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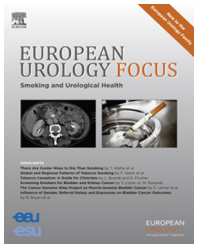

Bladder Cancer

Prognostic Significance of Neutrophilic Infiltration in Benign Lymph Nodes in Patients with Muscle-invasive Bladder Cancer

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Abstract

Background: Preclinical studies suggest that signal transducer and activator of transcription 3 (STAT3)-mediated recruitment of neutrophils to premetastatic tissue occurs prior to metastatic progression.
Objective: We sought to determine if neutrophilic infiltration in benign nodal tissue is associated with poor clinical outcome in patients with muscle-invasive bladder cancer.
Design, setting, and participants: Formalin-fixed, paraffin-embedded tissue was secured from 55 patients with muscle-invasive bladder cancer who had undergone cystectomy at our institution. Sections of benign lymph nodes were obtained and stained with primary antibodies against 3-fucosyl-N-acetyl-lactosamine, phosphorylated STAT3, and interleukin-17, the latter being a key mediator of neutrophil infiltration and STAT3 activation.
Outcome measurements and statistical analysis: The Kaplan–Meier method was used to interrogate differences in overall survival (OS) in patients with high versus low biomarker expression. Cohorts stratified by receipt and nonreceipt of neoadjuvant chemotherapy were separately explored.
Results and limitations: Of the 55 patients examined, 19 patients (35%) had no prior neoadjuvant chemotherapy. Amongst these patients, median OS was improved in patients with low 3-fucosyl-N-acetyl-lactosamine$^+$ cell counts (196 mo vs 37 mo; $p = 0.0062$) and low phosphorylated STAT3$^+$ cell counts (278 mo vs 106 mo; $p = 0.025$). In the same cohort, a trend towards improved OS in patients with low interleukin-17$^+$ cell count was observed (not reached vs 117 mo; $p = 0.18$). No differences in OS were noted in biomarker-based subgroups amongst patients that had received prior neoadjuvant chemotherapy.
Conclusions: The results herein support the hypothesis that bladder cancer metastasis may be driven by STAT3-mediated neutrophilic infiltration in premetastatic sites. Validation of these findings using tissues derived from a phase 3 surgical trial (Southwest Oncology Group 1011) is currently underway.
Patient summary: Lymph node metastases occur in up to 25% of patients with muscle-invasive cancer and it represents one of the most frequent sites of bladder cancer metastasis. This report provides preliminary evidence that neutrophil levels in benign lymph nodes may predict clinical outcome.

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1. Introduction

Since its inception by Paget [1] in 1889, the popularity of the "seed-and-soil" hypothesis has waxed and waned in oncology. The hypothesis refers to the dependency of a primary tumor (the "seed") upon the appropriate microenvironment (the "soil") for growth. In recent years, preclinical data supports a complex interplay between immune cells at premetastatic sites, leading to formation of a "premetastatic niche." Kaplan [2] proposed that vascular endothelial growth factor receptor-1-positive bone-marrow derived cells may be essential in establishing the niche. More recently, however, Kowanetz et al [3] have elegantly demonstrated that the niche may form in a vascular endothelial growth factor receptor-1 independent fashion. In murine models, it was shown that Ly6G + Ly6C + granulocytes (ie, neutrophils) migrate to premetastatic sites prior the formation of micrometastases, driven by Bv8-mediated activation of the prokineticin rector-1. Although neutrophils have been implicated as mediators of metastasis in several other preclinical studies, their role in forming the premetastatic niche has not been well documented in a clinical setting [4–6].

Bladder cancer represents a particularly relevant malignancy in which to explore this phenomenon. Several reports have linked neutrophil quantity and function to the pathophysiology of the disease. For instance, Gondo et al [7] have suggested that neutrophils may have a prognostic role in bladder cancer. Specifically, in a series of 189 patients with bladder cancer treated with cystectomy, the neutrophil-to-lymphocyte (NLR) ratio was noted to independently predict disease-specific survival. Separate preclinical studies have shown that the antitumor effect of bacillus Calmette–Guerin is contingent upon neutrophil trafficking to the bladder [8].

Given evidence supporting the role of neutrophils in bladder cancer pathogenesis, we sought to determine whether neutrophils might facilitate premetastatic niche formation in this disease. In particular, muscle-invasive bladder cancer represents an ideal setting in which to explore the niche as radical cystectomy and bilateral pelvic lymph node dissection is the standard of care. Lymph node metastases occur in up to 25% of patients with muscle-invasive cancer and they represent one of the most frequent sites of bladder cancer metastasis, and are therefore an ideal location to examine for the presence of the niche [9]. Underlying our work was the a-priori hypothesis that increased niche formation (ie, increased neutrophilic infiltration in lymph nodes) is associated with a worse prognosis. Given evidence from our group suggesting that phosphorylated signal transducer and activator of transcription-3 (pSTAT3) may play a role in neutrophil recruitment, we further assessed this moiety [10]. Interleukin-17, a cytokine mainly produced by a subset of T helper cells and innate lymphocytes to regulate recruitment of monocytes and neutrophils to the site of inflammation and a driver of STAT3-mediating signaling, was also assessed in the same tissues [11].

2. Materials and methods

2.1. Patient selection

The City of Hope Biospecimen Repository warehouses biologic specimens and associated pathologic data from consenting patients who have received diagnostic or therapeutic surgical procedures at the institution. Through a City of Hope Institutional Review Board-approved protocol (City of Hope Institutional Review Board 11210), we obtained permission to access paraffin-embedded nodal tissue from patients with: (1) histologically documented bladder cancer, (2) absent nodal metastases, and (3) cystectomy and lymphadenectomy occurring between January 1, 1990, and December 31, 2011. Variant histologies (eg, sarcoma, squamous cell carcinoma, or small cell carcinoma) were excluded in the current analysis. The Institutional Review Board waived the need for consent, and initially, tissue from a cohort of 20 consecutive patients was requested. When it was determined that none of these patients had received prior neoadjuvant therapy, the parent protocol was amended to include additional patients who had received prior neoadjuvant chemotherapy with either gemcitabine–cisplatin (GC) or methotrexate–vinblastine–adriamycin–cisplatin (MVAC). Of 61 patients included in a recently reportedly retrospective analysis of outcomes with GC or MVAC chemotherapy, tissue was available for 36 patients [12].

Up to 12 4-μm sections were obtained for each patient. Lymph nodes were randomly selected, with a preference for larger lymph nodes as indicated in available pathology reports (to maximize the yield of viable tissue). Tissues were deidentified, coded, and transferred to laboratory-based investigators (W.L. and R.J.). Only the principal clinical investigator (S.K.P.) and study biostatistician (X.L.) were in possession of a master list linking laboratory and clinical data.

2.2. Analysis of specimens

Paraffin-embedded sections were deparaffinized, antigen retrieved following the instructions in the manuals of the antibodies, blocked with normal serum, and stained with primary antibodies against 3-fucosyl-N-acetyl-lactosamine (CD15; 1:50, Clone Carb-3, DAKO, Carpinteria, CA, USA), pY705-Stat3 (1:50, Clone D3A7, Cell Signaling, Danvers, MA, USA), and IL-17 (1:100, Rabbit polyclone to IL-17, Abcam, Cambridge, MA, USA) overnight at 4 °C, which was followed by staining with secondary antibodies (1:50, 30 min at room temperature; Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) and counterstaining for nuclei. Tissue sections stained with isotype control or purified rabbit immunoglobulin-G using the identical concentrations and procedures revealed no positive staining, thus verifying the specificity of the staining results. Photos were taken from eight randomly selected fields for each slide at 40× magnification using the Nikon Eclipse TE2000-U microscope (Nikon Instruments, Amsterdam, Netherlands). Image-Pro Plus (MediaCybernetics, Rockville, MD, USA) software was used to count the number of stained cells as indicated in the figure legends for quantification purpose. An average positively-staining cell count for CD15, pSTAT3, and IL-17 was separately reported for each patient.

Clinical characteristics were compared amongst subgroups stratified by receipt and nonreceipt of neoadjuvant chemotherapy using the *t*-test for numerical variables and the Fisher's exact test for categorical variables. The median value for $CD15^+$, $pSTAT3^+$, and $IL\text{-}17^+$ cell counts was established in three separate groups: (1) patients who received no neoadjuvant therapy, (2) patients who received prior neoadjuvant therapy, and (3) the overall study population. The Kaplan–Meier method was used to compare survival in cohorts stratified by biomarker expression above and below these median values. Data were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

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