Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients

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Abstract

Studies aimed at elucidating the immunological and prognostic significance of HLA-DR expression on breast carcinoma cells have yielded contradictory results. To expand on previous studies, we have investigated the associations of tumor cell expression of HLA-DR and its related co-chaperones, invariant chain (Ii) and HLA-DM, with infiltrating inflammatory cells, in situ cytokine mRNA levels and prognosis and outcome in 112 breast carcinoma patients with a median follow-up of 59 months. While the majority of HLA-DR+ tumors co-express Ii, only a minority express HLA-DM. Tumor cell expression of HLA-DR and co-chaperones positively associated with both infiltrating CD4+ and CD8+ T-cell subsets (P < 0.01). Expression of HLA-DR and Ii associated with decreased estrogen receptor alpha levels and younger age at diagnosis, suggesting a role for hormones in the control of HLA class II expression in breast carcinoma. Patients with DR+Ii+DM- tumors had markedly decreased recurrence-free and disease-specific survival as compared with patients with DR+Ii+DM+ tumors (P < 0.05) and HLA-DR/co-chaperone expression was an independent predictor of survival by multivariate Cox regression analysis, controlling for standard prognostic indicators. Tumors that co-express HLA-DR, Ii and HLA-DM have increased levels of IFN-γ, IL-2 and IL-12 mRNA, suggesting improved survival of patients with DR+Ii+DM+ tumors may be attributable to Th1-dominated immunity. We conclude that expression of determinants of the immune response by tumor cells may influence breast tumor progression and patient outcome.

Introduction

Successful anti-tumor immunity is dependent on CD4+ T cells, which recognize tumor peptides, presented by HLA class II antigens (HLA-DR, -DP, -DQ) (1, 2). HLA class II antigen expression is generally restricted to professional antigen-presenting cells (APC) (dendritic cells, macrophages and B cells) and thymic epithelial cells but HLA class II antigens are expressed in a subset of benign and malignant breast tumors (3). As HLA class II antigens are not normally present on resting breast epithelial cells, in the context of breast carcinoma, HLA class II expression is likely dependent on the hormonal or cytokine milieu (4–8). Several groups have demonstrated that HLA class II-positive tumor cells can induce an anti-tumor T-cell response (9–11). Thus, one would expect to see a clear association between the two factors within breast carcinoma lesions; however, immunohistochemical studies attempting to relate the two have yielded discrepant results (12–15).

Similarly, the prognostic implications of HLA class II expression on breast carcinoma cells remain unclear as some studies have reported that HLA-DR expression on breast tumor cells associates with the favorable prognostic indicators of well-differentiated tumors (15, 16) and hormone receptor expression...
(17), while others found no such relationships (3, 18). The impact of HLA-DR expression on relapse-free and disease-specific survival (DSS) has also not been elucidated. Although HLA-DR expression associated with improved survival in a small subset of lymph node-negative patients (19), most have reported no association (18, 20, 21).

These conflicting findings may be resolved by addressing other parameters of the anti-tumor immune response. The aforementioned studies investigated associations of tumor cell HLA-DR expression with clinicopathological parameters, but did not address co-expression of the HLA class II co-chaperones invariant chain (li) and HLA-DM. Since li plays a critical role in HLA-DR trafficking (22), and HLA-DM facilitates peptide loading of HLA-DR molecules (23), discordant expression could have a detrimental effect on antigen presentation of tumor peptides by HLA-DR+ tumor cells. To date, there are no published reports of HLA-DM expression in breast carcinoma and two studies that examined co-expression of li and HLA class II found that a greater subset of breast carcinomas expressed li than HLA-DR, followed by HLA-DP and HLA-DQ (3, 24). In other carcinomas, high li expression is associated with poor prognosis and disease progression (25, 26), suggesting that li interferes with CD4+ T-cell recognition of endogenously derived tumor peptides. This is supported by in vitro experiments in which HLA class II-transfected tumor cells are unable to present tumor antigens to CD4+ T cells in the presence of li (10, 27).

Previously, we showed an association between HLA-DR and li expression on breast carcinoma cells with CD3+ T-cell infiltration (28). The effects of these factors on prognosis and outcome in breast carcinoma are expected to depend on the subsets of infiltrating inflammatory cells and the in situ cytokine milieu, which in turn influence class II expression on tumor cells and their ability to act as surrogate APC. Thus, in this study, we focused on the interrelationships of tumor cell expression of HLA-DR, li and HLA-DM with infiltrating cell subsets and prototypical Th1 (IFN-γ, IL-2, IL-12) and Th2 (IL-4, IL-10, transforming growth factor β [TGF-β]) cytokine mRNA levels. These factors have been further evaluated for their associations with clinicopathological parameters and survival in invasive breast carcinoma patients.

**Materials and methods**

**Patient sample**

A total of 112 primary breast carcinoma lesions were obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada), with approval of the local Human Investigation Committees. Tumor type was known for 108 tumors, comprising mostly of infiltrating ductal carcinoma (IDC, n = 85), 20 infiltrating lobular carcinoma (ILC) and 3 mixed IDC + ILC. Tumors spanned a wide range of grade (Grades 5–9), determined for 103 tumors, using the Nottingham grading system (29), and were classified as I (score 5, n = 15), II (scores 6–7, n = 51) and III (scores 8–9, n = 37). Tumor size was available for 109 tumors, categorized as small (<2 cm, n = 28) or large (>2 cm, n = 81). Clinical lymph node (LN) status was available for all but one patient, and 56 were LN+. A total of 108 tumors were staged according to the American Joint Committee on Cancer guidelines (30), of which 16 were of Stage I, 71 of Stage II, 18 of Stage III and 3 of Stage IV. The age of diagnosis was available for all patients and ranged from 32 to 86 years (median 60 years; mean ± SD = 59.3 ± 14.5 years). Estrogen receptor (ER) and progesterone receptor (PR) levels were determined by ligand binding assay and values ranged from 0 to 331 fmol mg⁻¹ (median 13.3 fmol mg⁻¹; mean ± SD = 35.4 ± 57.7 fmol mg⁻¹) and 0 to 1088 fmol mg⁻¹ (median 14.7 fmol mg⁻¹; mean ± SD = 59.6 ± 138.0 fmol mg⁻¹), respectively. Using a cutoff for negativity of less than 10 fmol mg⁻¹, 48 were ER− and 73 were PR−. Her-2/neu expression was assessed in 89 tumors, as described below, and 19 (21.3%) tumors over-expressed Her-2/neu. The median follow-up time was 59 months (mean ± SD = 57 ± 30 months; range 2–127 months), during which 34 patients died of breast cancer. The median time to recurrence was 51 months (mean ± SD = 49.2 ± 32.1 months), during which 45 patients experienced recurrences (32 distant, 8 regional and 5 distant + regional).

Tumor samples were assessed by immunohistochemistry (IHC) for HLA and co-chaperone expression and infiltrating cells and by RT–PCR for cytokine mRNA levels. In total, 77 tumors were examined by IHC and RT–PCR, 27 were assessed by IHC only and 8 had only cytokine information.

**mAbs**

mAbs (spent supernatants) were used to detect generic HLA class I (W6/32, 1:150, ATCC, Manassas, VA, USA) and HLA-DR (L243, 2.4 μg ml⁻¹, ATCC). Commercially available mAbs (BD Biosciences Pharmingen, Ontario, Canada) were used to detect li chain (clone LN2, 2.5 μg ml⁻¹), HLA-DM (clone M3P.DM1, 2.5 μg ml⁻¹), Her-2/neu (clone CB11, 1/100; clone N12, 2 μg ml⁻¹; Neomarkers, Quebec, Canada) and infiltrating CD3+ (clone UCHT1, 2.5 μg ml⁻¹, Pharmingen), CD4+ (clone RPA-T4, 0.625 μg ml⁻¹, Pharmingen), CD8+ (clone HIT8a, 0.625 μg ml⁻¹, Pharmingen), CD20+ (clone HI(FB1), 2.5 μg ml⁻¹, Pharmingen) and CD68+ (clone EBM11, 2.15 μg ml⁻¹, DakoCytomation, Ontario, Canada) cells. Tumor cells were identified using anti-cytokeratin mAb (clone AE1/AE3, 5 μg ml⁻¹, DakoCytomation). Negative controls consisted of isotype-matched non-specific mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

**IHC**

Serial frozen sections (8 μm) were fixed in acetone for 10 min at −20°C, shipped from the Manitoba Breast Tumor Bank and stored at −70°C until immunohistochemical staining. After thawing, drying and rehydrating in PBS (pH 7.4), sections were treated with 1.5% H₂O₂ in PBS for 30 min to remove endogenous peroxidases, and non-specific binding was blocked with 15% goat serum in PBS for 1 h. Sections were incubated for 1 h with primary antibody followed by incubation for 30 min with goat anti-mouse DAKO EnVision horseradish peroxidase-labeled polymer (DAKO Diagnostics Canada Inc., Ontario, Canada). Antibody binding was visualized by incubating with diaminobenzidine + H₂O₂ (Sigma, Ontario, Canada) for 5 min. The reaction was stopped with water and sections were counterstained in Mayer’s hematoxylin. Infiltrating mononuclear cells served as intrinsic positive controls.
for the immunoreactivity of the mAbs. Hematoxylin and eosin staining was performed on one section for each breast carcinoma and samples where tumor cells comprised <10% of the section area were excluded from analysis.

IHC interpretation
All slides were coded and independently examined by two readers, in the absence of information on prognostic parameters and HLA class II alleles. The percentage of tumor cells expressing HLA-DR, Li and HLA class I was coded based on comparison to expression levels on inflammatory cells within the same tissue section: – (0–24% tumor cells positive); −/+ (25–49% tumor cells as strong as inflammatory cells or 25–74% tumor cells weaker than inflammatory cells); + (50–100% tumor cells as strong as inflammatory cells or 75–100% tumor cells weaker than inflammatory cells). Since DR and Li are not normally expressed on breast epithelial cells, for categorical analysis, samples with DR or Li up-regulated in at least 25% of tumor cells (codes −/+ and +) were classified as positive. As HLA-DM was expressed at much lower levels than HLA-DR or Li on infiltrating inflammatory cells and tumor cells, we used a cutoff value of 10% for HLA-DM expression. For HLA class I, only those coded + were considered positive as samples coded – or −/+ were considered to have a substantial down-regulation of HLA class I. Likewise, only the percentage of tumor cells strongly positive for HLA class I was used for continuous variable analysis, while total percentage of tumor cells positive (weak or strong) was included for HLA-DR, Li and HLA-DM.

Infiltrating CD3+, CD4+, CD8+, CD20+ and CD68+ cells were coded based on examination of the entire section and estimation of relative numbers: – (no or a few scattered cells); −/+ (small numbers of scattered cells or occasional small aggregates); + (moderate numbers of scattered cells, numerous small aggregates or occasional large aggregates); ++ (large numbers of scattered cells or several large aggregates).

Tumor cell expression of Her-2/neu was determined in 89 tumors and coded as 0 (<10% with membrane staining), 1+ (≥10% with weak, incomplete membrane staining), 2+ (>10% with weak to moderate complete membrane staining) or 3+ (>10% with strong complete membrane staining). As some tumors coded 2+ by IHC will not show gene amplification by fluorescent in situ hybridization (31), only those tumors coded 3+ were considered clinically positive for statistical analysis.

RNA extraction and semi-quantitative RT-PCR
Total RNA was isolated from breast tumor tissues by homogenization using the Trizol method (Gibco BRL, Rockville, MD, USA), followed by treatment with DNA-free (Ambion, Austin, TX, USA) to remove any contaminating DNA. Reverse transcription was performed on 1 μg RNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Quebec, Canada). PCR was performed using a Biometra T Gradient thermocycler (Montreal Biotech Inc., Quebec, Canada) to amplify cDNA using primers for β-actin (32), IFN-γ, IL-2, IL-4, IL-10, IL-12 (33) and TGF-β1 (34), synthesized by Gibco BRL. All primer sequences were validated using published GenBank mRNA sequences: β-actin (GenBank accession number NM_00159; sense: ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG, anti-sense: CGT CAT ACT CCT GCC TGC TGA TCC ACA TCT GC); IFN-γ (GenBank accession number J00219; sense: AGT TAT ATC TTG GCT TTT CA, anti-sense: ACC GAA TAA TTA GTC AGC TT); IL-2 (GenBank accession number HSU25676; sense: ACT CAC CAG GAT GCT CAT AC, anti-sense: AGG TAA TCC ATC TGC TGA GA); IL-4 (GenBank accession number NM_00179; sense: CCA AGA AGC AGC TGA AG, anti-sense: TGG GTG TAT TTC GTT GTG TC); IL-4 (GenBank accession number M23442; sense: CCT CTG TTC TTG CTA GCA TG TGG GCC, anti-sense: CCA AGG TAC TCT GGT TGG CCT CCT TCA); IL-10 (GenBank accession number NM_00572; sense: ATG CCC CAA GAT GAC AAC CAA GAC CCA, anti-sense: TCT CAA GGG GTG GCT GGA TCA GGT GCA CCA); TGF-β1 (GenBank accession number X02812; sense: GCC CTT CGA ACC ACC TAT GC, anti-sense: AGG CTC CAA ATG TAG GGG CAG G). All PCR reactions were performed in a volume of 50 μl with 200 μM dNTPs (Gibco BRL) and 1 μl cDNA. PCR buffer contained 20 mM Tris–HCl (pH 8.4) and 50 mM KCl. Samples containing water instead of test cDNA were included as contamination controls and cDNA from the cell lines Jurkat E6-1 (IL-2, IL-4, IL-10, TGF-β), C10/MJ (IFN-γ) and YAR (IL-12) were used as positive controls. Primers were synthesized by Gibco BRL and included β-actin (32), IFN-γ, IL-2, IL-4, IL-10, IL-12 (33) and TGF-β1 (34). Primer sequences and product sizes are provided in Table 1. Primers were used at concentrations of 20 μM for β-actin and 10 μM for all others. MgCl2 (Gibco BRL) concentration was 1.5 mM for β-actin and IL-4 and 2 mM for all other reactions. Taq DNA polymerase (0.2 μl) (Gibco BRL) was used for β-actin reactions, all others used 0.25 μl. Reaction mixtures were subjected to amplification for 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C (IFN-γ and IL-2) or 65°C (all others) and extension at 72°C for 1 min, followed by a separate 5-min extension step at 72°C. The intensity of amplified products was semi-quantified and normalized as a percent of β-actin using the ChemiImager 4000 with AlphaEase 4.0 Software (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis
Contingency tables were analyzed using Pearson’s chi-square test or Fisher’s exact test for 2 × 2 tables when at least one expected count was ≤5 (two sided). Non-parametric Mann–Whitney (2 category variables) and Kruskal–Wallis (≥2 category variables) tests were used when assessing statistical significance of continuous variables, which were not normally distributed by Kolmogorov–Smirnov test. One-way analysis of variance and Fisher’s least significant difference post hoc test were used to assess diagnosis age, which was normally distributed.

Survival estimates were calculated using Kaplan–Meier method with log-rank (LR) statistic. Ninety-five percent confidence intervals (95% CI) around 5-year percent survival estimates were calculated using the standard error (SE) of the cumulative survival probability (95% CI = cumulative survival probability ± 1.96 × SE). Estimates were calculated as time to regional or distant metastasis for recurrence-free survival (RFS), time to distant metastasis for distant recurrence-free
survival (dRFS) and time to death from breast cancer for DSS. For DSS, patients who died of other causes were censored from analysis at time of death. Cox proportional hazards models were constructed for multivariate survival analyses using backward stepwise method. No patients were lost to follow-up and patients not experiencing the event were censored at the time of last follow-up or at 5 years for 5-year survival. For survival analysis, normalized cytokine units were stratified into high and low categories by comparing the fourth quartile to the first to third quartiles. For multivariate analysis, correlation matrices were constructed to ensure lack of collinearity of covariates. All analysis was performed using SPSS Version 11.5 statistical software.

Results

Tumor cells discordantly express HLA-DR and co-chaperones in breast carcinoma samples

HLA-DR and II were assessed in 104/112 tumors and HLA-DM was assessed in 102/112. Following exclusion of samples containing <10% tumor cells, information on HLA-DR/II and HLA-DM expression was available for 99 and 97 tumors, respectively. Using categorical classification, there were 36 HLA-DR+ (mean ± SD (x̄) = 75 ± 23.4%), 63 HLA-DR− (x̄ = 1.3 ± 4.1%), 51 II+ (x̄ = 78.6 ± 24.3%), 48 II− (x̄ = 2 ± 5.3%), 9 HLA-DM+ (x̄ = 28.9 ± 14.5%) and 88 HLA-DM− (x̄ = 0 ± 0%) tumors. Positive associations were observed between tumor cell expression of HLA-DR and II (χ² = 47.3, P < 0.001), HLA-DM and HLA-DR (Fisher’s exact test, P < 0.001) and HLA-DM and II (Fisher’s exact test, P = 0.003) (not depicted). In total, 46 were DR−II−DM−, 9 co-expressed all three antigens and discordant expression was observed in 42 tumors, with 25 DR+II+DM−, 1 DR−II+DM−, and 16 DR+II+DM+. Representative examples of breast tumor cells with the aforementioned HLA/co-chaperone phenotypes are depicted in Fig. 1A–D. As an indirect measure of integrity of the HLA loci, tumor cell expression of generic HLA class I antigens was determined. Comparison of the percentage of tumor cells with strong HLA class I expression within HLA-DR/co-chaperone expression categories revealed decreased HLA class I in both HLA-DR− categories (Fig. 2A), suggesting a negative effect on the HLA complex in HLA-DR− tumors.

HLA-DR and co-chaperone expression associates with a Th1 cytokine profile in breast carcinomas

Tumor cell expression of HLA-DR, II and HLA-DM positively associated with infiltrating CD3+, CD4+ and CD8+ T cells (Fig. 2B–D) but did not significantly differ with respect to infiltrating CD20+ and CD68+ cell numbers (Fig. 2E and F). All tumors that co-express HLA-DR and co-chaperones contain infiltrating CD3+ T cells comprising both CD4+ and CD8+ T-cell subsets (Fig. 3A and representative example depicted in Fig. 1B). Tumor-infiltrating T-cell populations associate with both T,1 and T,2 cytokine mRNAs in invasive breast carcinoma (S. A. Oldford, D. Codner and S. Drover, in preparation). Thus, relative amounts of T,1 and T,2 cytokine mRNAs were compared in tumors stratified by HLA-DR/co-chaperone expression. All DR+II+DM+ tumors had detectable IFN-γ, IL-2 and IL-12 mRNA (Fig. 3B) and levels of these T,1 cytokines were significantly increased in DR+II+DM+ tumors as compared with tumors that lack expression of one or more molecules (Fig. 3C). The prototypical T,2/T,3 cytokines, IL-4, IL-10 and TGF-β, did not significantly differ between expression categories (data not shown).

Associations of tumor cell HLA class II and co-chaperone expression with prognostic indicators in breast carcinoma patients

The clinical significance of tumor cell expression of HLA-DR and co-chaperones was assessed by examining associations with prognostic parameters. The age of diagnosis was decreased in patients with HLA-DR+ tumors, as compared with HLA-DR− tumors (Fig. 4A). Tumors that express HLA-DR and II in the absence of HLA-DM had significantly lower ER expression as compared with DR−II−DM− (Fig. 4B). Likewise, ER-negative tumors had increased HLA-DR (ER−: x̄ = 37.4 ± 39.8% versus ER+: x̄ = 20.9 ± 36.1%; P = 0.021) and II (ER−: x̄ = 54.5 ± 43.0% versus ER+: x̄ = 31.4 ± 39.4%; P = 0.003) expression (not depicted). Expression categories

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**Table 1. Multivariate cox proportional hazards regression models using backward stepwise method**

<table>
<thead>
<tr>
<th>Variables remaining at last step</th>
<th>Survival (dRFS)</th>
<th>dRFS</th>
<th>DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>PR &lt;10 fmol mg⁻¹ (versus &gt;10 fmol mg⁻¹)</td>
<td>2.29 (1.05–4.97)</td>
<td>0.037</td>
<td>2.63 (1.13–6.15)</td>
</tr>
<tr>
<td>TNM Stage III/IV (versus Stage I/II)</td>
<td>3.03 (1.41–6.52)</td>
<td>0.005</td>
<td>3.02 (1.26–7.23)</td>
</tr>
<tr>
<td>Her-2/neu Code 3 (versus Codes 0–2)</td>
<td>2.49 (1.18–5.25)</td>
<td>0.017</td>
<td>2.71 (1.17–6.25)</td>
</tr>
<tr>
<td>DR/DM category</td>
<td>0.001</td>
<td>0.001</td>
<td>0.031</td>
</tr>
<tr>
<td>DR+DM−</td>
<td>0.07 (0.01–0.52)</td>
<td>0.010</td>
<td>0.07 (0.01–0.58)</td>
</tr>
<tr>
<td>DR+DM+</td>
<td>0.37 (0.18–0.79)</td>
<td>0.010</td>
<td>0.31 (0.14–0.69)</td>
</tr>
<tr>
<td>DR−</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Model chi-square at last step (P value)</td>
<td>22.6 (&lt;0.001)</td>
<td>21.8 (0.001)</td>
<td>15.8 (0.003)</td>
</tr>
<tr>
<td>No. of events/no. at risk</td>
<td>35/82</td>
<td>28/82</td>
<td>25/82</td>
</tr>
</tbody>
</table>

*All regression models controlled for treatment, tumor type, ER, PR, diagnosis age, TNM stage and Her-2/neu expression status. bHazards ratio (95% confidence interval for hazards ratio), ns = non significant.*
did not significantly associate with PR, tumor grade, tumor stage, tumor size, LN status or Her-2/neu over-expression (Fig. 4C–F and data not shown). Although HLA-DR/co-chaperone expression categories did not associate with tumor differentiation, the percentage of tumor cells expressing Ii positively associated with tumor grade (Grade I: x% = 25.4 ± 38.8%; Grade II: x% = 39.7 ± 42.2%; Grade III: x% = 53.3 ± 42.9%; Kruskal–Wallis χ² = 6.6 P = 0.036, not depicted).

Tumor cell co-expression of HLA-DR and HLA-DM predicts improved patient survival

Kaplan–Meier survival analysis was performed to assess whether expression of HLA-DR or HLA class II co-chaperones associated with RFS, dRFS or DSS. As there was no significant difference in survival between II+ and II− tumors that did not express HLA-DR [DR−II−−versus DR−II+−−: LR = 0.7, P = 0.393 (RFS); LR = 0.4, P = 0.552 (dRFS); LR = 0.5, P = 0.461 (DSS)], the two subsets were grouped for analysis. Patients with DR+/DM+ tumors had prolonged RFS (Fig. 5A), dRFS (overall: LR = 9.3, P = 0.009; 5 years: LR = 9.8, P = 0.007, not depicted) and DSS (Fig. 5B), while those with DR+/DM− tumors had the shortest time to recurrence or breast cancer-specific death. Infiltrating cell populations did not associate with breast carcinoma patient survival (data not shown). However, significant differences in patient survival were observed when tumors were stratified using quartile cut points for cytokine mRNA levels. Patients with tumors containing high amounts of IFN-γ mRNA had prolonged RFS (Fig. 5C) and dRFS (overall: LR = 4.2, P = 0.041; 5 years: LR = 4.2, P = 0.041, not depicted). Patients with high IFN-γ mRNA levels also had improved DSS although the difference was not statistically significant (Fig. 5D). Patient survival did not differ in patients stratified by levels of IL-2, IL-12, IL-4, IL-10, or TGF-β mRNA (data not shown).

Cox proportional hazards models were constructed to determine if co-expression of HLA-DR and HLA-DM was an independent predictor of improved survival (Table 1). Cox regression models also contained treatment, tumor type, TNM stage, ER, PR, age of diagnosis and Her-2/neu status. Although histological tumor grade significantly associated with tumor type, LN, ER, PR and diagnosis age, it was not associated with overall or 5-year RFS, dRFS or DSS. Therefore, grade was not included in the multivariate models as the dependence among the covariates made the parameter estimates indeterminate, resulting in a lack of coefficient convergence. When controlling for the above prognostic factors, tumor cell HLA-DR/DMS expression was an independent predictor of RFS (P = 0.001), dRFS (P = 0.001) and DSS (P = 0.031). Tumor stage, PR and Her-2/neu status were also independent predictors for RFS and dRFS, while PR expression independently predicted DSS. Similar results were obtained for 5-year survival models (data not shown).

As fewer samples had information on cytokine mRNA, separate multivariate models were constructed to test the ability of IFN-γ to independently predict patient survival. When controlling for the aforementioned clinicopathological parameters, high IFN-γ displayed only a trend for improved RFS [at Step 1: HR (95% CI) = 0.38 (0.12–1.21), P = 0.101] and dRFS [at Step 1: HR (95% CI) = 0.33 (0.09–1.23), P = 0.099] and did not associate with DSS [at Step 1: HR (95% CI) = 0.32 (0.06–1.74), P = 0.188] (data not shown).

Discussion

This study was aimed at investigating relationships of tumor cell expression of HLA-DR and the co-chaperones, Ii and HLA-DM, with immune cell infiltration, the intra-tumoral cytokine profile and prognosis and outcome in breast carcinoma patients. HLA-DR, II and HLA-DM are typically coordinately regulated by the HLA class II transactivator (CIITA) (35); however, discordant expression by tumor cells was observed. Within HLA-DR+ tumors, 97% co-expressed II but only 26% were HLA-DM+. The underlying mechanisms responsible for discordant expression of HLA-DR and co-chaperones are currently unclear. II expression in the absence of HLA-DR and HLA-DM is likely attributable to CIITA-independent transcription of II, owing to unique cis-acting elements found within the II promoter (36). Thus, trans-acting factors produced during tumorigenesis may facilitate II expression, possibly explaining the positive association of tumor cell II expression with increased histological grade of differentiation. Tumor cell expression of HLA-DM did not occur independently of HLA-DR or II and was observed only in those tumors with high levels of the Tp1 cytokines IFN-γ, IL-2, and IL-12. Discordant HLA-DM expression in HLA-DR+ tumors may be due to regulatory defects in HLA-DM since its promoters contain additional cis-acting elements not shared by HLA class II or II promoters (37). As such, additional trans-acting regulatory factors may be necessary for HLA-DM transcription. In support of this, the kinetics of HLA-DM induction on synovial fibroblast lines is much slower than that of HLA-DR or II, in response to IFN-γ treatment (38).

Discordant HLA-DM expression in the majority of HLA-DR+ tumors may also reflect a tumor-escape mechanism similar to that reported for regulatory defects in HLA class I antigen processing machinery in breast carcinoma cell lines (BCCL). Down-regulated immunoproteasome subunits LMP-2, LMP-7, LMP-10 and transporter associated with antigen processing (TAP)-1 and TAP-2 molecules were documented in BCCL, and these defects could be restored via IFN-γ treatment (39). Thus, if regulatory mutations are present and cause negative HLA-DM expression, they may be overcome in the presence of high levels of intra-tumoral IFN-γ. In support of this, all DR+II+DM+ tumors had detectable IFN-γ and relative levels were significantly higher than in tumors that lack expression of HLA-DM. We have observed that high-dose IFN-γ treatment (500 U ml⁻¹ for 96 h) up-regulates HLA-DM protein expression in a majority of BCCL (7/11 tested), and HLA-DM induction by IFN-γ is clearly dose dependent (S. A. Oldford, A. D. Edgecombe and S. Drover, unpublished observations).

The negative association of HLA-DR and II expression with ER suggests that ER may negatively modulate HLA class II expression in breast carcinoma. Furthermore, the decreased age of diagnosis of patients with HLA class II-expressing tumors implies a role for circulating estradiol levels. This finding was intriguing as 17β-estradiol is known to negatively modulate HLA-DR expression. In particular, 17β-estradiol inhibits IL-1α and IL-1β induction of HLA-DR in ER+ human endometrial and BCCL in a dose-dependent manner (4) and...
HLA-DM expression in breast carcinoma
Fig. 1. Representative IHC examples of breast tumor cell expression patterns of HLA-DR, Ii and HLA-DM and tumor infiltration by CD4+ and CD8+ tumor infiltrating lymphocytes (TIL). Indirect IHC using mAb against HLA class I (mAb W6/32), HLA-DR (mAb L243), Ii (mAb LN2), HLA-DM (mAb MaP.DM1), CD4+ TIL (mAb RPA-T4) and CD8+ TIL (mAb HIT8a) was performed on acetone-fixed breast carcinoma tissue sections. Staining with IgG isotype control antibodies served as a negative control. Hematoxylin and eosin (H&E) staining illustrates invasive ductal morphology. Infiltrating inflammatory and stromal cells served as positive controls for the immunoreactivity of mAb (closed arrowheads). Representative tumor cells are depicted by open arrowheads. (A) Breast tumor cells lack expression of HLA-DR, Ii and HLA-DM and the tumor contains a paucity of CD4+ and CD8+ TIL. (B) Breast tumor cells co-express HLA-DR, Ii and HLA-DM and CD4+ and CD8+ cells were detected in and around tumor nests. (C) Breast tumor cells co-express HLA-DR and Ii but lack expression of HLA-DM and CD4+ and CD8+ cells were detected in and around tumor nests. (D) Breast tumor cells express the co-chaperone Ii but fail to express HLA-DR and HLA-DM and CD4+ and CD8+ cells were detected in and around tumor nests. Original magnifications of ×100 (H&E) and ×200 (all others).

Fig. 2. Associations of tumor cell HLA-DR and co-chaperones with HLA class I expression and infiltrating cells. (A) HLA class I expression is decreased in HLA-DR-negative tumors. Chi-square and P value in upper right corner correspond to comparison of all groups, using Kruskal–Wallis H test. Asterisks indicate significance in comparison of DR+Ii+DM+ tumors determined using Mann–Whitney U test (*P < 0.05, **P < 0.01). (B–F) Associations of infiltrating inflammatory cells with tumor cell expression of HLA-DR and co-chaperones in breast carcinoma were assessed using Kruskal–Wallis H test. (B) Infiltrating CD3+ cells associated with tumor cell expression of HLA-DR (P = 0.0003), Ii (P = 0.0008) and HLA-DM (P = 0.01). (C) Infiltrating CD4+ cells associated with tumor cell expression of HLA-DR (P = 0.0012), Ii (P = 0.0005) and HLA-DM (P = 0.01). (D) Infiltrating CD8+ cells associated with tumor cell expression of HLA-DR (P = 0.0024), Ii (P = 0.0018) and HLA-DM (P = 0.007). (E) Infiltrating CD20+ did not significantly associate with tumor cell HLA-DR (P = 0.391), Ii (P = 0.159) or HLA-DM (P = 0.179) expression. Only one tumor contained large numbers of CD20+ cells, so moderate and large numbers were grouped for comparison. (F) Infiltrating CD68+ cells did not significantly associate with tumor cell HLA-DR (P = 0.168), Ii (P = 0.056) or HLA-DM (P = 0.384) expression.
Fig. 3. Association of tumor cell HLA-DR/co-chaperone expression categories with infiltrating T cells and cytokines in invasive breast carcinoma. (A) Co-expression of HLA-DR, II and HLA-DM by tumor cells associates with increased numbers of CD3+ ($\chi^2 = 37.9, P < 0.0001$), CD4+ ($\chi^2 = 25.4, P = 0.003$) and CD8+ ($\chi^2 = 23.1, P = 0.006$) infiltrating cells. (B) All DR+II+DM+ breast tumors have detectable IFN-γ, IL-2 and IL-12 mRNA. RT-PCR was carried out using total RNA (1 µg) prepared from freshly frozen breast carcinoma tissue. β-Actin was amplified to confirm the integrity of the cDNA. Bands are representative of 1–3 RT-PCR reactions. Cell lines were used as positive controls for all RT-PCR reactions. (C) Tumors that co-express HLA-DR, II and HLA-DM have increased IFN-γ ($\chi^2 = 11.0, P = 0.012$), IL-2 ($\chi^2 = 7.4, P = 0.060$) and IL-12 ($\chi^2 = 9.1, P = 0.028$). Relative amounts of cytokine mRNA were normalized to β-actin and averaged for each breast carcinoma sample. Chi-square and $P$ value in upper right corner correspond to comparison of all three groups using Kruskal–Wallis H test. Asterisks indicate significance in comparison to DR+II+DM+ tumors determined using Mann–Whitney $U$ test ($*P < 0.05$, **$P < 0.01$).
17β-estradiol can down-modulate constitutive and IFN-γ-induced MHC class II expression in a variety of murine and human cell types (40). Its effects are independent of CIITA and involve ER binding to MHC class II promoters and activation of the c-Jun N-terminal kinase pathway, leading to histone hypoacetylation and decreased association of CREB-binding protein with the class II promoter (41). Based on our findings we suggest that the down-modulatory effect of estradiol on HLA-DR expression may be overcome in ER+/C0 breast carcinoma cells.

The association of tumor cell expression of li with a poor prognosis in this study and in other types of carcinoma (25, 26) is hypothesized to be due to inefficient presentation of endogenous tumor-antigen-derived peptides by HLA class II molecules. It is plausible that induction of HLA-DM expression by tumor cells allows for efficient exchange of li for endogenous tumor-antigen-derived peptides that have transited the endocytic pathway. HLA-DM is important for the exchange of the class II-associated li peptide (CLIP) for exogenous antigenic peptides (23, 42) and high levels of HLA-DR/CLIP complexes are expressed by mutant B cells deficient in HLA-DM (43). Similarly, we have observed increased surface HLA-DR/CLIP expression in a HLA-DM-deficient BCCL as compared with HLA-DM-expressing BCCL (S. A. Oldford, A. D. Edgecombe and S. Drover, unpublished observations). Thus, improved survival of patients with DR+/li+/DM+ tumors over patients with DR+/li+/DM− tumors may reflect differences in antigen presentation and the resulting T-cell response.

**Fig. 4.** Association of tumor cell HLA-DR/co-chaperone expression categories with prognostic parameters. (A) Diagnosis age is decreased in patients with DR+/li+/DM+ and DR+/li+/DM− tumors. (B) DR+/li+/DM− tumors have significantly decreased ER levels as compared to DR−li−DM− tumors. Tumor cell HLA-DR/co-chaperone expression categories did not significantly associate with PR levels (C), tumor differentiation (D), tumor stage (E) or Her-2/neu over-expression (F). F statistic or chi-square and P value in upper right corner correspond to comparison of all four groups using Kruskal–Wallis H test ($\chi^2$) or one-way analysis of variance (F statistic). Asterisks and P values indicate significance in comparison to DR−li−DM− tumors determined using Mann–Whitney U test for ER and Her-2/neu, and Fisher’s least significant difference test for diagnosis age (**P < 0.05, ***P < 0.01).
Indeed, experimental studies showed high levels of CLIP, whether exogenously added or endogenously expressed by APC, modulated antigen-specific effector T cells, inducing a shift from Th1 to Th2 responses (44, 45). Interestingly, other self-peptides did not have a polarizing effect on the effector CD4+ TH cell response. Meazza et al. (11) showed that CIITA-transfected TS/A mammary adenocarcinoma cells strongly up-regulated MHC class II antigens and Ii and were rejected in syngeneic mice in a CD4+ T cell-dependent manner. Recently, Thompson et al. (46) demonstrated that human breast carcinoma cells transduced with CIITA were able to present endogenous Her-2/neu peptides and activate human CD4+ T cells to secrete high levels of IFN-γ, with or without siRNA suppression of Ii. Although not investigated in either study, the improved immunogenicity of these tumor cells may be due to up-regulation of DM, which is also transcriptionally controlled by CIITA (35), thereby allowing for efficient exchange of CLIP for endogenous tumor antigen peptides. Indeed, high levels of CLIP on myeloid leukemia blast cells predicted poor patient survival and associated with decreased HLA-DM expression (47).

Fig. 5. Association of tumor cell HLA-DR/co-chaperone expression and IFN-γ mRNA levels with patient survival. (A) Patients with tumors that co-express HLA-DR, Ii, and HLA-DM have improved RFS as compared with patients with tumors that express HLA-DR and Ii in the absence of HLA-DM [5-year % survival (95% CI) = 89% (68–100%) for patients with DR+Ii+DM+ tumors, 39% (20–59%) for patients with DR+Ii+DM– tumors and 58% (44–71%) for patients with HLA-DR– tumors]. (B) Patients with tumors that co-express HLA-DR, Ii, and HLA-DM have improved DSS as compared with patients with tumors that express HLA-DR and Ii in the absence of HLA-DM [5-year % survival (95% CI) = 89% (68–100%) for patients with DR+Ii+DM+ tumors, 51% (31–71%) for patients with DR+Ii+DM– tumors, and 72% (60–84%) for patients with HLA-DR– tumors]. (C) Patients with tumors containing high IFN-γ mRNA levels have improved RFS [5-year % survival (95% CI) = 71% (49–93%) for patients with high IFN-γ, 48% (35–61%) for patients with low IFN-γ]. (D) Patients with tumors containing high IFN-γ mRNA levels display a trend for increased DSS (5-year % survival (95% CI) = 89%, 51% (31–71%) for patients with high IFN-γ, 50% (48–73%) for patients with low IFN-γ). LR statistic and P value in upper right corners correspond to overall comparison; LR and P value for 5-year survival rates are indicated on graph.
improved patient survival. In support of this, high levels of IFN-γ associated with decreased time to recurrence by univariate analysis (Fig. 5). Relative IFN-γ mRNA levels failed to independently predict patient survival in this study, possibly owing to the small sample size, but IFN-γ is an independent predictor of survival in other solid tumors (48).

The intermediate survival of HLA-DR− tumors may in part reflect the increased loss of HLA class I in HLA-DR− tumors (Fig. 2). In a large study of 439 breast carcinoma lesions, total loss of HLA class I independently predicted improved patient survival (49), although not investigated, this may reflect enhanced NK cell recognition or increased susceptibility to apoptosis (21). Decreased HLA class I expression in DR−li−DM− and DR−li+DM− tumors suggests a negative effect on chromosome 6p. Loss of heterozygosity (LOH) of chromosome 6p is likely to play a major role in the lack of HLA class II antigen expression, since this mechanism causes HLA class I haplotype loss in a high percentage of tumors and is frequently attributable to loss of an entire chromosome, as indicated by LOH at both 6p and 6q markers (50). This coupled with epigenetic mutations such as DNA methylation or histone deacetylation (39) likely explains the total loss of HLA observed in these tumors.

The results of this study suggest that coordinate expression of HLA-DR, li and HLA-DM by tumor cells is an indicator of improved prognosis in breast carcinoma. Furthermore, tumor cells that coordinately express HLA-DR and components of the HLA class II antigen processing machinery may function as effective APC, facilitating the induction of effective Tc1 anti-tumor immunity. Investigations such as this provide improved understanding of the immune response to carcinomas and suggest that targeting antigen processing aberrations may aid in the successful generation of immunotherapeutic approaches to cancer treatment.

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Abbreviations
BCCL  breast carcinoma cell lines
CI  confidence interval
CIITA  class II transactivator
CLIP  class II-associated li peptide
dRFS  distant recurrence-free survival
DSS  disease-specific survival
ER  estrogen receptor
IDC  infiltrating ductal carcinoma
IHC  immunohistochemistry
li  invariant chain
ILC  infiltrating lobular carcinoma
LN  lymph node
LOH  loss of heterozygosity
LR  log-rank
PR  progesterone receptor
RFS  recurrence-free survival

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