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Characterization of the Inflammatory Microenvironment and Identification of Potential Therapeutic Targets in Wilms Tumors[1, 2]

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Abstract

The role of inflammation in cancer has been reported in various adult malignant neoplasms. By contrast, its role in pediatric tumors has not been as well studied. In this study, we have identified and characterized the infiltration of various inflammatory immune cells as well as inflammatory markers in Wilms tumor (WT), the most common renal malignancy in children. Formalin-fixed paraffin-embedded blocks from tumors and autologous normal kidneys were immunostained for inflammatory immune cells (T cells, B cells, macrophages, neutrophils, and mast cells) and inflammatory markers such as cyclooxygenase-2 (COX-2), hypoxia-inducible factor 1α, phosphorylated STAT3, phosphorylated extracellular signal–related kinases 1 and 2, inducible nitric oxide synthase, nitrotyrosine, and vascular endothelial growth factor expression. Overall, we found that there was predominant infiltration of tumor-associated macrophages in the tumor stroma where COX-2 was robustly expressed. The other tumor-associated inflammatory markers were also mostly localized to tumor stroma. Hence, we speculate that COX-2–mediated inflammatory microenvironment may be important in WT growth and potential therapies targeting this pathway may be beneficial and should be tested in clinical settings for the treatment of WTs in children.

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Introduction

The importance of inflammation in tumor development is well known, and it is apparent that an inflammatory microenvironment is a key component of many tumors, even when a clinical association with inflammation is not yet demonstrated [1–3]. During the past decade, studies using cell-specific knockout animals have elucidated mechanisms by which inflammation leads to cancer [4]. Inflammation is initiated by the recruitment of a wide range of inflammatory immune cells, which induce tumor cells to produce inflammatory mediators such as chemokines and cytokines, reactive oxygen and nitrogen species, and various other bioactive molecules, which work in an autocrine and/or paracrine manner [2]. In some instances, genetic as well as epigenetic modifications can also establish an

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Table 1. Number of Sections that Stained Positive for Each Immune Cell Type and Inflammatory Marker Analyzed

CD3	CD20	TAM	TIN	MC	COX-2	HIF-1	p-Stat3	p-ERK1/2	iNOS	NT	VEGF
10/14	7/14	13/14	12/14	12/14	12/12	7/7	10/13	10/14	11/13	13/13	13/13

inflammatory microenvironment to promote tumor progression [1]. Thus, there exists a delicate balance between antitumor immunity and tumor-promoting immune activity within the tumor microenvironment, involving tumor cells, stroma (including fibroblasts and endothelial cells), and innate and adaptive immune cells.

The role of an inflammatory microenvironment in tumor development has been investigated primarily in adult-onset cancers, often those for which inflammation is a known risk factor. Little is known about the role of an inflammatory microenvironment in the development and growth of childhood tumors. Wilms tumor (WT) is a childhood cancer of the kidney that is thought to be largely a result of genetic alterations, variably including mutations in the *WT1*, *CTNNB1*, and/or *WTX1* genes. Vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 (HIF-1), two proteins that are upregulated in the inflammatory environment and recruit inflammatory immune cells, have been observed in WT [5]. In a comparative analysis with adult tumors, infiltration of macrophages [6] was reported along with ubiquitous expression of cyclooxygenase-2 (COX-2) in a small set of pediatric tumors (including five WTs), and this infiltration and expression were independent of the type of neoplasm [7,8]. However, these studies were restricted to only one inflammatory marker, and none of the studies provided a comprehensive view of the inflammatory microenvironment in pediatric tumors or correlated the presence of these markers with inflammation in WT.

To learn more about the role of the inflammatory microenvironment in the development of WT, we analyzed tumors for various inflammatory markers and inflammatory immune cells by immunohistochemical (IHC) staining. Overall, we found that WT exhibited infiltration of inflammatory immune cells and overexpression of several inflammatory transcription factors and other inflammatory markers compared with normal kidneys. Our data suggest that a COX-2–mediated inflammatory microenvironment may be important in WT tumorigenesis and that investigating the potential utility of therapeutic targeting of this environment is warranted.

Materials and Methods

Tissue Samples

Pretreatment tumor tissues and autologous normal kidney specimens were obtained from 16 WT patients aged 7 to 66 months at the time of diagnosis. Informed consent was obtained from each patient's parent or guardian. Studies were approved by the Institutional Review Board and in accordance with an assurance filed with and approved by the US Department of Health and Human Services. Eight of the patients were males and eight were females, and one patient had bilateral disease. Of these 16 patients, 4 were at stage IV, 4 were at stage III, 3 were at stage II, and 5 were at stage I of WT disease. Tissues were fixed in formalin and embedded in paraffin (FFPE) in preparation for analysis.

Mouse Tumor Tissues

A mouse model for the human WT has been generated in our laboratory [9] by *Wt1* gene ablation and *insulin-like growth factor 2* (*IGF2*) upregulation by conditional knockout strategy (*Wt1*$^{-/fl}$*H19*$^{+/-m}$*Cre-ER*^{TM} *or Wt1-IGF2* mice). These mice developed tumors at the age of 3 months on an average. The tumors and normal kidneys from its littermate controls were collected at the similar age and processed as mentioned earlier for histology and IHC analysis.

Histology and IHC of Immune Cell Markers

FFPE specimens were cut in 5-μm sections, which were stained with hematoxylin and eosin. For IHC analysis, FFPE sections were deparaffinized in xylene, rehydrated sequentially in ethanol (100%, 90%, and 70%), and placed into a 1% phosphate-buffered saline solution (PBS; pH 7.4). Tissues were analyzed for infiltration by T cells, B cells, macrophages, neutrophils, and mast cells (MCs). Inflammatory markers analyzed were COX-2, HIF-1, phosphorylated extracellular signal–related kinases 1 and 2 (p-ERK1/2), phosphorylated STAT3 (p-Stat3), inducible nitric oxide synthase (iNOS), nitrotyrosine (NT), and VEGF. Simultaneously, to prove the similar expression and infiltration pattern in the mouse model of WT, mouse tumor tissues and control kidneys were immunostained for inflammatory marker COX-2 and predominant inflammatory immune cells, macrophages (F4/80). Details of antibody staining and epitope retrieval are summarized in Table W1.

Tissues known to contain the antigen of interest were identified and used as positive controls to optimize IHC staining by testing antigen retrieval methods and times and by titrating antibody dilutions. A positive control tissue slide was included in each batch of immunostaining. Negative controls were tissue sections not treated with the primary antibody. The numbers of sections assessed for each tumor for different immune cells and inflammatory protein markers are indicated in Table 1. Because of limitations in the amount of tumor tissue available, IHC data could not be obtained for all tumors.

MC Staining

MC infiltration in tumors and normal kidneys was assessed by quantification of chloroacetate esterase (Cat. No. 91C kit; Sigma Chemical Co, St Louis, U.S.A.). Briefly, immediately before fixation, 1 ml of sodium nitrite solution was added to 1 ml of Fast Red Violet LB base solution in a test tube and mixed gently by inversion and allowed to stand for 2 minutes. This solution was added to 40 ml of prewarmed (at 37°C) deionized water and then to 5 ml of Trizmal 6.3 buffer concentrate; afterwards, 1 ml of naphthol AS-D chloroacetate solution was added to obtain a red colored solution that was transferred into a Coplin jar. Slides were fixed in citrate acetone formaldehyde solution at room temperature (23-26°C) for 30 seconds. Slides were rinsed in running water for 45 to 60 seconds and incubated in previously prepared red colored solution for 15 minutes in Coplin jar at 37°C protected from light. Slides were rinsed with deionized water for 2 minutes and counterstained by Mayer's hematoxylin (Fisher Scientific, Fair Lawn, NJ) and mounted by aqueous mounting media. After drying, slides were evaluated microscopically.

Double Immunofluorescence Analysis

To examine the co-distribution of inflammatory marker COX-2 and tumor-associated macrophage (TAM) infiltration in the tumor stroma, a double immunofluorescence staining was carried out.