Prognostic role of stromal tumor-infiltrating lymphocytes in locally advanced upper tract urothelial carcinoma: A retrospective multicenter study (TSU-02 study)

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ABSTRACT

Locally advanced upper urinary tract urothelial carcinoma (UTUC) exhibits high recurrence and metastasis rates even after radical nephroureterectomy. Adjuvant immunotherapy can be a reasonable option, and a simple, low-cost, and effective biomarker is further needed. Stromal tumor-infiltrating lymphocytes (sTILs) has been demonstrated as a prognostic and predictive biomarker in various tumor types, but not yet in locally advanced UTUC. In this multicenter, real-world and retrospective study, we tried to investigate the prognostic role of sTIL and its correlation with the PD-L1/PD-1/CD8 axis by reviewing the clinicopathologic variables of 398 locally advanced UTUC patients at four-high-volume Chinese medical centers. sTIL density was evaluated with standardized methodology on H&E sections, and patients were stratified by the cutoff of sTIL (50%). Results showed that high sTIL indicated improved survival (CSS, \( p = 0.022 \); RFS, \( p = 0.15 \); DFS, \( p = 0.004 \)), and was an independent predictor of better CSS (HR, 0.577; 95% CI, 0.391–0.851; \( p = 0.006 \)); RFS (HR, 0.613; 95% CI, 0.406–0.925; \( p = 0.020 \)); and DFS (HR, 0.609; 95% CI, 0.447–0.829; \( p = 0.002 \)). A strongly positive correlation between sTIL density and the expression level of PD-1/PD-L1/CD8 axis was observed. We also found that aristolochic acid (AA) exposure was associated with increased sTIL and elevated PD-L1 expression, indicating that AA-related UTUC might be a distinct subgroup with unique tumor microenvironment characteristics. Our results show that sTIL can be an easily acquired biomarker for prognostic stratification in locally advanced UTUC.

Introduction

Upper urinary tract urothelial carcinoma (UTUC) is a relatively rare disease, comprising only 5% to 10% of all urothelial carcinomas (UCs).\textsuperscript{1} However, the disease has a relatively high incidence in China, which may be related to the aristolochic acid (AA) consumption.\textsuperscript{2} For localized nonmetastatic UTUC, radical nephroureterectomy (RNU) with bladder cuff excision is the standard choice of treatment.\textsuperscript{3} However, recurrence and progression are frequently reported in advanced stages. The 5-year cancer-specific survival rate of patients has reached 73% after RNU in overall populations, whereas those of patients with advanced-stage and/or lymph node metastasis have markedly decreased (pT3, 54%; pT4, 12%; nodal involvement, 35%).\textsuperscript{4}

For advanced UTUC, platinum-based chemotherapy might be the first-choice adjuvant treatment, but its application after RNU is limited by impaired renal function.\textsuperscript{5} Current results on the application of adjuvant chemotherapy for UTUC remain controversial.\textsuperscript{6,4} Neoadjuvant chemotherapy exhibits potential, providing improved survival outcomes and resulting in substantial tumor downstaging.\textsuperscript{9–11} However, its clinical application is limited by the absence of definite preoperative pathologic information. Immunotherapy has recently emerged as a potential treatment for various malignancies, and PD-1/PD-L1 inhibitors have been approved as the first- or second-line treatment for UC by FDA.\textsuperscript{12–15} However, the majority of cases remain unresponsive to these agents, and the adverse events and financial issues need to be considered.

Stromal tumor-infiltrating lymphocytes (sTILs), which can be assessed simply, practically, and at a low cost by using hematoxylin-eosin (H&E) staining, has been established as a unique biomarker with independent favorable prognostic and predictive values in various tumor types.\textsuperscript{16} The presence of sTILs in UC has also drawn interest in recent decades. However, previous reports have mainly focused on the urothelial bladder cancer (UBC), and studies on locally advanced UTUC have rarely been reported. To elucidate the prognostic role of sTILs, and its relationship with the well-established biomarkers in tumor microenvironment in locally advanced UTUC, as well as to show the characteristic of sTIL in AA-related UTUC, we conducted this

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multicenter real-world study (TSU-02 study) in a large clinical cohort and presented the results of our exploratory analysis.

Materials and methods

Study design

The TSU study aims to evaluate the characteristics of the tumor microenvironment for patients with UTUC. The TSU-02 study mainly focuses on the prognostic and potential predictive roles of sTILs. We retrospectively reviewed charts from 1545 UTUC patients who underwent RNU from January 2006 to December 2017 in four high-volume Chinese medical centers, including Peking University First Hospital (PUFH), Sun Yat-sen Memorial Hospital (SYMH), Peking University Third Hospital (PUFH), and Fujian Provincial Hospital (FPH). Patients aged 18 years and older with a histologically confirmed diagnosis of locally advanced UTUC defined as ≥pT3 and/or pN+ were enrolled. Patients with distant metastasis at diagnosis and a history of other malignant tumors, as well as those received neoadjuvant chemotherapy, were excluded. The cases without available clinicopathological data, follow-up data or available H&E slides were also excluded. The establishment of clinical cohorts is illustrated in Figure 1. A total of 398 patients were ultimately enrolled.

Data including age, gender, AA exposure history, and oncologic variables were retrospectively collected. Tumor staging and grading were reviewed by experienced pathologists blinded to clinicopathological data and patient outcomes, and then evaluated in accordance with the 7th AJCC classification and the 2004 WHO classification. The study was approved by an independent ethics committee in each participating institution, conducted in accordance with the Declaration of Helsinki, and registered in the Chinese clinical trial registry (ChiCTR2000030308). Informed consent was obtained from all individual participants.

Outcomes

The primary endpoint of this study was cancer-specific survival (CSS), and the secondary endpoints were recurrence-free survival (RFS) and disease-free survival (DFS). CSS was defined as the duration from the date of surgery to the last follow-up or death due to UTUC, RFS was defined as the duration from surgery to the first documented urothelial cancer relapse, and DFS was defined as the duration from surgery to any urothelial cancer relapse or death from any cause, whichever occurred first.

Histological assessment of sTIL

The H&E-stained slides were evaluated by two independent pathologists blinded to all clinicopathological and survival data in each institution, under the guidelines of the international TIL working group. The slides used for assessment were expected to present the most invasive part (i.e., the slides used in routine pathology to determine the T status). When facing more than one slide from the most invasive part, the section with the highest sTIL density determined the final assessment. Briefly, sTIL was evaluated within the borders of the invasive tumor, including both central tumor as well as invasive margin, and quantified as a percentage of tumoral stromal area occupied by mononuclear inflammatory cells. A full assessment of average sTILs across the whole slide (not hotspots) was conducted. The results were reported by increments of 10%.
initially: a score of 10% indicates a sTIL percentage between 0% and 10%; a score of 20% indicates a percentage between 10% and 20%; going on up through 100%. Then a binary classification was used to categorize them into low-sTIL (sTIL density ≤ 50%) and high-sTIL (sTIL density > 50%) (Figure S1).

In addition, we evaluated both the number of TILs infiltrated into the tumor cell nests and stromal TILs in 100 randomly selected cases, and found that the number of intratumoral TILs was positively correlated with the number of sTILs, but had generally lower density (Figure S2). The same viewpoint has been proved by other studies. Therefore, we mainly focused on stromal TILs in current study.

**Immunohistochemical staining and evaluation**

Immunohistochemical (IHC) staining was performed on 4 μm serial sections of formalin-fixed, paraffin-embedded tumor tissues in SYMH cohort as previously described, using primary antibodies against PD-L1 (1:400, clone SP142, Spring Bioscience), PD-1 (1:100, clone EH33, Cell Signaling Technology), and CD8 (1:400, clone D8A8Y, Cell Signaling Technology). For the evaluation of IHC staining, all stained slides were scanned using a high-resolution digital slide scanner with up to ×400 magnification (Aperio Digital Pathology, Leica, Germany). PD-L1 expression was evaluated on tumor cells (TCs) and a 1% or greater PD-L1+ TC staining percentage was considered positive. Two independent pathologists blinded to clinicopathological data and patient outcomes conducted the evaluation.

**Statistical analysis**

The SPSS version 25.0 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. The Kaplan-Meier survival analysis was used to assess the correlations between sTIL categories with CSS, RFS, and DFS by using the Log-rank test. Univariate and multivariate Cox regression analysis were performed to evaluate the prognostic significance of each variable. We analyzed correlations between sTIL with pathological parameters by using Pearson’s chi-squared test and Pearson’s correlation test. To minimize the effect of inclusion bias, patients with AA exposure and non/possible AA exposure were propensity score-matched (PSM) at a 1:1 ratio, based on age, gender, multifocality, pathologic T stage, pathologic N stage and tumor grade. The two cohorts were matched using a caliper width of 0.2 of the standard deviation. For all analyses, the two-sided p < .05 was considered to indicate statistical significance.

**Results**

After the exclusion criteria were applied, 398 patients with locally advanced UTUC were included for final analysis. The patients consisted of 215 males and 183 females with a mean age of 65.5 years (range 20–92 years). Overall, 236 patients (59.3%) had low sTIL(≤50%) and 162 patients (40.7%) had high sTIL(>50%). The detailed clinicopathological parameters are listed in Table 1.

**Association of sTILs with patient survival in UTUC**

During a median follow-up of 55 months (interquartile range 32–71 months), 143 patients (35.9%) died and 106 patients (26.7%) exhibited disease recurrence. Kaplan-Meier analysis demonstrated that patients with high sTIL had significantly better cancer-specific survival than that of patients with low sTIL (p = .022) (Figure 3a). At the end of the 1-, 3- and 5-year follow-ups, the CSS rates were 90.4%, 78.7%, and 72.0%, respectively, in the high-sTIL group and 85.1%, 65.9% and 60.6%, respectively, in the low- sTIL group. A significantly better survival outcomes in RFS (p = .015) and DFS (p = .004) for patients with high-sTIL were also observed (Figure 3b,c).

Univariate analysis identified that sTIL (low or high), tumor size (>3 or ≤3 cm), T stage (1–2 or 3–4), and tumor grade (G1–2 or G3) were significantly associated with CSS. After adjusting for clinicopathological variables, sTIL (HR, 0.577; 95% CI, 0.391–0.851; p = .006), tumor size (HR, 1.521; 95% CI, 1.025–2.256; p = .037), T stage (HR, 9.458; 95% CI, 1.283–69.717; p = .027), and tumor grade (HR, 2.393; 95% CI 1.434–3.995; p = .001) were independent predictors of CSS in multivariate analysis (Table 2).

Univariate analysis and multivariate analysis confirmed that sTIL (HR, 0.613; 95% CI, 0.406–0.925; p = .020) and T stage (HR, 3.255; 95% CI, 1.320–8.028; p = .010) were independent predictors of RFS (Table 3). In addition, sTIL, tumor size, T stage and tumor grade were significantly associated with DFS in univariate analysis. Multivariate analysis further proved the independent prognostic roles for DFS of sTIL (HR, 0.609; 95% CI, 0.447–0.829; p = .002), T stage (HR, 3.126; 95% CI, 1.406–6.951; p = .005) and tumor grade (HR, 1.543; 95% CI, 1.078–2.211; p = .018) (Table 4).

**Correlations between sTIL and PD-L1/CD8 axis**

A significant correlation was determined between the PD-L1+ TC and sTIL, with high-sTIL showing more intense PD-L1+ TCs than low-sTIL (p = .012) (Figure 4a). Moreover, we found that sTIL density was positively correlated with the number of CD8+ lymphocytes (p = .017, Pearson’s correlation test) (Figure 4b,d) and PD-1+ lymphocytes (p = .005, Pearson’s correlation test) (Figure 4c,e).

**Correlations between sTIL and AA-related UTUC**

Among the patients, 16 were found to have a history of confirmed AA exposure. We found that patients with AA exposure were more likely to have high sTIL, compared with patients without AA exposure (p = .020, χ2 test) (Figure 5a). We further compared PD-L1 expression in 32 samples after PSM at a 1:1 ratio. Patients with AA exposure showed significantly higher PD-L1 expression in TCs (p = .003, χ2 test) (Figure 5b). The correlation between AA exposure and sTIL was further verified after PSM (p = .034, χ2 test). The distributions of sTIL, PD-L1 expression, and clinicopathological parameters of these 32 patients are presented in Figure 5c.
Table 1. Clinical and pathological characteristics of 398 patients.

| Variable                                                  | N(%)          |
|-----------------------------------------------------------|---------------|
| No. of patients                                           | 398           |
| Age, years (median, range)                                | 65.5 (20–92)  |
| Gender (Male vs. Female)                                  | 215/183 (54.0/46.0) |
| Side (Left vs. Right)                                     | 208/190 (52.3/47.7) |
| Main tumor location (Pelvic vs. Ureter)                   | 254/144 (63.8/36.2) |
| Main tumor size (>3 cm vs. ≤3 cm)                         | 227/171 (57.0/43.0) |
| Multifocality (Yes vs. No)                                | 93/305 (23.4/76.6) |
| Tumor stage (T1-2 vs. T3-4)                               | 40/358 (10.1/89.9) |
| Lymph node status (cN0/pN0 vs. pN +)                      | 292/106 (73.4/26.6) |
| Tumor grade (G1-2 vs. G3)                                 | 121/277 (30.4/69.6) |

Figure 3. Kaplan-Meier curves on patient survival by sTIL density. sTIL can predict (a) cancer-specific survival (p= .022), (b) recurrence-free survival (p= .015), and (c) disease-free survival (p = .004). P values were calculated by the log-rank test. Vertical tick marks represent censored subjects. Stromal tumor-infiltrating lymphocyte = sTIL.

Figure 2. Expression of PD-L1/PD-1/CD8 in locally advanced UTUC. (a) Representative images of immunohistochemical detection of PD-L1 (brown) in tumor cells (TCs). (b) Representative images of immunohistochemical detection of CD8+ lymphocytes and PD-1+ lymphocytes (brown). (scale bar, 100 μm for upper rows, 25 μm for lower rows), Programed death-1 = PD-1, Programed death-ligand 1 = PD-L1.

Discussion

UTUC, particularly the advanced type, is an aggressive disease with high recurrence and progression. However, current prognostic factors such as TNM stage, tumor grade, and molecular biomarkers have their own limitations. To evaluate whether the sTIL could potentially be used as a biomarker for clinical applications in advanced UTUC, we conducted a multicenter real-world study encompassing a large cohort of 398 RNU-treated locally advanced UTUC with a long-term median follow-up. We found that sTIL was positively correlated with higher survival and had strong correlation with several well-established immunotherapy biomarkers. We also found that AA-related UTUC exhibited distinct features with enhanced sTIL and PD-L1 expression, indicating its distinct tumor microenvironment characteristics. To the best of our knowledge, this study is the first to focus on the prognostic role of sTIL and its relationship with the immunotherapy biomarkers in locally advanced UTUC.

The presence of increased tumor-infiltrating lymphocytes (TILs) has been established as a prognostic factor in various tumors, including melanoma, breast cancer, gastrointestinal tract carcinoma, ovarian carcinoma and urothelial cancer. Previous studies on TILs in UC mainly focused on UBC, and various studies presented different results. Some studies reported that high TILs correlated with improved outcomes, whereas others considered TILs predicted a poor survival, and even some studies found that stromal TIL was not a prognostic indicator. Similarly, the role of TILs in UTUC remains inconclusive. Wang reported that high CD4+ T cells and CD8+ T cells could predict improved survival. Conversely, Nuku found that low stromal TIL combined with low PD-L1 expression by TC predicted increased survival. The variations in the results might be attributable to the distinct characteristics of the included populations, the relatively small size of the study samples, heterogeneity of urothelial carcinoma, and the complexity of the underlying immune regulatory pathway. In the current study, we used a large study sample from multi-centers to provide a higher level of evidence. The present study indicated that sTIL was an...
Table 2. Univariate and multivariate Cox regression analysis of factors associated with cancer-specific survival.

| CSS                  | Univariate | Multivariate |
|----------------------|------------|--------------|
|                      | HR         | 95.0% CI     | P value | HR         | 95.0% CI     | P value |
| Age (>65 vs ≤65)     | 1.332      | 0.924–1.920  | .124    | 1.320      | 1.025–2.56   | .037    |
| Gender (Male vs. Female) | 0.963      | 0.671–1.382  | .838    |            |              |         |
| Tobacco consumption (Yes vs. No) | 0.845      | 0.505–1.414  | .522    |            |              |         |
| Side (Left vs. Right) | 1.014      | 0.706–1.457  | .938    |            |              |         |
| Main tumor location (Ureter vs. Pelvic) | 1.055      | 0.728–1.527  | .777    |            |              |         |
| Main tumor size (>3 vs. ≤3 cm) | 1.091      | 1.344–2.950  | .001    | 1.521      | 1.025–2.56   | .037    |
| Multifocality (presence vs. absence) | 0.845      | 0.549–1.301  | .445    |            |              |         |
| Tumor stage (T3–4 vs. T1–2) | 19.487     | 2.719–139.663 | .003   | 9.458      | 1.283–69.717 | .027   |
| Lymph node status (pN+ vs. cN0/pN0) | 0.912      | 0.605–1.375  | .660    |            |              |         |
| Tumor grade (G3 vs. G1–2) | 3.214      | 1.941–5.321  | <.001   | 2.393      | 1.434–3.995  | .001    |
| sTIL (High vs. Low)  | 0.640      | 0.435–0.942  | .024    | 0.577      | 0.391–0.851  | .006    |

Variables with a P < .1 (univariate analysis) were analyzed further in the multivariate Cox regression. Statistical significance is defined as p < .05 (Bold).

Table 3. Univariate and multivariate Cox regression analysis of factors associated with recurrence-free survival.

| RFS                  | Univariate | Multivariate |
|----------------------|------------|--------------|
|                      | HR         | 95.0% CI     | P value | HR         | 95.0% CI     | P value |
| Age (>65 vs ≤65)     | 0.976      | 0.666–1.430  | .899    |            |              |         |
| Gender (Male vs. Female) | 1.162      | 0.763–1.644  | .562    |            |              |         |
| Tobacco consumption (Yes vs. No) | 0.659      | 0.360–1.205  | .175    |            |              |         |
| Side (Left vs. Right) | 1.350      | 0.915–1.993  | .131    |            |              |         |
| Main tumor location (Ureter vs. Pelvic) | 1.000      | 0.674–1.484  | .999    |            |              |         |
| Main tumor size (>3 vs. ≤3 cm) | 1.130      | 0.768–1.662  | .535    |            |              |         |
| Multifocality (presence vs. absence) | 0.874      | 0.553–1.380  | .564    |            |              |         |
| Tumor stage (T3–4 vs. T1–2) | 3.304      | 1.340–8.146  | .009    | 3.255      | 1.320–8.028  | .010    |
| Lymph node status (pN+ vs. cN0/pN0) | 0.746      | 0.472–1.178  | .209    |            |              |         |
| Tumor grade (G3 vs. G1–2) | 1.392      | 0.907–2.136  | .130    |            |              |         |
| sTIL (High vs. Low)  | 0.605      | 0.401–0.914  | .017    | 0.613      | 0.406–0.925  | .020    |

Variables with a P < .1 (univariate analysis) were analyzed further in the multivariate Cox regression. Statistical significance is defined as p < .05 (Bold).

Table 4. Univariate and multivariate Cox regression analysis of factors associated with disease-free survival.

| DFS                  | Univariate | Multivariate |
|----------------------|------------|--------------|
|                      | HR         | 95.0% CI     | P value | HR         | 95.0% CI     | P value |
| Age (>65 vs ≤65)     | 1.161      | 0.869–1.551  | .312    |            |              |         |
| Gender (Male vs. Female) | 1.098      | 0.823–1.464  | .527    |            |              |         |
| Tobacco consumption (Yes vs. No) | 0.809      | 0.534–1.226  | .318    |            |              |         |
| Side (Left vs. Right) | 1.095      | 0.820–1.462  | .538    |            |              |         |
| Main tumor location (Ureter vs. Pelvic) | 1.075      | 0.801–1.444  | .631    |            |              |         |
| Main tumor size (>3 vs. ≤3 cm) | 1.561      | 1.156–2.108  | .004    | 1.249      | 0.919–1.698  | .156    |
| Multifocality (presence vs. absence) | 0.967      | 0.693–1.348  | .842    |            |              |         |
| Tumor stage (T3–4 vs. T1–2) | 4.555      | 2.134–9.722  | <.001   | 3.126      | 1.406–6.951  | .005    |
| Lymph node status (pN+ vs. cN0/pN0) | 0.803      | 0.574–1.122  | .199    |            |              |         |
| Tumor grade (G3 vs. G1-2) | 1.915      | 1.360–2.696  | <.001   | 1.543      | 1.078–2.211  | .018    |
| sTIL (High vs. Low)  | 0.645      | 0.476–0.876  | .005    | 0.609      | 0.447–0.829  | .002    |

Variables with a P < .1 (univariate analysis) were analyzed further in the multivariate Cox regression. Statistical significance is defined as p < .05 (Bold).

Independent prognostic factor and high sTIL predicted improved outcomes in locally advanced UTUC. Adding this easily acquired and robust indicator into the clinical practice may make beneficial supplement to the current pathological system for UTUC.

The results of both PD-L1 and PD-1 inhibitor immunotherapy were encouraging in locally advanced or metastatic urothelial cancer, and higher response rate was observed in UTUC compared with UBC (39% vs.17%). However, the response rates were still far from satisfactory, and the majority of patients did not respond to checkpoint inhibitors. Previous reports indicated higher objective response rates in PD-L1 high urothelial tumors, leading to the approval of PD-L1 expression by FDA as a biomarker for frontline drug use. However, in the cases of insufficient TILs, PD-1/PD-L1 blockade is not likely to cause specific T-cell proliferation and increased effector function, even with high PD-L1 expression. Several studies demonstrated the predictive value of CD8+ T cells, as well as the TIME (tumor immunity in the microenvironment) classification, which can be a valuable indicator to guide immunotherapy on the basis of PD-L1 expression and the TILs density. The current study found that sTILs were strongly correlated with PD-L1 expression in TCs, as well as with CD8+ lymphocyte density and PD-1+ lymphocyte density. Due to the strong correlation between sTIL and PD-L1/PD-1/CD8 axis, we speculate that sTIL might become a potential biomarker to predict clinical response to immunotherapy in the future, although comparative analysis between the response and non-response cohorts must be performed before a conclusion can be obtained.

Exposure to AA is widespread in East Asia and has led to the relatively high prevalence of UTUC. Distinct from non-AA UTUC, AA-related UTUC exhibits unique clinicopathological features, such as prevalence in females, multifocality, and lower T stage, as demonstrated in both the present study and our
AA-related locally advanced UTUC might be a distinct population with potentially increased sensitivity to immunotherapy. In addition, the renal function of patients with AA-related UTUC was found to be poorer than that of patients with other types of UTUC and was less likely to receive chemotherapy, rendering immunotherapy a reasonable option. We suggest the use of AA-related factors in future trials studying immune checkpoint inhibitors for UTUC to further explore its distinct treatment strategy.

This study has several limitations. First, this is a retrospective study, and selection bias may exist. Second, although we conducted a multicenter cohort, the entire population is confined to the Chinese population, disregarding the effect of racial differences on the outcomes for patients with UTUC. In addition, this finding does not include more advanced ways to characterize UTUC, such as gene expression signatures and mutation-based subtyping, which might result in more refined subtypes with different immunobiology.

In summary, we evaluated the role of sTIL in a quite large clinical cohort for this rare and aggressive disease, locally advanced UTUC. We found that high sTIL is independently correlated with improved survival and there are strong correlations between sTIL and the PD-L1/PD-1/CD8 axis, suggesting that it can be an easily acquired biomarker for prognostic stratification and might have potential role in immunotherapy response prediction. Furthermore, we presume that AA-related UTUC patients with distinct tumor microenvironment characteristics might be good candidates for immunotherapy while further trials are needed. Our findings may make good supplements to the current pathological evaluation system and help distinguish the optimal treatment strategy for locally advanced UTUC.

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.

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