Low immune index correlates with favorable prognosis but with reduced benefit from chemotherapy in gallbladder cancer

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Abstract
Use of immune index is a new potential approach for cancer classification and prediction. To investigate the status and clinical effect of immune index in gallbladder cancer (GBC), 238 GBC patients from Zhongshan Hospital affiliated to Fudan University were involved in the present study, including 113 patients in a training set and 125 patients in a validation set. Five immune cells (macrophages, neutrophils, regulatory T cells, cytotoxic T cells and mast cells) were selected based on a literature review and the immune index for each patient was calculated using the LASSO regression. A low immune index (<1) was defined as immunotype A and a high immune index (≥1) was defined as immunotype B. The 5-year overall survival rate for immunotype A was higher than that for immunotype B in the training set and the validation set (70.0% vs 37.0%, \( P < 0.001 \); 68.9% vs 47.5%, \( P = 0.002 \); respectively). Moreover, the immune index showed higher prediction efficiency compared with all the single immune cells which we selected. When combined with the immune index, the areas under the curve (AUC) of the TNM staging system in both sets were elevated from 0.677 to 0.787 and from 0.631 to 0.694, respectively. Interestingly, gemcitabine-based chemotherapy only benefits stage II patients of immunotype B and stage III patients of both immunotype A and immunotype B (\( P = 0.015, P = 0.030, P = 0.011 \); respectively) but does not work in stage II patients of immunotype A (\( P = .307 \)). Taken together, the immune index could effectively predict prognosis and the benefits of gemcitabine-based chemotherapy and might improve on the TNM staging system.

KEYWORDS
chemotherapy, gallbladder cancer, immune cells, immune index, tumor microenvironment

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INTRODUCTION

Gallbladder cancer (GBC) is the sixth most common type of digestive tract carcinoma and the most common type of biliary cancer but is still a relatively infrequent malignancy, with a morbidity rate of 1/100,000-23/100,000.\(^1\)\(^-\)\(^2\) However, the incidence of GBC has steadily risen at the rate of 4% in the past 10 years.\(^3\) GBC is also a highly lethal disease, with a 5-year survival rate of 5%-10%. Complete surgical resection is the only curative therapy for GBC,\(^4\) with only 10% of GBC patients (with early-stage disease) having the opportunity to receive complete surgical resection.\(^5\) Therefore, chemotherapy is critical for GBC patients. Gemcitabine-based chemotherapy is the first-line treatment for advanced GBC patients in the National Comprehensive Cancer Network (NCCN) Guidelines.\(^6\)\(^,\)\(^7\) Nevertheless, some studies report the negative impacts of gemcitabine-based chemotherapy and potential shorter survival of GBC patients.\(^8\) The precise mechanism is unknown at present, but a practical risk stratification model for GBC to predict the prognosis after surgical resection and the benefit from gemcitabine-based chemotherapy is urgently needed.

The most widely used model for GBC is the TNM staging system published by the American Joint Committee on Cancer (AJCC). The TNM staging system classifies GBC patients by the extent and size of the primary tumor (T), the involvement of regional lymph nodes (N), and the absence or presence of distant metastases (M).\(^9\) Although the TNM staging system has been validated and accepted worldwide, heterogeneous prognosis still exists for each stage. With the increasing knowledge of the tumor microenvironment (TME) and the rapid development of tumor immunology, the crucial role of tumor-infiltrating immune cells in the TME is becoming clearer, improving the predictive efficiency of the TNM staging system.

As an essential hallmark of cancer, inflammation is the most common risk factor of GBC, which primarily presents as a gallbladder stone.\(^1\)\(^,\)\(^2\)\(^,\)\(^10\)\(^,\)\(^11\) Inflammation inside tumors is induced by tumor-infiltrating immune cells, which constitute the TME together with tumor-associated fibroblasts (TAF) and vascular endothelial cells (VEC).\(^12\)\(^-\)\(^14\) The tumor-infiltrating immune cells in the TME mainly include lymphoid lineage cells such as T cells and B-cells, and myeloid lineage cells such as macrophages, neutrophils and mast cells (MC).\(^12\)\(^-\)\(^14\) These immune cells could communicate with each other and play a promotive or contrary function on the initiation, growth and invasion of tumor cells, directly or indirectly.\(^15\)\(^-\)\(^19\) Furthermore, some research suggests that the immune elements in TME could modulate chemotherapy response.\(^20\)\(^-\)\(^22\)

In consideration of the important value of tumor-infiltrating immune cells, we evaluated the different kinds of immune cells, constructed an immune index model using multiple linear regression and explored its prognostic value for clinical outcomes, especially for gemcitabine-based chemotherapy. Our work illustrates the importance of tumor-infiltrating immune cells and develops a promising system to predict prognosis and chemotherapy benefit in GBC.

PATIENTS AND METHODS

2.1 Study design

The flow chart of the study is shown in Figure 1. This study was approved by the Institutional Review Board (IRB) of Fudan University Zhongshan Hospital and informed consent was obtained from each patient. A total of 238 consecutive patients with GBC undergoing radical resection or palliative surgery were enrolled in our study from Fudan University Zhongshan Hospital in China between 2004 and 2013. Among them, 113 patients with odd ID numbers were included in the training set and 125 patients with even ID numbers were included in the validation set. All the included patients met the following criteria: 8th AJCC TNM stage with confirmed postoperative histopathology diagnosis, available tumor specimens and complete follow-up data. The exclusion criteria included: loss of follow up, or survival less than 1 month after surgery. The following clinicopathological factors were collected: age at surgery, gender, TNM stage, tumor differentiation, surgical margin, microvascular invasion and chemotherapy status. The follow up of postoperative patients included: medical history (symptoms and physical examination), laboratory studies and imaging examination every 3 months in the first 2 years and every 6 months in the subsequent years. Overall survival (OS) was confirmed from the day of operation to the date of death or the latest follow up. Baseline characteristics and 5-year OS according to TNM stage and different sets are described in Table 1 and Figure S1.

2.2 Immunohistochemistry and evaluation of immune cells

Tissue microarray (TMA) was established using paraffin-embedded tumor specimens and mounted on glass slides with 2.0-mm tissue core. We selected six kinds of immune cells which might influence the prognosis of patients based on a literature review (ie, macrophages, neutrophils, regulatory T cells [Tregs], cytotoxic T cells [CTL], MC and natural killer cells [NK cells]) and marked them by immunohistochemistry stain of specific molecule markers. No NK cells were found after immunohistochemistry staining of two kinds of anti–CD56 antibody (Dako, clone 123C3; Abcam, ab9018) with different concentrations. The process of immunohistochemistry was described previously\(^23\) with single and specific antibodies (macrophage: anti–CD68 polyclonal antibody, Abcam, ab955, diluted 1:200; neutrophil: anti–CD66b polyclonal antibody, BD Biosciences, Clone G10F5, diluted 1:200; Treg: anti–Foxp3 polyclonal antibody, Abcam, ab22510, diluted 1:100; CTL: anti–CD8, DAKO, IR623, ready-to-use; mast cell: anti–tryptase polyclonal antibody, Abcam, ab2378, diluted 1:1000, respectively). Intensity of immune cells in TMA was defined as the mean number of positive markers per HPF (200X) from three random fields. Two independent pathologists (Dr Luo and Dr Chen) were blinded to the clinical information.
and assessed the intensity of different immune cells, respectively, and the counts were averaged.

2.3 | Least Absolute Shrinkage and Selection Operator

Least absolute shrinkage and selection operator (LASSO) is a co-regression analysis method that can reduce the dimension of original data and produce a statistical model. It performs both variable selection and regularization and enhances the prediction accuracy and interpretability of the statistical model. Because LASSO regression has a low requirement for original data, it has been applied in many medical modeling processes.\(^{24-27}\) We adopted LASSO regression to construct the prognostic model in our study.

2.4 | Statistical analyses

Continuous variables were calculated using Student’s t test, and categorical variables were analyzed using the \(\chi^2\) test. The Kaplan-Meier method, the log-rank test, and univariate and multivariate Cox proportional hazards models were applied to evaluate the prognostic value of the immune index model. The prognostic efficiency of different predictive models was assessed by receiver operating characteristic (ROC) analysis. Statistical analysis was performed with SPSS 23.0 (IBM), MedCalc 15.6.1 (MedCalc Software bvba; https://www.medcalc.org; 2015) and R software packages 3.4.2 with the “glmnet” package (The R foundation for Statistical Computing, https://www.r-project.org/). A two-sided \(P\)-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of immune cells

Tumor-infiltrating immune cells were assessed by immunohistochemical staining of specific polyclonal antibodies (Figure 2A). As shown in Figure 2B, different kinds of immune cells infiltrated into gallbladder tumor tissues in totally different ways. The distribution of macrophages was spindle-like, neutrophil distribution was TV-tower-like and Tregs distribution was lightning-rod-like. CTL distribution was in between macrophage and neutrophil distribution, and mast cell distribution was in between neutrophil and Treg distribution. Meanwhile, the cell counts per HPF of the same immune
cell ranged widely among patients, and the median counts of macrophages, neutrophils, Tregs, CTL and mast cells were 50.3, 11.3, 2.5, 20.5 and 13.3, respectively. We also explored the relationship between TNM stage and tumor-infiltrating immune cells. As shown in Figure S2, there was no difference between stages I-II and stages III-IV for the infiltration of macrophages, Tregs, CTL and mast cells (but there was a difference for neutrophils). Cell counts of infiltrating neutrophils in stages III-IV were significantly higher than those in stages I-II ($P < 0.001$).

Immune cells are not isolated from each other. As presented in Figure S3, significant Spearman’s rank correlation coefficients occurred between immune cells. There were significant positive correlations between macrophages, neutrophils and Tregs, and between CTL and mast cells. A significant negative correlation also existed between neutrophils and mast cells.

### 3.2 Construction of immune index model

To explore the predictive ability of the different immune cells, we divided the patients into high or low infiltration on the basis of median counts of tumor-infiltrating immune cells and conducted survival analysis. As shown in Figure S4, high infiltration of macrophages, neutrophils and Tregs predicted compromised prognosis ($P < 0.001$, $P < 0.001$, $P = 0.011$, respectively), while high infiltration of CTL and mast cells predicted favorable prognosis ($P = 0.002$ and $P = 0.008$, respectively).
We then constructed the immune index model for the training set. The coefficients for all five immune cells were calculated with LASSO regression when \( \log \lambda = -5 \) (Figure 2C), and the partial likelihood deviance for the selected lambda was 11.632 (Figure 2D). The formula was generated where immune index = \( 2^{\text{macrophage} \times 0.01105 + \text{neutrophil} \times 0.006284 + \text{Treg} \times 0.003582 - \text{CTL} \times 0.007434 - \text{mast cells} \times 0.0096} \). Each patient could obtain a unique immune index using the formula. Patients with a low immune index (<1) were defined as immunotype A, while other patients with a high immune index (≥1) were defined as immunotype B. The clinicopathological variables between immunotype A and immunotype B did not vary significantly, except for age (Table S1).

### 3.3 Correlation between immunotype and overall survival

To investigate the difference between immunotype A and immunotype B, we constructed a heat map according to the immune index, from low to high. As shown in Figure 3A, with the increase of the immune index and the conversion of immunotype, the main tumor-infiltrating immune cells gradually changed from CTL and mast cells to macrophages, neutrophils and Tregs in the training set. The validation set had the same trend. Following this, we assessed the influence of the immune index on patients’ prognosis using smooth hazard ratio (HR) curves of OS. As shown in Figure 3B, the Ln (HR) increased obviously with the gradual increase of the immune index in both sets. The HR and 95% confidence interval (CI) for both sets were 3.081(2.040-4.654), \( P < 0.001 \) and 2.207(1.361-3.581), \( P = 0.001 \), respectively.

As shown in Figure 3A, the 5-year OS of immunotype A vs immunotype B in both sets were 70.0% vs 37.0% and 68.9% vs 47.5%, respectively. To further verify the predictive value of the immune index model, we compared the OS based on immunotypes using survival curves. Immunotype B patients demonstrated a more lower OS than immunotype A patients in the training set (Figure 3C, \( P = 0.002 \)). The results were similar for the validation set (Figure 3C, \( P < 0.001 \)). We also conducted a subgroup analysis of OS according to immunotype in the combined set, and found that immunotype was an effective prognostic predictor for OS in all the subgroups (Figure S5).

Univariate and multivariate analysis was performed to further verify the clinical value of the immune index model. Immunotype proved to be an effective predictive factor in both sets (\( P = 0.001 \) and \( P = 0.004 \), respectively; Table 2). Subsequently, all the statistically
significant factors in univariate analysis were included in multivariate cox proportional hazards analysis, and immunotype (HR: 3.287; 95% CI: 1.720-6.278; \( P < 0.001 \); and HR: 2.334; 95% CI: 1.275-4.273; \( P = 0.006 \); respectively) and TNM stage (HR: 2.301; 95% CI: 1.312-4.035; \( P = 0.004 \); and HR: 1.798; 95% CI: 1.065-3.035; \( P = 0.029 \); respectively) were still independent prognostic indicators for OS in both sets (Table 2).

### 3.4 | Efficiencies of prognostic models

Hence, we have proved the prognostic value of the immune index model, but the efficiency of our model was still obscure. Therefore, we calculated the C-indexes of the immune index model and the single immune cell models. In the training set, the immune index model and all the single immune cell models were significant prognostic factors (all \( P < 0.05 \)), while the C-indexes of the single immune cell models (0.575-0.623) were apparently lower than that of the immune index model (0.693) (Tables 2 and S2). Moreover, we extended the application of our immune index model using ROC analysis. The area under the curve (AUC) of the TNM stage was only 0.677 (95% CI: 0.578-0.775) in the training set but was elevated to 0.787 (95% CI: 0.704-0.871) when the immune index model was integrated (Figure 3D). All the results were similar in the validation set. Accordingly, we found that our immune index model was more effective than any single immune cell model and might improve on the TNM staging system.

### 3.5 | Correlation between immunotype and chemotherapy

We evaluated the association between immunotype and gemcitabine-based chemotherapy, which was widely used in gallbladder cancer patients with stage II-IV disease. As shown in Figure 4A, the OS of the immunotype A patients in the training set was not improved (\( P = 0.345 \)) after gemcitabine-based chemotherapy, while the OS of the immunotype B patients was significantly elevated (\( P < 0.001 \)). Similar findings were also found in the validation set (Figure 4B). Treatment with gemcitabine-based chemotherapy was related to reduced risk of poor survival in immunotype B patients in both sets (HR, 0.421; 95%CI, 0.216-0.820; \( P = 0.008 \); and HR, 0.459; 95%CI, 0.236-0.894; \( P = 0.023 \); respectively), whereas similar risk reduction did not occur in immunotype A (HR, 0.663; 95%CI, 0.177-2.485; \( P = .544 \); and HR, 0.680; 95%CI, 0.206-2.246; \( P = .529 \); respectively) (Figure 4C).

We conduct a subgroup analysis to further explore the influence of immunotype on chemotherapy. As shown in Figure 4D-F, for stage II patients with immunotype B and stage III-IV patients with both immunotype A and immunotype B, gemcitabine-based chemotherapy could significantly improve the 5-year overall survival compared with those not receiving gemcitabine-based chemotherapy (\( P = 0.015 \), \( P = 0.030 \), \( P = 0.011 \); respectively). However, for stage II patients with immunotype A, gemcitabine-based chemotherapy could not improve the 5-year overall survival compared with those not receiving gemcitabine-based chemotherapy (\( P = .307 \)).
In this study, we integrated five tumor-infiltrating immune cells in GBC tissue, which we selected based on literature review, and constructed the immune index model using LASSO regression. Our model could effectively predict the prognosis and the gemcitabine-based chemotherapy benefit for GBC patients. More specifically, immunotype A patients with a low immune index (Macrophage\text{low} Neut\text{low} Treg\text{low} CTL\text{high} MC\text{high}) had a favorable survival rate, but there was a reduced benefit on 5-year OS from gemcitabine-based chemotherapy, especially for stage II patients. In contrast, immunotype B patients with a high immune index (Macrophage\text{hi} Neut\text{hi} Treg\text{hi} CTL\text{lo} MC\text{lo}) would have a lower survival rate but greater chemotherapy benefit (Figure S6). Compared with single immune cell models, our integrated model had a more precise prediction efficiency, and could elevate the discriminatory power of the TNM staging system.

Applying an "immunoscore," that is, using immunostaining of CD3\textsuperscript+ cells and CD8\textsuperscript+ cells, was first proposed by Galon.\textsuperscript{28} Based on the adaptive immune response, the immunoscore model proved to be a strong prognostic factor for survival of colon cancer in a multi-center clinical trial.\textsuperscript{29} Galon also suggested that additional markers could be added subsequently to refine the method even further.\textsuperscript{30,\textsuperscript{31}} Thanks to the great effort of scientists, the important roles of innate immune and myeloid-derived cells were discovered recently.\textsuperscript{32-40} However, an effective model for GBC based on both adaptive immune cells and innate immune cells, both lymphoid-derived cells and myeloid-derived cells, and both pro-tumor cells and anti-tumor cells, is urgently needed. Our immune index model for GBC was constructed with this background. Compared with Galon’s immunoscore model, our

### TABLE 2 Univariate and multivariate Cox regression analysis of overall survival

| Characteristic                  | Training set |                      |       |                      | Validation set |                      |       |
|---------------------------------|--------------|-----------------------|-------|----------------------|----------------|-----------------------|-------|
|                                 | hazard ratio | (95% CI)              | P     | hazard ratio         | (95% CI)       | P                     |       |
| **Univariate**                  |              |                       |       |                      |                |                       |       |
| Age at surgery, years:          |              |                       |       |                      |                |                       |       |
| >60 vs <60                      | 0.647        | (0.387-1.080)         | 0.097 |                      | 1.806          | (0.476-1.364)         | 0.424 |
| Gender: male vs female          | 1.266        | (0.726-2.206)         | 0.408 |                      | 0.873          | (0.496-1.539)         | 0.641 |
| TNM stage: III + IV vs I + II   | 2.092        | (1.225-3.575)         | 0.007 |                      | 1.915          | (1.136-3.229)         | 0.015 |
| Differentiation: poor vs well-moderately | 1.617        | (0.947-2.761)         | 0.080 |                      | 1.505          | (0.885-2.558)         | 0.133 |
| Surgical margin: positive vs negative | 2.343        | (1.186-4.630)         | **<0.001** |                      | 1.861          | (0.911-3.800)         | 0.090 |
| Microvascular invasion: present vs absent | 1.703        | (0.977-2.969)         | 0.062 |                      | 1.018          | (0.578-1.979)         | 0.951 |
| Immunotype: Type B vs Type A    | 2.892        | (1.532-5.462)         | **<0.001** |                      | 2.449          | (1.340-4.477)         | **<0.001** |
| Macrophages: high vs low        | 1.013        | (1.006-1.021)         | **<0.001** |                      | 1.008          | (1.001-1.014)         | 0.023 |
| Neutrophils: high vs low        | 1.016        | (1.009-1.022)         | **<0.001** |                      | 1.006          | (1.001-1.011)         | **<0.001** |
| Tregs: high vs low              | 1.027        | (1.012-1.042)         | **<0.001** |                      | 1.005          | (0.998-1.013)         | 0.0858 |
| CTLs: high vs low               | 0.990        | (0.980-0.999)         | 0.029 |                      | 0.991          | (0.984-0.998)         | 0.029 |
| Mast cells: high vs low         | 0.988        | (0.979-0.997)         | 0.015 |                      | 0.989          | (0.979-0.999)         | 0.038 |
| **Multivariate**                |              |                       |       |                      |                |                       |       |
| TNM stage: III + IV vs I + II   | 2.301        | (1.312-4.035)         | 0.004 |                      | 1.798          | (1.065-3.035)         | 0.029 |
| Surgical margin: positive vs negative | 1.748        | (0.861-3.548)         | 0.124 |                      |                |                       |       |
| Immunotype: type B vs type A    | 3.287        | (1.720-6.278)         | **<0.001** |                      | 2.334          | (1.275-4.273)         | **<0.001** |

Bold indicates statistically significant values P < .05.
immune index model includes additional types of immune cells and is constructed with a more complicated formula, so that it is more comprehensive and can reflect the TME more precisely, although the complicated formula might be a hinderance for widespread clinical application.

In our immune index model, macrophages, neutrophils and Tregs were negative predictors while CTL and mast cells were positive predictors. This result is in accord with previous literature. CTL are the main effector cells in the TME, which can induce tumor cell apoptosis by expressing FasL or secreting perforin, granzyme or IFN-γ. Mast cells have been reported to activate CTL by releasing TNF-α and OX40L, while Tregs can directly inhibit the killing function of CTL in a cell-cell contact-dependent manner. Macrophages can inhibit the effector function of CTL directly through secretion of PD-L1, IL-10, TGF-β or ROS, or indirectly by recruiting Tregs or inhibiting the recruitment and recognition of CTL. Neutrophils can secrete ROS, RNS and neutrophil elastase (NE), IL-1RA, ARG-1 to promote tumor initiation and tumor growth. The role of these immune cells in the TME and their different distribution in patients might be the reason why GBC patients with the same stage had totally different prognoses.

Interestingly, we found positive correlations between the poor predictors (macrophages, neutrophils and Tregs) and between the favorable predictors (CTL and mast cells). There was also a negative correlation between mast cells and neutrophils. The driving factor of this result was hard to determine in our study, but it proved that the immune cells in the TME did not exist independently. Correspondingly, these immune cells might keep communicating with each other through direct contact or various cytokines, as mentioned above.

The gemcitabine-based chemotherapy has been proved to prolong the survival time of advanced GBC patients and has remained the first-line regimen since 2004. However, there is conflicting evidence regarding the role of gemcitabine for GBC. In a randomized controlled trial, Edeline (2019) reported that the gemcitabine-based treatment not only did not bring a survival benefit for GBC but might even promote tumor recurrence and shorten patients’ survival. In our opinion, this situation might be related to the patient’s immune
index. In our study, gemcitabine-based chemotherapy could significantly improve the 5-year OS for stage II patients with immunotype B and stage III-IV patients with both immunotype A and immunotype B. Nevertheless, for stage II patients with immunotype A, gemcitabine-based chemotherapy did not improve the 5-year overall survival rate and even led to (insignificant) worse survival. It is reported that favorable anticancer immunity occurred after chemoradiotherapy, and elevated CDB8 density after chemoradiotherapy was associated with a favorable clinical outcome.55 While the effect of chemotherapy on the elevation of CD8 density might be rather weak for the immunotype A (CTLhigh) patients in stage II, insufficient benefit but more side effects might be the possible reasons for the worse prognosis of immunotype A patients who received chemotherapy in stage II compared to patients without chemotherapy. This result is helpful for the clinical decision of who should receive gemcitabine-based chemotherapy, and for the prevention of excessive toxicity and unnecessary resource consumption. However, we also noticed that the 5-year OS of immunotype B patients receiving effective chemotherapy was still lower compared with immunotype A patients who benefit from gemcitabine-based chemotherapy. Therefore, new treatment options for them, such as effective targeted therapy and immunotherapy, are urgently needed.

The present study has some limitations. First, this study is a single-center and retrospective study with a relatively small number of patients. Therefore, more prospective studies or multi-center studies are needed to verify our results. Second, this study only included five immune cells, and some other immune cells that could affect patients' prognosis might be missed because of the lack of single and specific markers. We will update our model with further development of cell identification methods in the future.

In conclusion, we constructed a new immune index model of GBC by integrating multiple tumor-infiltrating immune cells. Our immune index model is practical for predicting the prognosis of GBC patients and might improve on the current TNM staging system. Furthermore, our data suggest that the immune index model might be useful for determining which patients are most likely to benefit from gemcitabine-based chemotherapy.

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CONFLICT OF INTEREST
The authors declare no potential conflicts of interest.

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REFERENCES
1. Lazzano-Ponce EC, Miquel JF, Munoz N, et al. Epidemiology and molecular pathology of gallbladder cancer[J]. CA Cancer J Clin. 2001;51(6):349-364.
2. Wistuba II, Gazdar AF. Gallbladder cancer: lessons from a rare tumour[J]. Nat Rev Cancer. 2004;4(9):695-706.
3. Are C, Ahmad H, Ravipati A, et al. Global epidemiological trends and variations in the burden of gallbladder cancer[J]. J Surg Oncol. 2017;115(5):580-590.
4. Rakic M, Patrilj I, Kopijar M, et al. Gallbladder cancer[J]. Hepatobiliary Surg Nutr. 2014;3(5):221-226.
5. Sheth S, Bedford A, Chopra S. Primary gallbladder cancer: recognition of risk factors and the role of prophylactic cholecystectomy[J]. Am J Gastroenterol. 2000;95(6):1402-1410.
6. Valle J, Wasan H, Palmer DH, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer[J]. N Engl J Med. 2010;362(14):1273-1281.
7. National Comprehensive Cancer Network (NCCN). Clinical Practice Guidelines in Oncology. Hepatobiliary Cancers, Version 2. 2019. https://www.nccn.org/professionals/physician_gls/default.aspx#hepatobiliary. Accessed March 6, 2019.
8. Edeline J, Benabdellah M, Bertaut A, et al. Gemcitabine and oxaliplatin chemotherapy or surveillance in resected biliary tract cancer (PRODIGE 12-ACCORD 18-UNICANCER G1): a randomized phase III study[J]. J Clin Oncol. 2019;37(8):658-667.
9. Amin MB, Edge S, Greene F, et al. AJCC Cancer Staging Manual, 8th edn. New York: Springer; 2017.
10. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation[J]. Cell. 2011;144(5):646-674.
11. Miquel JF, Covarrubias C, Villarol E, et al. Genetic epidemiology of cholesterol cholelithiasis among Chilean Hispanics, Amerindians, and Maoris[J]. Gastroenterology. 1998;115(4):937-946.
12. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment[J]. Cancer Cell. 2012;21(3):309-322.
13. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis[J]. Nat Med. 2013;19(11):1423-1437.
14. Fukumura D, Kloepper J, Amoozgar Z, Duda DG, Jain RK. Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges[J]. Nat Rev Clin Oncol. 2018;15(5):325-340.
15. Bets BC, Veerapathran A, Pidala J, et al. Targeting Aurora kinase A and JAK2 prevents GVHD while maintaining Treg and antitumor CTL function[J]. Sci Transl Med. 2017;9(372).
16. Speiser DE, Ho PC, Verdiol G. Regulatory circuits of T cell function in cancer[J]. Nat Rev Immunol. 2016;16(10):599-611.
17. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more[J]. Nat Rev Cancer. 2016;16(7):431-446.
18. Rigoni A, Colombo MP, Pucillo C. Mast cells, basophils and eosinophils: from allergy to cancer[J]. Semin Immunol. 2018;35:29-34.
19. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy[J]. Nat Rev Immunol. 2019.
20. McCoy MJ, Hemmings C, Miller TJ, et al. Low stromal Foxp3+ regulatory T-cell density is associated with complete response to neoadjuvant chemoradiotherapy in rectal cancer[J]. Br J Cancer. 2015;113(12):1677-1686.
21. Zhang H, Liu H, Shen Z, et al. Tumor-infiltrating neutrophils is prognostic and predictive for postoperative adjuvant chemotherapy benefit in patients with gastric cancer[J]. Ann Surg. 2018;267(2):311-318.
22. Wang JT, Li H, Zhang H, et al. Intratumoral IL17-producing cells infiltration correlate with antitumor immune contexture and improved response to adjuvant chemotherapy in gastric cancer[J]. Ann Oncol. 2019;30(2):266-273.
23. Wang J, Bo X, Suo T, et al. Tumor-infiltrating neutrophils predict prognosis and adjuvant chemotherapeutic benefit in patients with biliary cancer[J]. Cancer Sci. 2018;109(7):2266-2274.

24. Bloniarz A, Liu H, Zhang CH, Sekhon JS, Yu B. Lasso adjustments of treatment effect estimates in randomized experiments[J]. Proc Natl Acad Sci USA. 2016;113(27):7383-7390.

25. Davies H, Glodzik D, Morganella S, et al. HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures[J]. Nat Med. 2017;23(4):517-525.

26. Backes Y, Elias SG, Groen JN, et al. Histologic factors associated with need for surgery in patients with pedunculated T1 colorectal carcinomas[J]. Gastroenterology. 2018;154(6):1647-1659.

27. Fu H, Zhu Y, Wang Y, et al. Identification and validation of stromal immunotype predictive survival and benefit from adjuvant chemotherapy in patients with muscle-invasive bladder cancer[J]. Clin Cancer Res. 2018;24(13):3069-3078.

28. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and localization of immune cells within human colorectal tumors predict clinical outcome[J]. Science. 2006;313(5795):1960-1964.

29. Pages F, Mlecnik B, Marliot F, et al. International validation of the consensus immunoscore for the classification of colon cancer: a prognostic and accuracy study[J]. Lancet. 2018;391(10135):2128-2139.

30. Galon J, Pages F, Marincola FM, et al. The immune score as a new possible approach for the classification of cancer[J]. J Transl Med. 2012;10:1.

31. Galon J, Mlecnik B, binde A, et al. Towards the introduction of the ‘Immunoscore’ in the classification of malignant tumours[J]. J Pathol. 2014;232(2):199-209.

32. Demaria O, Cornen S, Daeren M, Morel Y, Medzhitov R. Natural killer cells control tumor growth by sensing a growth factor[J]. EMBO J. 2015;34(17):2219-2236.

33. Shito E, Hase K, Hashiguchi Y, et al. CD8+ and FOXP3+ tumor-infiltrating lymphocytes as a predictor of adjuvant chemotherapeutic benefit in patients with biliary tract cancer patients[J]. Cancer Sci. 2018;109(7):2266-2274.

34. Lu L, Barbi J, Pan F. The regulation of immune tolerance by CD24 signalling through FOXP3[J]. Nat Immunol. 2019;20(3):327-334.

35. Liew PX, Kubes P. The Neutrophil’s role during health and disease[J]. Nat Rev Immunol. 2006;6(12):945-955.

36. Barkal AA, Brewer RE, Markovic M, et al. CD24 signalling through cytokine-dependent transcriptional repression of CD8+ T cell cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells[J]. Cancer Cell. 2014;26(5):623-637.

37. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm[J]. Nat Immunol. 2010;11(10):889-896.

38. Mollinedo F. Neutrophil degranulation, plasticity, and cancer metastasis[J]. Trends Immunol. 2019;40(3):228-242.

39. Blaisdel A, Crequer A, Columbus D, et al. Neutrophils oppose uterine epithelial carcinogenesis via debridement of hypoxic tumor cells[J]. Cancer Cell. 2015;28(6):785-799.

40. Antonio N, Bonnellyke-Behrndtz ML, Ward LC, et al. The wound inflammatory response exacerbates growth of pre-neoplastic cells and progression to cancer[J]. EMBO J. 2015;34(17):2219-2236.

41. Park JO, Oh DY, Hsu C, et al. Gemcitabine plus cisplatin for advanced biliary tract cancer: a systematic review[J]. Cancer Res Treat. 2015;47(3):343-361.

42. Thomassen DS, Schumacher TN. T cell dysfunction in cancer[J]. Cancer Cell. 2018;33(4):547-562.

43. Wang JB, Tanaka A, Sakaguchi S. Human FOXP3+ regulatory T cell heterogeneity and function in autoimmunity and cancer[J]. Immunity. 2019;50(2):302-316.

44. Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3[J]. Nat Rev Immunol. 2017;17(11):703-717.

45. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment[J]. Trends Immunol. 2012;33(3):119-126.

46. Shiao SL, Ruffell B, DeNardo DG, Faddegon BA, Park CC, Coussens LM. TH2-polarized CD4+ T cells and macrophages limit efficacy of radiotherapy[J]. Cancer Immunol Res. 2015;3(5):518-525.

47. Ruffell B, Chang-Strachan D, Chan V, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells[J]. Cancer Cell. 2014;26(5):623-637.

48. Supporting Information Additional supporting information may be found online in the Supporting Information section.