Immune infiltration in p53abn endometrial carcinomas

Authors

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Abstract

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## 1 Introduction

Endometrial cancer is the second most common type of gynecologic malignancy worldwide and the most common gynecologic malignancy in North America (Siegel et al. 2024). Molecular classification has separated endometrial cancers into 4 prognostically distinct subtypes: POLE (polymerase epsilon-mutated), MMRd (mismatch repair-deficient), p53abn (p53 abnormal) and NSMP (no specific molecular profile, (Getz et al. 2013; A. Talhouk et al. 2015; Aline Talhouk et al. 2017; Kommoss et al. 2018). p53abn cancers, the worst prognostic subtype, comprise only 15% of all endometrial cancers but account for 50-70% of mortalities (A. Talhouk et al. 2015; On-Castillo et al. 2020; Jamieson et al. 2021; Siegenthaler et al. 2022), with extrauterine involvement in over 50% of cases (Kommoss et al. 2018; Momeni-Boroujeni et al. 2021). Most patients recur within five years on standard-of-care carboplatin-paclitaxel chemotherapy with or without radiotherapy, highlighting the need for alternative therapeutic options (On-Castillo et al. 2020; Ott et al. 2017; Jamieson et al. 2021).

Increased CD8+ cytotoxic T and CD4+ helper T tumor-infiltrating lymphocytes (TILs) are associated with longer overall and recurrence-free survival in endometrial cancer (Jong et al. 2009), while immunosuppressive regulatory T cells are associated with shorter survival (Willvonseder et al. 2021). TILs are known to respond to and track tumor-associated antigens (Wick et al. 2014) to eradicate cancer cells. In response, cancer cells activate immunosupressive pathways, including the immune checkpoint molecule programmed death-ligand 1 (PD-L1), to evade immune surveillance (Chan et al. 2019). Treatment with immune checkpoint inhibitors reactivates exhausted T cells and is particularly effective in tumors with many neoantigens due to high mutational burden (Chan et al. 2019). In endometrial cancer, POLE and MMRd tumors have over 10 times as many mutations as p53abn and NSMP tumors (Getz et al. 2013) and correspondingly higher TIL densities (Aline Talhouk et al. 2019). While systemic therapy is typically unnecessary in POLE cancers due to exceptionally favorable outcomes with hysterectomy alone, anti-PD1 immune-checkpoint inhibitors have demonstrated promising efficacy in advanced, recurrent, or persistent MMRd endometrial cancers after multiple lines of therapy (Konstantinopoulos et al. 2019; Antill et al. 2021).

More recently, Mirza et al. (2023) showed that the anti-PD1 antibody dostarlimab was associated with significantly improved outcomes not only in MMRd but also MMRp (MMR proficient, non-POLE) endometrial cancer when added to standard-of-care chemotherapy. While the benefit of dostarlimab in MMRp cancers was less than in MMRd cancers, subgroup analysis showed that the benefit in MMRp was driven by p53abn cases (ESMOMirza2023). These findings contrast to those observed in high-grade serous ovarian cancer (HGSC), a p53-mutated gynecologic cancer with similar histologic and genomic characteristics to p53abn endometrial cancer (Getz et al. 2013), where despite well-documented correlations between TIL and prolonged survival (L. Zhang et al. 2003; Nielsen et al. 2012; Kroeger, Milne, and Nelson 2016) immune checkpoint inhibition has shown limited efficacy (Homicsko and Coukos 2015; Gaillard, Secord, and Monk 2016; Matulonis et al. 2019). The factors underlying this promising response to immune checkpoint inhibitors in p53abn endometrial cancer remain poorly understood.

PARP inhibitors are another class of targeted therapies under investigation in p53abn endometrial cancer (Bosse et al. 2022). Spurred by remarkable clinical responses in HGSC (Moore et al. 2018), PARP inhibitors have become standard-of-care in *BRCA1*/*BRCA2*-mutated or homologous recombination deficient cancers across multiple cancer types (Friedlander, Lee, and Tew 2023). Compared to HGSC, HRD is less common in p53abn endometrial cancer, with approximately 25% of cases showing evidence of HRD and fewer than 5% with *BRCA* mutations (Wallbillich, Morris, and Ali-Fehmi 2020). In HGSC, HRD tumors have higher immunogenicity than non-HRD tumors, and markers of adaptive immunity are associated with longer overall survival in HRD but not non-HRD tumors (Allen W. Zhang et al. 2018). Understanding the relationship between HRD and the immune microenvironment in p53abn endometrial cancers may help inform combination PARP inhibitor and immunotherapy clinical trials (Konstantinopoulos et al. 2022).

To understand the clinical relevance of TILs in p53abn endometrial cancer, we systematically profiled the immune microenvironment of 256 clinically annotated p53abn endometrial cancers with multiplex immunofluorescence. We evaluated the expression patterns of PD1, PDL1 and IDO1, three pharmacologically actionable immunosuppressive molecules with translational relevance to current clinical trials in endometrial cancer. Finally, we investigated the relationship between TILs, homologous recombination deficiency, and HER2 in p53abn endometrial cancer.

## 2 Methods

### 2.1 Data acquisition

#### 2.1.1 Sample acquisition

Ethics approval for this study was obtained from the University of British Columbia (UBC) Research Ethics Board (REB NUMBERS). The cohort consisted of 256 treatment-naive p53-abnormal endometrial carcinomas collected between XXXX and XXXX in Vancouver and in the Cross Canada endometrial cancer cohort. CONSENT? Clinicopathologic and outcome data were collected by chart review.

### 2.2 Experimental methods

#### 2.2.1 Tissue microarray construction

Representative samples of endometrial carcinomas from our Vancouver cohort were cored (0.6 mm) in duplicate and arrayed as described previously (A. Talhouk et al. (2015)). CROSS CANADA COHORT? TMAs were cut at WHAT thickness for immunofluorescence.

#### 2.2.2 Multiplex immunofluorescence

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### 2.3 Computational analysis

#### 2.3.1 Cell and region counting

Two multiplex immunofluorescence panels to classify TIL types (CD3, CD8, FOXP3, CD20, CD79a) and quantify immunosuppressive markers (PD1, PDL1, IDO1, CD8, CD68) were performed on each tissue microarray. For each immunofluorescence panel, 3 tissue segmentation and cell phenotyping algorithms were trained in HALO using 10 representative images for each algorithm. Regions were classified as epithelial, stromal, glass, or other (including necrosis) for tissue segmentation. The mean and standard deviation across all algorithms was calculated for each core, and any cores with a standard deviation of >5 were flagged for manual review by a pathology resident (SM). Final epithelial and stromal areas were calculated by subtracting glass and other areas from tissue area.

Phenotypes were defined according to combinations of positive and negative markers in each cell. Cytotoxic T cells were defined by positivity for both CD3 and CD8, helper T cells by positivity for CD3 and negativity for CD8 and FOXP3, regulatory T cells by CD3 and FOXP3 positivity with CD8 negativity, B cells by positivity for both CD20 and CD79a, and plasma cells by CD79a positivity in the absence of CD20. Cells were classified according to cytoplasmic and/or nuclear positivity for each marker. Each phenotype was scored independently by 3 algorithms, and averages across all algorithms were calcualted separately for epithelial and stromal regions. Standard deviations across algorithms of >5 were flagged for manual review by a technologist and pathology resident (SM). All immune cells touching tumor cells were considered epithelial.

#### 2.3.2 Mixture modeling of cell counts

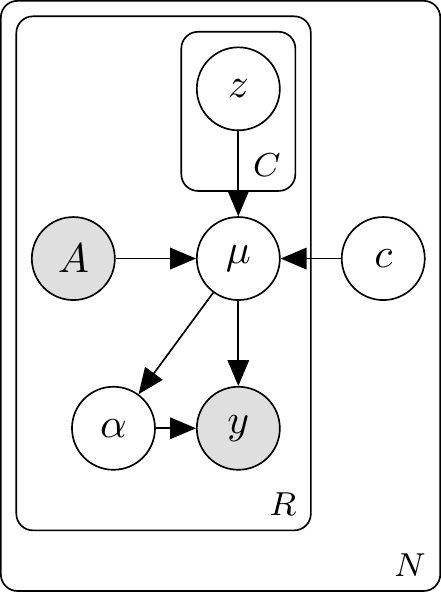
TIL count for a given core , cell type , and region (tumour/stroma) were described as follows:

where the mean follows:

where is the inferred cluster of core , is the area of region in core , and is the mean density (count divided by area) value across all cores . The Gamma distribution was parametrized in terms of a mean and scale parameter, with the scale parameter set to 100 to allow for a fairly uninformed prior.

To reduce the dimensionality of the parameter space, the dispersion parameter was formulated semi-parametrically in terms of a set of Gaussian radial-basis kernels with parameters and , for a specified number of centers uniformly distributed between 0 and the maximum number of counts (Kapourani and Sanguinetti 2016; A. W. Zhang et al. 2019), with the default number of centers set equal to 20:

where and follow relatively agnostic lognormal priors as above.



Source: [Article Notebook](https://Irrationone.github.io/tfri_halo/index.qmd.html)

Inference was performed in pymc v5.9.1 (Abril-Pla et al. 2023). To determine the optimal number of clusters , a Dirichlet process prior was first fitted to a version of the model marginalized over cluster membership . At a minimum threshold of 0.05 for cluster proportion, two clusters with proportions of at least 0.05 were inferred (first cluster with mean proportion 0.588, 95% CI 0.512-0.656; second cluster with mean proportion 0.347, 95% CI 0.289-0.407). The lower bound of the 95% CI for the third most prevalent cluster was 0.004 (equivalent to 1 out of the 256 input samples), supporting the presence of only 2 clusters. Thus, the model was fitted with 2 clusters () to sample cluster membership .

#### 2.3.3 Statistical analysis

All statistical analysis was performed in R (v4.3.2). The Mann-Whitney U test was used to evaluate significance in two-way comparisons. Comparisons for categorical count data were evaluated for significance with Fisher’s exact test. Multiple testing correction was performed with the Holm method. Statistically significant results were considered those with *P* < 0.05.

P values for Kaplan-Meier analyses were computed with log-rank tests. Cox proportional hazards analysis was performed in R with the survival package (v3.5-7). Proportional hazards assumptions were evaluated with weighted Schoenfeld residuals (Grambsch and Therneau 1994). Posterior distribution samples were used to calculate confidence intervals and *P* values.

#### 2.3.4 Code availability

Code associated with this project is publicly available at <https://github.com/Irrationone/tfri_halo>.

## 3 Results

### 3.1 Cohort

We assembled a cohort of 256 treatment-naive p53abn endometrial carcinomas diagnosed between XXXX and XXXX ([Table 1](#tbl-cohort-overview), OTHER REF) (Aline Talhouk et al. 2019). All cancers were classified according to ProMisE (A. Talhouk et al. 2015) by p53 and MMR immunohistochemistry in addition to next-generation sequencing for POLE and p53 mutations. Histotypes included were serous (n=136), endometrioid (n=52), carcinosarcoma (n=31), clear cell (n=15), mixed (n=17), undifferentiated/dedifferentiated (n=2) and other (n=3). All patients received treatment in accordance with standard-of-care at the time of diagnosis, with most patients receiving adjuvant chemotherapy and a smaller proportion receiving adjuvant brachytherapy or radiotherapy ([Table 1](#tbl-cohort-overview)). No patients received immunotherapy.

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| Table 1: Cohort statistics   |  | | --- | | (a) | |

Source: [Cohort-level analysis](https://Irrationone.github.io/tfri_halo/notebooks/cohort_analysis.qmd.html#cell-tbl-cohort-overview)

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| Figure 1: Cohort overview |

Source: [Cohort-level analysis](https://Irrationone.github.io/tfri_halo/notebooks/cohort_analysis.qmd.html#cell-fig-cohort-track)

We performed multiplex immunofluorescence and automated image analysis to segment tumors into epithelial (intratumoral) and stromal regions and quantify CD8+ T cells (CD3+CD8+), CD4+ T cells (CD3+CD8-FOXP3-), T regulatory cells (CD3+CD8-FOXP3+), B cells (CD20+CD79a+) and plasma cells (CD20-CD79a+) ([Section 2](#sec-methods)). Cells within epithelial and stromal regions were counted separately, and automated counts were manually verified. At least two TMA cores were sampled from each tumor to account for spatial heterogeneity. Sarcomatoid areas in carcinosarcomas were considered epithelial. Densities for all cell types in epithelial and stromal regions were highly correlated ([Figure S1](#suppfig-densities-correlation)). Representative H&E and immunofluorescence images with corresponding epithelial-stromal region segmentation and cell type identification results are shown in [Figure 2](#fig-halo-images).

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| Figure S1 |

Source: [Clustering results](https://Irrationone.github.io/tfri_halo/notebooks/clustering.qmd.html#cell-suppfig-densities-correlation)

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| Figure 2 |

Source: [Article Notebook](https://Irrationone.github.io/tfri_halo/index.qmd.html)

### 3.2 TIL-rich tumors are associated with longer survival

Next, we clustered tumors based on epithelial and stromal counts normalized by region area with a negative binomial mixture model. The optimal number of clusters, as determined by a Dirichlet process prior, was two: a TIL-rich and a TIL-poor cluster ([Section 2](#sec-methods)). TIL-rich tumors were highly infiltrated by both epithelial and stromal T and B cells, including CD3+CD8+ cytotoxic T cells, CD3+CD8-FOXP3- T helper cells, CD3+CD8-FOXP3+ T regulatory cells, CD79a+CD20+ B cells, and CD79a+CD20- plasma cells. TIL-rich tumors had more stroma than TIL-poor tumors (*P =* 1.98e-03). Vascular density was similar in TIL-rich and TIL-poor tumors (*P =* 0.224). Contrary to observations in high-grade serous ovarian carcinoma, where tumors with both epithelial and stromal TIL are an immunologically and genomically distinct subgroup from those with stroma-restricted TIL (Allen W. Zhang et al. 2018), we were not able to identify a distinct subgroup of p53abn endometrial cancers with stroma-restricted TIL. However, TIL-rich tumors had significantly higher epithelial but not stromal CD8/Treg ratios, suggestive of tumor-specific T cell activation ([Figure 3](#fig-densities-tilclust)) (Jong et al. 2009; Yamagami et al. 2011).

We next assessed whether TIL subgroup was associated with survival. In univariate Kaplan-Meier analyses, there was no significant association between TIL cluster and overall, progression-free, or disease-specific survival (all *P >* 0.175). However, TIL cluster was significantly associated with prolonged overall and disease-specific survival in multivariate Cox proportional-hazards analysis accounting for age at diagnosis, FIGO stage (Creasman 2009), and adjuvant treatment ([Table 2](#tbl-cox-hazards)). TIL cluster was also associated with longer progression-free survival, but this was not statistically significant (P = 0.15). Age was associated with shorter overall survival. None of the adjuvant treatment modalities tested were significantly associated with longer survival. The discordance in the effect of TIL between univariate and multivariate analyses was at least partially explained by differences between limited and advanced stage disease. Univariate Kaplan-Meier analyses stratified by stage highlighted that the association between TIL cluster and survival was most pronounced in patients with stage III disease ([Figure S2](#suppfig-km-stage)). Neither TIL subgroup nor individual TIL densities were correlated with HER2 status by immunohistochemistry or shallow whole-genome sequencing. Thus, TILs are associated with longer survival in p53abn endometrial carcinoma.

As p53abn endometrial carcinomas represent a mixture of different histotypes, we investigated whether histotype modifies the association between TIL and survival. TIL subgroup was significantly associated with histotype (P = 6.51e-03), with most carcinosarcomas falling in the TIL-poor group (23/31), compared to 97/224 of non-carcinosarcoma histotypes (adjusted P = 0.011). Other non-serous histotypes were not significantly associated with histotype. To account for the differences in immune composition between carcinosarcomas and other p53abn endometrial cancer histotypes, we included carcinosarcoma as an explanatory variable in our multivariate analysis. The association between TIL subgroup and overall survival remained significant (P < 0.044), suggesting that TIL subgroup is prognostic independently of histotype. Carcinosarcomas were associated with shorter overall, progression-free and disease-specific survival, consistent with prior findings (Chuyao Zhang et al. 2015; Bernardini et al. 2016; Raffone et al. 2022), but this was not statistically significant (all P > 0.067).

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| Figure 3: Clusters and association with survival |

Source: [Clustering results](https://Irrationone.github.io/tfri_halo/notebooks/clustering.qmd.html#cell-fig-densities-tilclust)

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| Table 2: Cox hazards table   |  | | --- | | (a) | |

Source: [Clustering results](https://Irrationone.github.io/tfri_halo/notebooks/clustering.qmd.html#cell-tbl-cox-hazards)

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| Figure S2 |

Source: [Clustering results](https://Irrationone.github.io/tfri_halo/notebooks/clustering.qmd.html#cell-suppfig-km-stage)

### 3.3 Overexpression of immune checkpoint molecules in TIL-rich samples

Next, we profiled immune checkpoint expression to determine the extent of exhaustion within the tumor microenvironment. Consistent with our previous findings (Aline Talhouk et al. 2019), TIL-rich tumors contained more CD8+PD1+ and CD8+PD1- T cells ([Figure 4](#fig-tilclust-ardensities)). Additionally, there was relative enrichment of PD1+ cytotoxic T cells, suggestive of T cell exhaustion ([Figure 4](#fig-tilclust-ardensities)).

PDL1 is expressed by both tumor and immune cells, particularly macrophages, and binds to PD1 on T cells to suppress the immune response (Tucci et al. 2019). Mirroring the pattern seen with PD1 in cytotoxic T cells, PDL1-positive macrophages were enriched in TIL-rich tumors ([Figure 4](#fig-tilclust-ardensities)). In contrast, there was no significant difference in PDL1-negative macrophages between TIL-rich and TIL-poor tumors, irrespective of IDO1 status. Our results highlight upregulation of the PD1-PDL1 axis in TIL-rich p53-abnormal endometrial carcinomas which may be therapeutically exploitable with immune checkpoint blockade.

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| Figure 4 |

Source: [Adaptive response](https://Irrationone.github.io/tfri_halo/notebooks/adaptive_response.qmd.html#cell-fig-tilclust-ardensities)

### 3.4 Mutational signatures and TILs in endometrial cancer

Finally, we evaluated the relationship between TIL subgroups and mutational processes. Using shallow whole genome sequencing data-derived copy number signatures that we previously described in endometrial cancer [CITE], we correlated signature exposures to TIL subgroup for n=126 p53abn endometrial carcinomas. None of the copy number signatures, including the HRD signature VS3 (CHECK WITH DAWN), were significantly associated with TIL subgroup in p53abn endometrial carcinoma. Densities for individual TIL types in epithelial and stromal regions were not significantly correlated with any mutational signature, and none of the mutational signature exposures were significantly associated with survival in multivariate Cox analyses including TIL cluster. Thus, mutational signatures show different patterns of co-segregation with TILs and prognostic significance in p53abn endometrial carcinoma compared to HGSC (Allen W. Zhang et al. 2018).

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| Figure 5: Mutational signatures |

Source: [Mutational signatures](https://Irrationone.github.io/tfri_halo/notebooks/mutational_signatures.qmd.html#cell-fig-mutational-signatures)

## 4 Discussion

Historically considered one of the less immunogenic subtypes of endometrial cancer, p53abn tumors have received minimal attention in immunotherapy research. Initial trials showed only modest response rates to pembrolizumab in advanced and recurrent MMRp endometrial cancer (Ott et al. 2017), mirroring similar results in epithelial ovarian cancer (Matulonis et al. 2019). More recently, dostarlimab in addition to standard-of-care chemotherapy demonstrated benefit in both MMRd and MMRp cancers (Mirza et al. 2023), with p53abn cases responding substantially better than NSMP cases [ESMOMirza2023]. We systemically profiled the immune microenvironment in one of the largest cohorts of p53abn endometrial cancers to date, revealing 2 immunologically distinct subgroups defined by extensive and limited infiltration of T cells, B cells, and macrophages. Over half of p53abn cancers are highly infiltrated by TIL, challenging the belief that p53abn cancers are immune depleted (Mendiola et al. 2022). Cytotoxic T cells, but not immunosuppressive T regulatory cells, co-localized with tumor cells in TIL-rich tumors. TIL-rich tumors expressed high levels of PD1 and PDL1, providing mechanistic rationale for the effectiveness of anti-PD1 inhibitors. TIL-rich tumors were also associated with longer overall and disease-specific survival, in contrast to prior findings in smaller cohorts (Aline Talhouk et al. 2019). Notably, TILs were more strongly associated with survival in patients with advanced disease, who have the most presing treatment requirements. These findings may inform clinical trials of immunotherapies in early-stage endometrial cancer (NCT04634877, NCT04214067), where TILs were not significantly associated with longer survival.

The pervasiveness of TIL-rich p53abn endometrial cancers underscores potential shortcomings of molecular subtype-based strategies for selecting patients for immunotherapy (Ott et al. 2017; Yamashita et al. 2017). While TIL-rich tumors are more frequent in MMRd (78% (Aline Talhouk et al. 2019)) than other subtypes, 53% of p53abn tumors in our study were also TIL-rich. Histotype likely has limited importance, as apart from carcinosarcomas, which were mostly TIL-poor, histotype was not associated with TIL status. NSMP, the most TIL-poor molecular subtype (Aline Talhouk et al. 2019; Chong Zhang, Wang, and Wu 2023), derived correspondingly limited benefit from dostarlimab in the RUBY trial [ESMOMirza2023]. Thus, TIL response, not molecular subtype alone, may be more informative for stratifying endometrial cancer patients for immunotherapy.

In our study, IDO1 expression was not associated with TIL-rich tumors, contrasting with PD1 and PDL1 expression patterns. This may indicate a different mechanism for IDO1 induction in the tumor microenvironment. While IDO1+ macrophages were less prevalent than PDL1+ macrophages, their presence irrespective of overall TIL infiltration may make IDO1 a suitable target for immunotherapy in a subset of TIL-poor tumors.

Our data show for the first time the relationship between immune response and mutational processes in p53abn endometrial cancer. Despite genomic similarities between HGSC and p53abn endometrial cancer (Getz et al. 2013; Allen W. Zhang et al. 2018), TILs were not correlated with mutational signatures, including HRD, in p53abn endometrial cancer. In contrast to HGSC, p53abn endometrial cancer may elicit TIL responses through mechanisms independent of HRD. Other mutational processes generating widespread genomic instability in endometrial cancer [CITE DAWN] may elicit TIL responses through common cGAS-STING pathway activation (Deng et al. 2014; Woo et al. 2014). Additionally, TILs were not correlated with HER2 status by immunohistochemistry or whole-genome sequencing. Immunotherapies, anti-HER2 therapies, and PARP inhibitors targeting HRD tumors may represent orthogonal approaches effective in different groups of p53abn endometrial cancers, with some tumors susceptible to multiple agents.

As the use of immunotherapies extends to MMRp endometrial cancers, the immune microenvironment must be considered in addition to molecular subtype as a relevant factor. Our findings highlight properties of the immune microenvironment that may portend susceptibility to immune checkpoint susceptibility in p53abn endometrial cancers, and how these relate to other clinically actionable targets being explored in clinical trials.

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