Prognostic Values of CD38⁺CD101⁺PD1⁺CD8⁺ T Cells in Pancreatic Cancer

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ABSTRACT
Programmed death-1 (PD-1), a key immune checkpoint molecule, has been developed as an oncotherapy target for various carcinomas. However, treatment with anti-PD-1 elicited only a minimal effect in pancreatic ductal adenocarcinoma (PDAC). Subsequent studies revealed the existence of a subset of PD-1⁺ T cells coexpressing CD38 and CD101, representing a fixed dysfunctional subpopulation that are not able to be rescued by anti-PD-1 immunotherapy. However, whether this subpopulation of PD-1 expressing CD8⁺ T cells could be useful in predicting PDAC stage or prognosing survival is unknown. In this study, we used flow cytometry and immunofluorescence assay to analyze the expression of CD38 and CD101 in 183 clinical PDAC samples, including 84 of peripheral blood and 99 of surgical tissues. High coexpression of CD38/CD101 on peripheral PD-1⁺CD8⁺ T cells or tumor-infiltrating lymphocytes (TILs) was found to be most significantly correlated with Tumor/Node/Metastasis (T/N/M) classification and clinical stage, in contrast PD-1⁺CD8⁺ T cells could not correlate with T classification. CD38/CD101 co-repression on TILs also correlated with the poor survival in these PDAC patient samples. Our data suggest that CD38/CD101 might represent a more helpful biomarker than PD-1 alone for diagnosis and prognosis of PDAC.

KEYWORDS Pancreatic ductal adenocarcinoma; PD-1; CD38; CD101; prognosis

Introduction
Pancreatic cancer is currently one of the most aggressive cancers, and features a highly immunosuppressive tumor microenvironment (Kamisawa et al., 2016). In general, this cancer type leads to almost 220,000 deaths per year worldwide, having a 5-year survival rate of only 7–8% (Raimondi et al., 2009, Siegel et al., 2016). The pancreatic ductal adenocarcinoma (PDAC) form develops from noninvasive precursor lesions, with the vast majority originating as pancreatic intraepithelial neoplasias. The poor prognosis of PDAC is
largely due to its clinical silence at the early stage, and the ongoing lack of efficient methods for early diagnosis impedes progress in this realm (Wolfgang et al., 2013).

Surgical resection remains the only potentially curative therapy, but even it does not improve patient prognosis appreciably (Hartwig et al., 2013). PDAC research has thus focused on the promise of immunotherapy. A particular challenge to this approach, however, are the contradictory facts that immunotherapy is based on tumor-specific T cells (Chen and Mellman, 2013) but an immunosuppressive microenvironment, such as in PDAC, facilitates immune escape of tumors (Gajewski et al., 2006, Laheru and Jaffee, 2005). An essential checkpoint factor in the immunosuppressive pathway is the programmed death-1 (PD-1) protein. In general, PD-1 shows a gradual up-regulation in T cells after antigen recognition and activation, serving to restrict the extent of T cell activation, keeping it within normal range (McGray et al., 2014). PD-1 binding to its cognate ligand, PD-L1, will lead to T cell exhaustion. This effect not only suppresses the overall antitumor activity of T cells (McGray et al., 2014) but also exacerbates the immunosuppressive microenvironment, facilitating tumor growth and progression.

The PD-1-targeted therapies developed to date have demonstrated clinical success for multiple cancer types, including PDAC, as they effectively enhance T cell responses (Callahan et al., 2016, Pauken and Wherry, 2015). However, not all patients experience the clinical benefit from anti-PD-1 therapy. In fact, more than 80% of reported patients have shown no response to this therapy, indicating that not all tumor-specific T cells are rescued after the blockade of immunosuppressive PD-1 signaling. Thus, there is a critical need to determine the requirements for optimal T cell rescue; such information will improve current therapies and help in identifying useful predictive biomarkers.

Last year, Philip et al. (2017) reported that mouse tumor-infiltrating lymphocytes (TILs) differentiate through two independent chromatin states, namely a plastic dysfunctional state, from which T cells can be rescued, and a fixed dysfunctional state, in which the cells are resistant to reprogramming by immune checkpoint blockade. Interestingly, the authors also identified CD38 and CD101 as surface markers of some TILs in mouse and human tumors, with their expression being associated with chromatin state. Moreover, expression of these antigens distinguished reprogrammable PD1 hi dysfunctional T cells from non-reprogrammable ones. The subpopulation of PD-1 hi CD8+ TILs with low levels of CD38 and CD101 expression were shown to regain the ability to produce interferon gamma (IFNγ) and tumor necrosis factor alpha upon in-vitro interleukin-15 (IL-15) stimulation; in contrast, the CD38 hi CD101 hi TIL subpopulation did not, suggesting that these markers could potentially be used to identify T cells that are amenable to therapeutic reprogramming in mouse and human tumors (Philip et al., 2017). The data from this study also suggested that these markers could be used for the prognosis of patients after tumor resection.

To this end, we designed the study presented herein to first measure the expression level of CD38 and CD101 on the peripheral PD1+CD8+ T lymphocytes (by flow cytometry) and on the TILs (by immunofluorescence, IF) of PDAC patients. The study then explored the association between CD38/CD101 coexpression and clinicopathological parameters, including survival rate, and sought to confirm whether coexpression of CD38 and CD101 on peripheral PD1+CD8+ T cells or TILs could be used as a novel predictive biomarker for prognosis of PDAC. Collectively, the knowledge gained from this study of human PDAC will provide guidance for future development of selective immunotherapies to treat this disease.
Materials and methods

Ethics statement

The research protocol was reviewed and approved by the Research Ethics Committee of Southwest Hospital, Third Military Medical University. All experiments were conducted in accordance with approved guidelines of Southwest Hospital, Third Military Medical University. All participants provided written informed consent for scientific research and publication of the resultant data (anonymized).

Patients

Peripheral blood samples were obtained from 84 patients with PDAC and 22 healthy controls visiting the Southwest Hospital for treatment and annual checkup, respectively, between January 2017 and January 2018. The sampled PDAC population included 34 females and 50 males, ranging in age from 38-years old to 89-years old. In addition, PDAC surgical resection specimens were purchased from Outdo Biotech (Shanghai, China), in the form of a tissue microarray consisting of 99 patients’ tumor tissues and matched adjacent normal tissues (controls). The tissue microarray was analyzed by IF. The patients’ demographics and tumor pathological diagnoses were analyzed according to seventh edition of AJCC 2017 Tumor/Node/Metastasis (TNM) classification (Table 1).

Flow cytometric analysis

Cells from suspensions of peripheral blood monocytes were stained with anti-human CD3-PerCP (300428, clone: UCHT1; BioLegend, San Diego, USA; eBioscience, San Diego, USA), CD8-FITC (11-0086-42, clone: OKT8; eBioscience), PD-1-PE/Cy7 (329918, clone: EH12.2H7; BioLegend), CD38-PE (12-0388-42, clone: HB7; eBioscience), and CD101-APC (331007, clone: BB27; BioLegend) antibodies, by means of 30-min incubation on ice in the dark. Isotype control antibodies were used to ensure accurate compensation and to set gates. The stained cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, USA; FlowJo 10.0.6 software, Palo Alto, USA), and the data were analyzed by FlowJo 10.0.6 software.

Table 1. Patients’ demographic and tumor characteristics.

| Clinicopathological parameters                  | Peripheral blood samples, n = 84 | Tissue samples, n = 99, incl. 74 deaths |
|------------------------------------------------|----------------------------------|----------------------------------------|
| Sex, male/female                                | 50/34                            | 63/36                                  |
| Age in years                                    | 38–89, median (57.2)             | 34–85, median (59.5)                   |
| Tumor size, ≤2.0 cm/>2.0 cm                     | 36/48                            | 7/92                                   |
| Histological grade, well/poor + moderate        | 22/62                            | 23/76                                  |
| Tumor classification, T1–T2/T3–T4               | 53/31                            | 78/21                                  |
| Node classification, N0/N1                      | 39/45                            | 55/44                                  |
| Distant metastasis, M0/M1                       | 40/44                            | 79/20                                  |
| Clinical stage, I–II/III–IV                     | 37/47                            | 54/45                                  |
**IF analysis**

Colocalization of CD38 and CD101 was assessed by IF staining of the tissue microarrays. Briefly, the paraffin-embedded tissue microarrays were first dewaxed and rehydrated through a gradient alcohol series; next, endogenous peroxidase activity was blocked and antigen retrieval was performed (heating to 95–98°C in citric acid buffer, pH 6.0, for 15 min). The subsequent blockade of nonspecific sites (by goat serum) was followed by the IF staining, which was carried out by overnight coincubation with mouse anti-CD38 antibody (ZM-0422, clone: SPC32; ZSGB-BIO, Beijing, China; Bioss Antibodies Inc, Beijing, China) and rabbit anti-CD101 antibody (bs-10727R, polyclonal; Bioss Antibodies Inc.) in working solution of 1:200 dilution. Detection was carried out the next morning with donkey anti-mouse IgG H&L Alexa Fluor 555 (A-31570; Invitrogen, San Diego, USA) and goat anti-rabbit IgG H&L Alexa Fluor 488 (A-11008; Invitrogen). IF-negative controls were generated by use of PBS in lieu of the primary antibodies.

The tissue distributions of CD38 and CD101 and their colocalization were digitally photographed using an optical microscope (BX51; Olympus, Tokyo, Japan). This step was performed by two authors (Yi Zhang and Jian Zhou), blinded to the clinical data and who were working independently. Image-ProPlus software (Media Cybernetics, Inc., Rockville, USA) was employed to quantitatively evaluate positive staining, using at least five high-power fields (10 × eyepiece and 20 × objective) for each sample assessed. The number of CD38+CD101+ T cells was calculated in each field and the averages were compared.

**Statistical analysis**

All data were analyzed by SPSS Statistics software (v24.0; IBM Corp., New York, USA) and GraphPad Prism software (v6.0), San Diego, USA. The patients were divided into high- and low-expression groups according to the median value. Student’s t-test was used to analyze the comparisons between two groups, and the χ2 test was used to analyze the correlations between each cell subset and clinicopathologic parameters in PDAC patients. Receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were used to compare the discrimination capacity for each of the CD8+PD-1+ T cell subsets. Survival analyses were carried out by the Kaplan–Meier method and log-rank test. Multivariate analyses were performed by the Cox proportional hazards regression model. p Values less than 0.05 were considered statistically significant.

**Results**

**CD38/CD101 were markedly up-regulated on peripheral PD-1+CD8+ T cells in PDAC patients**

PD-1, an essential checkpoint factor in the immunosuppressive pathway and an acceptable marker of immune exhaustion, has been reported as over-expressed in PDAC patients compared to healthy donors (Shen et al., 2017). Accordingly, we first verified the expression of PD-1 on peripheral CD8+ T lymphocytes from our study participants with PDAC by flow cytometry with the indicated gating strategy (Figure 1a). The PDAC patients showed significantly higher levels of PD-1 expression on the CD8+ T cells than the healthy donors (Figure 1b). Further examination of the expression of CD38 and CD101 on PD-1+CD8+ T cells indicated that though
the frequency of CD38\(^{-}\)CD101\(^{+}\)PD-1\(^{+}\)CD8\(^{+}\) T cells was higher in PDAC patients, the CD38\(^{-}\)CD101\(^{+}\)PD-1\(^{+}\)CD8\(^{+}\) T cells were dramatically increased in the PDAC patient samples, as compared with the samples from healthy controls. However, CD38\(^{-}\)CD101\(^{-}\)PD-1\(^{+}\)CD8\(^{+}\) T cells were dramatically decreased in the PDAC patient samples. In addition, we did not observe the statistical difference of the frequency of CD38\(^{-}\)CD101\(^{-}\)PD-1\(^{-}\)CD8\(^{+}\) T cells between PDAC patients and the control (Figure 1c).
Clinical significance of CD38/CD101 and PD-1 expression on peripheral CD8^+ T cells in PDAC patients

To determine the clinical significance of each PD-1^+CD8^+ T subset with or without CD38 and CD101 expression in PDAC patients, a retrospective analysis was performed. The chi-square test was used to assess correlations between the frequency of each subset and clinicopathological parameters, including age, sex, histological stage, tumor size, tumor site, T/N/M stage, and clinical stage. Results indicated that none of the PD-1^+CD8^+ T cell subsets correlated with age, sex, histological stage, tumor size, or tumor site among the PDAC patients. Moreover, among all subsets, the coexpression of CD38/CD101 in PD-1^+CD8^+ T lymphocytes was the most significantly correlated subset with T classification, N classification, M classification, and clinical stage (p < 0.05) (Table 2 and Table S1). Though PD-1 expression on CD8^+ T cells was also markedly correlated with N/M classifications and clinical stage, it did not correlate with T classification (Table 2).

For each subset of the PD-1^+CD8^+ T lymphocytes, we also performed ROC analysis to compare their clinicopathological significance; the ROC curves for PD-1^+CD8^+ T cells and PD-1^+CD38^+CD101^+CD8^+ T cells are shown in Figure S1, but the ROC curves for other subsets are not shown. The AUC was determined to further assess and compare the correlation significance of each subset with PDAC T/N/M classifications and clinical stage (Table S2).

Table 2. Correlation of PD-1 expression or PD-1/CD38/CD101 coexpression with clinical parameters in PDAC patients.

| Clinicopathological parameters | PD-1 (MV = 12.32%) | PD-1/CD38/CD101 (MV = 15.90%) |
|-------------------------------|--------------------|---------------------------------|
| Age (years)                   |                    |                                 |
| <65                           | 53 24 29           | 27 26                           |
| ≥68                           | 31 18 13           | 15 16                           |
| Gender                        |                    |                                 |
| Male                          | 50 28 22           | 26 24                           |
| Female                        | 34 14 20           | 16 18                           |
| Tumor site                    |                    |                                 |
| Head                          | 57 29 28           | 31 26                           |
| Body + tail                   | 27 13 14           | 11 16                           |
| Histological grade            |                    |                                 |
| Well                          | 22 10 12           | 14 8                            |
| Poor + moderate               | 62 32 30           | 28 34                           |
| Tumor size (cm)               |                    |                                 |
| ≤2.0                          | 36 21 15           | 22 14                           |
| >2.0                          | 48 21 27           | 20 28                           |
| Tumor classification          |                    |                                 |
| T1 – T2                       | 53 30 23           | 33 20                           |
| T3 – T4                       | 31 12 19           | 9 22                            |
| Node classification           |                    |                                 |
| N0                            | 39 26 13           | 25 14                           |
| N1                            | 45 16 29           | 17 28                           |
| Distant metastasis            |                    |                                 |
| M0                            | 40 28 12           | 27 13                           |
| M1                            | 44 14 30           | 15 29                           |
| Clinical stage (AJCC)         |                    |                                 |
| I – II                        | 37 26 11           | 27 10                           |
| III – IV                      | 47 16 31           | 15 32                           |

Analyzed by χ² test; MV = median value; * represent Significant differences (p < 0.05) are indicated in bold.
As compared to other subsets, peripheral PD-1+CD38+CD101+CD8+ T cell percentages have the best ability for discriminating T/N/M classifications and clinical stage of PDAC, with an associated AUC equal to 0.912, 0.931, 0.861, and 0.848, respectively. All these results suggest, the increased CD38/CD101 coexpression in peripheral PD-1+CD8+ T cells might be involved in immune escape and metastasis of PDAC.

**CD38/CD101 expression on TILs in PDAC patients**

TILs serve not only as predictors of cancer prognosis but also as therapeutic targets, and have potential as biomarkers of response to cancer immunotherapy (Lee et al., 2016). Considering that CD38 and CD101 are mainly expressed on T cells, indicating activation of such (Gouttefangeas et al., 1994, McGray et al., 2014, Quarona et al., 2013), and that PD-1 is over-expressed on TILs in cancer patients, we examined the coexpression of CD38 and CD101 by IF staining to first generally examine the presence of CD38+CD101+PD-1+ T cells in PDAC as compared to healthy tissues. Specifically, we examined the expression of CD38 and CD101 in TILs using double-IF staining of 99 pairs of resection samples (tumor tissues and matched adjacent nontumor tissues) from PDAC patients (representing 36 females and 63 males, with age range of 34–85 years). The results indicated that CD38 and CD101 proteins were mainly expressed on the membrane of TILs, with some displaying colocalization on the membrane; in contrast, there were almost no CD38+CD101+ lymphocytes infiltrated in the matched adjacent nontumor tissues (Figure 2a). Furthermore, those tumor tissues having higher clinical stage showed more infiltrated CD38+CD101+ cells than those at lower clinical stage (Figure 2b). Statistical analysis showed that the CD38+CD101+ TIL counts per five microscopic fields in patients were 11.9 ± 6.82 (mean ± SD) with clinical stage III–IV, as compared to 3.27 ± 2.97 in those with clinical stage I–II (Figure 2b; p < 0.05).

These data suggested that the level of coexpression of CD38/CD101 in TILs was much higher in PDAC terminal-stage tissues than in early-stage PDAC tissues. Furthermore, the chi-square test was used to assess correlations between the CD38/CD101 coexpression level and clinicopathological parameters. Consistent with the results in peripheral blood, the coexpression level of CD38/CD101 in PDAC tissues was largely significantly correlated with T classification, N classification, M classification, and clinical stage (Table 3; p < 0.05). No significant correlations were observed between proportions of each subset and age, sex, histological stage, tumor size, or tumor site.

**Prognostic significance of CD38/CD101 coexpression on TILs in PDAC patients**

To determine the prognostic value of CD38/CD101 coexpression for PDAC patients, we used the Kaplan–Meier method and log-rank test to analyze the relationship between the coexpression and clinical follow-up information of 74 died patients (Table 1). The patients were divided into high- and low-expression groups, according to the median value of CD38/CD101 coexpressing cells count from above IF staining assay. Results showed that high CD38/CD101 expression was associated with short overall survival (log-rank: p < 0.001), indicating an association with poor prognosis (Figure 3a). Furthermore, we examined the correlation between CD38/CD101 coexpression and overall survival in PDAC patients with different clinical stages. Kaplan–Meier and log-rank test analyses showed that higher level of CD38/CD101 coexpression correlated with
shorter overall survival, independent of clinical stage (Figure 3b). In addition, a multivariate survival analysis by Cox proportional hazard modeling confirmed that CD38/CD101 expression, T classification, N classification, M classification, and clinical stage were independent predictors of overall survival in the 99 PDAC patients examined by microarray (Table 4). These results further indicated that increased CD38/CD101 coexpression on TILs was an adverse factor in PDAC, suggesting its potential as a novel predictor for prognosis in patients with PDAC.

Discussion

Over-expression of PD-1 by cancer antigen-reactive T cells has been noted in a number of human solid cancers, including melanoma and non-small cell lung cancer (Ma et al., 2017), and PD-1 targeted treatments have shown promising clinical perspectives in these cancer types (Callahan et al., 2016, Pauken and Wherry, 2015). PD-1 over-expression has also been
detected in PDAC; its general role as an essential checkpoint factor in the immunosuppressive pathway makes it of interest for pathogenic and therapeutic studies of this cancer type (Shepard and Bonney, 2013). Shen et al. (2017) have already demonstrated that high and refractory expression of PD-1 on CD8+ T cells, in both TILs and peripheral blood, is associated with poor survival of pancreatic cancer patients. In addition, PD-1/PD-L1

Table 3. Correlation of CD38/CD101 coexpression on TILs in tumor tissues of PDAC patients with major clinical parameters.

| Clinicopathological parameters | n       | Low | High | p Value |
|-------------------------------|---------|-----|------|---------|
| Age(years)                    |         |     |      |         |
| <65                           | 58      | 25  | 33   | 0.130   |
| ≥65                           | 41      | 24  | 17   |         |
| Gender                        |         |     |      |         |
| Male                          | 63      | 32  | 31   | 0.732   |
| Female                        | 36      | 17  | 19   |         |
| Tumor site                    |         |     |      |         |
| Head                          | 65      | 30  | 35   | 0.358   |
| Body + tail                   | 34      | 19  | 15   |         |
| Histological grade            |         |     |      |         |
| Well                          | 23      | 8   | 15   | 0.107   |
| Poor + moderate               | 76      | 41  | 35   |         |
| Tumor size(cm)                |         |     |      |         |
| ≤2.0                          | 7       | 4   | 3    | 0.675   |
| >2.0                          | 92      | 45  | 47   |         |
| Tumor classification          |         |     |      |         |
| T1 – T2                       | 78      | 43  | 35   | 0.031*  |
| T3 – T4                       | 21      | 6   | 15   |         |
| Node classification           |         |     |      |         |
| N0                            | 55      | 33  | 22   | 0.019*  |
| N1                            | 44      | 16  | 28   |         |
| Distant metastasis            |         |     |      |         |
| M0                            | 79      | 45  | 34   | 0.003*  |
| M1                            | 20      | 4   | 16   |         |
| Clinical stage(AJCC)          |         |     |      |         |
| I – II                        | 54      | 35  | 19   | 0.001*  |
| III – IV                      | 45      | 14  | 31   |         |

Analyzed by χ2 test; MV = median value; * represent significant differences (p < 0.05) are indicated in bold

Figure 3. Survival rate analysis. (a). High coexpression level of CD38/CD101 on TILs in tumor tissue was significantly correlated with poor survival of PDAC patients. (b). Correlation between CD38/CD101 coexpression and overall survival rate in PDAC patients was independent of clinical stage.
signaling has prompted research interest for its potential as a predictor of prognosis for PDAC patients (Geng et al., 2008, Okudaira et al., 2009, Shen et al., 2017). Indeed, it has already been demonstrated that blockade of PD-L1 in a mouse model can efficiently inhibit pre-established pancreatic cancer by increasing IFN-γ production and decreasing IL-10 production (Loos et al., 2008, Okudaira et al., 2009), providing rationale for development of an immunotherapy targeting the PD-1/PD-L1 pathway to treat pancreatic cancer. The subsequent clinical trials, however, have indicated that such (mono)therapy has a poor efficacy (Feng et al., 2017). Theories on the mechanism(s) underlying this limited efficacy include immunosuppression caused by a high tumor burden and the intrinsic nonimmunogenicity of pancreatic cancer (Feng et al., 2017).

The latest reviews suggest that anticancer immunity can be classified into three phenotypes (Chen and Mellman 2017, Herbst et al., 2014, Kim and Chen, 2016): the immune-desert phenotype; the immune-excluded phenotype; and the inflamed phenotype. The immune-desert and immune-excluded phenotypes are both considered noninflamed phenotypes; noninflammatory tumors generally express cytokines involved in immune suppression or tolerance. Almost all reported PDAC samples have showed the noninflamed phenotypes; moreover, the functional immune cells in pancreatic cancer parenchyma are insufficient, though there may be abundant immune cells penetrating into the stroma surrounding a nest of pancreatic cancer cells (Chen and Mellman 2017, Feng et al., 2017). For instance, the CD8⁺ TILs may also fall into a dysfunctional state, such as occurs in hyperexhaustion, and this might partly explain the failure of anti-PD-1/PD-L1 therapy in PDAC. Thus, how to discriminate whether exhausted CD8⁺ TILs could be rescued or the dysfunctional state could be reversed to provide antitumor immunity is a key objective of research into PDAC immunotherapy development.

Table 4. Multivariate Cox regression analysis of prognostic parameters for survival in PDAC patients.

| Prognostic parameter | Multivariate analysis |
|----------------------|----------------------|
|                      | HR       | 95%CI     | p       |
| Age                  |          |          |         |
| <65 vs. ≥65          | 1.059    | 0.629–1.784 | 0.829   |
| Sex                  |          |          |         |
| Male vs. female      | 1.088    | 0.538–1.882 | 0.817   |
| Tumor site           |          |          |         |
| Body + tail vs. head | 0.699    | 0.417–1.171 | 0.174   |
| Histological grade   |          |          |         |
| Well vs. poor + moderate | 0.893  | 0.533–1.492 | 0.666   |
| Tumor size           |          |          |         |
| ≤2.0 vs. >2.0        | 0.953    | 0.645–1.629 | 0.862   |
| CD38/CD101 coexpression |        |          |         |
| Low vs. high         | 1.957    | 1.020–2.577 | 0.002   |
| Tumor classification  |          |          |         |
| T3 – T4 vs. T1 – T2  | 1.853    | 1.085–3.179 | 0.024   |
| Node classification   |          |          |         |
| N1 vs. N0            | 2.228    | 1.284–3.866 | 0.004   |
| Metastasis classification |      |          |         |
| M1 vs. M0            | 2.050    | 1.129–4.765 | 0.018   |
| Clinical stage        |          |          |         |
| III – IV vs. I – II  | 3.046    | 1.539–6.029 | 0.001   |

Significant differences (p < 0.05) are indicated in bold. HR: hazard ratio.
Recently, two research groups reported focused studies on the multiplicity of PD-1 expressing CD8\(^+\) TILs (Philip et al., 2017, Schietinger et al., 2016). The collective results indicated that the PD-1\(^-\)CD8\(^+\) TILs could be divided into two functional groups, according to the expression levels of CD38 and CD101 (i.e. CD38\(^\text{low}\)CD101\(^\text{low}\) and CD38\(^\text{hi}\)CD101\(^\text{hi}\) PD-1\(^-\)CD8\(^+\) T cells). Intriguingly, the cytolytic function of the CD38\(^\text{low}\)CD101\(^\text{low}\) cells could be rescued by in-vitro stimulation, while that of the CD38\(^\text{hi}\)CD101\(^\text{hi}\) cells could not. Thus, CD38 and CD101 may be useful for identifying T cells that are amenable to therapeutic reprogramming in mouse and human tumors, and their expression levels on PD-1 expressing CD8\(^+\) T cells might reflect immunological status, suggesting their potential as novel biomarkers for prognosis of PDAC.

The results from our present study agreed with the literature (Geng et al., 2008, Okudaira et al., 2009, Shen et al., 2017), in that significant correlations were found between PD-1 expression and a clinico-pathological parameter; specifically, in our study, the correlation involved N/M classifications and clinical stage, but not T classification. However, when we further explored the multiplicity of PD-1\(^-\)CD8\(^+\) T cells according to the expression of CD38 and CD101, we found a dramatically increased expression of CD38\(^\text{hi}\)CD101\(^\text{hi}\)PD-1\(^-\)CD8\(^+\) T cells and a markedly decreased expression of CD38\(^\text{low}\)CD101\(^\text{low}\)PD-1\(^-\)CD8\(^+\) T cells for peripheral blood of PDAC patients. Moreover, the coexpression of CD38/CD101 in PD-1\(^-\)CD8\(^+\) T lymphocytes was most significantly correlated with T/N/M classifications and clinical stage. When we compared the clinico-pathological significance of each PD-1\(^-\)CD8\(^+\) T subset, we found that the peripheral CD38\(^\text{hi}\)CD101\(^\text{hi}\)PD-1\(^-\)CD8\(^+\) T cell percentages had the best ability to discriminate T/N/M classifications and clinical stage. IF staining of the tissue microarrays further confirmed the coexpression of CD38/CD101 on TILs of PDAC patients. Although CD38/CD101 dual staining could not warrant their unique expression on PD-1\(^-\)CD8\(^+\) TILs, however, what’s interesting is that the survival rate analysis revealed that patients with high frequency of CD38\(^\text{hi}\)CD101\(^\text{hi}\) TILs had significantly poorer overall survival, as compared with low-frequency patients. Multivariate analysis further supported the notion that CD38 and CD101 proteins (coexpression) could represent an independent risk factor of poor prognosis. Taken together, all the results from our present study suggest CD38/CD101 as a better progression indicator and prognosis predictor than PD-1 alone in PDAC.

CD38 is a 45-kDa transmembrane glycoprotein with ectoenzymatic activity. As a surface antigen that is expressed or re-expressed on T cells, it is an activation marker of T cells (de Carvalho et al., 2016, Quarona et al., 2013). CD101, on the other hand, is a 240-kDa human cell-surface type-1 glycoprotein, the expression of which is positively modulated following lymphocyte activation (Gouttefangeas et al., 1994). Very few studies to date have reported on the effects of CD38/CD101 on CD8\(^+\) T cells. However, considering the accumulated data and our experimental data, we speculate that the sustained expression of CD38 and CD101 might indicate the exhaustion status of CD8\(^+\) T cells. For instance, in rheumatoid arthritis, CD8\(^\text{hi}\)CD101\(^-\) T cells exhibited greater cytotoxic activity than CD8\(^\text{hi}\)CD101\(^+\) T cells (Boulouc et al., 2000, Jovanovic et al., 2011). PD-1 is an accepted marker of immune cell exhaustion, and its coexpression with CD38 has been used for predicting the exhaustion level of CD8\(^+\) T cells in human immunodeficiency virus-infected patients who exhibited increased levels of exhausted CD8\(^+\) T cells (CD8\(^+\)CD38\(^+\)HLA-DR\(^+\)PD-1\(^+\)) (Eckard et al., 2016). Therefore, the coexpression of CD38 and CD101 with PD-1 in CD8\(^+\) T cells observed in this study probably reflects ultimately exhausted T cells having a fixed dysfunctional state; as such, marked increase of this subpopulation would predict poor prognosis after reaction.
surgery. This notion is further supported by our data in TILs, which showed that the level of CD38/CD101 coexpression was much higher in terminal-stage PDAC tissues than in early-stage PDAC tissues.

Along with the significantly increased CD38^+CD101^+PD-1^-CD8^+ T cells, we also observed a dramatic decrease in the frequency of CD38^-CD101^-PD-1^-CD8^+ T cells in PDAC patients compared with healthy controls. This subpopulation was reported as capable of being rescued by in-vitro stimulation and anti-PD-1 therapy (Philip et al., 2017, Schietinger et al., 2016). Nonetheless, we did not find any statistically significant correlation between this subpopulation and PDAC clinicopathological parameters. It is possible that the reprogrammable CD38^-CD101^-PD-1^-CD8^+ T cells, together with the non-reprogrammable CD38^-CD101^-PD-1^-CD8^+ T cells, are suppressed under the tumor microenvironment (Philip et al., 2017, Schietinger et al., 2016). In such a case, the potential reprogrammable ability of CD38^-CD101^-PD-1^-CD8^+ T cells in PDAC patients would only be demonstrated with PD-1/PD-L1 immunotherapy. Therefore, high frequency of CD38^-CD101^-PD-1^-CD8^+ T cells in tumor patients might be a predictive candidate for immune checkpoint therapy, rather than a predictive marker for PDAC clinical parameters.

In conclusion, this study first demonstrated that the high coexpression of CD38/CD101 on PD-1^-CD8^+ T cells was most significantly correlated with T/N/M classifications, clinical stage, and poor survival in PDAC patients, in contrast PD-1 expression alone could not correlate with T classification, in both TILs and peripheral blood cells. These results indicate that CD38/CD101 protein coexpression might represent a more useful predictor of diagnosis and prognosis for PDAC patients than PD-1 alone.

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ZM carried out the flow cytometry and immunofluorescence staining assays. YJ, ZY, LY, and ZJ performed the clinical data collection and statistical analysis. GW and WH were responsible for clinical sample collection. NB and RZ designed the study and drafted the manuscript. All authors read and approved the final manuscript.

**Competing interests**

All authors declare no financial or commercial conflict of interest.

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References

Bouloc A, Boulland ML, Geissmann F, et al. (2000). CD101 expression by Langerhans cell histiocytosis cells. Histopathology, 36(3), 229–232.

Callahan MK, Postow MA, Wolchok JD. (2016). Targeting T cell co-receptors for cancer therapy. Immunity, 44(5), 1069–1078. doi: 10.1016/j.immuni.2016.04.023.

Chen DS, Mellman I. (2013). Oncology meets immunology: the cancer-immunity cycle. Immunity, 39(1), 1–10. doi: 10.1016/j.immuni.2013.07.012.

Chen DS, Mellman I. (2017). Elements of cancer immunity and the cancer-immune set point. Nature, 541(7637), 321–330. doi: 10.1038/nature21349.

de Carvalho PG, de Oliveira Rodrigues R, Ribeiro Da Silva SF, et al. (2016). CD38+CD8+ and CD38+CD4+ T cells and IFN gamma (+874) polymorphism are associated with a poor virological outcome. Immunol Invest, 45(4), 312–327. doi: 10.3109/08820139.2016.1157603.

Eckard AR, Rosebush JC, Lee ST, et al. (2016). Increased immune activation and exhaustion in HIV-infected youth. Pediatr Infect Dis J, 35(12), e370–e377. doi: 10.1097/INF.0000000000001326.

Feng M, Xiong G, Cao Z, et al. (2017). PD-1/PD-L1 and immunotherapy for pancreatic cancer. Cancer Lett, 407, 57–65. doi: 10.1016/j.canlet.2017.08.006.

Gajewski TF, Meng Y, Harlin H. (2006). Immune suppression in the tumor microenvironment. J Immunother, 29(3), 233–240. doi: 10.1097/01.cji.0000199193.29048.56.

Geng L, Huang D, Liu J, et al. (2008). B7-H1 up-regulated expression in human pancreatic carcinoma tissue associates with tumor progression. J Cancer Res Clin Oncol, 134(9), 1021–1027. doi: 10.1007/s00432-008-0364-8.

Gouttefangeas C, Jacquot S, Meder E, et al. (1994). Differential proliferative responses in subsets of human CD28+ cells delineated by BB27 mAb. Int Immunol, 6(3), 423–430.

Hartwig W, Werner J, Jager D, et al. (2013). Improvement of surgical results for pancreatic cancer. Lancet Oncol, 14(11), e476–e485. doi: 10.1016/S1470-2045(13)70172-4.

Herbst RS, Soria JC, Kowanetz M, et al. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature, 515(7528), 563–567. doi: 10.1038/nature14011.

Jovanovic DV, Boumsell L, Bensussan A, et al. (2011). CD101 expression and function in normal and rheumatoid arthritis-affected human T cells and monocytes/macrophages. J Rheumatol, 38(3), 419–428. doi: 10.3899/jrheum.100676.

Kim JM, Chen DS. (2016). Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). Ann Oncol, 14(11), e476–e485. doi: 10.1093/annonc/mdw217.

Herbst RS, Soria JC, Kowanetz M, et al. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature, 515(7528), 563–567. doi: 10.1038/nature14011.

Jovanovic DV, Boumsell L, Bensussan A, et al. (2011). CD101 expression and function in normal and rheumatoid arthritis-affected human T cells and monocytes/macrophages. J Rheumatol, 38(3), 419–428. doi: 10.3899/jrheum.100676.

Kamisawa T, Wood LD, Itoi T, Takaori K. (2016). Pancreatic cancer. Lancet, 388(10039), 73–85. doi: 10.1016/S0140-6736(16)00141-0.

Kim JM, Chen DS. (2016). Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). Ann Oncol, 27(8), 1492–1504. doi: 10.1093/annonc/mdw217.

Laheru D, Jaffee EM. (2005). Immunotherapy for pancreatic cancer – science driving clinical progress. Nat Rev Cancer, 5(6), 459–467. doi: 10.1038/nrc1630.

Lee N, Zakka LR, Mihm Jr. MC, Schatton T. (2016). Tumour-infiltrating lymphocytes in melanoma prognosis and cancer immunotherapy. Pathology, 48(2), 177–187. doi: 10.1016/j.pathol.2015.12.006.

Loos M, Giese NA, Kleeff J, et al. (2008). Clinical significance and regulation of the costimulatory molecule B7-H1 in pancreatic cancer. Cancer Lett, 268(1), 98–109. doi: 10.1016/j.canlet.2008.03.056.

Ma H, Mao G, Zhang G, Huang H. (2017). The expression and clinical signification of PD-1 in lymph nodes of patients with non-small cell lung cancer. Immunol Invest, 46(7), 639–646. doi: 10.1080/08820139.2017.1341521.

McGray AJ, Hallett R, Bernard D, et al. (2014). Immunotherapy-induced CD8+ T cells instigate immune suppression in the tumor. Mol Ther, 22(1), 206–218. doi: 10.1038/mt.2013.255.

Okudaira K, Hokari R, Tsuzuki Y, et al. (2009). Blockade of B7-H1 or B7-DC induces an anti-tumor effect in a mouse pancreatic cancer model. Int J Oncol, 35(4), 741–749.

Pauken KE, Wherry EJ. (2015). Overcoming T cell exhaustion in infection and cancer. Trends Immunol, 36(4), 265–276. doi: 10.1016/j.it.2015.02.008.
Philip M, Fairchild L, Sun L, et al. (2017). Chromatin states define tumour-specific T cell dysfunction and reprogramming. Nature, 545(7655), 452–456. doi: 10.1038/nature22367.

Quarona V, Zaccarello G, Chillemi A, et al. (2013). CD38 and CD157: a long journey from activation markers to multifunctional molecules. Cytometry B Clin Cytom, 84(4), 207–217. doi: 10.1002/cyto.b.21092.

Raimondi S, Maisonneuve P, Lowenfels AB. (2009). Epidemiology of pancreatic cancer: an overview. Nat Rev Gastroenterol Hepatol, 6(12), 699–708. doi: 10.1038/nrgastro.2009.177.

Schietinger A, Philip M, Krisnawan VE, et al. (2016). Tumor-specific T cell dysfunction is a dynamic antigen-driven differentiation program initiated early during tumorigenesis. Immunity, 45(2), 389–401. doi: 10.1016/j.immuni.2016.07.011.

Shen T, Zhou L, Shen H, et al. (2017). Prognostic value of programmed cell death protein 1 expression on CD8+ T lymphocytes in pancreatic cancer. Sci Rep, 7(1), 7848. doi: 10.1038/s41598-017-08479-9.

Shepard MT, Bonney EA. (2013). PD-1 regulates T cell proliferation in a tissue and subset-specific manner during normal mouse pregnancy. Immunol Invest, 42(5), 385–408. doi: 10.3109/08820139.2013.782317.

Siegel RL, Miller KD, Jemal A. (2016). Cancer statistics, 2016. CA Cancer J Clin, 66(1), 7–30. doi: 10.3322/caac.21332.

Wolfgang CL, Herman JM, Laheru DA, et al. (2013). Recent progress in pancreatic cancer. CA Cancer J Clin, 63(5), 318–348. doi: 10.3322/caac.21190.