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Research paper

Surface profiles of live colorectal cancer cells and tumor infiltrating lymphocytes from surgical samples correspond to prognostic categories

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ABSTRACT

Extensive surface profiles of colorectal cancer (CRC) cells and tumor infiltrating lymphocytes (TIL) have been obtained from 45 surgical resection samples. Live cells were captured on an antibody microarray and stained with fluorescently-labeled antibodies. Minimal panels of 11 CRC antigens (CD13, CD24, CD26, CD49d, CD138, CD166, CA-125, CA19-9, EGFR, Galectin-4 and HLA-DR) and 11 T-cell antigens (CD10, CD11b, CD11c, CD25, CD31, CD95, CD151, CD181, Galectin-4, CA19-9, TSP-1) provide signatures for relapse and survival. Hierarchical clustering of profiles from CRC cells and TIL identified groups of patients for survival, systemic relapse and death. The groups from CRC and TIL profiles for systemic relapse showed 79.2% concordance, enabling prediction of relapse after surgery. The results demonstrate communication between CRC cells and TIL.

1. Introduction

The microenvironment of colorectal cancer (CRC) cells can influence whether they will metastasize or remain dormant (Ribatti et al., 2006; Gout and Huot, 2008; Witz, 2009). The presence of tumor infiltrating lymphocytes (TIL) within CRC may diminish metastasis formation, improving survival (Pages et al., 2010). Interactions between CRC cells and TIL occur via trans-membrane proteins. For example, HLA-DR is required for tumor-associated antigen recognition by $CD4^+$ T-cells. Increased HLA-DR expression on CRC cells can stimulate $CD4^+$ T-helper cells and natural killer cells by presenting tumor-associated antigens resulting in interferon-γ production, and is an independent factor for better survival outcomes (Matsushita et al., 2006). Surface antigens on CRC cells may interact with partner molecules on other cell types, affecting the phenotype of the cancer and cells within the microenvironment.

Abbreviations: CRC, colorectal cancer; TIL, tumor infiltrating lymphocytes; CD, cluster of differentiation; HLA, human leukocyte antigen; ACP, Australian Clinico-Pathological; EpCAM, epithelial cell adhesion molecule; NLR, neutrophil to lymphocyte ratio; CA19-9, carbohydrate antigen 19-9; TSP-1, thrombospondin 1; TGF β1, transforming growth factor beta 1.

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In recent years, proteomic analyses have been used to discover prognostic 'markers' for CRC (Pei et al., 2007; Alfonso et al., 2008). However, no single marker has improved the prognostic power of current clinico-pathological staging, or gained wide acceptance for use in routine pathology. A CD (cluster of differentiation) microarray containing 82 antibodies has been developed that provides extensive surface profiles of all the common leukemias, enabling their classification based solely on 'surface signatures' (Belov et al., 2006). We have modified this microarray by addition of 40 antibodies against CRC surface proteins, identified from the literature. The 122-antibody DotScan™ CRC microarray consists of the 82-antibodies recognizing a range of lineage-specific leukocyte CD antigens that are adhesion molecules, receptors and markers of inflammation and immune response (Belov et al., 2006), with a satellite array of 40 antibodies for CRC surface proteins. In this paper, profiles of surface proteins were determined on live CRC cells and TIL, isolated from disaggregated primary tumors. These profiles have been used to identify signatures on CRC and TIL that predict systemic relapse, i.e., distant metastasis and cancer-specific death in CRC patients (overall survival).

2. Materials and methods

2.1. Samples from CRC resection specimens

Fresh CRC specimens were collected from 45 patients at the Royal Prince Alfred Hospital (Camperdown, NSW, Australia) and Concord Repatriation General Hospital (Concord West, NSW, Australia) with informed consent under Protocol No. X08-164. Each CRC sample was macro-dissected from resected specimens that were verified as CRC by histopathology and staged and graded according to the Australian Clinico-Pathological (ACP) staging system for CRC (Davis and Newland, 1983). The 4 main group stages of this system (A, B, C, D) are directly equivalent to the stages (I, II, III, IV) of the American Joint Committee on Cancer/International Union against Cancer (AJCC/UICC) Pathological Tumor–Node–Metastasis (pTNM) system (Fielding et al., 1991). Surface antigen profiles of Stages A, B, C and D tumors were subjected to statistical analysis. Normal mucosa was taken from the same patient sample at least 10 cm from the site of the tumor as a control. Patients were monitored for systemic relapse and survival (median follow-up time 45 months) from the date of resection. Detailed patient information is listed in Table 1.

Table 1
Clinico-pathological characteristics of the CRC patients.

	No. of patients (%) (n = 45)
Gender	
Male	21 (47%)
Female	24 (53%)
Age	
<68	18 (40%)
≥68	27 (60%)
Australian clinico-pathological (ACP) stage	
A	9 (20%)
B	20 (44%)
C	12 (27%)
D	4 (9%)
Direct spread	
Submucosa	2 (4%)
Muscularis propria	11 (24%)
Subserosa	26 (58%)
Serosa	6 (13%)
Presence of inflammation	
Present	21 (47%)
Absent	24 (53%)
Tumor infiltrating lymphocytes (TIL)	
Present	5 (11%)
Absent	40 (89%)
Circulating neutrophil to lymphocyte ratio (NLR)	
<5	16 (36%)
≥5	29 (64%)
Adjuvant therapy	
No	24 (53%)
Yes	18 (40%)
Unknown	3 (7%)

2.2. Surface profiling of CRC cells and TIL

Tissue disaggregation, cell fractionation, cell capture on DotScan™ CRC microarrays and fluorescence multiplexing with labeled antibodies were carried out as previously described (Zhou et al., 2010, 2011). Captured TIL and epithelial/CRC cells were stained with Phycoerythrin (PE)-anti-CD3 and Alexa 647-anti-EpCAM antibodies, respectively, on the same DotScan microarray. Microarrays were then scanned on a Typhoon 8600 Variable Mode Imager (Amersham-Pharmacia, Castle Hill, NSW, Australia) using a 532 nm laser/580 BP30 emission filter for PE and a 633 nm laser/670 BP30 emission filter for Alexa 647. Images were analyzed using DotScan™ software, and the dot fluorescence intensities for each microarray were imported into MultiExperiment Viewer (MeV) version 4.4 from the TM4 Microarray Software Suite. Cell binding densities were corrected for background and isotype-control binding and the duplicate microarray data were averaged and $\log_2$-transformed. Data obtained from 45 tumors and matched controls, consisting of CRC and TIL profiles, were median normalized separately and a paired 2-tailed Student's t-test assuming equal variances was used to identify differentially abundant antigens between CRC cells and normal intestinal mucosa. The analysis was repeated for TIL and T-cells from normal mucosa, using the same microarrays and captured cells visualized with PE-anti-CD3 antibody.

2.3. Antigen analysis

Patient follow-up information (relapse and survival) was obtained for the 45 patients. Antigens associated with metastasis were identified by Cox proportional hazards regression analysis from the 45 CRC surface profiles. Stage D patients were used to define metastatic potential and provide an accurate representation of the metastatic antigen profile. Stage A/B/C patients who subsequently developed systemic relapse may have had occult metastases at the time of operation not diagnosed but found subsequently during follow-up. The analysis was repeated with 41 TIL surface profiles. Four of the TIL profiles that were excluded as fluorescence multiplexing were unable to detect distinct profiles. Antigens associated with cancer-specific death were identified from the 45 CRC surface profiles by Cox proportional hazards regression analysis. Patients who had succumbed to CRC-related death were included in the analysis while those who died from other causes were also included but had their survival times censored

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