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Original article

Infiltration of CD3$^+$ and CD68$^+$ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors[1]

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Abstract

Objectives: Urothelial carcinoma (UC) aggressiveness is determined by tumor inherent molecular characteristics, such as molecular subtypes, as well as by host reactions directed toward the tumor. Cell types responsible for the host's response include tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs). The aim of the present investigation was to explore the immunological response in relation to UC molecular subtypes and to evaluate the prognostic effect of TIL and TAM counts in tissue sections from muscle-invasive (MI) tumors.

Methods and materials: Tissue microarrays with 296 tumors spanning all pathological stages and grades were analyzed with antibodies for CD3, CD8, FOXP3, CD68, and CD163. Cases were classified into the following molecular subtypes: urobasal, genomically unstable, and squamous cell carcinoma–like using a combination of immunohistochemistry and histology. The Cox regression and Kaplan-Meier analyses were performed with progression-free survival and disease-specific survival as end points.

Results: UC molecular subtypes demonstrate different degrees of immunological responses; the urobasal subtype induces a weak response, the genomically unstable subtype induces an intermediate response, and the squamous cell carcinoma–like subtype induces a strong response. These subtype specific responses are independent of tumor stage and include both TILs and TAMs. The presence of infiltrating CD3$^+$ TILs was significantly associated with good prognosis in the MI cases ($P < 0.01$). This positive association was modulated by the presence of CD68$^+$ TAMs. The strongest association with poor survival was observed for a high ratio between CD68 and CD3 ($P = 7 \times 10^{-5}$).

Conclusion: UC molecular subtypes induce immunological responses at different levels. A high CD68/CD3 ratio identifies a bad prognosis group among MI UC cases.

Keywords: Bladder cancer; Molecular subtypes; T cells; CD3 antigen; CD68 antigen; Prognosis

1. Introduction

The inherent molecular characteristics of urothelial carcinomas (UCs) determine their aggressiveness. For instance, low-stage low-grade UCs with a small propensity to progress show very different molecular profiles compared to muscle-invasive (MI) cases [1]. However, molecular profiles also differ among pathologically similar tumors. We recently showed that UCs may be classified into

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3 major molecular subtypes, urobasal (Uro), genomically unstable (GU), and squamous cell carcinoma–like (SCCL) tumors using immunohistochemistry (IHC) [2] and that Ta tumors are dominated by the Uro cases, T1 tumors by Uro and GU cases, and MI tumors by GU and SCCL cases. The tumor subtypes show distinct molecular profiles at both RNA [3] and protein [2] levels, as well as distinct survival characteristics. However, the outcome of a given tumor is also affected by host reactions toward the tumor cells. Tumor-infiltrating lymphocytes (TILs) are a heterogeneous group of immune cells that are abundant in tumors of different origin. Cytotoxic T cells (CTLs) are the main effector cells in antitumor T-cell immunity, and their activity is modulated by other T-cell subtypes, such as T-helper cells and regulatory T cells (Tregs). The effect of a particular immune response is determined by the balance between the various T-cell subtypes involved. It has previously been shown that tumor infiltration by both CTLs ($CD8^+$) and T cells in general ($CD3^+$) promotes survival in patients with UC [4,5]. Another important immune cell population present in tumors is tumor-associated macrophages (TAMs). As for T cells, macrophages may have activating (M1), or suppressive (M2) immune functions. TAMs have previously been suggested to be M2-like [6]. They secrete a wide range of cytokines and have an important role in immune suppression, angiogenesis, and tumor progression. Consequently, TAMs may have an overall tumor growth–promoting effect, counteracting the action of the T-cell response [7,8]. Thus, in the present investigation, we have evaluated the level of both TILs and TAMs in 296 cases of UC and related the immune response to UC molecular subtypes and clinical outcome.

2. Materials and methods

2.1. Patient and sample selection

Tumor biopsies from transurethral resection of 296 patients diagnosed with UC in the Southern Sweden Healthcare Region between 2001 and 2009 were included. Patient and tumor data are summarized in Table 1. Of the patients with non-MI (NMI) tumors, 56 received bacillus Calmette-Guérin (BCG) treatment (6 instillations), of the patients with MI tumors 52 were treated with radical cystectomy, and of these 4 with additional neoadjuvant chemotherapy. The majority of the cases included were primary tumors; approximately 1 in 5 tumors (54 cases) had a history of bladder cancer. Median follow-up time was 51 months for patients with NMI disease and 70 months for patients with MI disease. The investigation was approved by the regional ethics committee (no. 2010/5). The tissue sections were reevaluated by a uropathologist (G.C.) using TNM 2009 [9], as staging and grading was performed according to the World Health Organization 1999 system.

Table 1
Distribution of molecular subtype and patient/tumor data across pathological stages

	Ta, $n = 112$	T1, $n = 89$	MI, $n = 93$	Tx, $n = 2$
Molecular subtype, no. of cases				
Urobasal	98	34	11	2
GU	9	43	46	0
SCC-like	0	4	34	0
Not classified	5	8	2	0
Patient/tumor data				
Age, mean years	69.1	70.8	71.7	75.0
Gender, no. of cases				
Male	77	72	69	2
Female	35	17	24	0
Tumor grade, no. of cases				
G1	42	0	0	0
G2	62	31	10	1
G3	8	58	83	1

2.2. Tissue microarrays and IHC

Tissue microarray blocks were constructed from 1.0-mm punches of formalin-fixed paraffin-embedded specimens of transurethral resection of the bladder using a manual array (TMA arrayer, Pathology Devices, Inc, Westminster, MD). Tissue punches were collected from areas with the highest grade on the corresponding sections and from areas without necrosis. Tissue microarray (TMA) sections were stained with antibodies against CD3, CD8, FOXP3, CD68, and CD163. As negative controls, the primary antibodies were omitted for each staining. Antibodies for CD3 (clone, F7.2.38; mouse; dilution, 1:200; product M7254; Dako), CD8 (clone, C8/144B; mouse; dilution, 1:50; product M7103; Dako), FOXP3 (clone, 236A/E7; mouse; dilution, 1:1,000; product ab20034; Abcam), CD163 (clone, 10D6; mouse; dilution, 1:250; product NCL-CD163; Novocastra), and CD68 (clone, EBM11; mouse; dilution, 1:1,500; product M0718; Dako) were used. All markers showed discrete cellular-staining patterns. Each TMA core was given a score of 0 to 5 based on the average count of positive cells per tissue area. The scores 0 to 5 corresponded approximately to the bins 0 to 20, 20 to 50, 50 to 100, 100 to 300, 300 to 500, and >500 positive cells per TMA core. The range of scoring intervals was defined to capture the observed variation for each individual marker. For each marker, 1 case per bin was manually counted, and these cases were used as a reference in the evaluation process. The evaluations were done manually by 2 observers. When the opinions differed, both the observers discussed the case and reached an agreement. For most cases ($n = 287$), 2 cores were available, and the mean of the 2 cores was used. Marker scores were recorded for the entire core and separately for the intratumoral and stromal compartments when the distinction between both the compartments was clear. The evaluations were performed on digitalized