Abstract

Purpose: The development of local and distant recurrences is a major problem in the treatment of rectal cancer patients. In this study, we investigated whether epithelial human leukocyte antigen-DP (HLA-DR) expression allowed discrimination between high and low tumor recurrence rates, and analyzed the mechanism behind its expression.

Experimental Design: The role of IFN-γ in HLA-DR expression was studied in rectal cancer cell lines and tumors by promoter-specific analyses of class II transactivator (CIITA). The predictive value of epithelial HLA-DR expression was investigated by immunohistochemical evaluation of 1,016 rectal tumors, obtained from a large prospective trial. Associations with recurrences and survival were determined by univariate and multivariate log-rank testing.

Results: HLA-DR was induced by IFN-γ in rectal cancer cell lines. Activity of the IFN-γ-inducible pIV-CIITA promoter correlated with epithelial HLA-DR expression in rectal tumors. Patients with HLA-DR–positive tumors developed less frequent local and distant recurrences (1.6% versus 9.1% \( P = 0.0015 \) and 15.3% versus 29.9% \( P < 0.0001 \), respectively, after 5 years of follow-up) and had better survival (78.6% versus 61.3%, \( P < 0.0001 \)) than patients with HLA-DR–negative tumors. Epithelial HLA-DR was more often found in lower tumor-node-metastasis (TNM) stages. Next to TNM and circumferential resection margin, HLA-DR expression was independently associated with lower distant recurrence rates and prolonged survival.

Conclusions: Epithelial HLA-DR expression can be used as a marker to discriminate patients with high or low risk of developing recurrences. The possible involvement of IFN-γ, the relationship with lower TNM stages, and the independent effect on recurrence development together suggest that the host immune response plays an important role in controlling tumor cells.

In the treatment of rectal cancer patients, local and distant recurrences are a major problem because these are associated with both high mortality and morbidity. The introduction of total mesorectal excision (TME) surgery in combination with preoperative radiotherapy has been shown to be useful in reducing local recurrences. However, more than 25% of the patients develop metastases within 5 years after surgical treatment with curative intent (1, 2).

We and others have shown that (colo)rectal cancer patients develop less frequent recurrences when high numbers of infiltrating immune cells are present in the tumor (3–6). An optimal antitumor immune response usually requires activation of CD4+ and CD8+ T lymphocytes with a tumor-associated antigen. CD8+ T cells are activated by antigen presented by human leukocyte antigen (HLA) class I molecules whereas CD4+ T cells are activated by antigen presented by HLA class II molecules, normally expressed on professional antigen-presenting cells such as dendritic cells and macrophages. We have recently shown that a subgroup of tumors expressed high levels of HLA classes I and II and several other immune-related genes. Importantly, most of these tumors expressed the HLA class II protein HLA-DR on epithelial cells (7). These findings suggest an important role for the host immune response in targeting tumor cells, implying a better prognosis for patients with tumors expressing epithelial HLA-DR. Indeed, most studies on colorectal cancer patients have shown that epithelial expression of HLA-DR relates to a better prognosis (8–11). Because of the limited amount of data for rectal cancer patients, it is unclear whether the prognostic value of HLA-DR observed in colorectal cancer patients (with 33–44% rectal cancer patients) can also be applied specifically to rectal cancer patients. We therefore analyzed the expression of epithelial HLA-DR in a large number of rectal tumors, obtained from a prospective trial randomizing...
surgery alone or short-term preoperative radiotherapy followed by surgery (1). The standardized high-quality surgery technique done in this trial decreased the number of recurrences due to inadequate surgery (12), and therefore allowed a reliable investigation of the influence of intrinsic tumor characteristics, such as HLA-DR expression, on recurrences and survival. In addition, we analyzed the involvement of IFNγ in epithelial HLA-DR expression in vivo.

Materials and Methods

Cell lines. Colon cancer cell line HT29, rectal cancer cell lines SW837 and SW1463, and Ramos B-cell line were all cultured in Iscove’s modified Dulbecco’s medium (Cambrex) supplemented with glutamine, penicillin/streptomycin, and 8% heat-inactivated FCS, at 37°C. Where indicated, exponentially growing cells were treated with 100 units/mL human recombinant IFNα.

HLA-DR expression

Materials and Methods

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Visualization of HLA-DR on surface of cell lines. For these experiments, IFNγ-treated or untreated growing cells were harvested, washed, and resuspended in PBS supplemented with 0.5% bovine serum albumin and 0.05% sodium azide (PBA). Cells were incubated with a monoclonal FITC-labeled anti-HLA-DR antibody at 1:50 (BD Biosciences). Propidium iodide was added to allow exclusion of dead cells.

Promoter-specific reverse transcription-PCR for class II transactivator. Isolation of RNA was done as previously described (13). Complementary DNA was synthesized from 1-µg aliquots of total RNA using random primers (Ambion) and SuperScript III (Invitrogen) according to the manufacturer’s instructions. Class II transactivator (CIITA) products were amplified as previously described with the following primers: pIV-CIITA, sense 5′-AGCTGCGCGGGAGGGA-GAGGCCACC-3′ and antisense 5′-CATACCTGTCTCCAGTTCGCGA-TAATGG-3′; pII-CIITA, sense 5′-GATTCCTACACAATGCGTTGCCTGG-3′ and antisense as for pIV-CIITA (14). As a control, glyceraldehyde-3-phosphate dehydrogenase was amplified with the following primers: sense 5′-AAGTGAAGGTGCAGGTACAC-3′ and antisense 5′-TGGAAGTATGGATGATT-3′.

Western blotting. Adherent cells were washed and scraped in 20 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl (TBS). Cells were lysed in cold cell lysis buffer (Cell Signaling Technology) supplemented with complete protease inhibitor cocktail (Roche Applied Science). After clearing the lysates by high-speed centrifugation, protein concentrations were determined with bicinchoninic acid protein assay (Pierce Biotechnology). Equal amounts of total protein were resolved on SDS-PAGE gels and blotted on Hybond-P (Amersham). Ponceau S stainings were done to verify protein quantities transferred onto the membranes. Membranes were blocked with 5% nonfat dry milk in TBS supplemented with 0.2% Tween 20 (TBS-T) for 2 h. Primary antibody incubations were carried out in 1% milk/TBS-T overnight at 4°C and followed by three washes with TBS-T. Membranes were incubated with antirabbit IgG-horseradish peroxidase conjugates (Amersham) for 45 min, washed thrice, and developed with ECL Plus reagents (Amersham). The primary antibodies used were rabbit monoclonal antibodies recognizing human phospho-Tyr701 signal transducer and activator of transcription 1 (STAT1; Cell Signaling Technology) at 1:3,000; STAT1 at 1:1,000 (Cell Signaling Technology); IFN regulatory activator of transcription 1 (STAT1; Cell Signaling Technology) at 1:1,000; and goat serum in PBS/1% bovine serum albumin for 30 min was followed by overnight incubation with the prediluted monoclonal HLA-DR antibody (LN3; Signet Laboratories). Sections were incubated for 1 h with biotinylated goat anti-mouse (1:200; DakoCytomation) followed by incubation with streptavidin complexed with biotinylated peroxidase (DakoCytomation) and developed with 3,3-diaminobenzidine (DakoCytomation). Finally, sections were counterstained with Mayer’s hematoxylin. As a negative control, PBS alone was used in place of the primary antibody.

Scoring. For each tumor, one 2-mm-diameter punch was evaluated.

Immunohistochemistry. Stainings were done as previously described (7). Briefly, 4-µm sections were deparaffinized and endogenous peroxidise activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was done by boiling for 10 min in 0.01 mol/L citrate buffer (pH 6.0). Preincubation with 20% normal goat serum in PBS/1% bovine serum albumin for 30 min was followed by overnight incubation with the prediluted monoclonal HLA-DR antibody (LN3; Signet Laboratories). Sections were incubated for 1 h with biotinylated goat anti-mouse (1:200; DakoCytomation) followed by incubation with streptavidin complexed with biotinylated peroxidase (DakoCytomation) and developed with 3,3-diaminobenzidine (DakoCytomation). Finally, sections were counterstained with Mayer’s hematoxylin. As a negative control, PBS alone was used in place of the primary antibody.

Results

Role of IFNγ in epithelial HLA-DR expression in rectal cancer cell lines. We have recently shown, following gene expression analysis on 47 nonirradiated rectal tumors, that a subgroup of tumors expressed HLA-DR, a member of the HLA class II
proteins, in epithelial cells (7). To obtain insight into the mechanism of epithelial HLA-DR expression, we first tested whether IFN-γ is able to induce HLA-DR expression in vitro in rectal cancer cells, as has been described for colon cancer cells (17). Indeed, fluorescence-activated cell sorting analysis showed up-regulated surface expression of HLA-DR following treatment with IFN-γ. Specfically, IFN-γ induced HLA-DR expression in SW1463 cells, which do not normally express HLA-DR, and markedly enhanced the HLA-DR expression in SW837 cells above the endogenous expression (Fig. 1A).

IFN-γ induces HLA-DR through the STAT1 signaling pathway, which promotes transcription of IRF1, leading to transcription of CIITA (18). Western blot analyses of rectal cancer cells treated with IFN-γ indeed showed rapid phosphorylation of STAT1 and up-regulation of IRF1 after 6 h on treatment (Fig. 1B). Together, the data show that IFN-γ is able to induce epithelial HLA-DR expression in these rectal cancer cells, and therefore suggest that epithelial HLA-DR expression in tumors might be driven by IFN-γ.

Role of IFN-γ in epithelial HLA-DR expression in rectal tumors. To investigate the role of IFN-γ in epithelial HLA-DR expression in vivo, we examined the promoter activity of CIITA in rectal tumors. CIITA is controlled by four independent promoters, of which promoter pIV has been described to regulate IFN-γ-induced CIITA expression, and pIII to regulate constitutive CIITA expression such as in B cells and some melanomas (19–21). PCR analyses, with promoter-specific primers, enabled us to determine the promoter activity in rectal tumors and showed both active pIII and pIV in HLA-DR-positive tumors. Whereas pIII-CIITA was detected in some HLA-DR-negative tumors, activity of pIV-CIITA could not be found in HLA-DR-negative tumors (Fig. 2, left). In addition, analysis of IFN-γ-treated cells showed that both promoters, pIII and pIV, were activated on IFN-γ treatment. Although SW837 displayed pIV-derived CIITA in the absence of IFN-γ, its activity was markedly enhanced on IFN-γ treatment (Fig. 2, right). Together, these observations suggest that IFN-γ is likely involved in epithelial HLA-DR expression in vivo.

Correlation between epithelial HLA-DR expression and patients' characteristics. We previously analyzed 47 rectal cancer patients for epithelial HLA-DR expression and showed epithelial HLA-DR expression in 9 tumors (7). Interestingly, none of the patients with HLA-DR-positive tumors developed recurrences after 3 years of follow-up, whereas 19% of the patients with HLA-DR-negative tumors developed recurrences within this period of time. The number of patients is obviously too small to provide reliable data. To further investigate this, we evaluated HLA-DR expression in 1,016 paraffin-embedded rectal tumors obtained from a prospective trial randomizing for preoperative radiotherapy, followed by surgery. Most tumors (66%) did not show any HLA-DR expression in the epithelial compartment. These were grouped together with the 12% of tumors that showed <20% positivity and considered HLA-DR negative. The 216 (21%) tumors that expressed
Tumors were classified as being HLA-DR positive if at least 20% of epithelial cells stained positive. To evaluate the consistency of the data, the analyses have been repeated with the cutoff values $\geq 5\%$ and $\geq 50\%$. These analyses showed similar results (i.e., significantly lower recurrence rates and better survival for patients with HLA-DR–positive tumors).

The curves in Fig. 3A showed that epithelial HLA-DR can discriminate patients with high or low recurrence risks. Nevertheless, several important variables favor the HLA-DR–positive group (Table 1). Curves that are corrected for the effects of TNM, CRM, and treatment are displayed in Fig. 3B. These curves also showed better prognosis for patients with HLA-DR–positive tumors.

Stratified analysis based on the treatment regimen revealed that both nonirradiated and irradiated patients had lower distant recurrence rates and better survival when the tumor stained positive for HLA-DR (Table 2). We also observed better local control for HLA-DR–positive tumors in irradiated patients ($P = 0.011$), and a similar trend in nonirradiated patients ($P = 0.064$). Interestingly, none of the irradiated patients with HLA-DR–positive tumors developed local recurrences within 5 years of surgery. Stratification based on TNM showed a comparable trend for TNM stage I/II and TNM stage III/IV tumors: lower recurrence rates and better survival for the HLA-DR–positive group. Although the predictive value of epithelial HLA-DR on recurrence rates was not statistically significant for the CRM-positive tumors (local recurrences, $P = 0.11$; distant recurrences, $P = 0.10$), it was associated with a better survival ($P = 0.01$) for this group of patients. For the CRM-negative tumors, positive HLA-DR was significantly associated with lower recurrence rates and prolonged survival.

The independent predictive value for HLA-DR was tested in multivariate Cox regression analysis with backward stepwise elimination in the context of the following variables: CRM, TNM stage, differentiation, distance to anal verge, and treatment. No significant interactions were observed between the variables. Although statistically not significant, epithelial HLA-DR expression tended to be predictive for local recurrences (hazard ratio, 2.4; $P = 0.07$) in addition to CRM, TNM, and treatment. Importantly, HLA-DR expression on epithelial tumor cells had independent prognostic value for distant recurrence rate (hazard ratio, 1.6; $P = 0.01$) and survival (hazard ratio, 1.4; $P = 0.01$) in addition to TNM and CRM (Table 3).

### Discussion

In this study, we found that HLA-DR was expressed on epithelial cells in $\sim 20\%$ of rectal cancers, and showed that rectal cancer patients with HLA-DR–positive tumors had better survival than patients with HLA-DR–negative tumors. In addition, epithelial HLA-DR expression correlated with a reduction in both local and distant recurrence rates.

The expression of HLA-DR was inducible by IFNγ in rectal cancer cell lines, which is consistent with published data for colon cancer cell lines (17). The influence of IFNγ on HLA-DR expression in vivo was previously suggested following detection of IFNγ mRNA in HLA-DR–positive tumors (11). Nevertheless, the presence of IFNγ can also be a consequence of epithelial HLA-DR because HLA-DR–expressing tumor cells recruit and activate CD4+ T helper 1 and natural killer cells, resulting in local production of IFNγ (22, 23). To obtain more insight into HLA-DR expression in vivo, we analyzed CIITA promoter.
activity and showed that activity of pIV correlated with HLA-DR expression, in contrast to that of pIII, which is involved in constitutive expression (20, 21). This suggests that epithelial HLA-DR expression in vivo is regulated by CIITA via the IFN-\(\gamma\)-inducible pIV promoter. It therefore follows that HLA-DR expression could result from the presence of IFN-\(\gamma\).

Epithelial HLA-DR expression as prognostic factor has previously been described for colorectal cancer patients (8–11).
However, stratified analyses according to tumor location revealed better prognosis for HLA-DR–positive tumors in colon cancer patients but not in rectal cancer patients (9). The prognostic value of HLA-DR obtained in colorectal patients might be skewed by its association with microsatellite instability (9), which is a rare event in rectal tumors (24, 25). To our knowledge, this is the first comprehensive study investigating a correlation between epithelial HLA-DR expression and survival of rectal cancer patients. The high number of patients studied and the standardized high-quality TME surgery enabled us to show remarkably lower recurrence rates and better survival for rectal cancer patients with HLA-DR–positive tumors. A role for IFN-γ has been described, as it affects multiple genes involved in apoptosis, cell growth, and genetic instability (10, 26–28). Other studies focused on immunologic tumor control. Animal experiments showed that CIITA-transfected tumor cells were rejected via activation of the host immune response (22, 23). In vitro experiments with human tumor cells also showed enhanced immunogenicity on (IFN-γ-induced) expression of CIITA (29, 30). In agreement with this, we and others have shown that high amounts of tumor infiltrating lymphocytes correlate with a better prognosis in (colo)rectal cancer patients (3, 4, 6). In our study, HLA-DR–positive tumors were more often found in low TNM stages. This might be explained by reduced proliferation of HLA-DR–positive cells (10), as well as by immunologic control of the primary tumor. The less frequent distant recurrence development supports the hypothesis that HLA-DR–positive tumor cells exert an immunologic control. Whereas HLA-DR expression independently predicted lower distant recurrence rates and prolonged survival, its predictive value for local recurrences was not statistically significant in the context of TNM, CRM, and treatment. This loss of significance indicates different regulation of local versus distant recurrences. Whereas the immune response plays an important role in preventing HLA-DR–positive cells from metastasizing to lymph nodes or other organs, local control also depends on other mechanisms, such as the effect of radiotherapy.

TNM stage, CRM, and radiotherapy were independently associated with local control, whereas HLA-DR only showed a trend toward better local control. These data indicate that radiation and a negative CRM could be more important factors than epithelial HLA-DR expression in preventing local recurrences. The role of HLA-DR expression may, however, be underestimated due to the small number of events.

In conclusion, epithelial HLA-DR expression is associated with a reduced recurrence rates and better survival. Because this expression is an intrinsic tumor characteristic, evaluation of HLA-DR expression can be used to identify patients with high risk for disease recurrences who need adjuvant treatment (31). In addition, we showed that epithelial HLA-DR expression in rectal tumors is likely to result from the presence of IFNγ. It is also known that HLA-DR expression enhances the immune system and results in local IFNγ production. This implies a self-amplifying system between HLA-DR and IFNγ. If this is the case, then the induction of HLA-DR could be a promising therapeutic strategy. A recently published phase II trial in metastatic melanoma patients showed a long-lasting expression of HLA-DR on IFNγ treatment (32), indicating that up-regulation of HLA-DR can indeed be achieved by administration of IFNγ in vivo. In these melanoma patients, HLA-DR induction did not correlate with tumor regression. However, melanomas are not directly comparable to rectal tumors because they are known to be highly immunogenic and have several mechanisms to escape immune destruction (26, 33–38). It remains to be elucidated whether treatment-induced HLA-DR expression will improve prognosis to comparable levels as endogenous epithelial HLA-DR expression for rectal cancer patients. In light of our study, future investigation into the therapeutic benefit of HLA-DR induction for the treatment of rectal cancer patients is warranted.

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**References**


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**Table 3. Prognostic values of epithelial HLA-DR expression for distant and local recurrences and survival in multivariate analysis**

<table>
<thead>
<tr>
<th>HR (95% CI)</th>
<th>Local recurrence</th>
<th>Distant recurrence</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>2.4 (0.9–6.0)</td>
<td>1.6 (1.1–2.4)</td>
<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>1</td>
<td>1.4 (1.1–1.9)</td>
</tr>
<tr>
<td>CRM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>1</td>
<td>1.7 (1.3–2.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>1.9 (1.1–3.3)</td>
<td>1.8 (1.3–2.4)</td>
<td>1</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>1</td>
<td>3.0 (2.2–4.0)</td>
</tr>
<tr>
<td>II</td>
<td>3.9 (1.3–11.7)</td>
<td>2.5 (1.6–4.1)</td>
<td>1.3 (1.1–1.7)</td>
</tr>
<tr>
<td>III</td>
<td>8.2 (2.9–23.4)</td>
<td>6.4 (4.1–10.0)</td>
<td>1</td>
</tr>
<tr>
<td>Distance to anal verge, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>ns</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>5–10</td>
<td>1.4 (1.0–2.0)</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>&lt;5</td>
<td>1.8 (1.3–2.5)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TME</td>
<td>1.8 (1.0–3.0)</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>RT + TME</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** P values and hazard ratios with 95% confidence intervals were determined by Cox regression analysis. "ns" indicates that this variable was not statistically significant in the backward stepwise logistic regression analysis and was therefore eliminated.
are predicted by the nonspecific immune response; specific immune response has only a systemic effect—a histopathological and immunohistochemical study. BMC Cancer 2001;1:7.


Epithelial Human Leukocyte Antigen-DR Expression Predicts Reduced Recurrence Rates and Prolonged Survival in Rectal Cancer Patients

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