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ARTICLE

Molecular Diagnostics

TIGIT and PD-1 expression atlas predicts response to adjuvant chemotherapy and PD-L1 blockade in muscle-invasive bladder cancer

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BACKGROUND: TIGIT and PD-1 are checkpoint receptors that could regulate the functional status of immune cells through independent pathways. However, the clinical significance of immune classification based on TIGIT and PD-1 expression remains unclear in muscle-invasive bladder cancer (MIBC).
METHODS: Patients with MIBC from four independent cohorts were categorised into three clusters. Survival analysis conducted through Kaplan–Meier curves and Cox regression model. Immune contexture was measured by immunohistochemistry and CIBERSORT algorithm. Twenty-five fresh tumour tissue samples were utilised to evaluate functional state of CD8$^+$ T cells by flow cytometry.
RESULTS: Cluster I (TIGIT^{low}PD-1^{low}) contained widely poor immune infiltrates with higher FGFR3 mutation, Cluster II (TIGIT^{low}PD-1^{high}) exhibited a highly infiltrated contexture with increased cytolytic CD8$^+$ T cells and had the best prognosis, Cluster III (TIGIT^{high}) presented a suppressive tumour microenvironment (TME) featured by exhausted CD8$^+$ T cells and basal molecular subtype. Patients of Cluster III had the worst survival but could benefit more from adjuvant chemotherapy and anti-PD-L1 immunotherapy, and also presented limited FGFR3 signalling signature but activated immunotherapeutic and EGFR-associated pathway.
CONCLUSIONS: TIGIT/PD-1-based risk stratification with distinct immune and molecular features could be served as a predictor for systematic therapeutic response including adjuvant chemotherapy and immunotherapy in MIBC patients.

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INTRODUCTION

Bladder cancer is the most common genitourinary malignancy, which accounts for nearly 170,000 deaths worldwide annually [1]. Muscle-invasive bladder cancer (MIBC) represents ~20% of newly diagnosed cases and has inferior prognosis. Despite continuous improvement of surgical treatment like radical cystectomy (RC), about 50% of patients eventually relapse due to distant metastasis. Even if administrated with cisplatin-based chemotherapy, the 5-year survival rate of patients is still less than 50% [2]. MIBC is found to harbour higher tumour mutation burden (TMB), which allows clinicians to apply immune checkpoint blockade (ICB) therapies targeting PD-1/PD-L1 axis to these patients [3]. Nevertheless, the response rates of this treatment were relatively low due to the heterogeneity of tumour microenvironment (TME) [4, 5]. Hence, it is clinically required to accurately characterise what kinds of patients would benefit more from systemic therapies.

Immune checkpoints, such as PD-1 and TIGIT, are induced after T-cell receptor (TCR) stimulation and mediate T cell suppression and dysfunction. The potential role of immune checkpoints in mediating tumour immune evasion in MIBC and their interaction from systemic therapy remain poorly understood. Agents targeting programmed cell death protein 1 (PD-1) achieved great success in immune checkpoints therapy [6]. Despite PD-1 related pathways' role in T cell exhaustion and tumour immunosuppression, PD-1 itself serves as a marker of effector T cell rather than an exhaustion-specific molecule [7]. T cell Ig and ITIM domain (TIGIT) is an inhibitory receptor expressed by T cells and NK cells binding the adhesion molecules CD155/CD112 on tumour cells and antigen-presenting cells [8]. Our group revealed that TIGIT could depress CD8$^+$ T cells' tumoricidal capacity turning which to exhausted T cell phenotype and further mediate immunosuppressive micro-environment in MIBC patients [9]. Recent researches and trials have found that TIGIT and PD-1 dual checkpoint blockade could enhance antitumor immunity and patient's survival [10, 11]. However, there have been few studies investigating the clinical value of the combination of TIGIT and PD-1 expression.

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In this study, we evaluated the expression of immune checkpoints TIGIT and PD-1 in MIBC patients. The stratification based on TIGIT and PD-1 expression could accurately differentiate prognosis of MIBC patients and stratify patients into distinct groups with different therapeutic sensitivity to ACT and PD-L1 inhibitor immunotherapy. TIGIT and PD-1 expression impacted the characteristics of tumour immune microenvironment and function status of $CD8^+$ T cells. Moreover, the new stratification was closely associated with MIBC molecular classification and therapy-related signatures. These findings suggested that the potential of TIGIT and PD-1 expression in survival prediction and therapeutic guidance.

METHODS
Study cohort
This study enrolled five independent cohorts, including Zhongshan (ZSHS) cohort, Fudan University Shanghai Cancer Center (FUSCC) cohort, The Cancer Genome Atlas (TCGA) cohort, IMvigor210 cohort and Flow cytometry (FCM) cohort. Supplementary Fig. 1 summarised the selecting procedure of studying cohorts, the details of which were shown as follows.

This study was approved by the Clinical Research Ethics Committee of Zhongshan Hospital and the Ethics Committee of Fudan University Shanghai Cancer Center. 393 bladder cancer patients treated with radical cystectomy (RC) from two independent hospitals were enrolled in this cohort study. 215 patients were treated at Zhongshan Hospital of Fudan University from 2002 to 2014 (ZSHS cohort) and 178 patients were treated at Fudan University Shanghai Cancer Center from 2008 to 2012 (FUSCC cohort). 60 non-muscle invasive bladder cancer (NMIBC) patients and 13 non-urothelial carcinoma patients were excluded in ZSHS cohort. 35 NMIBC patients, 6 non-urothelial carcinoma patients and 18 patients with unavailable follow-up data were excluded in FUSCC cohort. At last, since a specimen was lost on the tissue microarray in each cohort because of immunohistochemistry detachment, 259 eligible MIBC patients were included (ZSHS cohort, $n = 141$; FUSCC cohort, $n = 118$). Among them, 119 patients of the 2 cohorts received adjuvant cisplatin-based chemotherapy and lasted at least one therapeutic cycle. The follow-up time points were every 3 months in the first year, every 6 months for next 2 years and once per year afterwards according to the European Association of Urology (EAU) guideline for MIBC [12]. Clinical history recording, physical examination and laboratory test were implemented for each patient at every visit. Follow-up data collection was finished before July 2016. The overall survival (OS) was defined as the period from the date of RC to the date of death and the recurrence-free survival (RFS) was defined as the time from the date of RC to the first recurrence or the last follow-up. Patients' clinicopathological characteristics of ZSHS and FUSCC cohorts were shown in Supplementary Table 1.

The TCGA data including mRNA sequencing data, clinical information and mutation information of bladder cancer (BLCA) was downloaded from Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov). 4 NMIBC patients, 10 patients receiving neoadjuvant chemotherapy and 7 patients with unavailable survival or sequencing data were excluded from the original 412 patients. IMvigor210 was a single-arm phase 2 study to investigate atezolizumab in 348 patients with metastatic urothelial carcinoma (mUCC). All data of IMvigor210 trial were downloaded from "http://research-pub.gene.com/IMvigor210CoreBiologies" through the "IMvigor210CoreBiologies" R package. Fragments per kilobase of exon model per million mapped fragments (FPKM) value was adopted for the RNA-seq data. Cutoff value was determined by the median value of TIGIT and PD1 mRNA expression. Patients' clinicopathological characteristics of IMvigor210 cohort were shown in Supplementary Table 2. The involved signatures were defined by previous studies and scored by the average of log2(related genes' FPKM+1) [13].

Immunohistochemistry (IHC) and Flow cytometry (FCM)
IHC staining was performed on tissue microarray (TMA) with formalin-fixed, paraffin-embedded surgical specimens as described previously [14]. The IHC antibodies were listed in Supplementary Table 3. All TMA slides were evaluated under Leica DM6000 B Microsystems by two pathologists independently who were blind to clinical data. The mean value of the number of the positive cells extracted from the typical view of three sections in high-power field (HPF, *200 magnification) was calculated and used for analysis. For $TIGIT^+$ cells and $PD-1^+$ cells, the correlation between the two pathologists' evaluation from the same side were 0.965 (95% CI:

0.937–0.986) and 0.921 (95% CI: 0.869–0.959), respectively. Cutoff value was determined by the median value of $TIGIT^+$ cells and $PD-1^+$ cells infiltration density in ZSHS cohort, which were 9 cells/HPF and 12 cells/HPF, respectively. The cutoff values were used in FUSCC cohort as well. The immunological phenotype was based on the prevalence of $CD8^+$ cells as well as the pattern of infiltration with respect to malignant epithelial cells in HPF. Tumours were categorised as "desert" when the abundance of $CD8^+$ cells was less than ten cells in an area of tumour and tumour-associated stroma tissue. Tumours were categorised as "excluded" if $CD8^+$ cells were more than ten cells and exclusively seen in stroma immediately adjacent to the main tumour mass. Tumours were categorised as "inflamed" if $CD8^+$ cells were more than 10 cells and seen in direct contact with malignant epithelial cells either in the form of spillover of stromal infiltrates into tumour cell aggregates or of diffuse infiltration of $CD8^+$ cells in aggregates or sheets of tumour cells.

FCM was performed with cell suspensions as described before [15]. Fresh samples of 25 MIBC patients were collected in Zhongshan Hospital, Fudan University Shanghai Cancer Center and Ruijin Hospital from June 2019 to Feb 2020. The FCM antibodies were listed in Supplementary Table 4. Flow cytometry data were analysed by FlowJo software (Tree Star, San Carlos, CA). The groups of high and low $TIGIT^+$ cells and $PD-1^+$ cells were determined by the median value of percentage of $TIGIT^+$ cells or $PD-1^+$ cells /$CD45^+$ cells. Finally, we trichotomized these 25 patients into three clusters: Cluster I ($TIGIT^+$ $CD45^+$ cells^low & PD-1^+ $CD45^+$ cells^low, $n = 8$), Cluster II ($TIGIT^+$ $CD45^+$ cells^low & PD-1^+ $CD45^+$ cells^high, $n = 4$), and Cluster III ($TIGIT^+$ $CD45^+$ cells^high, $n = 13$).

Statistical analysis
CIBERSORT was constructed to quantify relative proportions of 22 immune cell types known as LM22 in TCGA cohort. Heatmap data was processed by Z-score. Gene set enrichment analysis (GSEA) was performed by exhausted $CD8^+$ T cells signature to identify the function status of $CD8^+$ T cells. The "maftools" R package was used to identify the mutation status. Results were shown as mean ± SD in this study. Kruskal–Wallis H test, Wilcoxon signed-rank test, Chi-square test and Spearman correlation analysis were used in this study. OS and RFS were determined by Kaplan–Meier method, which was evaluated by log-rank tests. Multivariate and univariate analyses of cox regression model were applied to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). A two-tailed P value <0.05 was considered statistically significant in our study. All statistical analyses were conducted using IBM SPSS Statistics 25.0, R4.0.4 and Graph Pad Prism Software 8.0.

RESULTS
Prognosis of MIBC patients could be predicted according to the levels of TIGIT and PD-1 expression
The distribution of $TIGIT^+$ cells and $PD-1^+$ cells was evaluated on TMA using IHC (representative stained images are illustrated in Supplementary Fig. 2). We found that $PD-1^+$ cells infiltration had no prognostic value in patients with high $TIGIT^+$ cells infiltration (Supplementary Fig. 3). Thus, we trichotomized the patients based on $TIGIT^+$ cells and $PD-1^+$ cells infiltration into three clusters: Cluster I ($TIGIT^+$ cells^low & PD-1^+ cells^low), Cluster II ($TIGIT^+$ cells^low & PD-1^+ cells^high), and Cluster III ($TIGIT^+$ cells^high), in both ZSHS and FUSCC cohorts (Fig. 1a, b). The comprehensive description of the association between the novel stratification and patients' clinicopathological characteristics was shown in Supplementary Table 1. To further elucidating the clinical merit of the stratification, Kaplan–Meier analyses were conducted to compare the OS and RFS among three clusters. We found patients from Cluster II aired the best prognosis while patients from Cluster III possessed the worst prognosis (ZSHS: OS: $P = 0.006$, RFS: $P = 0.010$; Fig. 1c; FUSCC: OS: $P = 0.003$, RFS: $P = 0.024$; Fig. 1d). In multivariate and univariate models with classical prognostic factors, the novel stratification was found to be significantly associated with OS and RFS, independent of other clinical parameters, such as AJCC stage, pathological N stage, grade and lymphovascular invasion (LVI) in both ZSHS and FUSCC cohorts (Supplementary Tables 5, 6). Therefore, our findings illustrated that this new combined classification based on TIGIT and PD-1 expression was proved to be an independent prognostic factor of MIBC patients.
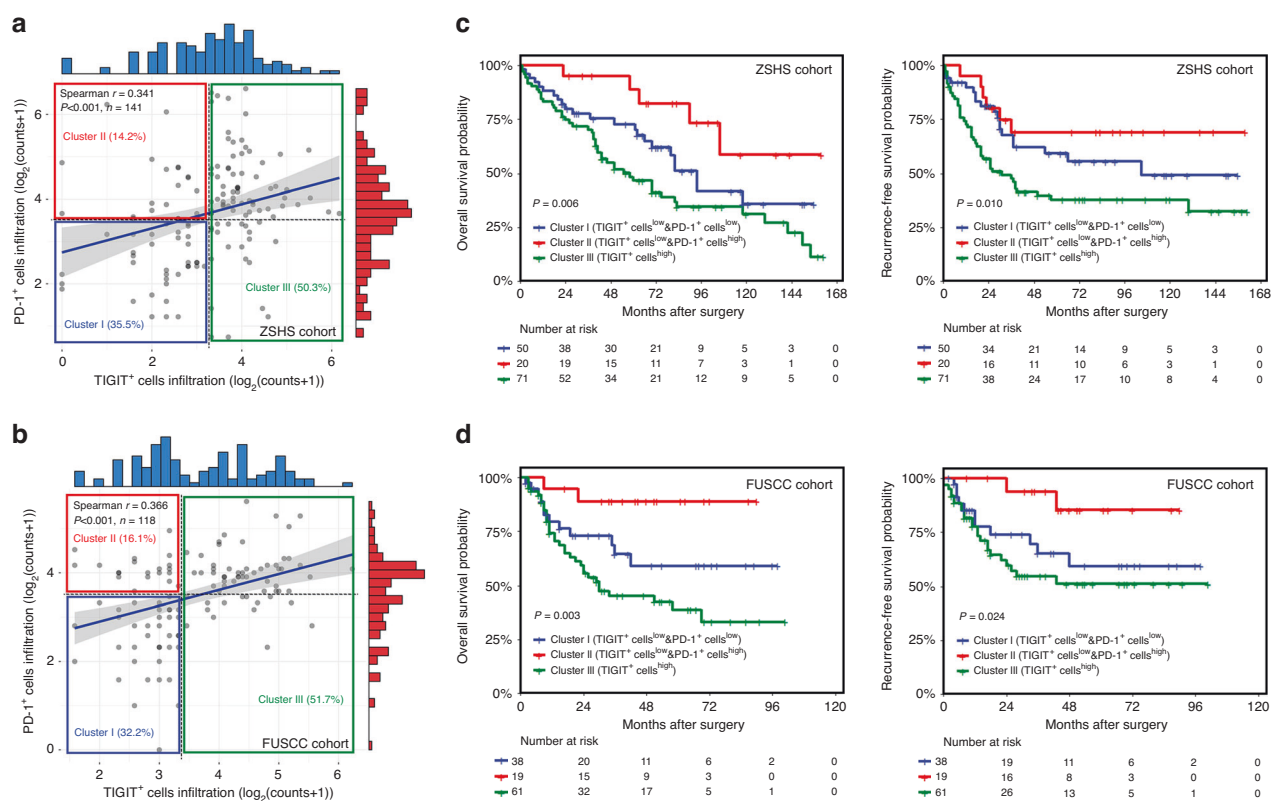
Fig. 1 Stratification based on TIGIT and PD-1 expression could predict prognosis in MIBC patients. a, b In ZSHS cohort (**a**) and FUSCC cohort (**b**), patients were divided into three clusters: cluster I (TIGIT$^+$ cells^{low} & PD-1$^+$ cells^{low}), cluster II (TIGIT$^+$ cells^{low} & PD-1$^+$ cells^{high}), and cluster III (TIGIT$^+$ cells^{high}). Spearman rank correlation test was applied. **c, d** Kaplan–Meier curves for overall survival (left) and recurrence-free survival (right) were compared among three clusters in ZSHS cohort (**c**) and FUSCC cohort (**d**). Log-rank test was performed. ZSHS, Zhongshan Hospital of Fudan University; FUSCC, Fudan University Shanghai Cancer Center.

Predictive value of stratification based on TIGIT and PD-1 expression for ACT responsiveness in MIBC patients

For MIBC, cisplatin-based ACT for post-operation patients has been applied as a standard treatment regimen [12]. Thus, we evaluated whether patients of various clusters presented corresponding ACT responsiveness. Both cohorts were combined for following investigation. Although ACT failed to improve OS or RFS in pT2 patients (OS: $P = 0.111$, RFS: $P = 0.248$; Fig. 2a, b), after dividing patients into the above three clusters, we found that patients with high TIGIT$^+$ cells infiltration could benefit from ACT and live longer (Cluster III: OS: $P = 0.001$, RFS: $P = 0.036$; Fig. 2c, d), while no survival benefit was observed in patients of remained clusters (Cluster I & II: OS: $P = 0.511$, $P = 0.637$, RFS: $P = 0.523$, $P = 0.333$, respectively; Fig. 2c, d). Analysis for the interaction reflected that our stratification could effectively distinguish patients who truly respond to ACT ($P = 0.032$, $P = 0.032$, respectively; Fig. 2c, d). Univariate cox regression analysis was performed to assess the relationship between the novel stratification and ACT benefit, which suggested that pT2 MIBC patients with TIGIT$^+$ cell high infiltration could receive more benefit from adjuvant chemotherapy (OS: HR, 0.287, 95%CI, 0.128–0.644, $P = 0.002$; RFS: HR, 0.415, 95%CI, 0.177–0.972, $P = 0.043$), whereas there was no improvement with ACT application in pT3/4 or all patients according to the stratification (Supplementary Table 7).

Characterisation of the immune microenvironment based on TIGIT and PD-1 expression in MIBC patients

Accumulating evidence exhibited that components in the tumour microenvironment can reprogram tumour progression and change the response to therapies [16]. To discover the potential mechanism beneath this prognosis stratification, we subsequently performed CIBERSORT to decipher the immune contexture among three clusters in TCGA cohort. Notably, we found that patients of Cluster II and III showed a higher level of immune cells infiltration, including CD8$^+$ T cells, CD4$^+$ T cells, follicular helper T cells (Tfhs), regulatory T cells (Tregs), gamma delta T cells, NK cells, tumour-associated macrophages (TAMs), dendritic cells (DCs) and mast cells (Fig. 3a). We then conducted IHC staining of tumour infiltrating immune cells and validated the aforementioned results in ZSHS cohort (Fig. 3b). Most solid tumours presented one of three distinct immunological phenotypes (immune inflamed, immune excluded or immune desert) [17]. In ZSHS cohort, we found that a large proportion of patients (75%) exhibited the non-inflamed phenotype in Cluster I; by contrast, half of patients displayed the inflamed phenotype in Cluster II and III ($P = 0.040$, Fig. 3c), consistent with the abundance of cytolytic lymphocytes in both cohorts. Meanwhile, we observed that Cluster III possessed higher immunosuppression signature (CXCL12, TGFB1, TGFB3 and LGALS1) in TCGA cohort [18] (Fig. 3a). Similarly, patients in Cluster III expressed increased TGF-β and IL-10 at the protein level of ZSHS cohort, which were considered as immunosuppressive cytokines (Fig. 3b). We concluded from the results above that Cluster I patients possessed decreased immune infiltrating contexture, Cluster II patients however exhibited relatively favourable one and Cluster III patients were related with immune enriched but suppressive microenvironment.

These data indicated that Cluster I patients possessed a desolation immune infiltration characteristic, Cluster II patients exhibited a highly infiltrated contexture and Cluster III patients were related with immune enriched but suppressive microenvironment.

Intriguingly, most patients in Cluster III were linked to an inflamed immune contexture with substantial CD8$^+$ T cells subpopulation,
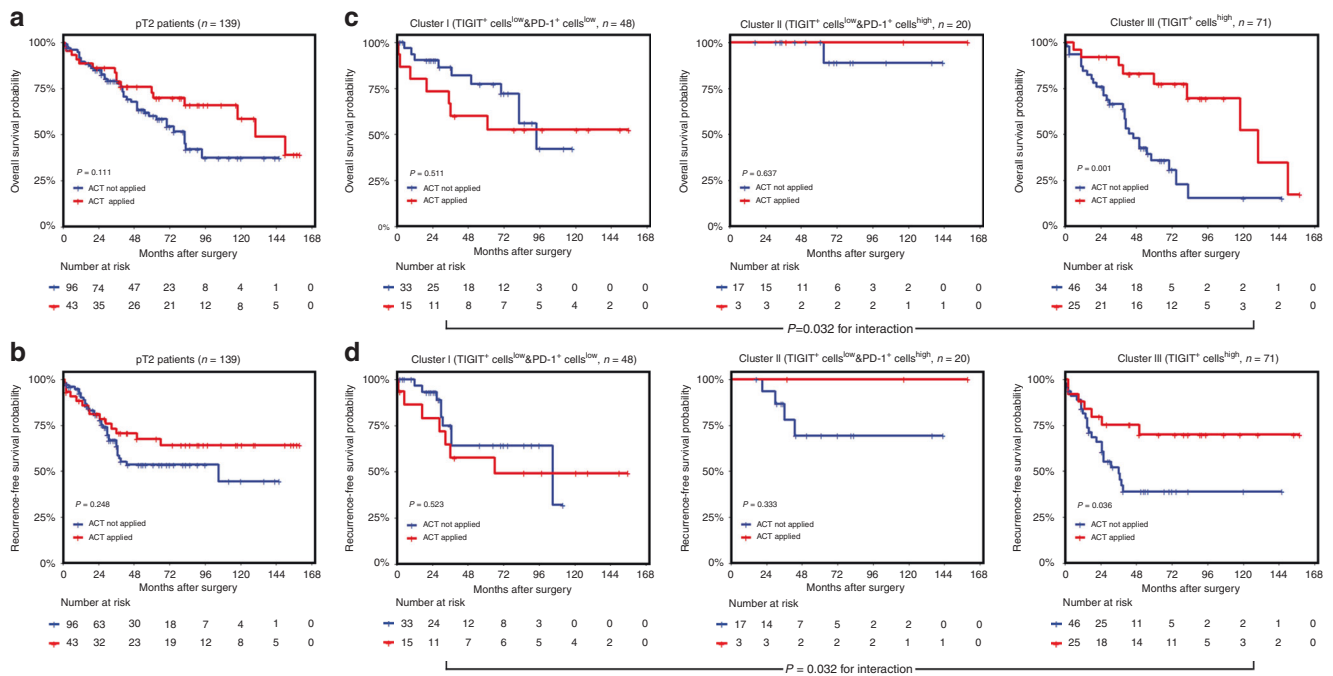
Fig. 2 Predictive value of stratification based on TIGIT and PD-1 expression for ACT responsiveness. a, b Kaplan–Meier curves for overall survival (**a**) and recurrence-free survival (**b**) with ACT application strata in pT2 MIBC patients. **c, d** Kaplan–Meier curves for overall survival (**c**) and recurrence-free survival (**d**) with ACT application strata among cluster I, cluster II and cluster III in patients with pT2 MIBC. Log-rank test was performed for Kaplan–Meier curves. pT, pathology Tumour; ACT, adjuvant chemotherapy.

albeit with the worst outcomes, which motivated us to investigate whether the function of CD8$^+$ T cells could be influenced based on three clusters. GSEA analysis revealed that abundance of exhausted CD8$^+$ T cells in Cluster III patients were much higher than Cluster I and II [19] (Cluster III vs. I: NES = 2.48, Cluster III vs. II: NES = 2.33; Fig. 3d). FCM analyses using 25 fresh resected samples were conducted to further investigate the functional status of CD8$^+$ T cells in MIBC patients (Cluster I: $n = 8$; Cluster II: $n = 4$; Cluster III: $n = 13$). Results confirmed that CD8$^+$ T cells infiltrated in Cluster III presented decreased effector cytokines (GZMB: $P = 0.035$, PRF-1: $P = 0.028$; Fig. 3e) but elevated checkpoints (TIM-3: $P = 0.029$, LAG-3: $P = 0.037$; Fig. 3f), suggesting an exhausted immunological phenotype, while in Cluster II patients CD8$^+$ T cells exerted cytolytic influence on TME owing to increased GZMB and PRF-1. In Cluster I, the expression level of both effector cytokines and checkpoints was relatively low, representing a naïve phenotype of CD8$^+$ T cells. To sum up, the heterogeneous functional status of CD8$^+$ T cells was found in three clusters mentioned above, which might be a direct result of the different expression level of PD-1 and TIGIT.

Stratification based on TIGIT and PD-1 expression correlated with molecular features in MIBC patients
Molecular features of MIBC might be of great importance in designing personalised treatment and heralding therapeutic responses, which has been proved by accumulating studies [20]. The overview of mutation and molecular subsets were visualised among three clusters by constructing the oncoPrint (Fig. 4a). We observed a significant association of consensus classification (LumP, LumNS, LumU, Stroma-rich, Ba/sq and NE-like) in different clusters [21]: specifically, patients of Cluster III were more likely to be classified to Ba/sq subtype ($P < 0.001$). Patients with basal bladder tumours exhibiting an immune enriched state had worse outcomes, which was consistent with our previous results. We then characterised the prevalence of the top altered genes within three clusters. Remarkably, abundant *FGFR3* mutations were significant in Cluster I patients ($P = 0.008$), agreed with features of luminal subtype. Moreover, we detected a higher tumour

mutation burden (TMB) in Cluster III patients ($P = 0.048$). Cluster III was also significantly associated with DNA damage repair gene mutations like *BRCA1* and *TP53* (*BRCA1*: $P = 0.044$; *TP53*: $P = 0.001$; Supplementary Fig. 4).

TIGIT expression may guide the application of anti-PD-L1 therapy in MIBC patients
Given that molecular subtypes have been associated with differential responses to target and immune treatments, we next focused on panels of therapy-associated genes among three clusters [13]. In both TCGA and IMvigor210 cohorts, Cluster I patients, characterised by high level of FGFR3-coexpressed gene signature score (TCGA: $P = 0.001$; IMvigor210: $P < 0.001$; Supplementary Fig. 5A, D), might be sensitive to FGFR3-targeted therapy. In comparison, the EGFR signatures were found stagewise elevated among Cluster I, II and III (TCGA: EGFR signalling: $P < 0.001$, EGFR ligands: $P < 0.001$; IMvigor210: EGFR signalling: $P < 0.001$, EGFR ligands: $P < 0.001$; Supplementary Fig. 5B, E), suggesting the promising prospect of EGFR-targeted drugs for Cluster III patients. Especially, Cluster III patients exhibited activated immunologic pathway (immune checkpoints: $P < 0.001$, antigen presenting: $P < 0.001$; Supplementary Fig. 5C), which implied its positive correlation with the efficacy of immunotherapy. Furthermore, we enrolled IMvigor210 cohort containing 348 mUCC patients upon PD-L1 blockade (atezolizumab). We found that TIGIT high subgroup exhibited a significantly improved response regardless of PD-1 expression ($P = 0.022$; Fig. 4b, Supplementary Fig. 5F). Besides, patients characterised by high PD-L1 expression and inflamed immune phenotype were more distributed in the Cluster III with high expression of TIGIT (PD-L1 IC: $P < 0.001$, Immune phenotype: $P < 0.001$). Kaplan–Meier analyses also confirmed that Cluster III had better OS after the application of anti-PD-L1 treatment ($P = 0.026$; Fig. 4c). Previous studies have revealed the association of clinical response to ICB therapies with existing biomarkers including high TMB, high PDL1 expression in a number of cancer types [22], supporting our findings that Cluster III patients might derive more benefits from anti-PDL1 treatment.
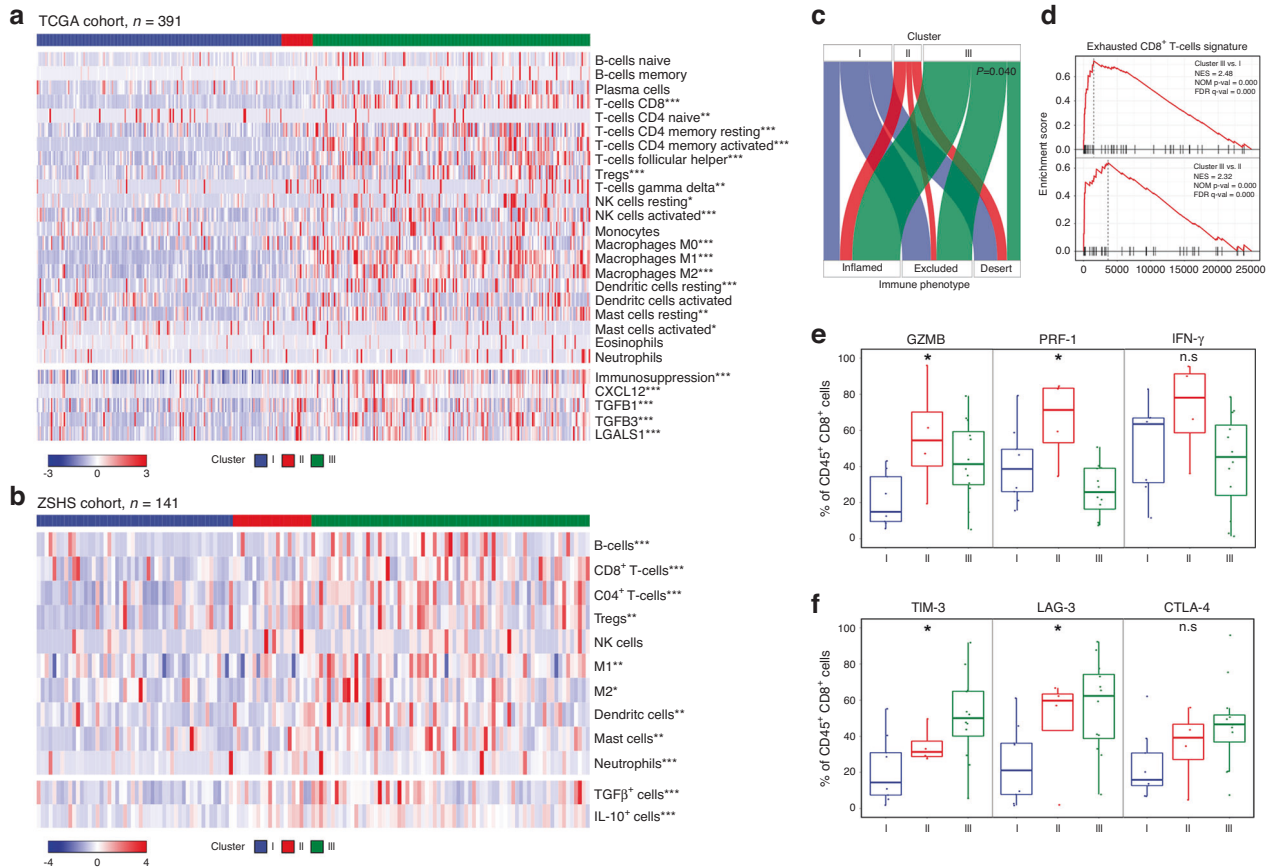
Fig. 3 Characterisation of the immune microenvironment based on TIGIT and PD-1 expression. a Heat-map displaying immune cell compositions demonstrated by CIBERSORT and immunosuppression genes (CXCL12, TGFB1, TGFB3, LGALS1) in TIGIT/PD-1-stratified clusters in TCGA cohort. **b** Heat-map displaying immune cell compositions and immunosuppressive cytokines (TGFβ and IL-10) in TIGIT/PD-1-stratified clusters in ZSHS cohort. **c** Analysis of immunological phenotypes (immune inflamed, immune excluded, immune desert) according to TIGIT/PD-1-stratified clusters in ZSHS cohort. **d** Gene set enrichment analysis (GSEA) plots of gene expression changed in Cluster III compared with Cluster I and II. **e** Expression of effector cytokines (GZMB, PRF-1, IFN-γ) in CD45$^+$ CD8$^+$ T cells among three clusters. **f** Expression of checkpoints (TIM-3, LAG-3, CTLA-4) on CD45$^+$ CD8$^+$ T cells among three clusters. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Kruskal–Wallis H test and Pearson's chi-square test.

DISCUSSION

The expression of immune checkpoint receptors can be of great importance for maintaining immunological balance in TME. For example, the delicate balance between effector and regulatory T cells might be broken without the help of according immune checkpoints because the proper contraction of effector T cell responses and the proper function of Treg cells to control effector T cells are ensured by immune checkpoints expression [23]. TIGIT and PD-1 are immune checkpoints that generally give rise to immune evasion in malignant tumours. Despite the positive correlation between TIGIT and PD-1 expression, the roles of the two co-inhibitory receptors remained obscure. In previous studies, TIGIT was mainly located on Tregs and exhausted T cells and, to a lesser extent, on effector T cells [24]. For PD-1, it was detected at high levels in both exhausted and effector T cells and was recognised as a hallmark for both early antigen-specific T cell activation and later chronic stimulation [25]. This discrepancy indicated that the biologic behaviour and prognostic value were distinct between TIGIT and PD-1. Interestingly, associations between TIGIT or PD-1 expression levels and survival have been reported in MIBC with inconsistent findings [9, 26]. In our research, we categorised MIBC patients into three clusters prognostically based on TIGIT and PD-1 expression in two independent cohorts. PD-1 expression identified a better prognosis subset in TIGIT low patients (Cluster II). TIGIT high subgroup characterised by the worst clinical outcomes, however, could benefit more from

adjuvant chemotherapy and anti-PD-L1 immunotherapy (Cluster III). These findings highlighted the importance of the novel framework for the combination of TIGIT and PD-1 as a potential prognostic and predictive marker for MIBC, and that the immunotherapeutic potential of TIGIT alone or in combination with PD-1/PD-L1 axis was worthy of further exploration.

It has been shown that various immune cell populations could influence efficacy of anti-tumour therapies, including chemotherapy, targeted therapy and immunotherapy [27]. Our previous studies have observed the impact of the component of TME with the resounding success achieved by anti-tumour therapy [28]. In this research, these three clusters possessed distinct immunological phenotypes [17]: i) tumours with decreased immune infiltration (Cluster I, TIGIT^{low}PD-1^{low}), ii) tumours with an immune enriched contexture contributed with infiltrated cytolytic T cells (Cluster II, TIGIT^{low}PD-1^{high}), iii) tumours with an immunosuppressive TME due to abundant exhausted CD8$^+$ T cells (Cluster III, TIGIT^{high}). Despite the fact that T cell exhaustion was associated with overexpression of immune checkpoints, evidence substantiated the association of T cells quantity and function with higher PD-1 expression level [29]. So, it is doubtful whether a binary classification of cells being dysfunctional or not is justified. According to the expression of effector molecules and inhibitory receptors, we supposed that T cell was activated of different degrees among all the clusters, which could be identified as naïve-like, pre-dysfunctional and dysfunctional state [30]. This means
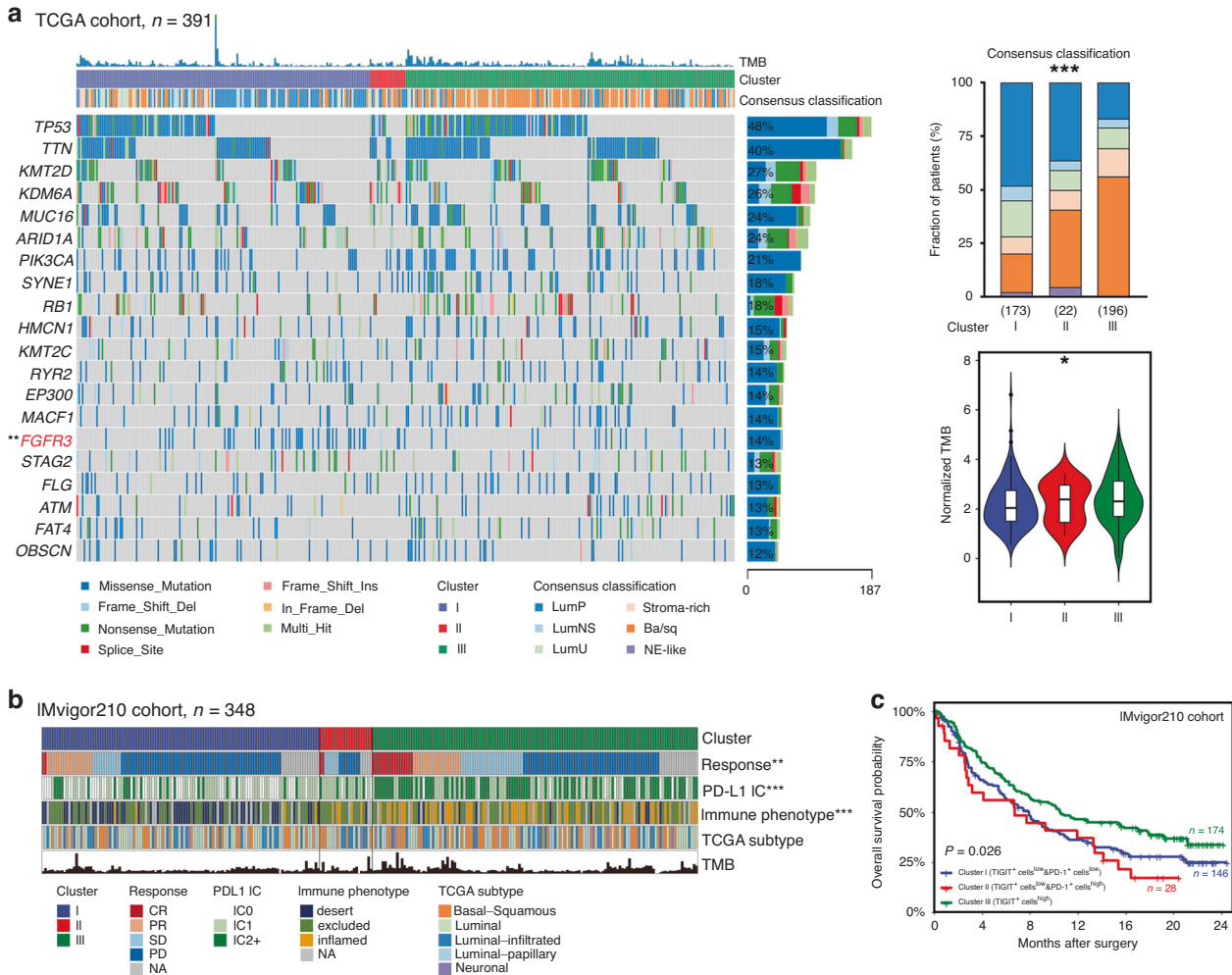
Fig. 4 Stratification based on TIGIT and PD-1 expression correlated with molecular features and immunotherapeutic implication. a The genomic OncoPrint displaying the top 20 most frequently mutated genes ranked by their frequency of mutation and categorised by variant type in patients with MIBC. The upper bar categorises the patients by TIGIT/PD-1-stratified clusters and consensus classification (LumP, LumNS, LumU, Stroma-rich, Ba/sq and NE-like) (left). Analysis of consensus classification and TMB according to TIGIT/PD-1-stratified clusters (right). **b** Relationship between TIGIT/PD-1-stratified clusters and key immunotherapeutic biomarkers in IMvigor210 cohort. **c** Kaplan–Meier curves for overall survival were compared among three clusters in IMvigor210 trial. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Kruskal–Wallis H test and Pearson's chi-square test. Log-rank test was performed for Kaplan–Meier curves. TMB, tumour mutation burden; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; IC, immune cells.

that it is the gradient of cell states rather than discrete populations being consistent with different level of TIGIT and PD-1, which resulted in cells that reside along a continuum of dysfunction. Similarly in hepatocellular carcinoma, PD-1 was only modestly upregulated during the transition between the cytotoxic and exhausted states, whereas TIGIT expression was found to be significantly higher in exhausted subsets [31]. Besides, the presence of TIGIT is shared in Tregs, dysfunctional CD8 T cells and dysfunctional NK cells compared with PD-1 [29, 32], supporting our results that Cluster III patients possessed most inhibitory immune contexture by T-cell suppression and exhaustion while Cluster II patients were shown to generate a highly immunogenic state.

In view of the diversity in cell subtypes and states, an understanding of the effect of systemic therapies on patients with different immune and molecular features is of importance. The molecular characterisation of MIBC demonstrates two major phenotypes, similar to the established subtypes of breast cancer [21, 33]. Luminal tumours are generally immunologically cold with increased overall survival. Nevertheless, despite adverse outcomes in patients with basal subtype, tumours possessed better

responsiveness to both chemotherapy and immunotherapy possibly owing to the inflamed immune contexture [34]. Additionally, TMB has preceded PD-L1 in treating metastatic bladder cancer according to the FDA [35]. Moreover, previous evidence has suggested that patients with high PD-L1 expression and inflamed TME could benefit most from checkpoint blockade [22, 33]. In our study, we found Cluster III patients featured by inflamed immune contexture, higher PD-L1 expression and higher TMB were correlated with basal subtype and benefited more from cisplatin-based chemotherapy and anti-PD-L1 immunotherapy. Cluster I patients with non-inflamed phenotype were positively associated with luminal-like subtype and best survival. Novel biomarker and combinatorial immunomodulatory treatments were of urgent need and were verified by the recent clinical trial involving ICB therapies. Based on the combination of TIGIT and PD-1 expression, we shed light on the integration of immune and molecular classification to refine patient selection in clinical application.

In conclusion, our results firstly proved that TIGIT/PD-1-based stratification with distinct immune and molecular features could be served as an independent companion predictor for clinical

outcome. Furthermore, our novel immune classification based on TIGIT and PD-1 might optimise patient personalised adoption of system therapy involving ACT and PD-L1 inhibitor, which deserved more investigation in future clinical practice.

DATA AVAILABILITY

All data generated that are relevant to the results presented in this article are included in this article. Other data that were not relevant for the results presented here are available from the corresponding author Dr. Xu upon reasonable request.

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AUTHOR CONTRIBUTIONS

Z Liu, H Zeng, K Jin, Y Yu and R You for acquisition of data, analysis and interpretation of data, statistical analysis and drafting of the manuscript; H Zhang, C Liu, X Su, S Yan, Y Chang, L Liu and L Xu for technical and material support; J Xu, Y Zhu and Z Wang for study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding and study supervision. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University (No. B2015-030) and the Ethics Committee of Fudan University Shanghai Cancer Center (No. 050432-4-1212B). Written informed consent was obtained from each patient.

CONSENT TO PUBLISH

Consent for publication was obtained from each author.

COMPETING INTERESTS
The authors declare no competing interests.

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