Morphological Evidence of an Activated Cytotoxic T-Cell Infiltrate in EBV-Positive Gastric Carcinoma Preventing Lymph Node Metastases

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Abstract: Recently, we showed that Epstein-Barr virus (EBV)-positive gastric carcinoma (GC) forms a distinct clinicopathologic entity with a better prognosis due to lower incidence of lymph node metastases (LN+). Here we investigated whether in EBV-positive GC more pronounced activation of cellular immune responses is associated with absence of (micro)metastases. Twenty EBV-positive primary tumors (PT) (9 LN+) were matched with 28 EBV-negative GC (11 LN+) for T- and N-stage, gender, and age. The PT (n = 28) and its LNs were analyzed by EBER RNA in situ hybridization and by immunohistochemistry for MHC class I and II expression, for CD3, CD8, CD4, CD20, CD56, CD83, and Granzyme B (GzB) expression. In LN metastases of EBV-positive GC, the EBV genome is maintained, excluding tumor escape by virus deletion. All GC express MHC class I independently of EBV status. In comparison with EBV-negative GC, EBV-positive GC have higher expression of MHC class II on the tumor cells ($P = 0.029$) and a more extensive infiltrate ($P < 0.0001$) of activated GzB+ CD8+ T cells ($P = 0.028$), which is most abundant in those EBV-positive tumors that do not metastasize ($P < 0.0001$). In addition, in EBV-positive GC without metastases, the infiltrate contains higher numbers of mature dendritic cells (DC) ($P = 0.018$). At present, the antigenic target has to be determined. These data support the notion that local triggering of cellular immune responses in EBV-positive GC prevents lymph node metastasis formation.

Key Words: Epstein-Barr virus, gastric carcinomas, cellular infiltrate

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Worldwide, the human γ-herpes virus Epstein-Barr (EBV) is present in approximately 10% of gastric adenocarcinomas not otherwise specified.[19] We have recently shown a similar prevalence of 7.2% for the Dutch population.[24]

EBV-positive and EBV-negative gastric carcinomas (GCs) show distinct characteristics at the molecular level, including different chromosomal aberrations,[29] different and lower frequencies of microsatellite instability[5] and allelic loss,[26] more CpG methylation,[6,10] and different protein expression profiles.[12]

Moreover, in a large Dutch cohort of GC patients from the D1D2 trial,[3,8] we have shown that EBV-positive GC indeed should be considered as a distinct clinicopathologic entity of gastric adenocarcinomas characterized by a lower frequency of lymph node (LN) metastases, resulting in a better disease-free survival and longer disease-free period for those patients with an EBV-positive carcinoma compared with patients with EBV-negative GCs.[24]

We hypothesized that EBV in the tumor cells induces an immunologic response that prevents formation of (micro)-metastases resulting in a lower incidence of LN metastases in EBV-positive GCs. Furthermore, LN metastases in EBV-positive GC then could occur by deleting EBV.

Several studies indeed describe a statistically larger infiltrate in EBV-positive primary tumors.[4,7,12,20] Two more in-depth studies are performed in Japanese patient populations on 8 and 24 EBV-positive primary GCs.[11,16] Both describe a significant CD8+ cellular infiltrate surrounding the primary tumors.

In the present study, we investigated our hypotheses by performing immunohistochemical staining and EBER RNA in situ hybridization on the tissue sections from patients of the D1D2 trial. This clinical trial was performed in the Netherlands between 1989 and 1993, aiming to analyze whether extensive LN dissection influenced long-term survival after surgery with curative intent.[3,8] We studied both the primary GCs and the LNs with and without metastases of selected EBV-positive (LN− 11/LN+ 9) and EBV-negative cases (LN− 17/LN+ 11), matched for T and N stage, age, and sex.

MATERIALS AND METHODS

Clinical Materials

Patients were originally enrolled for the D1D2 trial between 1989 and 1993 in different hospitals in the Netherlands.[3,8] For the original analysis and all subsequent analyses, permission was obtained from the medical ethical committees

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of the participating hospitals. Prior analysis of the EBV status of the primary tumors by EBER RNA in situ hybridization (EBER RISH) resulted in 41 EBV-positive carcinomas in the D1D2 cohort.[23,24] For the present study, we selected EBV-positive GCs (n = 20) from which paraffin-embedded LNs were available. At least 1 EBV-negative control (n = 28) was matched with an EBV-positive case for T and N classification, gender, and age according to the pathology database.[3] EBV-positive cases of the original cohort (n = 21) were excluded when no paraffin-embedded material was available for analysis of either the primary tumor or LN from any matched control.

EBER RNA In Situ Hybridization

All LNs were analyzed for the presence of EBV by EBER RISH using EBER PNA-probe (DAKO Cytomations, Denmark). Paraffin-embedded formalin-fixed tissue sections of 5 μm were mounted on Superfrost-plus slides. After deparaffination, endogenous peroxidase activity was quenched by incubation in 1% H_2O_2 in methanol. Slides were washed in ethanol and air-dried. Sections were incubated for 90 minutes with EBER probe (DAKO Cytomations) at 55°C in humidified atmosphere or with phosphate-buffered saline for the negative controls. Samples were washed for 25 minutes with stringent wash buffer at 55°C containing 50 mM Tris, 0,15 M NaCl, pH 10. Samples were washed with phosphate-buffered saline, 0.005% Tween at RT. Immunodetection was performed using the Labvision (Labvision, United States) detection system and visualized with aminoethylcarbazol.

Immunohistochemistry

Immunohistochemistry was performed on the primary tumors and the LNs of all cases for CD20, CD3, CD8 (DAKO Cytomations), CD4, CD56, CD83, Granzyme B (GrB7) (Sanbio, the Netherlands), HLA-A (HCA2), HLA-B and C (HC10) (kindly provided by J Neefjes, Dutch Cancer Institute, the Netherlands), β2-microglobulin (A072) (DAKO Cytomations), and MHC II (LN3) (Biotest Seralc, Belgium). Presence or absence of tumor in the LNs was confirmed on parallel hematoxylin and eosin-stained slides. Paraffin-embedded sections were mounted onto Superfrost plus slides. All staining procedures were performed according to standard protocols in our laboratory. Antigen retrieval for CD3, CD4, CD83, Gbr7, and HCA2 was performed in EDTA buffer pH 9 in the microwave oven, and for CD56, CD20, and LN3 in citrate buffer pH 6 in the microwave oven. Antigen retrieval for CD8 and HC10 was performed in citrate pH 6 in the autoclave. No antigen retrieval was required for β2m. Tissue sections were incubated with the different primary antibodies, detected with either Labvision or Envision (DAKO Cytomations) and visualized by diaminobenzidine.

Data Evaluation

All slides were semi-quantitatively evaluated under conventional light microscopy by two investigators (J.v.B., E.B.).

EBV presence as determined by EBER RISH was scored as either positive (+) or negative (−).

The MHC class I complex was considered present if both the heavy (HC10 or HCA2) and the light chain (β2m) were expressed on the cell surface of the tumor cells. MHC class I and MHC class II (LN3) expression was scored as follows: 0, negative to sporadic positive cells; 1, <50% tumor cells positive; 2, ≥50% of the tumor cells positive. For statistical analyses, MHC class I and II was considered absent (0) or present (1 and 2).

Density of the infiltrate was determined based upon CD3 staining because T cells were by far the most abundant infiltrating cells. Density of infiltrate was expressed as small (1), intermediate (2), moderate (3), and extensive (4). CD8+ and CD4+ T cells were estimated as the ratio (CD8:CD4). A ratio of ≥ 10 was considered as maximum. For statistical analyses, data was grouped as: 1, ratio < 1; 2, ratio = 1; and 3, ratio > 1.

GrB7+ cells were expressed as estimated percentage of CD8+ cells expressing Granzyme B7. For statistical analysis, percentages were grouped as: 1, <10%; 2, 10% to 50%; 3, >50%.

Presence of CD20+, CD56+, and CD83+ cells were estimated as: 0, negative to sporadic cells; 1, moderate; and 2, large infiltrate present.

Statistical Analysis

EBV status was correlated with the presence of the different markers, separately for both the primary tumors and metastases. Next it was examined whether the markers within the EBV-positive and EBV-negative group were associated with formation of metastases and whether markers were different within the group of tumors with (LN+) and without metastases (LN−) in correlation with EBV status. In addition, patients with metastases were analyzed for loss (1), no change (2), or gain (3) of marker on metastases versus primary tumor. Pearson χ^2 tests were applied, as indicated in *Results*. If the tables contained empty cells a permutation procedure was used to compute the *P* value. The number of permutations was set at 100,000.

RESULTS

In the present study, primary tumor and one metastasis in an LN were analyzed for the different markers described. In all cases, both LN+ and LN− and an LN without a metastasis was studied. The total study population comprised of 48 primary GCs (20 EBV-positive vs. 28 EBV-negative) of which 20 cases had one or more LN metastases (9 EBV-positive vs. 11 EBV-negative). These tumors were previously classified according to Laurén (Tables 1, 2) and included 40 intestinal type tumors (19 EBV-positive and 21 EBV-negative) and 8 of diffuse type (1 EBV-positive vs. 7 EBV-negative) histology. The LN metastases were histologically indistinguishable from their primary tumor (Tables 1, 2).

EBV Is Still Present in the Lymph Node Metastases of EBV-Positive Primary Tumors

To investigate whether EBV-positive carcinomas metastasize due to loss of EBV from the tumor cells, we analyzed all LNs (n = 48) for the presence of EBV by EBER RISH. The study included 9 LNs with LN metastases from an EBV-positive primary tumor. In the nuclei of all tumor cells of these metastases, EBER transcripts were detected (Fig. 1B). The staining pattern was comparable with that of the primary tumor

TABLE 1. Clinical and Immunohistochemical Data on EBV-Positive Tumors

	TN	Lauren	Differentiation	MHC I PT	MHC I Meta	MHC II PT	MHC II Meta	Infiltrate PT	Infiltrate Meta	CD8:CD4 PT†	CD8:CD4 Meta	GrB7+ CD8 PT‡ (%)	GrB7+ CD8 Meta (%)
1	T1N0	Intestinal	Moderately	+		+		Large		10:1		25	
2	T1N0	Intestinal	Moderately	+		+		Large		10:1		5	
3	T1N0	Intestinal	Moderately	−*		+		Large		10:1		5	
4	T1N0	Intestinal	Moderately	+		+		Very large		2:1		33	
5	T1N1	Intestinal	Moderately	−*	−*	−	−	Small	Small	10:1	Missing	5	5
6	T1N1	Intestinal	Poorly	−*	−*	−	−	Small	Small	5:1	5:1	30	30
7	T2N0	Intestinal	Poorly	−		−		Very large		3:1		25	
8	T2N0	Intestinal	Poorly	+		+		Very large		10:1		25	
9	T2N0	Intestinal	Moderately	+		+		Large		10:1		1	
10	T2N0	Intestinal	Poorly	+		−		Very large		10:1		1	
11	T2N0	Intestinal	Poorly	−*		−		Large		10:1		5	
12	T2N0	Intestinal	Moderately	+		+		Intermediate		10:1		30	
13	T2N0	Intestinal	Poorly	+		+		Large		2:1		30	
14	T2N1	Intestinal	Poorly	−*	+	−	+	Small	Large	10:1	3:1	25	0
15	T2N1	Intestinal	Moderately	+	−*	+	−	Small	Intermediate	2:1	10:1	30	0
16	T3N1	Intestinal	Poorly	+	−	−	−	Intermediate	Intermediate	10:1	10:1	50	50
17	T3N2	Intestinal	Poorly	+	+	+	−	Very large	Very large	10:1	Missing	75	Missing
18	T3N3	Intestinal	Poorly	+	−	−	−	Intermediate	Intermediate	10:1	10:1	5	5
19	T4N1	Diffuse	Poorly	+	+	+	−	Very large	Large	10:1	10:1	75	1
20	T4N2	Intestinal	Poorly	+	+	+	+	Very large	Very large	2:1	5:1	40	50

PT, primary tumor; Meta, lymph node metastasis.
*β2m IH negative.
†(1), <1; (2), 1; (3), >1: for statistical analysis.
‡(1), <10%; (2), 10%–50%; (3), >50%: for statistical analysis.

(Fig. 1A). In addition, no EBER transcripts were detected in any of the LNs of EBV-negative primary tumors with LN metastases (N = 11), nor in the LNs without metastases (17 EBV-negative primary GC and 11 EBV-positive primary GC). Thus, the EBV genome is maintained in LN metastases of EBV-positive primary gastric adenocarcinomas.

Antigen Presentation: MHC I and MHC II Expression on Primary Tumors

T cells play a pivotal role in the human immune system: the CD4+ cells to provide "help" to both the cellular and humoral immune response by release of cytokines and the CD8+ effector T cells to kill infected or malignant cells. T cells recognize antigens on the surface of cells in the context of MHC class I or II cells for CD8+ and CD4+ cells, respectively. To analyze whether tumor cells are capable of presenting antigens to the T cells, we analyzed the expression of MHC class I and II on the tumor cells.

MHC class I staining of the inflammatory cells and MHC class II staining of macrophages was used as internal positive control for all slides. A tumor was considered MHC I positive when at least one heavy chain (either HC10 or HCA2) was expressed in combination with the light chain (β2m). Most primary tumors (62.5%) expressed MHC I on their membrane, and this was independent of EBV-status of the tumor (70% EBV-positive GC and 57.1% EBV-negative GC; not significant). If tumors were MHC class I negative, this was mostly due to the absence of the light chain.

MHC class II expression was statistically significant more frequent on EBV-positive primary tumors (12 of 20; 60%) than on EBV-negative primary tumors (8 of 28; 28.6%) ($P = 0.029$) (Fig. 2). EBV-positive tumors without metastases showed a trend toward more frequently MHC class II expression than EBV-negative tumors without metastases ($P = 0.053$) (Fig. 2). No difference was observed between EBV-positive and EBV-negative tumors with LN metastases. Within either the EBV-positive or EBV-negative group, no significant differences were observed for those cases that have metastases and those that have not. Representative staining for MHC class II is shown in Figure 1C. An MHC class II negative tumor is shown in Figure 1D.

In summary, MHC class I is expressed on most primary tumors and is independent of EBV status, but MHC class II expression is more prevalent on EBV-positive tumors. In particular, MHC class II is more prevalent on those EBV-positive carcinomas that do not have LN metastases.

Density of T-Cell Infiltrate of the Primary Tumors

After analysis of antigen presentation capacity of GC tumor cells, we analyzed the density of the leukocyte infiltrate and the composition thereof. Density of the infiltrate was determined based upon CD3 staining because T cells were the most abundant infiltrating cells. The infiltrate in EBV-positive primary tumors was significantly larger than those surrounding EBV-negative primary tumors ($P = 0.0001$) (Fig. 1E). This difference in density of infiltrate between EBV-positive and EBV-negative tumors is most pronounced in the tumors that do not metastasize (LN− GCs) ($P = 0.0001$) but fails to reach statistical significance in the tumors that metastasize (LN+ GCs) ($P = 0.085$). Within the EBV-positive group (EBV+

TABLE 2. Clinical and Immunohistochemical Data on EBV-Negative Tumors

	TN	Lauren	Differentiation	MHC I PT	MHC I Meta	MHC II PT	MHC II Meta	Infiltrate PT	Infiltrate Meta	CD8:CD4 PT‡	CD8:CD4 Meta	GrB7+ CD8 PT (%)§	GrB7+ CD8 Meta (%)
1	T1N0	Intestinal	Good	−*		−		Small		10:1		5	
2	T1N0	Intestinal	Moderately	−*		−		Small		10:1		33	
3	T1N0	Intestinal	Moderately	+		−		Intermediate		10:1		5	
4	T1N0	Intestinal	Poorly	−*		−		Small		10:1		0	
5	T1N0	Intestinal	Moderately	−*		−		Small		1:1		10	
6	T1N1	Intestinal	Moderately	+	+	−	−	Small	Small	10:1	10:1	0	25
7	T1N1	Intestinal	Poorly	+	+	−	+	Small	Small	10:1	10:1	5	30
8	T1N1	Intestinal	Moderately	−*	−*	−	−	Small	Intermediate	10:1	Missing	80	Missing
9	T2N0	Intestinal	Moderately	−*		−		Small		10:1		0	
10	T2N0	Intestinal	Poorly	+		+		Very large		10:1		1	
11	T2N0	Intestinal	Poorly	+		+		Small		5:1		10	
12	T2N0	Intestinal	Moderately	+		+		Very large		1:1		50	
13	T2N0	Intestinal	Poorly	−*		+		Small		10:1		75	
14	T2N0	Diffuse	Poorly	+		−		Intermediate		10:1		0	
15	T2N0	Diffuse	Poorly	−*		−		Small		10:1		10	
16	T2N0	Diffuse	Poorly	+		−		Small		1:3		0	
17	T2N0	Intestinal	Poorly	−*		−		Intermediate		10:1		70	
18	T2N0	Intestinal	Moderately	+		+		Very large		1:1		80	
19	T2N0	Intestinal	Poorly	+		+		Intermediate		10:1		1	
20	T2N0	Diffuse	Poorly	−*		−		Small		5:1		33	
21	T2N1	Intestinal	Moderately	+	+	−	−	Intermediate	Intermediate	10:1	1:2	0	33
22	T2N1	Intestinal	Moderately	+	Missing	−	−	Small	Small	1:1	5:1	0	0
23	T2N1	Diffuse	Poorly	−	+	−	+	Small	Intermediate	10:1	10:1	1	1†
24	T3N1	Intestinal	Good	+	+	−	+	Small	Small	1:1	1:1	25	33
25	T3N1	Intestinal	Poorly	+	−*	+	+	Small	Small	5:1	5:1	80	0
26	T3N2	Intestinal	Moderately	−*	−*	−	−	Small	Small	2:1	3:1	30	5
27	T3N3	Diffuse	Poorly	+	+	−	−	Small	Small	1:2	5:1	5	1
28	T4N2	Diffuse	Poorly	+	+	+	+	Small	Small	1:1	5:1	1	0

PT, primary tumor; Meta, lymph node metastasis.
*β2m IH negative.
†CD56+ infiltrate.
‡(1), <1; (2), 1; (3), >1: for statistical analysis.
§(1), <10%; (2), 10%–50%; (3), >50%: for statistical analysis.

GCs), a significantly more extensive infiltrate was observed in the primary tumors without LN metastases compared with the tumors with LN metastases ($P = 0.003$). Presence of metastases was independent of the density of the infiltrate within the EBV-negative primary tumors.

In summary, EBV-positive tumors are characterized by a significantly more extensive T-cell infiltrate and the most abundant infiltrate is observed in the EBV-positive tumors that do not metastasize.

Composition of the Cellular Infiltrate

Next, we analyzed the tumors for the relative contribution of CD8+ and CD4+ T cells to the T-cell infiltrate and the presence of mature dendritic cells (DC), B cells, and NK cells. The percentage of Granzyme B expressing CD8+ cells was analyzed as a marker for their functional activation.

Overall, in 83% of the primary GCs CD8+ cells were more prevalent in the T-cell infiltrate than CD4+ cells (40 of 48; 83% with ratio > 1) irrespective of infiltrate density or EBV-status of the tumor. However, in the EBV-positive primary tumors, the ratio CD8:CD4 was always >1, whereas in 6 EBV-negative primary tumors equal amounts of CD8 and CD 4 (ratio = 1, n = 6) and in 2 EBV-negative primary tumors excess of CD4+ cells was observed ($P = 0.022$). Activated cytotoxic T cells were scored as the percentage CD8+ cells expressing Granzyme B. EBV-positive tumors had more GrB7+ CD8+ T cells (10%–50%) ($P = 0.028$) (Fig. 1E, F).

CD83-positive mature DCs were present in 25% of the primary GC. Mature DCs were more frequently present in EBV-positive tumors than in EBV-negative tumors without metastases ($P = 0.018$). This difference is not observed in primary tumors that have metastasized to the LNs.

In the primary tumors, no CD56-positive infiltrating cells were present, whereas CD20-positive B cells were sporadically present in most primary tumors.

In summary, EBV-positive primary GC are characterized by a larger activated CD8+ infiltrate than their EBV-negative counterparts. Those EBV-positive primary tumors that do not metastasize harbor more CD83+ mature dendritic cells than EBV-negative tumors without metastases.

Overall data on primary carcinomas are summarized in Table 3.

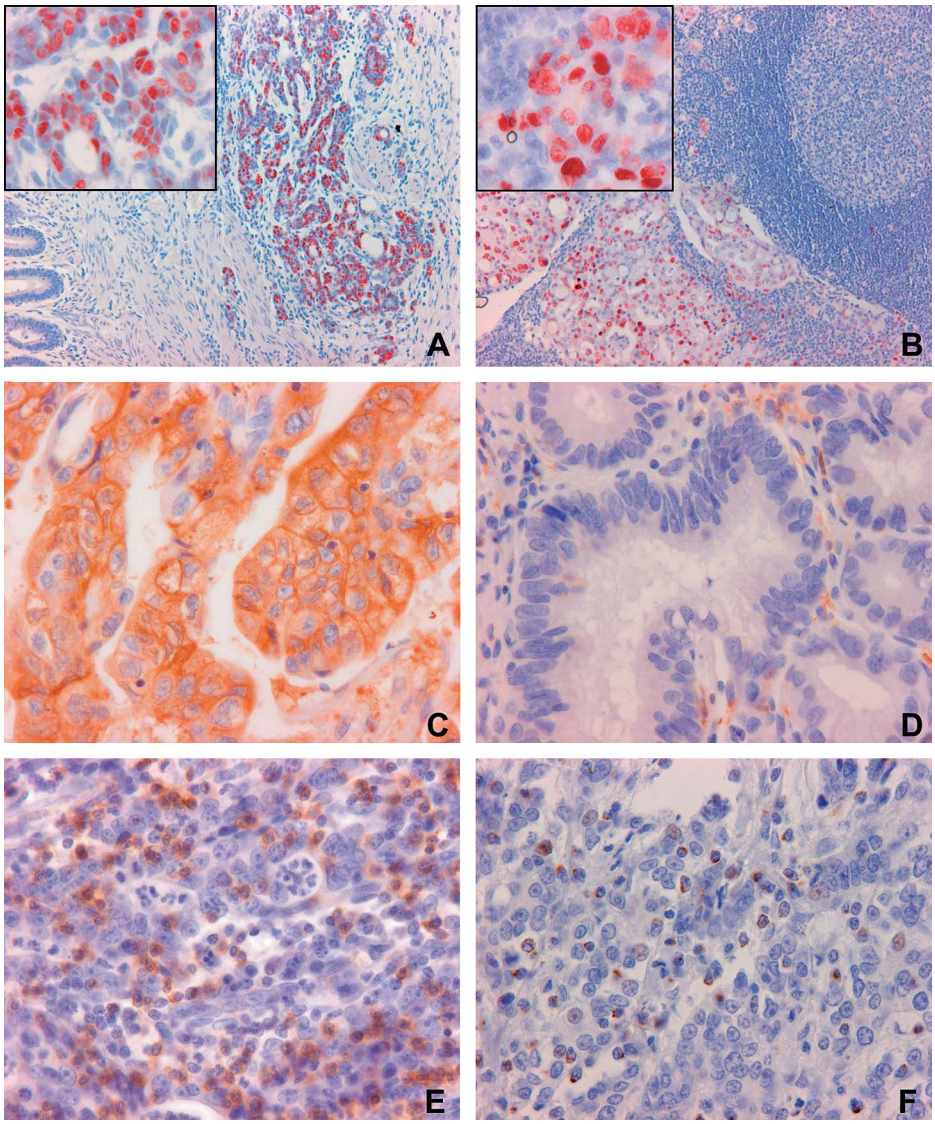

FIGURE 1. EBER RISH on an EBV-positive primary tumor (A) and a lymph node metastasis (B) of the same tumor. EBER 1/2 transcripts are detected in the nuclei of the carcinoma cells. EBER 1/2 transcripts are not present in the normal gastric epithelium (A), nor in the lymphocytes in the lymph node (B). In the left corner, tumor cells in the primary tumor (A) and lymph node metastases (B) are shown at larger magnification. Representative immunohistochemical staining for strong MHC class II expression in an EBV-positive GC (C) and absence of MHC class II expression in the EBV-negative tumor. Macrophages stain as positive control (D). Infiltrate in EBV-positive GC is characterized by cytotoxic CD8+ T cells. Representative immunohistochemical staining is shown for CD8 (E) and Granzyme B (F).

Density T-Cell Infiltrate Correlates With MHC Class I and MHC Class II Expression

We further analyzed density of infiltrate in relation to MHC class I and MHC class II expression. Interestingly, independent of EBV status of the tumor, the presence of MHC class I and MHC class II expression on the primary tumors correlated with more extensive infiltrate ($P = 0.019$ and $P = 0.0001$, respectively). However, MHC class I or MHC class II expression on the tumor cells did not correlate with TNM-stage or N-stage.

LN Metastases

The LN metastases were histologically indistinguishable from their primary tumor. Mature CD83+ dendritic cells were present in more LN metastases than primary tumors (overall, 50% vs. 25%, respectively). Eighty percent of metastases of EBV-positive tumors had CD83+ mature dendritic cells compared with 30% of metastases of EBV-negative tumors ($P = 0.037$). No significant changes were observed compared with those described for the primary tumors in MHC class I, MHC class II expression, infiltrate intensity, and composition of the infiltrate.

Lymph Nodes

In addition, of all cases at least one LN without metastasis and, if applicable, one with a metastasis were analyzed. No differences were observed in composition of cells or their markers in the "normal LNs" of either the EBV-positive or EBV-negative primary tumors, nor were any differences observed in the LNs containing metastases. Immune cells were compartmentalized in B-cell and T-cell areas and there were no indications for more T-cell activation or activation of DC.

DISCUSSION

Recently, we described that EBV-positive primary GC forms a distinct clinicopathologic entity, which is characterized by significantly less LN metastases resulting in a better

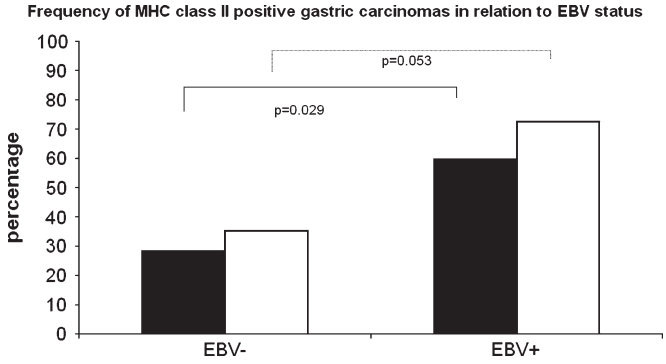

FIGURE 2. Frequency of MHC class II-positive primary gastric carcinomas in relation to EBV status. Filled bars: all primary GC cases; open bars: LN− primary GC cases.

disease-free survival for these patients.[24] We hypothesize that EBV plays a dual role in gastric carcinogenesis in which it induces both a late hit in the transgression from dysplasia toward carcinoma[28,30] and also induces an immunologic response to prevent formation of (micro)-metastases. Here we show that presence of EBV correlates with high expression of MHC class II on tumor cells and is associated with a large cellular infiltrate surrounding the primary tumor. The infiltrate consists mainly of activated (ie, GrB7+) CD8+ cells with more frequently CD83+ mature dendritic cells compared with their EBV-negative counterparts. Moreover, our finding that EBV-positive GC with metastases have a less extensive infiltrate than EBV-positive GC without metastases support the notion that the presence of the immune cells reflects an immune response, capable of preventing LN metastases. No evidence was found for loss of EBV in metastases of EBV-positive GC.

The dual effect of EBV on the tumor cells, ie, promoting tumor growth and evoking an active immune response, continues to be present during progression of the disease. No evidence was found for down-regulation of MHC class I on tumor cells by EBV, which would prevent a cytotoxic CD8+ immune response (reviewed by Algarra et al[1]) because MHC class I expression was detectable in the majority of cases.

In addition, released antigens or apoptotic bodies derived from GC tumor cells can be taken up by professional antigen presenting cells to be presented to CD4+ T cells on MHC class II molecules. These cells provide help to promote activation and longevity of CD8+ cytotoxic T cells. Interestingly, the CD83+ mature dendritic cells are more prominent in the EBV-positive tumors. Saiki et al[16] and Kijima et al[11] both describe considerable S-100-positive DC in EBV-positive carcinomas, but no correlation was made with presence of metastases. We observed the most extensive DC infiltrate in EBV-positive tumors that did not metastasize. In addition, DC recruitment to the metastasis was specific for EBV-positive cases. These findings suggest that more efficient presentation of antigens occurs in the EBV-positive tumors thus stimulating local cellular immune responses which may have a beneficial effect as reflected by less LN involvement and improved disease-free survival.

On a subset of the GCs, MHC class II molecules are expressed heterogeneously. Whereas up-regulation is observed in some EBV-negative tumors, this effect is more prominent on the EBV-positive tumors. This is in agreement with observations by Saiki et al in 8 EBV-positive and 12 EBV-negative Japanese GC cases.[16] MHC class II molecules are normally expressed by specialized antigen presenting cells including B cells, DC, macrophages, and thymic epithelial cells,[25] but can also be induced on T cells after activation and on non-professional antigen-presenting cells, including epithelial cells, in an environment rich in inflammatory cytokines, such as interferon-γ.[9] A source for inflammatory cytokines in the stomach might be an infection with *Helicobacter pylori*. These bacteria are a major cause of gastritis[2,17] and are associated with gastric cancer.[22] Because MHC class II expression is more prevalent on the EBV-positive GCs, viral antigens might attribute to the up-regulation.

Alternatively, the induction of MHC class II molecules on gastric epithelium in the context of gastritis might provide a route of entrance for EBV. At present, the receptor of EBV on epithelial cells is unknown. An α5β1 integrin mediated entrance is recently described by Tugizov et al.[21] However, MHC class II is required as a co-receptor for entrance of the virus into B cells.[13] Therefore, up-regulation of MHC class II may facilitate EBV entrance in epithelial cells.

The CD8+ immune effector cells are significantly more present in the EBV-positive GC than in the EBV-negative

TABLE 3. Summary of Characteristics of Primary Gastric Carcinomas

		LN–	LN+	EBV+	EBV–
	EBV+ vs EBV–	EBV+ vs EBV–	EBV+ vs EBV–	LN– vs LN+	LN– vs LN+
MHC I	NS	NS	NS	NS	NS
MHC II	0.029	0.053	NS	NS	NS
Infiltrate density	<0.0001	<0.0001	NS	0.003	NS
Composition					
CD8:CD4	0.022	NS	NS	NS	NS
GrB7+ CD8+ cells	0.028	NS	NS	0.073	NS
CD83+ mature DCs	NS	0.018	NS	NS	NS
CD20+ B cells	NS	NS	NS	NS	NS
CD56+ NK cells	NS	NS	NS	NS	NS

NS, not significant.

GC, confirming previously published data.[11,16] This abundant infiltrate is not only found in tumors previously characterized as lymphoepithelioma-like carcinoma but is a general feature of EBV-positive gastric adenocarcinomas.

The target antigen triggering the immune response remains undefined. The EBV transcription pattern is restricted to the noncoding BARTs and EBERs, and EBNA1, LMP2, and BARF1 transcripts.[18,28] At present, protein expression in GC cells is only demonstrated for EBNA1,[14,30] but this protein efficiently escapes from endogenous MHC class I presentation and therefore is a poor target for CD8+ cytolytic T cells.[27] Okugawa et al have recently described cytotoxic T cells primed with LMP2a peptide that were able to kill EBV-positive GC cells in vitro.[15] However, thus far we have not been able to demonstrate LMP2A and BARF1 protein expression in biopsies of EBV-positive GC (van Beek et al, submitted). Definition of the target antigen driving these immune responses could provide a basis for vaccination and immunotherapy.

In summary, we show that in EBV-positive GC, in contrast to EBV-negative GC matched for T- and N-stage, gender, and age of the patients, morphologic evidence is present for a cytotoxic immune response, which counteracts the development of LN metastases. This immune response appears to be continuously activated because EBV is always present in the tumor cells and their metastasis, and no evidence was found for decreased MHC class I expression on the tumor cells. Therefore, EBV-positive GC can be considered as an experiment of nature in which EBV on one hand promotes tumor growth but on the other hand induces an immune response that prevents LN metastases.

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