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ANATOMICAL PATHOLOGY

Immunological environment in colorectal cancer: a computer-aided morphometric study of whole slide digital images derived from tissue microarray

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Summary

Cancer research has moved from investigating tumour cells to including analysis of the tumour microenvironment as well. The aim of this study was to assess the cellular infiltrate of colorectal cancer (CRC) using computer-aided analysis of whole slide digital image derived from tissue microarray (TMA). TMA slides from 31 CRC patients were immunostained for forkhead box protein 3 (FOXP3) and immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) at four sites: centre (C) and invasive front (F) of the tumour, proximal non-metastatic draining lymph node (N−), tumour-draining lymph node with metastasis (N+) and healthy mucosa at 10 cm from the cancer (M). We analysed the proportion of IDO+ tissue areas in the lamina propria or in the non-epithelial area of the lymph node and in epithelial cells in each site. The normal mucosa of patients operated on for benign disease was also analysed. The proportion (%) of FOXP3+ tissue area in C, F, N−, N+ and M were 2.3 ± 1.8, 2.6 ± 2.9, 6.0 ± 2.9, 14.2 ± 5.8 and 1.2 ± 0.8 ($p < 0.001$). The proportion (%) of IDO+ tissue area in the lamina propria of C, F, N−, N+ and M were 1.6 ± 3.1, 1.1 ± 1.3, 3.4 ± 2.5, 9.1 ± 8.5 and 6.7 ± 5.4 ($p < 0.001$). IDO+ tissue area in the lamina propria was not significantly different between healthy mucosa of patients with cancer than without (1.8 ± 3 vs 1.1 ± 0.95). The proportion of IDO positive tissue area in the epithelium was significantly higher in healthy mucosa of patients with cancer than without (5.4 ± 13.8 vs 2.1 ± 2.4). The FOXP3+ tissue area was increased in healthy mucosa of CRC patients in comparison with healthy mucosa of patients with colorectal resection for disease other than cancer: 1.20 ± 1.81 versus 0.81 ± 0.51 ($p < 0.05$). The proportion of IDO+ tissue area in lymph node (N−) was correlated with the proportion of FOXP3+ tissue area in tumour area ($r = 0.44$, $p < 0.01$). TMA technique permits simultaneous analysis of FOXP3+ and IDO+ cells at different sites including tumour, draining non-metastatic lymph node, metastatic lymph node and normal mucosa.

Key words: Digital imaging; colorectal cancer; microenvironment; regulatory T cells; tissue microarray; FOXP3; IDO.

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INTRODUCTION

Colorectal cancer (CRC) is the most common gastrointestinal malignancy and one of the leading causes of cancer-related deaths worldwide.[1] Factors such as tumour invasion and frequency of lymph node or distant metastases determine the prognosis of the disease. Recently, studies of inflammatory cells in cancer have gained increasing interest, particularly with regard to tumour invasiveness and metastases. The composition of the microenvironment of several tumour types, especially CRC, could serve as prognostic factors,[2,3] although others advocate that the TNM stage should remain the preferred prognostic system.[4] More research on the inflammatory infiltrate in the tumour and the microenvironment both in the tumour and lymph node could help to improve our understanding of tumour genesis.[5,6] The inflammatory infiltrate consists of different B and T lymphocytes, mast cells, macrophages, antigen-presenting cells (APC), fibroblasts, and endothelial cells. Among these cells, regulatory T lymphocytes (Tregs) play a crucial part in the inflammatory response in CRC[7–10] and can be detected by immunohistochemistry, using forkhead box protein 3 (FOXP3) as a marker.[11] Moreover, considerable evidence now supports the concept that cells expressing indoleamine 2,3-dioxygenase (IDO) can suppress T-cell responses and promote tolerance.[12] In CRC, high expression of IDO by colorectal tumour cells enables certain cancer subsets to avoid immune attack and defeat the invasion of T cells via local tryptophan (Trp) depletion.[13]

The first aim of our study was to describe precisely the proportion of FOXP3+ and IDO+ cells at the centre of the tumour and invasive front, proximal draining lymph node, and healthy mucosa. Our histopathology department has

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developed a computer-aided analysis of whole slide digital image derived from tissue microarray (TMA).[14] This new analysis overcomes the considerable challenges of classic methods due to tumour heterogeneity, the extreme difficulty of reproducing semi-quantitative analysis results, and the time-consuming nature of analysing whole-tissue samples. The second aim of this study was to compare IDO+ infiltration in the tissues with plasma IDO concentration, evaluated with serum kynurenine (Kyn) level and the Kyn/Trp ratio.

MATERIALS AND METHODS

Patients

This prospective longitudinal observational study was conducted in a single institution, Pitié-Salpétrière Hospital, Paris, France, with approval of the local ethics committee.

Thirty-one patients who were operated on for CRC in our institution between March 2010 and September 2011 were included in the study. Prior to TMA construction, all diagnoses were established according to classical histopathological criteria. Analysis of mismatch repair status by immunohistochemistry was performed as described elsewhere.[8] A patient was considered MSI+ if there was DNA microsatellite instability (MSI), meaning deficiency in the mismatch repair system, and MSI– if no MSI existed. Tumour micro-invasive status was defined by the presence of vascular emboli, lymphatic invasion, and perineural invasion (collectively referred to as VELIPI); patients were scored from 0 to 3 according to the status of each parameter (0 negative, 1 positive). Demographic parameters and the main characteristics of patients' tumours are reported in Table 1.

Construction of TMA

For each paraffin-embedded tissue donor block, five tissue cylinders with a diameter of 0.6 mm were punched from morphologically representative areas at four sites: centre of the tumour (C), invasive front of the tumour (F), a proximal tumour-draining lymph node without metastasis (N–), metastatic tumour-draining lymph node (N+), and healthy mucosa (M) located 10 cm from the tumour. These cylinders were brought into a recipient paraffin block using a semi-automated tissue arrayer (Alphelys, France). Healthy mucosa of patients operated on for diseases other than cancer was also included in the same TMA to analyse IDO and FOXP3 expression in comparison with that of CRC patients.

Table 1 Demographic and histological characteristics of the 31 patients with CRC

Characteristics	No. patients
Age, years, mean ± SD	63 ± 12
Sex ratio (M/F)	19/12
T stage	
T1	1
T2	2
T3	19
T4	9
N stage	
N0	15
N1	8
N2	8
M stage	
M0	23
M1	8
MSI status	
+	3
–	28
VELIPI status	
0	13
1	8
2	3
3	7
TNM staging	
I	4
IIA	12
IIC	1
IIIB	3
IIIC	3
IVA	4
IVB	4

TNM staging according the AJCC 8th edition.
MSI, microsatellite instability; VELIPI, venous embolism, lymphatic invasion, perineural invasion.

Immunohistochemistry

The immunostaining procedure was performed on formalin fixed, deparaffinised, 4 µm thick TMA sections using a biotin-free polymeric visualisation system (Ultravison LP, ref TL-015-HD; Lab Vision, USA). The following primary antibodies were used at the indicated dilutions: mouse monoclonal anti-IDO antibody (dilution 1:100; clone 10.1; Chemicon-Millipore, France); mouse monoclonal anti-human FOXP3 (dilution 1:200; clone 236A/E7; Abcam, UK). Matched isotype control antibody was used to choose the best IDO antibody dilution and minimise staining background. The antigen retrieval reaction was performed in a water bath, using EDTA buffer (pH 8.0) for 20 min at 97°C. The slides were then incubated with the peroxidase block supplied by the manufacturer of the visualisation system for 10 min. Next, the slides were incubated with the primary antibodies for 60 min at room temperature. The incubation time was 10 min for each step of the visualisation system. Chromogen diaminobenzidine supplied by the manufacturer was applied for 5 min, and counterstaining with haematoxylin for 1 min.

Computer-aided morphometric study of whole slide digital images from TMA

TMA glass slides stained with FOXP3 or IDO were scanned to produce digital slides (0.25 µm/pixel at 40× magnification) using the Aperio Slide Scanning System (ScanScope CS; Aperio Technologies, USA). The whole slide digital images were viewed on a Barco Coronis fusion 6MP high-definition screen (Barco NV, Belgium) to determine the detection thresholds, and images were analysed with ICS software (Tribvn SA, France). This software is able to discriminate the immunostained area on the basis of red, green, and blue colour segmentation and to calculate the immunoreactive area. Given that an accurate optical count of immunoreactive cells was not feasible in the presence of very dense infiltrates, we did not attempt to convert area values to number of cells. We manually distinguished two types of IDO positive tissue area: on one hand, dendritic cells located in the lamina propria of normal mucosa or in the stroma of carcinomatous samples (expressed as a fraction of the area occupied by lamina propria or stroma) and, on the other hand, normal or neoplastic epithelial cells (expressed as a fraction of the area occupied by epithelial cells). We determined in C, F and M, the proportion of IDO+ area of each type. FOXP3 was expressed as a fraction of the TMA spot area. Concerning the lymph nodes, FOXP3 and IDO were expressed as a fraction of the TMA spot area. Finally, we obtained for each case, the mean of the results calculated for five TMA spots for each site (C, F, N–, N+, M) for FoxP3+ cells, IDO+ dendritic cells, and IDO+ epithelial (normal or neoplastic) cells, respectively.

Measurements of serum Trp and Kyn

Blood samples (sheltered from light) were obtained using a standard phlebotomy protocol immediately before each patient was anaesthetised. At the time of blood sampling, the patients had not received any treatment, including surgery, chemotherapy, or radiation therapy. No patients had hepatic or renal insufficiency or neuro-psychiatric disease. The samples were rapidly separated, and serum samples were frozen at −70 °C until batch processing. The concentrations of Trp and Kyn were measured simultaneously by high-performance liquid chromatography with fluorescence detection, according to the protocol of Yamada *et al.*[15] The IDO activity was determined by dividing the serum concentration of Kyn by that of Trp (Kyn/Trp).

Statistical analysis

For the between site comparison, within the CRC patient group, we tested the overall effect of the site by a one way analysis of variance (ANOVA) and compared the site two by two with a post-hoc test of Tukey.

For the between group comparison, we used a two sample Wilcoxon test and computed p values adjusted using Bonferroni method.

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