showing both decreased and increased nuclear IRF1 positivity in tumor cells. H, Changes of infiltrating CD8+ T-cell number after 10F RT in tumors with various dynamic change patterns of nuclear IRF1 positivity in tumor cells. I, The change of tumor-infiltrating CD8+ T-cell number after 10F RT is displayed on the bottom panel as a waterfall plot, ranking from the greatest decrease on the left to the greatest increase on the right. The short-term (immediately after EBRT) response to CCRT for each individual patient is shown on the top panel. CR in gray; PR in black. J, The percentage of patients with a tumor CR or PR in patients with or without decreased CD8+ T-cell number after 10F RT. The cutoff for CD8+ T-cell density was set at ~9.6 cells/mm², which was determined by using the ROC curve method. K, mRNA expression correlation between IRF1 and CD8A in TCGA dataset of patients with cervical cancer. N = 294. Patients from TCGA dataset who had received RT (N = 175) were stratified into subgroups according to their tumor mRNA expression level (high in red, low in blue) of CD8A or IRF1. OS curves of these subgroups of patients and comparison of median OS (in months [m]) are plotted in L and M. Data in bar charts show the mean ± SD. Mean comparisons were performed using the paired Student’s t test in A, the Wilcoxon matched-pairs signed rank test in B, and one-way ANOVA with Tukey multiple comparisons test in H. Ratio comparison in two groups (J) was performed by using the χ² test. The correlation between positivity of various markers was evaluated by Pearson correlation (K). Survival curves for different groups of patients were generated using the Kaplan–Meier method (L and M). The log-rank test was used to compare the median OS time (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results

Patient clinical characteristics

Between September 2018 and May 2020, 58 consecutive patients with primary cervical SCC were evaluated. Of those, 48 were enrolled in this prospective trial (flow diagram in Supplementary Fig. S3). All 48 patients completed the full course of CCRT. A total of 41 had tumor biopsies and PBMC collection before and after 10F RT. Tumor biopsies from 11 patients contained only necrotic or inflammatory tissues or were of poor quality, preventing further assessment. The remaining 30 patients were included as are summarized in Supplementary Table S3. The majority (90%) had locally advanced disease. Following EBRT, 57% (17/30) of patients had a CR, while the remaining patients had a PR. The number of leukocytes (neutrophils, lymphocytes, and monocytes) in the peripheral blood was reduced after EBRT (Supplementary Fig. S4).

Changes in tumor-infiltrating CD8+ T cells: correlation with nuclear IRF1 staining and tumor response

There was a wide range of CD8+ T-cell densities in the tumors as well as divergent responses after radiation (Fig. 2A). The patients could be divided into subgroups (decrease, no change, and increase) based upon the change in CD8+ T-cell density within the tumor after RT (Fig. 2B and C). We evaluated the relationship between CD8+ T-cell density and a series of immunologically relevant markers (Supplementary Figs. S5 and S6). There was a strong correlation between the number of tumor-infiltrating CD8+ T cells and nuclear IRF1 staining in tumor cells (Fig. 2D and E). The change of CD8+ T cells during CCRT also correlated with nuclear IRF1 in tumor cells (Fig. 2F and G). The number of tumor-infiltrating CD8+ T cells increased by approximately 28 cells/mm² (median) after RT in patients with tumor cells in which nuclear IRF1 positivity was also upregulated in comparison with tumors with no significant change in CD8+ T-cell density after RT (0 cells/mm²) in which there was also no significant change of IRF1 positivity. Those tumors with a decrease (on average of 135 cells/mm²) also had reduced nuclear IRF1 in tumor cells (Fig. 2H). These data suggest that IRF1-mediated signaling in tumor cells influences the infiltration and/or survival of CD8+ T cells into tumors during CCRT.

The change in tumor-infiltrating CD8+ T cells after 10F RT correlated with patient short-term response to CCRT. PR was associated with change of CD8+ T-cell density in tumors, change of LMP7, tumor size, and platelet-neutrophil ratio at baseline (Supplementary Table S4). However, only change of CD8+ T cells stood out as an independent factor associated with the risk of PR in multivariable analysis (Supplementary Tables S5–S7). We ranked patients according to the change of T-cell number in their tumors in parallel with their short-term response (Fig. 2I). The majority of patients with PR clustered with decreased CD8+ T-cell number after CCRT. Patients...
with decreased numbers of tumor-infiltrating CD8+ T cells after CCRT had significantly higher risk (75% vs. 22%, P = 0.0080) of PR compared with patients without (Fig. 2J). Consistent, significant correlation was found between the expression of CD8A and IRF1 in cervical cancer in TCGA database (Fig. 2K). More importantly, in patients treated with RT, high expression of CD8A was associated with better OS (Fig. 2L). A similar result was found in comparing patients with high versus low expression of IRF1 in their tumors (Fig. 2M). Macrophages were assessed by CD68 and showed an increase with CCRT in 52% of patients (Supplementary Fig. S7A–S7C). The macrophages in irradiated tumors were polarized toward the M1 phenotype in 53% of patients and M2 in 26% of patients after RT (Supplementary Fig. S7D–S7H). Thus, macrophages also had a heterogeneous response. Taken together, these data suggest that CD8A+ T cell-mediated immunity has a role in tumor response to CCRT in cervical cancer.

### IFN gene expression signature

There was similar heterogeneity in the IFN gene expression signature. Overall, there was no average difference in the IFN gene expression signature before and after 10F RT in both the tumors and the PMBCs (Fig. 3A and D). However, substantial changes were found in subgroups of patients (Fig. 3B and E). The IFN gene signature mainly either increased or decreased after 10F RT in both tumors and PMBCs and these responses were correlated (Fig. 3C, F and H). The baseline IFN gene signature showed a trend of correlation (Fig. 3G).

The IFN response in PMBCs could be triggered by direct exposure of PMBCs as they circulate in the radiation field. Alternatively, the PMBCs might be exposed to IFN generated by the tumor in response to radiation. Direct exposure would be predicted to lead to DDR expression. To distinguish these possibilities, we analyzed the correlation between the IFN gene signature and a DDR signature in PMBC and several other markers in tumors (Fig. 3M). No correlation was found between the PMBC IFN and DDR signatures suggesting that IFN signaling in the PMBCs was not due to direct exposure to radiation.

We then looked for a correlation between tumor cell response to radiation and the PMBC IFN gene signature. We used nuclear IRF1 as a marker for tumor cell response to radiation perhaps by IFN generation. Nuclear IRF1 in tumor cells correlates with the PMBC IFN signature during CCRT (Fig. 3K). The dynamic changes of these two markers also displayed significant correlation (Fig. 3L). Upregulation of nuclear IRF1 staining in tumor cells was associated with induction of the IFN gene signature in PMBCs after 10F RT (Fig. 3N). These data are consistent with the notion that DNA damage inducing treatments, RT and chemotherapy induce IFNs in cancer cells in an IRF-dependent manner (19, 31). It is striking, however that this occurred in only a subset of the patients. IRF1 mediates type III IFN induction in HT29, a human colorectal cancer cell line, after ionizing radiation (19). Similarly, induction of IFNB1 by ionizing radiation requires intact IRF1 signaling in cancer cells (Fig. 3O). Collectively, these results suggest that IFN induction mediated by IRF1 in tumor cells make a substantial contribution to the IFN response in circulating PMBCs during CCRT.

### Immune marker expression in tumor cells

We assessed the expression of some IFN-responsive markers, which could affect tumor immune response, including PD-L1, SERPINB9, IRF1, STAT1, HLA-A, HLA-B/C, β2M, LMP2, LMP7, and TAP1 (Fig. 4A, B, and D). PD-L1 expression in tumor cells (>1%) was found in 83.3% of patients both before after 10F RT. While there was a trend of an increase in the average positivity of PD-L1 across the whole cohort immediately after 10F RT, the difference was not statistically significant (30% vs. 24%, P > 0.05). The majority of tumor samples had tumor cell expression of SERPINB9, with an average positivity of 56% before treatment and 47% during CCRT.

Both STAT1 and IRF1 are important transcriptional factors for mediating the IFN response. Their presence in the nucleus could reflect activation of signaling pathways leading to IFN transcription. Nuclear STAT1 was found in 57% of tumor cells before treatment, while only 16% of displayed nuclear IRF1 staining. Both markers showed a trend of increasing during CCRT.

The antigen presentation machinery (APM) consists of a variety of components, including molecules involved in protein degradation (e.g., LMP2 and LMP7), peptide transportation (e.g., TAP1) and presentation on the cellular surface (e.g., HLA-A, HLA-B, HLA-C, and β2M). The majority of tumor cells, both before and after 10F RT, expressed these components with the exception of TAP1, which was found in only 5% of tumor cells at baseline. Upon RT, the average positivity of TAP1 in tumor cells increased to 17%. The dynamic change of TAP1 expression correlated with that of nuclear IRF1 (r = 0.41, P < 0.05). No significant change was noted in the other APM components examined.

Many of the markers in individual patients exhibited patterns of change similar to the infiltration of CD8 T cells (increase, no change, and decrease) after 10F RT (representative images in Supplementary Fig. S8, heatmap graph in Fig. 4C, summary of change pattern proportion in Fig. 4E). The comparisons of marker expression in the different subgroups of patients are shown in Fig. 4F–O with similar results with the H score (Supplementary Fig. S9). Markers with both significant increases or decreases in subgroups of patients included nuclear IRF1, PD-L1, SERPINB9, nuclear STAT1, and HLA-B/C. Around 40% of patients exhibited upregulation of PD-L1 expression (17% vs. 50%, P < 0.001) in tumor cells, while 20% of patients showed decreases (41% vs. 6%, P < 0.05; Fig. 4H). CD74 (a protein often expressed by tumor cells that inhibits phagocytosis by macrophages) was found in the majority of tumor cells with variable staining intensity at baseline and substantial changes after irradiation in a subgroup of patients (Supplementary Fig. S10; ref. 32). Significant induction of TAP1 (3% vs. 38%, P < 0.001) was found in 37% of patients, while the tumors the other patients did not show substantial changes (Fig. 4F). Significant downregulation of HLA-A and β2M was observed in 37% and 20% of patients, respectively (Fig. 4K and M). The expression of LMP2 and LMP7 was unresponsive to RT and was found in approximately 95% of cancer cells before and after RT (Fig. 4N and O). Overall, CCRT led to expression changes of key immune markers involved in immune surveillance that might affect a therapy-induced antitumor immune response.

### PD-L1 expression correlates with tumor cell nuclear IRF1 staining and the IFN gene signature of PMBCs during CCRT

PD-L1 expression on cancer cells is an indicator of tumor response to αPD-L1/PD-1 antibodies in some situations. The expression positivity of PD-L1 in tumor cells correlated with the IFN gene signature in tumors (r = 0.39, P < 0.01) and PMBCs (r = 0.45, P < 0.01) before CCRT (Fig. 5A; Supplementary Fig. S11). Following 10F RT, PD-L1 positivity showed a significant correlation (r = 0.48, P < 0.01) with nuclear IRF1 positivity (Supplementary Fig. S11) and a trend of association with others, including the nuclear STAT1 and IFN gene signature in tumors or in PMBCs. Patients with high (>10%) nuclear IRF1 positivity in tumor cells had more than two times higher expression positivity of PD-L1 (44% vs. 20%, P < 0.01) in tumor cells than those with low nuclear IRF1 positivity (representative images in Fig. 5C and quantification in Fig. 5D). The dynamic change of PD-
Figure 3.
IFN gene signature expression during CCRT in tumors and PMBCs. IFN gene signature expression in both tumors and PMBCs before and after 10F RT is summarized in A and D. The comparisons of IFN signature (both in tumors and PMBCs) before and after 10F RT with different dynamic change patterns (decrease, no change, and increase) are summarized in B and E. The case numbers (N) for each change pattern are as indicated. Definition of change patterns for IFN gene signature: Increase (in red), gene expression after 10F RT increased by >0.5-fold compared with that before; decrease (in blue), gene expression after 10F RT decreased by >0.5-fold compared with that before; no change (in gray): the difference of gene expression after RT ≤0.5-fold. The percentages of various dynamic change patterns of CD8\(^+\) T cells and IFN gene signature are summarized in C and F. PMBC and tumor IFN gene signature expression before and after 10F RT, and their dynamic changes are plotted in G–I. (Continued on the following page.)
L1 positivity was associated with nuclear IRF1 positivity in tumor cells, tumor-infiltrating CD8 T-cell number and the PMBC IFN gene signature (Fig. 5A). Consistently, augmented PD-L1 expression was associated with upregulation of nuclear IRF1, CD8 T-cell number, the PMBC IFN signature and to lesser extent, nuclear STAT1 positivity (heatmap graph in Fig. 5B). Patients with increased IRF1 positivity or the PMBC IFN signature had greater upregulation of PD-L1 than patients without (Fig. 5E and F). The probability of PD-L1 induction is significantly higher in patients with augmentation of their PMBC IFN signature than patients without (83% vs. 7%, P < 0.0001; Fig. 5G).

Indeed, following 10F RT, the average tumor cell PD-L1 positivity was 50% in patients with an increased PMBC IFN gene signature compared with 19% in patients with decreases (P < 0.05; Fig. 5H). The H score also correlated with nuclear IRF1 H score, CD8 T-cell number and the PMBC IFN signature (Supplementary Fig. S12). Patients with an increased PMBC IFN gene signature had significantly higher risk of PD-L1 H score upregulation in tumor cells than patients without (50% vs. 7%, P < 0.05). In summary, the expression of PD-L1 in tumor cells is correlated with IRF1-mediated signaling, numbers of tumor infiltrating CD8 T-cells and the PMBC IFN gene signature.

**SERPINB9 expression correlates with STAT1-mediated signaling in tumor cells**

SERPINB9 is an emerging target for cancer immunotherapy. It mediates resistance to radiation-induced immunity and to ICB in both experimental settings and retrospective clinical data (22, 33, 34). SERPINB9 expression correlates with STAT1-mediated signaling in tumor cells in biopsies from patients with cervical cancer (Fig. 6A–C). High nuclear STAT1 positivity is associated with greater expression of SERPINB9 in tumor cells after 10F RT (representative images in Fig. 6D; quantification in Fig. 6E and F). SERPINB9 expression was also found in immune cells, including T cells and dendritic cells. We compared the expression intensity of SERPINB9 between these two cellular components in patients and categorized them into three subgroups (representative images in Fig. 6G; quantification in Fig. 6H). Greater expression of SERPINB9 in cancer cells than inflammatory cells was found in 24% of patients, while 30% of patients displayed higher staining intensity in inflammatory cells than in cancer cells. The majority (46%) of patients had comparable expression levels of SERPINB9 in both cellular components.

In addition, STAT1-mediated signaling was associated with expression of APM components in tumor cells, in particular HLA-A, HLA-B/C, β2M, and LMP7 (Supplementary Fig. S13).

**Discussion**

Here we assessed evidence for an immune response and IFN signaling in biopsies before and during therapy of cervical cancer, a cancer often immunogenic due to HPV. Most notably, we found substantial heterogeneity in CD8 T-cell infiltration and in IFN signaling after 10F of CCRT with increases, no change, and decreases all represented. OS of patients with cervical cancer, who received RT, correlates strongly with tumor CD8A expression in TCGA database and in this study patients with decreased tumor-infiltrating CD8 T-cells during CCRT had a significantly higher risk of PR than of CR. Low CD8 T-cell infiltration also correlates with increased risk of relapse (35, 36). Therefore, our results suggest that the response of tumor-infiltrating CD8 T-cells in cervical cancer treated with CCRT contributes to therapeutic efficacy. Whether this early CD8 T-cell response reflects long-term local tumor control remains to be determined. The heterogenous T-cell response we observed might be related to timing as the infiltration of CD8 T-cells probably varies during treatment with diminished levels at least in mice, early after radiation followed by expansion (22, 37). Dorda-Estremera and colleagues found an initial decline of CD8 T-cell number in the first week, which was followed by variable expansion later in irradiated cervical cancers consistent with the results here (38).

Activation of IFN signaling after RT has been observed in many murine tumor models (4, 20, 39). However, this activation did not occur in many of our patients with cervical cancer. Only one-third of patients showed augmented IFN gene expression or nuclear STAT1 and IRF1 staining after 10F RT. Lack of response might be due to deficiencies of key mediators in IFN signaling. cGAS-STING or IFNAR1 signaling is frequently suppressed in tumors (40–42). In addition, mutation of these mediators is possible and has been shown for STING (43). In addition, HPV-derived E7 inhibits the cGAS-STING pathway in human cervical cancer cells (44). Here only 16% of cervical cancer cells expressed IRF1, a key transcription factor in IFN induction, a limitation that might contribute to the impaired IFN response. Activation of negative feedback loops in response to IFNs can inhibit IFN-mediated signaling (39, 45). Further necrosis in tumors following CCRT could affect the overall IFN response. Still it is striking that a positive IFN response to CCRT was found in less than 40% of these patients as compared with results using animal models. These data highlight the danger of extrapolation from animal model-derived results.

The benefit from adding ICB to RT may depend on the expression of immune checkpoints. A total of 40% of patients in this study displayed PD-L1 induction in tumor cells during CCRT. The combination of CD47-SIRPα blockade and RT could be synergistic resulting in enhanced antitumor T-cell immunity (32, 46). We separated patients into subgroups based on changes of PD-L1 positivity, CD8 T-cell number, CD47 H score, and CD68+ cell number (Supplementary Fig. S14). If cancers with increases of either the immune checkpoint (PD-L1 or CD47) or the effector cells (CD8+ or CD68+ cells) would benefit from blockade of CD47 or PD-L1 then CCRT might benefit from additional ICB in 59% of patients: 11% from αPD-L1/PD-1, 26% from αCD47/SIRPα, and 22% from both (Supplementary Fig. S14). SERPINB9 was frequently expressed in the samples we examined, validating it as a potential target for cancer immunotherapy (22). Consistently, emerging data suggest that SERPINB9 can protect cancer cells and immunosuppressive cells from granzyme B–mediated killing, which can be targeted simultaneously in vivo via a small-molecule...
Figure 4.

Immune marker expression positivity in tumor cells before and during CCRT. Heatmaps of expression positivity of indicated immune markers in tumor cells before (A) and after 10F RT (B), and their dynamic changes (C). Patients were ranked according to their mean changes of positivity from the highest on the left to the lowest on the right. The vertical color bars display the range of values. N = 30. D, Summary of the positivity of these markers in tumor cells before and after 10F RT. E, Summary of the dynamic change (10F vs. 0F) patterns (increase, no change, and decrease) of these markers. Increase (in red): positivity increased by >10%; decrease (in blue): positivity decreased by >10%; no change (in gray): changes of positivity ≤10%. Comparisons of marker expression positivity before and after 10F RT in subgroups with different dynamic change patterns (decrease, no change, and increase) are summarized in F-O. The markers and case numbers (N) for each change pattern are indicated. Mean comparisons in D (paired groups with normal distribution) were performed by using the paired Student t test. Mean comparison in F-O (paired groups with uncertain distribution normality) was performed by using the Wilcoxon matched-pairs signed rank test. ns: P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; na: the comparison is not applicable. P values are not shown if they > 0.05 in D.
Figure 5.
PD-L1 expression positivity correlates with tumor cell nuclear IRF1 staining and the IFN gene signature expression of PMBCs during CCRT. A, Heatmap of Pearson correlation coefficients between the expression of PD-L1 (positivity in tumor cells before and after 10F RT and their dynamic change) and various markers. The change of PD-L1 positivity in tumor cells after RT is displayed on the bottom panel as a waterfall plot, ranking from the greatest decrease on the left to the greatest increase on the right. The dynamic change pattern of expression positivity or level of indicated markers in each patient is shown as a heatmap on the top panel (decrease in blue, no change in gray, and increase in red). C, Representative IHC section staining (low vs. high) of PD-L1 (top) and IRF1 (bottom) in tumor biopsies from patients after 10F RT. The image of IRF1 staining on the right is the same as that shown in Fig. 2G. D, Quantification of PD-L1 positivity in tumor cells with low (≤10%) or high (>10%) nuclear IRF1 positivity in tumor biopsies from patients after 10F RT. E, Changes of PD-L1 positivity in tumor cells with or without increased nuclear IRF1 activity in tumor biopsies. The change of tumor cell PD-L1 positivity, the percentage of patients with PD-L1 induction (increased positivity in tumor cells), and the tumor cell PD-L1 positivity after 10F RT in patients with different change patterns of PMBC IFN signature are summarized in F–H, respectively. Data in bar charts represent mean ± SD. Mean comparison was performed by using the Mann-Whitney test (two groups) or one-way ANOVA with Tukey multiple-comparisons test (more than two groups). Ratio comparison was performed by using the χ² test. The correlation between positivity of various markers was evaluated by Pearson correlation (*, P < 0.05; **, P < 0.01; *** P < 0.001. P > 0.05 was not shown in heatmaps).
SERPINB9 inhibitor (47). TAP1 is one component of APM frequently deficient in cervical cancer cells and its deficiency can mediate tumor evasion (48, 49). Very few patients enrolled in this study exhibited TAP1 expression in tumor cells at baseline. However after 10F, 37% of tumors displayed significant induction of TAP1 in an IRF1-dependent manner suggesting its induction might contribute to antitumor immunity.

In addition, our work suggests that the IFN signature in PMBCs may reflect that of the tumor and then reflect tumor expression changes in PD-L1. Induction of serum IFNβ correlated with an abscopal response in patients with late-stage NSCLC who underwent SBRT in combination with CTLA-4 blockade (17). If the PMBC IFN gene signature mirrors that in the tumor, it could be used to monitor therapy and select patients for combinational therapeutics.
In summary, we demonstrate that CCRT leads to differential tumor immunogenicity in patients with cervical cancer. Increases in tumor infiltrating CD8+ T cells correlate with IFN-1-mediated signaling in tumor cells and short-term response to CCRT. The dynamic and differential tumor immunogenicity in patients with cervical cancer in response to CCRT suggest that RT induction of immunity is limited to a subset of patients and may reflect the heterogeneity of intratumoral induction of IFN.

**Authors’ Disclosures**

No disclosures were reported.

**Authors’ Contributions**

Jianzhou Chen: Conceptualization, data curation, software, formal analysis, supervision, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing. C. Chen: Resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. Z. Zhan: Resources, investigation. L. Zhou: Resources, investigation. Jie Chen: Resources, investigation. Q. Cai: Resources, investigation. Y. Wu: Resources, validation, investigation. Z. Su: Investigation, project administration. C. Zeng: Resources, investigation.

**References**


**Acknowledgments**

This study was funded by Cancer Research UK grant C5255/A15935 (to R. Muschel); Shantou University Medical College Clinical Research Enhancement Initiative, N0201424 (to C. Chen and J. Chen); Collaborative and Creative Center, Molecular Diagnosis and Personalized Medicine, Shantou University, Guangdong, China (to C. Chen); Science and Technology Special Fund of Guangdong Province of China, 2019-132 (to C. Chen); and Strategic and Special Fund for Science and Technology Innovation of Guangdong Province of China, 180918114960704 (to C. Chen). We thank Geng Wang from Department of Pathology, Jiuycning Chen, Chaosheng Hong, Jiediao Lin from Oncological Research Lab, Cancer Hospital of Shantou University Medical College, and the core facility team in the Central Laboratory of Shantou University Medical College for technical support.

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Received December 18, 2020; revised February 1, 2021; accepted March 22, 2021; published first March 25, 2021.


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Clin Cancer Res  Published OnlineFirst March 25, 2021.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-20-4521

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