Gender-specific Stratification of Survival Following Immune Checkpoint Inhibitor Therapy Based on Intratumoral Expression of a B cell Gene Signature

Adam K. Aragaki a,b, Yuezhou Jing a,b, Jean Hoffman-Censits a,b,c, Woonyoung Choi a,b, Noah M. Hahn a,b,c, Bruce J. Trock a,b, David J. McConkey a,b,c, Burles A. Johnson III a,b,*

a Greenberg Bladder Cancer Institute, Johns Hopkins University, Baltimore, MD, USA; b The James Buchanan Brady Urological Institute, Johns Hopkins University, Baltimore, MD, USA; c The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, USA

Abstract

Background: There is a great need to identify biomarkers that can accurately identify patients who will obtain the most clinical benefit from immune checkpoint inhibitor (ICI) therapy. While high intratumoral B cell gene expression correlated with an ICI response in melanoma, whether it adds predictive value in other cancers is unknown.

Objective: To examine the relationship between B cell gene signature (BCGS) expression and overall survival (OS) following ICI treatment.

Design, setting, and participants: A total of 348 patients with advanced urothelial carcinoma from the IMvigor 210 phase 2 clinical trial of atezolizumab and 406 patients with muscle-invasive bladder cancer from The Cancer Genome Atlas (TCGA) were included.

Outcome measurements and statistical analysis: We analyzed tumor RNA sequencing data of included patients to examine the relationships between a BCGS and clinical outcomes.

Results and limitations: Tumors with high levels of B cell and CD8+ T cell gene signatures (BCGS/CD8TGS or B8T high/high) were associated with the longest OS of all B8T groups. Moreover, the B8T cell signature stratified patients whose tumors had a high tumor mutational burden or high programmed death ligand 1 (PD-L1) into subsets with differential OS outcomes. Whereas the B8T high/high tumors were associated with the best clinical outcomes in ICI-treated men, they were not associated with better OS in women. Conversely, women with B8T high/high tumors had the best clinical outcomes in non–ICI-treated muscle-invasive bladder cancer.

Conclusions: These data suggest that the B8T signature can enhance OS stratification in patients with advanced urothelial carcinoma who are treated with ICI therapy and that sex-specific differences in the tumor immune microenvironment may drive disparate outcomes.
1. Introduction

Immune checkpoint inhibitor (ICI) therapies have transformed cancer care by improving overall survival (OS), particularly in patients with metastatic solid tumors [1]. Unfortunately, only a minority of patients with metastatic solid tumors have durable responses [2]. While several biomarkers enrich for clinical benefit, including tumor mutational burden (TMB), intratumoral CD8+ T cell gene signature (CD8TGS), and programmed death ligand 1 (PD-L1), only mismatch repair deficiency [6] predicts objective response rates (ORRs) in a majority of patients [4,7–10]. Therefore, much effort is focused on the development of ICI-predictive biomarkers, in order to direct ICIs to patients with the best chance of benefit [11]. In patients with advanced melanoma treated with ICI agents, high intratumoral B cell gene signature (BCGS) expression was associated with an improved Response Evaluation Criteria in Solid Tumors (RECIST) response [12]. However, whether a high BCGS correlates with an ICI response in other tumors, and whether a BCGS strengthens the ability of other biomarkers (eg, CD8+ T cells or TMB) to predict an ICI response, are unknown.

Here, we analyzed tumor RNA sequencing (RNAseq) data from the phase 2 IMvigor210 study, where patients with locally advanced or metastatic bladder cancer who were cisplatin ineligible or developed tumor progression despite cisplatin-based chemotherapy were treated with the anti–PD-L1 antibody atezolizumab [13,14]. Our goal was to examine the relationship between BCGS and CD8TGS expression (BCGS/CD8TGS or B8T signature), OS following ICI treatment, and whether sex-based differences in immunity [15] impacted outcomes.

2. Patients and methods

2.1. Patients

Detailed patient data for the IMvigor210 [13,14] and The Cancer Genome Atlas (TCGA) [16] studies are in the original publications (Supplementary Tables 1 and 2). Additional information is provided in the Supplementary material.

2.2. Data availability

The IMvigor210 trial data (EGAD00001003977) were received upon request from Genentech [5], and the TCGA dataset was accessed through the Genomic Data Commons. The code used to generate figures can be found at https://github.com/KaiAragaki/aragaki_b8t_2021_figures.

2.3. Data analysis

Detailed data analysis including references to code used is described in the Supplementary material.

2.4. Statistical analysis

The two-sample, two-tailed t test was performed to compare signature means. For survival analyses, the log-rank test was used. For pairwise tests, Benjamini-Hochberg adjusted p values are given. In all cases, p < 0.05 was used as a cutoff for statistical significance. R (version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria) was used to perform all analyses [17].

3. Results

3.1. High intratumoral B8T segregates OS outcomes in response to ICI

To elucidate the impact of B cells on OS in response to ICI treatment, we analyzed tumor RNAseq data from the IMvigor210 trial [13,14]. Using validated BCGS [18] and CD8TGS [13] (Supplementary Table 3), we found that there were bimodal distributions of both gene signatures (GSs; Fig. 1A). Patients with tumors with high BCGS or CD8TGS expression (as previously demonstrated [5]) had improved OS (Fig. 1B). This association extended to post-ICI tumor ORRs, in which case patients with complete responses to ICI treatment had higher BCGS or CD8TGS than patients with partial responses (Supplementary Fig. 1A and 1B). However, when we plotted BCGS versus CD8TGS expression, we found that some BCGS high tumors were CD8TGS low, and vice versa (Fig. 1C). This suggested the existence of four biologically distinct tumor subsets, based on high or low BCGS and CD8TGS expression.

As responses appeared to cluster among BCGS high CD8TGS high (B8T high/high) tumors (Fig. 1C), we plotted OS for each of the four subsets according to B8T expression. Consistent with the results suggesting high intratumoral B cells and CD8+ T cells associated with improved OS in non–ICI-treated melanoma patients [19], we found that ICI-treated patients with B8T high/high bladder cancer had the longest OS and disease control rate (Fig. 1D and Supplementary Fig. 1C). However, high BCGS (or CD8TGS) expression did not always confer an OS benefit, because patients with B8T high/low and B8T low/high tumors had poor OS that was comparable with the OS in patients with B8T low/low “immune desert” tumors (Fig. 1D). Enhanced survival with B8T high/high tumors was most striking in patients who received platinum-based chemotherapy (Supplementary Fig. 1D), while chemotherapy-naïve patients with B8T high/high and B8T low/low tumors performed similarly well (Supplementary Fig. 1E). To determine whether stroma content correlated with immune infiltration as defined by B8T subtypes, we used the Estimation of STromal and Immune cells in MalignanTumors using Expression data (ESTIMATE) algorithm to...
infer the stromal content for each tumor [20]. B8T low/low tumors had a significantly lower stromal score than all other groups, while B8T high/high tumors had a significantly higher stromal score (albeit a modest difference between B8T high/high and other immune infiltrated groups; Supplementary Fig. 1F). Overall, high coexpression of BCGS and CD8TGS correlates with improved OS in patients treated with ICIs, but high individual expression of either the BCGS or the CD8TGS associates with poorer outcomes.

3.2. B8T high/high stratifies OS in response to ICI treatment for TMB high tumors

We confirmed that patients with high TMB tumors had higher ORRs (Supplementary Fig. 2A) and longer OS (Fig. 2A) than patients with low TMB tumors in the IMvigor 210 cohort, consistent with previous observations [5]. We used TMB ≥10 mutations per megabase as the cutoff for high TMB, as this is the tumor-agnostic threshold for use of ICI therapy in patients not otherwise eligible for ICI or another treatment [7]. Although high TMB enriches for patients who have increased OS in response to ICI treatment, many patients with high TMB do not have increased OS; thus, TMB alone does not adequately differentiate those with long-term OS benefit. As tumors with TMB low and TMB high phenotypes exhibited similar heterogeneity in B8T expression (Fig. 2B and Supplementary Fig. 2B), we hypothesized that the B8T signature would stratify tumors with TMB high and TMB low molecular phenotypes into subsets associated with different OS rates.

To test this, we plotted OS of TMB high patients stratified by B8T signature status and found that the B8T high/high subset had the longest OS (Fig. 2C). The B8T signature did not stratify TMB low patients into cohorts with a statistically significant difference in OS (p = 0.079; Fig. 2D). As patients with low TMB exhibit relatively low survival, it may be more difficult to discern statistically significant differences among the four groups within this attenuated range. The pattern is qualitatively similar to that in TMB high tumors, with higher OS among B8T high/high tumors, but no clear differences among the remaining three groups.

TMB levels stratified B8T high/high tumors (Fig. 2E) into subsets that were also associated with different OS. TMB appeared to stratify B8T low/low tumors into groups with different OS, but this was not statistically significant (Supplementary Fig. 2C). To determine whether combining TMB high and B8T high/high status may identify patients with superior OS, we plotted OS for patients with TMB high and B8T high/high tumors, patients with TMB high without B8T high/high status (non-high/high B8T), and patients with B8T high/high but low TMB. Patients with tumors that were both TMB high and B8T high/high had significantly
greater OS (Fig. 2F). These results demonstrate that the combination of TMB high and B8T high/high status may be a useful biomarker for identifying who will best respond to ICIs.

### 3.3. B8T high/high segregates OS in ICI-treated patients with PD-L1 2+ tumors

High tumor microenvironment PD-L1 identifies patients more likely to respond to ICI treatment in several solid tumors [9]. Consistent with previous reports [5,13], patients in the IMvigor210 trial whose cancers displayed high PD-L1 immune cell (IC) expression (IC 2+) experienced longer OS (Fig. 3A) and higher ORRs (Supplementary Fig. 3A) than patients with intermediate (IC 1) or low (IC 0) expression. While the patients with B8T high/high tumors within the PD-L1 IC 2+ cohort associated with superior OS (Fig. 3B), the B8T high/high signature did not stratify the PD-L1 IC 0/1 group (Fig. 3C). Although the B8T signature did not stratify outcomes in patients with PD-L1 low tumors into groups with significantly different OS, B8T seemed to stratify patients with PD-L1 IC 0/1 tumors into cohorts with higher OS (B8T high/high and B8T low/low) and lower OS (B8T low/high and B8T high/low). The lack of significance may reflect smaller numbers, as the low numbers of patients in the PD-L1 0−1+/B8T low/low and PD-L1 0−1+/B8T high/low cohorts made it difficult to make conclusions.

PD-L1 expression segregated outcomes within the B8T high/high tumors, because patients with B8T high/high tumors and PD-L1 IC 2+ expression had longer OS than those with PD-L1 IC 0−1 tumors (Fig. 3D). B8T high/high and B8T low/high tumors were enriched for PD-L1 IC 2+ staining, while other tumors were enriched for PD-L1 IC 0−1 (Supplementary Fig. 3B).

To further elucidate the significance of these biomarkers, we performed univariable and multivariable analyses for OS (Supplementary Tables 4 and 5). As expected, we found that a high tumor neoantigen burden and PD-L1 IC 2+ expression were associated with improved OS on multivariable analyses. Notably, B8T high/high tumors (and B8T low/low tumors) were associated with significantly longer OS versus the B8T high/low subtype on multivariable analyses.

### 3.4. Tumors with B8T high/high expression and high TMB are associated with superior OS

To determine the relative impact of the B8T high/high signature, high TMB, and PD-L1 IC 2+ to predict OS in patients in the IMvigor210 study, we examined the effects of each biomarker individually and in combination. Because
these groups overlap, statistical comparisons are not appropriate, so we provide the 2 yr OS rate. We found that the B8T high/high signature, high TMB, and PD-L1 IC 2+ were each associated with similar 2-yr OS (Fig. 3E). Upon combining biomarkers, we found patients with B8T high/high + PD-L1 IC 2+ tumors, or high TMB + PD-L1 IC 2+ tumors, had similar 2-year OS that was longer than in patients captured by single biomarkers (Fig. 3E). Patients with B8T high/high + high TMB tumors had the longest OS (69% at 2 years), which was not improved upon by combining all three biomarkers (Fig. 3E).

To determine whether the B8T high/high signature pinpointed patients not identified by other biomarkers, we plotted all patients who were positive for one or more biomarkers (and for whom information for all three biomarkers were present) in a Venn diagram (Fig. 3F). Although there was significant overlap among groups, there was a cohort of patients with B8T high/high tumors who were not positive for the other biomarkers (25/272 tumors evaluable for all three biomarkers, or ~9%). Further, ~30% (14/45) of patients whose tumors coexpressed the B8T high/high signature and high TMB did not express IC 2+ levels of PD-L1, suggesting that restricting treatment to patients whose tumors expressed all three biomarkers would have excluded patients with superior OS outcomes.

To further evaluate the utility of the B8T high/high signature, high TMB, and PD-L1 IC 2+ to predict OS individually and in combination, we generated time-dependent summary receiver operating characteristic curves and associated area under the curve (AUC) values for each of these scenarios. The AUC was greater for the B8T plus TMB curve than for individual and dual combination biomarkers at each time point (Supplementary Fig. 3C). Consistent with the 2-year OS noted in Figure 3E, addition of PD-L1 IC to B8T plus TMB did not improve the AUC result. We also graphed AUC as a function of time, and the B8T plus TMB curve appeared similar to the B8T plus TMB plus PD-L1 IC curve (Supplementary Fig. 3D).

3.5. Gender dependency of the prognostic impact of B8T high/high expression

Large meta-analyses have yielded inconsistent results with regard to whether or not ICI therapy produces decreased clinical benefit in women with solid tumors compared to men [21,22]. Women have complex sex-specific immune-related differences when compared with men [15], which may impact ICI therapeutic responses. Thus, we examined whether the prognostic impact of the B8T signature varied according to gender. There was no significant gender-
specific OS difference in the IMvigor210 cohort (Fig. 4A), and we observed equivalent bimodal distributions in the expression of the BCGS and CD8TGS in tumors from men and women (Supplementary Fig. 4A). While men with BBT high/high tumors had longer OS than the other men (Fig. 4B), women with BBT high/high tumors had similar OS to other women (Fig. 4C; women with BBT low/high tumors not shown due to low numbers). This phenomenon was not specific to the presence of B cells or CD8+ T cells alone, because men (but not women) with a high BCGS or high CD8TGS had longer OS than those with low expression of these signatures (Supplementary Fig. 4B and 4C). Although it did not reach statistical significance, men with BBT high/high tumors, but not low/low tumors, appeared to have longer OS versus women with similar BBT expression profiles (Supplementary Fig. 4D and 4E). These findings correlated with the decreased ORRs in women with BBT high/high tumors when compared with men (Supplementary Fig. 4E).

When examining multivariable hazard ratios stratified by sex (Supplementary Tables 6 and 7), males with BBT high/low or BBT low/high tumors had nearly twice the death rate as that in males with BBT low/low tumors, whereas males with BBT high/high tumors had nearly identical OS to males with BBT low/low tumors. Males and females with BBT low/low tumors had significantly improved OS versus those with BBT high/low tumors. Males, but not females, with tumors with a high neoantigen burden or PD-L1 IC+ had significantly improved OS on multivariable analysis.

3.6. BBT confers sex-specific OS benefit in ICI-naïve urothelial carcinoma

To determine whether the BBT signature was associated with OS benefit in ICI-naïve patients with urothelial cancer, we examined the TCGA muscle invasive bladder cancer (MIBC) dataset [16]. Similar to the IMvigor210 cohort, we found bimodal distributions of intratumoral BCGS and CD8TGS expression (Supplementary Fig. 5A). Neither the BCGS (Supplementary Fig. 5B), CD8TGS (Supplementary Fig. 5C), or the BBT signature (Fig. 5A) stratified OS in patients with MIBC. When we separated tumors based on sex, we observed bimodal distributions of BCGS and CD8TGS (Supplementary Fig. 5D), and found that women (Fig. 5B), but not men (Fig. 5C), with BBT high/high tumors had the best sex-specific OS. BBT low/low tumors had a significantly lower stromal score (using the ESTIMATE algorithm [20])
than all other groups, while BBT high/high tumors had a significantly higher stromal score (Supplementary Fig. 5E). Finally, we performed univariable and multivariable analyses for OS benefit in these patients (Supplementary Tables 8–11). On multivariable analyses, we found that women, but not men, with BBT high/high tumors had significantly greater OS than those with BBT low/low tumors (Supplementary Tables 10 and 11).

### 4. Discussion

Identification of gender-related differences in biological mechanisms and therapy response is a top priority in cancer research. Bladder cancer is a prime example of a disease with clear gender distinctions, as the incidence of bladder cancer in men is approximately three-fold higher than it is in women [23], who often present with more aggressive disease [24,25]. Here, we present preliminary evidence that bladder cancer immune landscapes are subject to gender-related differences in clinical behavior. Using RNAseq data, we show for the first time that GS associated with B cells and CD8+ T cells (BBT) exhibit essentially binary expression patterns in MIBC and advanced bladder cancer. In the immunotherapy-naïve TCGA cohort, these gene expression patterns were prognostic in women (but not in men), whereas in patients treated with atezolizumab, the patterns were prognostic in men (but not in women). Although the mechanisms underlying these differences are currently unknown, our results are consistent with a recent study [21] (albeit controversial [22]) that concluded that men derived more benefit than women from ICI therapy. Whether the patterns observed here extend to other ICI-responsive cancers is under investigation.

Despite intense efforts to identify biomarkers to predict responses to ICIs, only tumor MMR deficiency accurately identifies majority subsets of patients who will achieve an objective response [6], whereas other biomarkers do not [4.7–10]. Here, we present evidence that the BBT signatures provide additional, independent prognostic value when added to the currently available biomarkers, PD-L1 and TMB. Moreover, patients with BBT high/high and TMB high tumors who received ICIs had the greatest OS at 2 years, and addition of PD-L1 IC 2+ to BBT high/high and TMB high did not add prognostic value for patients who received ICIs. Finally, our results indicate that current biomarkers (e.g., PD-L1 and high TMB) do not account for a significant percentage of tumors that are only BBT high/high. Thus, we may be identifying a previously unaccounted for subset of patients with favorable OS in response to ICIs.

Although anti-PD pathway treatment is approved by the US Food and Drug Administration for metastatic bladder cancer in the second-line setting, and as first-line treatment for platinum-ineligible patients or cisplatin-ineligible patients with PD-L1 combined positive score >10, there are several cases where these data may influence clinical treatment, if this biomarker was approved for decision-making purposes. If a patient had a tumor that was TMB high and BBT high/high, a clinician may be prompted to initiate single-agent ICI in the first-line setting even if a patient was cisplatin eligible, or cisplatin ineligible but carboplatin eligible regardless of PD-L1 status. In addition, if a patient had a TMB high + BBT high/high tumor but mild or controlled autoimmune disease, the risk/benefit ratio may favor giving anti-PD pathway treatment at some point in the treatment course, whereas otherwise a clinician would likely not chance this.

Commercial genomic testing firms are now performing RNAseq in parallel with DNA exome sequencing in CLIA environments. If our findings are reproduced in independent cohorts and prospective studies, integration of high-quality RNAseq and BBT testing into clinical diagnostic workflows should be feasible. The BCGS utilized in these
studies correlates with flow cytometry for B cells, and CD8α gene expression correlates with CD8 immunohistochemical (IHC) staining and flow cytometry for CD8+ T cells [18]. One caveat is that bulk GS expression profiling does not account for variation in levels of gene expressed by a single cell. Alternatively, IHC detection of CD8 (for CD8+ T cells) and CD20 or CD19 (for B cells) may be a cost-effective method of adopting the BBT analysis into standard workflows. Dual CD8+ T cell and B cell IHC has identified patients with superior OS among those with non–ICI-treated stage III melanoma [19], suggesting that this approach is feasible.

Our results build on recent studies demonstrating that high intratumoral B cell gene expression correlated with response to ICI therapy in advanced melanoma [12]. Notably, our data also show that high BCGS and CD8TGS expression was associated with reduced OS in the subset of ICI-naïve women and ICI-treated men whose tumors did not contain high expression of both GSs. Speculatively, the results suggest that while the presence of B cells and CD8+ T cells is consistent with an antitumor response and improved OS, the presence of either cell type alone is insufficient to drive a robust antitumor response. The BBT high/low tumor phenotype may result from immune suppressive, or regulatory, B cells (Bregs) that block CD8+ T cell expansion in preclinical cancer models [26–28]. The BBT low/high phenotype may be driven by the presence of CD8+ T cells with different function versus CD8+ T cells in BBT high/high tumors. Consistent with this, single-cell RNAseq data from human melanoma tumors reveal that CD8+ T cells from B cell–rich tumors have different gene expression profiles than CD8+ T cells from B cell poor tumors [19], including a memory TCF7+ T cell profile in B cell–rich tumors [19] that correlates with response to ICI [29]. The high number of CD8+ T cells in BBT low/high tumors may be due to the presence of CD8+ regulatory T cells [30]. Further functional characterization of the tumor-infiltrating B and T cells within these BBT subtypes may lead to novel therapeutic targets for future clinical evaluation.

Strengths of this paper include analyses of data from two high-quality datasets, a focus on OS as the clinical outcome, and demonstration that the BBT signature adds prognostic value to established biomarkers. Its major weakness is the lack of confirmatory analyses in independent cohorts. Based on the effects observed here, validation would require at least one cohort that contains at least 50 women with ICI-naïve cancers and another with at least 100 men treated with an anti-PD(L)1 antibody. Unfortunately, the large gender discrepancy in bladder cancer incidence has made existing public datasets underpowered with respect to women. We are working with collaborators to obtain candidate validation datasets that are sufficiently large to meet the above criteria.

5. Conclusions

The data suggest that the BBT signature can enhance OS stratification in patients with advanced urothelial carcinoma who are treated with ICI therapy and that sex-specific differences in the tumor immune microenvironment may drive disparate outcomes.

Author contributions: Burles A. Johnson III had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Johnson.

Acquisition of data: Aragaki, Johnson.

Analysis and interpretation of data: Aragaki, Johnson, McConkey, Jing, Trock.

Drafting of the manuscript: Johnson.

Critical revision of the manuscript for important intellectual content: Johnson, Aragaki, Hoffman-Censits, Choi, Hahn, McConkey.

Statistical analysis: Aragaki, Jing, Trock.

Obtaining funding: Johnson, Hahn, McConkey.

Administrative, technical, or material support: None.

Supervision: None.

Other: None.

Financial disclosures: Burles A. Johnson III certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: Noah M. Hahn reports research funding from AstraZeneca, Incyte, Genentech, BMS, Merck, Seattle Genetics, Astex, Principia Biopharma, Pieris, and HTG Molecular Diagnostics; consulting income from Merck, Genentech, GlaxoSmithKline, Ferring, Champions Oncology, Health Advances, Key-quest Health, Guidepoint Global, Seattle Genetics, Incyte, TransMed, CicloMed, Janssen, Pfizer, and Boehringer Ingelheim; and honoraria from Bladder Cancer Academy. David J. McConkey reports grant support from AstraZeneca, and serves on the advisory boards of Janssen, Rainier, and H3 Biomedicine. Burles A. Johnson, Adam K. Aragaki, Jean Hoffman-Censits, Noah M. Hahn, and David J. McConkey have declared the BBT technology and may receive future income as a result.

Funding/Support and role of the sponsor: The authors gratefully acknowledge support from the Johns Hopkins Greenberg Bladder Cancer Institute, the Bladder Cancer Advocacy Network New Discoveries Young Investigator Award (to Burles A. Johnson III), NIH NCI R01 CA235681 (to Noah M. Hahn), and the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Support Grant NIH NCI P30 CA06973. The described extended funding organization had no role in the generation of the paper. The RNAseq dataset from the IMvigor210 trial was obtained from Genentech under a data access agreement, but it did not have a role in the research we performed or the generation of the paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.euo.2021.07.003.

References


