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Invariant chain expression in colon neoplasms

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Abstract Invariant chain (Ii) is a chaperone molecule that inhibits binding of endogenous antigens to class II molecules. High levels of Ii in cancer cells may prevent tumour antigen expression with class II and render the tumour less immunogenic. To correlate the expression of Ii and class II molecules in colon carcinomas with the density of tumour infiltrating lymphocytes (TILs), surgical specimens from a total of 48 patients with well- (WDAC), moderately (MDAC) and poorly differentiated adenocarcinomas (PDAC), adenoma with high-grade dysplasia (AdHGD) and adenomas were immunostained for Ii and class II antigen (HLA-DR). Aggregates of TILs were graded in H&E-stained sections. Normal colon epithelium was negative for Ii and HLA-DR. Invasive carcinomas showed a linear increase in the expression of Ii in the progression from low- to high-grade tumours, while there was no significant difference in HLA-DR expression across the groups. Invasive carcinomas showed a disproportionate increase in Ii over HLA-DR. Frequency of TILs showed inverse correlation with expression of Ii and tumour grade. This is the first demonstration that expression of Ii increases in the progression from low- to high-grade colon neoplasms and is most marked in the poorly differentiated carcinomas. Ii expression by carcinomas is inversely related to the frequency of TILs. The findings suggest that increased Ii renders the tumour less immunogenic and less likely to stimulate a host immune response.

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Introduction

Tumour-host interactions influence the growth of neoplasms, and it has been suggested that patients with tumours containing more infiltrating lymphocytes (TILs) tend to have a better prognosis than those with fewer TILs [22].

One factor that might influence the host lymphocytic response to tumour is the expression of tumour antigens with class II molecules on the cell surface. Another factor that might influence the binding of peptides to class II molecules is the class II-associated invariant chain (Ii). It is a type-II membrane glycoprotein synthesized as different isoforms required for class II assembly in endoplasmic reticulum (ER). Ii plays a central part in the intracellular transport of class II molecules. When it remains associated with these molecules during their transport from the ER to the *trans*-golgi network, they are unable to bind endogenously derived peptides present in these compartments [2, 3, 6]. Increased expression of Ii in malignant neoplasms may thus prevent presentation of endogenous tumour antigens by class II molecules, and this in turn would prevent host immune response against the tumours.

Some human tumours, including colon carcinoma, express class II molecules (HLA-DR) [8, 13, 18]. However, class II expression in tumour cells does not always correlate with prognosis [5, 17, 18]. We aimed to correlate the expression of Ii and HLA-DR in colon neoplasms, including adenoma, adenoma with high-grade dysplasia and colon carcinoma, with the density of TILs. Our results showed that expression of Ii increased from well- to poorly differentiated carcinoma, and the carcinomas with high expression of Ii had fewer lymphocytic aggregates. These data support the speculation that expression of Ii prevents tumour cells from presenting their endogenous tumour antigens to T-lymphocytes and contributes to a

reduced host immune response to colon carcinomas. They also imply that Ii may be a potential marker for more aggressive behaviour of colon carcinoma.

Materials and methods

Colonic resections or biopsy specimens with well- (WDAC, *n*=8), moderately (MDAC, *n*=12) and poorly differentiated adenocarcinomas (PDAC, *n*=5), adenoma with high-grade dysplasia (AdH-GD, *n*=8), adenoma (*n*=14), and normal colon (NC, *n*=8) were identified in the surgical pathology files available at the University of Massachusetts Medical Center from 1991 to 1997. Cases with slides and blocks available were included in the study. Carcinomas included tumours in stage 0 (*n*=8); stage I (*n*=3); stage II (*n*=10); stage III (*n*=8); and stage IV (*n*=4). The 15 cases of adenoma included villous adenomas (VA, *n*=5), tubulovillous adenoas (TVA, *n*=5), and tubular adenomas (TA, *n*=5). Nine sections of normal bowel were obtained from the margins of the resection specimens or from the biopsies. All H&E-stained slides of the lesions were reviewed, and one representative section was selected from each case for immunostaining. Tumour grade was assessed according to standard criteria [15]. Tumour stage was obtained from surgical pathology reports.

The formalin-fixed, paraffin-embedded sections were cut at 4 μm, heated at 60° C for 30 min, then deparaffinized and hydrated through a series of xylenes and alcohols. Optimum pretreatment and dilutions were determined by testing with both known positive and negative material. Mouse monoclonal antibody against Ii (CD74) and HLA-DR (DAKO, Carpinteria, Calif.) at dilutions of 1:200 and 1:250, respectively, with antigen retrieval gave us the best signal-to-noise ratio in the positive test material and no staining in the negative test material. The slides were microwaved with a proprietary antigen retrieval solution (citrate buffer; BioTek Solutions, Santa Barbara, Calif.) for 5 min in an 800-W microwave oven. Following replenishment of this solution the slides were microwaved again for an additional 5 min and then allowed to cool for 20 min. The slides were stained on a BioTek Solutions TechMate 1000 automated immunostainer using an avidin-biotin complex (ABC) staining procedure (BioTek). Following a hydrogen peroxide block of endogenous peroxide and a serum-blocking step, the slides were incubated with the primary antibody for 45 min, followed by brief buffer washes and then incubation in a cocktail of biotinylated anti-mouse IgG/IgM and anti-rabbit IgG (BioTek) for 30 min. The sections were then washed, incubated in avidin–biotin complex (BioTek) for 30 min, washed, then reacted with diaminobenzidine and hydrogen peroxide to visualize the end-product. Sections were counterstained with haematoxylin. A duplicate set of slides was also stained in exactly the same manner except that normal mouse serum was substituted for the primary antibody, to serve as a negative control.

The expression of Ii and HLA-DR was graded as 0=no positive cells; 1+=<10% of tumour cells positive; 2+=10–30% of tumour cells positive; 3+=>30% of tumour cells positive. For data analysis, grade 2+ and 3+ staining was regarded as significant. TILs were evaluated around the periphery of the tumours and invasive nests of carcinoma cells, but not near areas of ulceration of tumour necrosis. Lymphoid aggregates were evaluated as follows: 0=no lymphoid aggregates or at most one single, small lymphoid aggregate in each tumour section; 1+=occasional, usually small lymphoid aggregates with rare or absent germinal centres; and 2+=numerous, large lymphoid aggregates with frequent germinal centres [11].

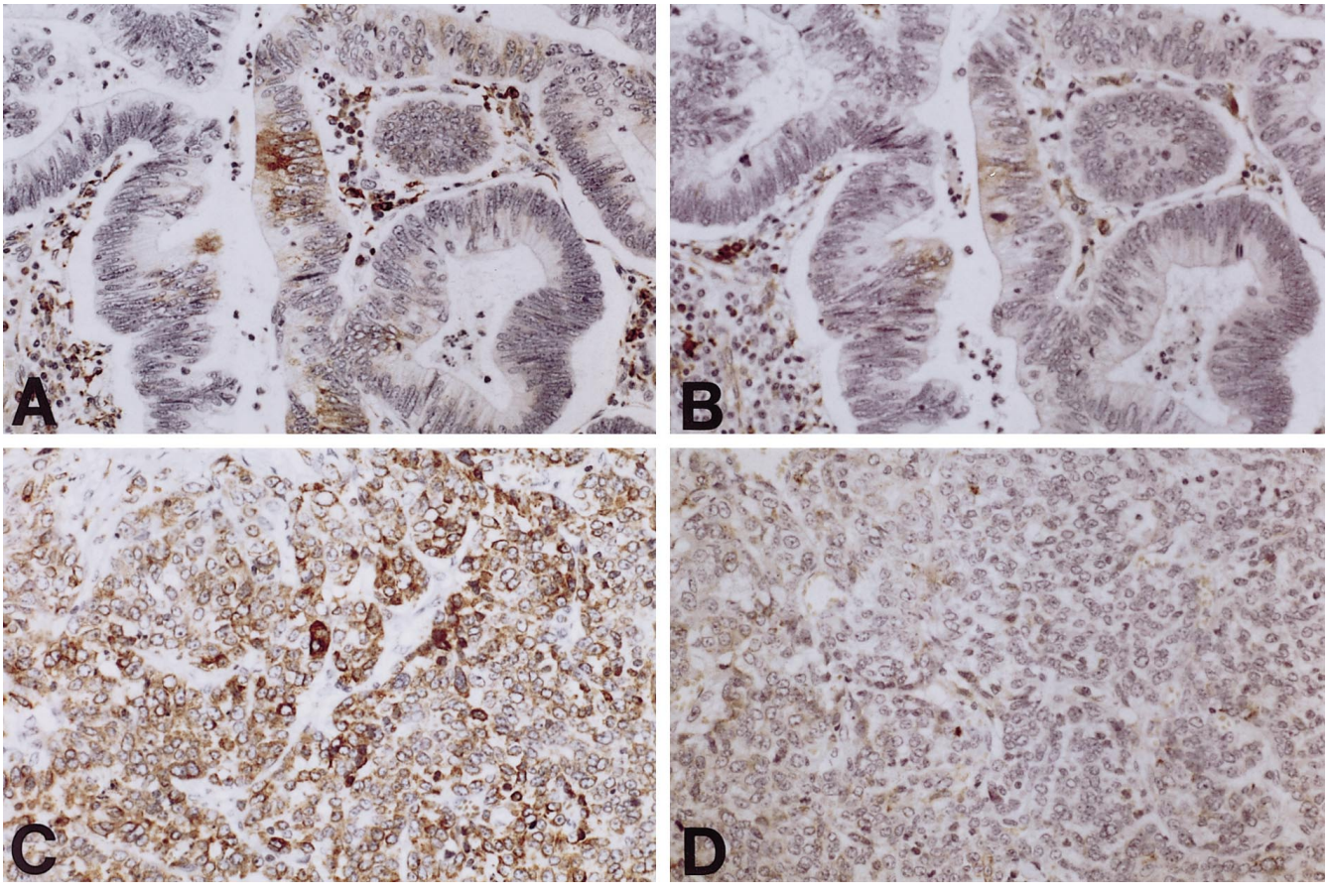

Fig. 1 Microscopic sections showing **A** invariant chain (Ii) and **B** class II antigen (HLA-DR) locally expressed in a well-differentiated adenocarcinoma of the colon, and **C** Ii diffusely positive and **D** HLA-DR positive in a few cells in a poorly differentiated adenocarcinoma of the colon

For statistical analysis, 2+ and 3+ positive staining for Ii and HLA-DR was considered to be a significant level of staining. The percentages of tumours with 2+ and 3+ positive staining for Ii and HLA-DR and the frequency of tumours with 2+ TILs were compared Chi-square tests and a regression analysis for linear fit.

Results

There was no expression of Ii and HLA-DR in normal colonic epithelium. Lymphocytes were strongly positive for Ii and HLA-DR.

Ii and HLA-DR were detected within the cytoplasm and on the cell membrane in epithelial cells of the adenomas, AdHGDs, and carcinomas. Staining was patchy in all adenomas, AdHGDs and WDACs (Fig. 1A, B). Most of the adenomas that were positive for HLA-DR, were also positive for Ii (Figs. 2, 3). In the AdHGD cases, Ii and HLA-DR were expressed in both the adenoma and high-grade dysplasia in situ components. In MDAC and PDAC, Ii and HLA-DR staining showed a more diffuse pattern of positive staining (Fig. 1C, D). However, Ii showed stronger staining than HLA-DR (Fig. 1C, D) and some cells were positive only for Ii. No adenoma cells and carcinoma cells were positive for HLA-DR without expression of Ii.

The frequency of cases with 2 and 3+ Ii, and HLA-DR is shown in Table 1. Low frequency of expression of Ii and HLA-DR was found in the adenomas, adenocarcinomas in situ (AdCISs) and WDACs (Table 1, Figs. 2, 3). There was no significant difference in expression of Ii and HLA-DR between adenomas and AdCISs. Invasive carcinomas, unlike adenoma and carcinoma in situ, showed a disproportionate increase in Ii over HLA-DR (Fig. 3). Furthermore, there was a significant increase in the Ii expression from well to poorly differentiated carcinomas (P<0,05, Fig. 3) (R-square=0.96). Frequency of expression of Ii with 2+ and 3+ was 100% in poorly differentiated carcinoma. However, there was no significant difference in the HLA-DR values across the groups

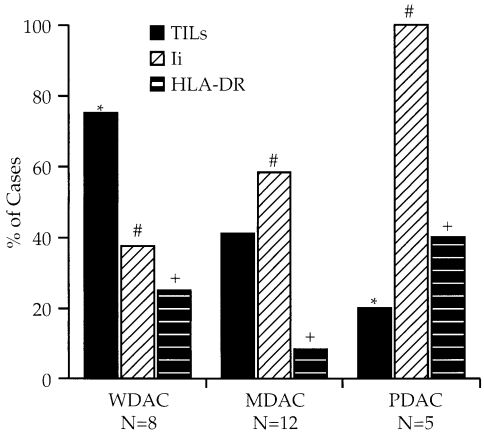

Fig. 3 Bar graph showing the percentage of invasive carcinomas with 2+ and 3+ Ii and HLA-DR expression and 2+ tumour-infiltrating lymphocytes (*TILs*), by tumour grade. The frequency of TILs was greater in well-differentiated adenocarcinomas (*WDAC*) than in poorly differentiated adenocarcinomas (*PDAC*). (*=P<0.05). TILs also showed inverse correlation with tumour grade (R-square=0.98). There was a significant difference in the Ii values across the groups (#P<0.05). Invasive carcinomas showed a linear increase in the expression of Ii in the progression from low- to high-grade tumours (R-square=0.96). Expression of Ii was always higher than that of HLA-DR in each category. There was no significant difference (+) in the HLA-DR values across the groups (*MDAC* moderately differentiated adenocarcinoma)

Table 1 Expression of Ii and HLA-DR in colonic adenomas and carcinomas (*AdHGD* adenoma with high-grade dysplasia, *WDAC* well-differentiated adenocarcinomas, *MDAC* moderately differentiated adenocarcinomas, *PDAC* poorly differentiated adenocarcinomas)

Category	No. of 2+ and 3+ cases	
	Ii	HLA-DR
Adenoma (n=15)	2 (13%)	2 (13%)
AdHGD (n=8)	3 (38%)	2 (25%)
WDAC (n=8)	3 (38%)	2 (25%)
MDAC (n=12)	7 (58%)	1 (8%)
PDAC (n=5)	5 (100%)	2 (40%)

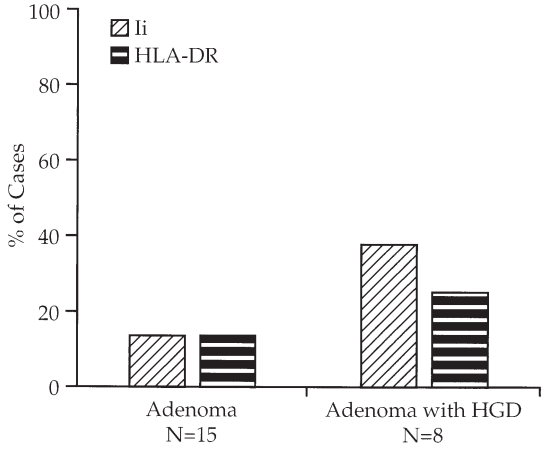

Fig. 2 Bar graph showing the percentages of adenomas and adenomas with high-grade dysplasia (*HGD*) stained 2+ and 3+ for Ii and HLA-Dr. There was no significant difference between adenomas and adenomas with high-grade dysplasia (*HGD*)

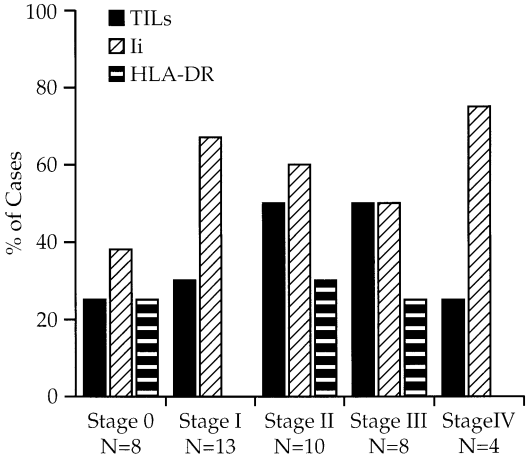

Fig. 4 Bar graph showing the percentage of invasive carcinomas with 2+ and 3+ and HLA-DR expression and 2+ TILs by tumour stage. There was no correlation between Ii and HLA-DR expression, or between TILs and tumour stage

(Fig. 3). The frequency of 2+ TILs was greater in WDAC than in PDAC ($P<0.05$, Fig. 3; R-square=0.98).

In addition to tumor grade, the expression of 2+ and 3+ Ii and HLA-DR and the frequency of TILs was analyzed with respect to stage. No significant relationship was found (Fig. 4).

Discussion

Our study shows that expression of Ii increases in the progression from low- to high-grade colon neoplasms and is most marked in the poorly differentiated carcinomas. Ii expression by carcinomas is inversely related to the frequency of TILs. These findings suggest that increased Ii renders the tumour less immunogenic and less likely to stimulate a host immune response.

Immune responses mediated by T lymphocytes against tumours are directed against tumour-specific antigens. These antigens are recognized by CD8+ cytotoxic T cells (CTLs) in the context of MHC class I molecules and by CD4+ T helper (Th) cells in the context of MHC class II molecules, TILs mainly contain CD4+ Th, CD8+ CTLs and macrophages [11]. Generation of tumour-specific CD4+ Th cells enables tumour-specific CD8+ T cells to function more efficiently. It has been shown that in vivo depletion of CD4+ cell results in host susceptibility to tumour, indicating that these cells are required for resistance [16]. Ostrand-Rosenberg et al. demonstrated that tumour cells transfected with syngeneic MHC class II genes were rejected by autologous mice, presumably because tumour antigens were displayed to host lymphocytes by class II molecules. However, tumour cells transfected with class II plus Ii were not rejected and were highly malignant, presumably because expression of tumour antigens with class II was prevented by Ii [2, 7]. In vitro studies confirmed this hypothesis, as it was clearly shown that peptide binding was inhibited when class II molecules were associated with Ii [21]. Bodmer et al. demonstrated that endogenous epitopes were presented more efficiently in Ii knock-out mice; the presentation of these epitopes was inhibited when Ii was expressed [4].

The animals models demonstrated that the Ii molecule has an important role in oncogenesis. However, little is known about Ii expression in human colon carcinomas and its relationship with tumour behaviour. To our knowledge this is the first observation that Ii is related to tumour grade and TILs in human colon carcinoma. Both tumour grade [9, 10, 12, 19] and TILs [11, 22] have been shown to be significant predictors of survival and independent prognostic factors in colorectal adenocarcinoma; TILs can provide important prognostic information in colon cancer and can be incorporated into a staging system and used in the evaluation for adjuvant therapy [14]. Our study shows a relationship between the frequency of TILs and Ii expression by tumour cells and suggests a possible mechanism by which Ii may influence the behaviour of colon carcinomas. One may speculate that Ii prevents presentation of tumour antigen by HLA-DR to stimulate tumour-specific CD4+ Th cells. Down-regulation of tumour-specific CD4+ Th cells would decrease the proliferation of TILs and would prevent the immune system from inhibiting tumour growth. Higher expression of Ii with lower frequency of TILs then suggests that Ii may be a potential marker for poor biological behaviour of colon carcinoma. Degener et al. [8] reported that Ii was expressed by colon carcinoma, but did not, however, find a correlation between Ii expression and tumour grade, and they found Ii overexpression in adenoma. A technical difference between our study and theirs may contribute to this discrepancy: they used frozen sections and VIC-Y1 antibody (anti-N-terminus of Ii), and we used an antibody that was anti-C-terminus of Ii.

Although the stage at diagnosis is the most important prognostic factor in colon carcinoma, we found no correlation between expression of Ii and tumour stage. However, multiple factors determine tumour stage, including the time preceding diagnosis and prior therapy. This correlation will need to be addressed in larger studies.

The relationship between Ii and HLA-DR may have implications for the development of immunotherapy for colon carcinoma, since previous reports have shown that strong HLA-DR expression in colon carcinoma is associated with a good prognosis [1]. Tumour cells transfected with syngeneic MHC class II genes for treatment of establish tumour and metastatic disease have been developed [20]. However, Ostrand-Rosenberg et al. have recently shown that if tumors coexpress class II with Ii they are not effective immunogens [2]. Our data support their findings: poorly differentiated carcinomas express more Ii and HLA-DR is not correlated with tumour grade. Even though there is a high level of HLA-DR in tumour cells, they cannot induce a host immune response if they have a high level of Ii expression. Transfection of class II without Ii or antisense to Ii could be an effective approach for tumour immunotherapy. Furthermore, if cell-based vaccines aimed at stimulating tumour-specific CD4 T helper cells for colon carcinoma are to be adopted clinically, they should express MHC class II molecules without coexpression of Ii.

In summary, in a group of colonic adenomas, adenomas with high-grade dysplasia and adenocarcinomas, Ii expression increased progressively from the adenomas to the poorly differentiated carcinomas, while the level of expression of HLA-DR did not. The frequency of TILs, assessed as the number of lymphoid aggregates around the tumours, was inversely and significantly correlated with the level of Ii expression. These findings suggest that strong expression of Ii by tumour cells may prevent display of tumour antigens with HLA-DR and allow escape from host immune surveillance.

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