Circulating Antibodies against Epstein–Barr Virus (EBV) and p53 in EBV-Positive and -Negative Gastric Cancer

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ABSTRACT

Background: Epstein–Barr virus (EBV)-positive gastric cancers have clinicopathologic differences from EBV-negative tumors and lack TP53 mutation. Serologic profiles may inform viral contribution to carcinogenesis.

Methods: We compared humoral responses of EBV-positive (n = 67) and EBV-negative (n = 137) patients with gastric cancer from the International EBV-Gastric Cancer Consortium. Serum antibodies against four EBV proteins, nuclear (EBNA), viral capsid (VCA), early-diffuse (EA-D), and Zta replication activator (ZEBRA), and to p53 were assessed by multiplex assays. OR of antibody level tertiles (T1–T3) were adjusted by logistic regression. We also conducted a meta-analysis of reported anti-p53 seropositivity in gastric cancer.

Results: Consistent with EBV’s ubiquity, 99% of patients were seropositive for anti-EBNA and 98% for anti-VCA, without difference by tumor EBV status. Seropositivity varied between patients with EBV-positive and EBV-negative tumors for anti-EA-D (97% vs. 67%, respectively, P < 0.001) and anti-ZEBRA (97% vs. 85%, respectively, P = 0.009). Adjusted ORs (vs. T1) for patients with EBV-positive versus EBV-negative tumors were significantly elevated for higher antibodies against EBNA (2.6 for T2 and 13 for T3), VCA (1.8 for T2 and 2.4 for T3), EA-D (6.0 for T2 and 44 for T3), and ZEBRA (4.6 for T2 and 12 for T3). Antibodies to p53 were inversely associated with EBV positivity (3% vs. 15%; adjusted OR = 0.16, P = 0.021). Anti-p53 prevalence from the literature was 15%.

Conclusions: These serologic patterns suggest viral reactivation in EBV-positive cancers and identify variation of p53 seropositivity by subtype.

Impact: Anti-EBV and anti-p53 antibodies are differentially associated with tumor EBV positivity. Serology may identify EBV-positive gastric cancer for targeted therapies.

Introduction

Chronic Helicobacter pylori infection is the primary cause of gastric cancer (1), the third leading cause of cancer death worldwide (2). Epstein–Barr virus (EBV) is also implicated in gastric carcinogenesis, as about 9% of gastric tumors harbor monoclonal viral episomes (3). EBV-positive gastric tumors have demographic and clinicopathologic differences from EBV-negative tumors. Tumor EBV positivity is increased with male sex, smoking, nonantral localization, and post-gastrectomy (3, 4). In addition, patients with EBV-positive gastric tumors have better overall survival as compared with those with EBV-negative tumors (5). The Cancer Genome Atlas project (6) identified EBV-positive tumors as one of four molecular subtypes of gastric cancer. EBV-positive tumors are characterized by recurrent PIK3CA mutation, absence of TP53 mutation, JAK2 amplification, and extreme DNA hypermethylation. EBV-positive gastric cancer is classically considered to exhibit type I viral latency with EBV protein expression largely restricted to EBNA-1. However, several lytic proteins and transcripts have been also found (BZLF1, BcLF1, BLLF1, BHRF1, BRLF1, BMRF1) in EBV-positive tumors (7).

EBV infection occurs ubiquitously in the world’s population. Primary infection is followed by lifelong persistence of proviral DNA in B-lymphocytes, recognized by a restricted humoral response (8). EBV infection may reactivate from latency by switching to the lytic replication cycle. Circulating viral particles trigger immunologic response, generating additional anti-EBV antibodies. Following primary infection, antibodies to viral capsid antigen (VCA) are produced within a few days and peak after 3 weeks, then subsequently decline but persist for life. Antibodies to EBV nuclear antigen (EBNA) are not seen during acute infection, but develop 2 to 4 months afterwards and persist as markers of exposure. Antibody titers against early antigen (EA) rise on primary infection and in pathologic states of EBV reactivation (8). EA
Anti-EBV Antibodies and EBV-Positive Gastric Cancer

Materials and Methods

NCI International EBV-Gastric Cancer Consortium analysis

Study population

Five gastric cancer case series from Korea (n = 63), Japan (n = 28), Poland (n = 41), Mexico (n = 27), and Honduras (n = 45) in the U.S. NCI’s International EBV-Gastric Cancer Consortium were included in this analysis. For each series, serum samples from all available EBV-positive cases and a subset of EBV-negative cases were selected, frequency matched for sex, age at diagnosis (±5 years), anatomic subsite, and year of diagnosis (±2 years). This study comprises a total of 67 EBV-positive and 137 EBV-negative tumors. Each contributing study received local institutional review board approval, and written informed consent was obtained from all patients.

Tumor EBV status

For all cases, the presence of EBV in cancer cells had been previously assessed by gold standard in situ hybridization for EBV-encoded RNA and an RNA preservation control in paraffin-embedded tissue, as described previously (4, 12, 13).

Antibody measurements

The EBV antigens selected for analysis are representative of the different infection phases (i.e., primary infection, latency, and reactivation). IgG antibodies to a fragment of EBNA1 (C-terminal part AA 325–641, EBV strain B-95-8), VCA p18, full-length EA-diffuse (EA-D), ZEBRA (EBV strain M-ABA; ref. 14), and p53 (full-length, native; ref. 15) were measured by fluorescent bead-based multiplex serology and quantified as median fluorescence intensity (MFI). Briefly, full-length proteins or peptides were expressed in Escherichia coli in fusion with an N-terminal GST domain. Glutathione cross-linked to casein was covalently bound to the antigen on the different bead types by their internal color and quantified by fluorescence intensity of at least 100 beads of each bead type. Bound antibodies were stained by biotinylated anti-human-Ig and streptavidin-R-phycoerythrin. Beads were washed and resuspended in fusion with an N-terminal GST domain. Glutathione cross-linked to casein was covalently bound to the antigen on the different bead types by their internal color and quantifies the antibody bound to the antigen on the different bead types via the median R-phycoerythrin fluorescence intensity of at least 100 beads of each bead type. The cutoff MFI values for seropositivity were 100 MFI for EBNA-1 and 15 for ZEBRA, EA-D, VCAp18, and p53. Serum samples were tested in one batch, using the same lot of custom reagents. Laboratory staff was blinded to tumor EBV status. In addition to the in-house controls, we inserted six-coded replicates across plates. The coefficients of variation for these quality control samples were 7% for EBNA-1, 6% for EA-D, 7% for VCAp18, and 18% for ZEBRA. All six replicate pairs were reproducibly seronegative for anti-p53.

Statistical analysis

Correlations among antibody levels were evaluated by Spearman’s rank correlation. Antibody positivity in patients with EBV-positive and EBV-negative gastric tumors was compared using the Pearson χ² test. EBV antibody levels were also divided into tertiles based on distributions among all patients. Unconditional logistic regression models were used to estimate ORs with 95% confidence intervals (CI) of EBV-positive versus EBV-negative gastric cancer for seropositivity to each protein. ORs were adjusted for country, year of diagnosis (tertiles), age at diagnosis (linear), sex, and anatomic subsite (cardia, noncardia, overlapping subsites, or unspecified). A P value less than 0.05 was considered statistically significant and all tests were two-sided. Statistical analyses were performed in Stata version 15 (Stata Corp.).

Meta-analysis

Search strategy and selection criteria

The literature database PubMed (National Library of Medicine) was searched for observational studies evaluating prevalence of anti-p53 antibodies in patients with gastric cancer, published in any language up to April 30, 2019. The following broad search strategy was used: [stomach neoplasms and (p53 or anti-p53) and (antibodies or autoantibodies)].

Data extraction

Two investigators (MCC and MS) independently reviewed titles and abstracts for selection of potentially relevant articles; any disagreement was resolved by consulting a third reviewer (IZ). Citations of retrieved articles were reviewed for studies that may have been missed or absent from the database query. The following information was abstracted from each selected article: first author, year of publication, study location (country), year of sample collection, participant age (range or mean) and sex (proportion of males), number of gastric cancer cases, prevalence of anti-p53, and method of antibody assessment.

Statistical analysis

We used random effects models (16) to summarize prevalences of anti-p53 antibodies. Between-study heterogeneity was assessed for statistical significance using the Q test and quantified with the I² statistic as low (<25%), moderate (25%–50%), or high (>50%; ref. 17). Meta-analyses were performed with Stata version 15 (StataCorp) using the macro metaprop (18). A P value less than 0.05 was considered statistically significant and tests were two-sided.

Results

NCI International EBV-Gastric Cancer Consortium analysis

All pair-wise correlations among the four anti-EBV antibodies were statistically significant in the combined group of patients. The correlation coefficients ranged from 0.6 (anti-ZEBRA vs. anti-EA-D) to 0.3.
Ninety-nine percent of patients were seropositive for anti-EBNA and 98% for anti-VCA, without difference by tumor EBV status. Seropositivity varied between patients with EBV-positive and EBV-negative tumors for anti-EA-D (97% vs. 67%, respectively, \( P < 0.001 \)) and anti-ZEBRA (97% vs. 85%, respectively, \( P = 0.009 \)). In analyses based on tertiles, each viral antibody was associated with tumor EBV positivity (Fig. 1). Adjusted ORs for EBV-positive versus EBV-negative tumors were significantly elevated for patients with higher levels (vs. T1) of antibodies against EBNA (13.0 for T3), VCA (2.4 for T3), EA-D (6.1 for T2 and 44.5 for T3), and ZEBRA (4.6 for T2 and 12.4 for T3).

Antibodies to p53 were detected in 11% of the gastric cancer patients overall, including 3% of those with EBV-positive vs. 15% with EBV-negative tumors (adjusted OR = 0.16, \( P = 0.021 \)). Antibodies to p53 were not statistically significantly correlated with anti-EBV antibodies in the combined set (\( \rho \) coefficients ranged from 0.02 to −0.13).

**Meta-analysis**

The literature search identified a total of 111 reports mentioning anti-p53 antibodies in gastric cancer. After excluding 97 irrelevant publications (mainly reports of anti-p53 IHC staining), 14 full-text reports were retrieved for further evaluation; six additional publications regarding anti-p53 antibody prevalence across multiple cancer sites were identified from a previous meta-analysis (Supplementary Table S1; ref. 19). Thus, there were a total of 20 reports (19 written in English and 1 in Polish) published between 1997 and 2017 regarding the seroprevalence of anti-p53 antibodies in patients with gastric cancer (Supplementary Figure). Fifteen studies were conducted in Asian countries and five in European countries. The total sample size ranged from 25 to 501 gastric cancer cases. Two reports each presented data on two independent populations. Eighteen (90%) studies assessed IgG anti-p53 antibodies by ELISA, one study used a Luminex-based multiplex assay, and one study used immunofluorescence.

None of the studies reported separately values for patients with EBV-positive gastric cancer. Across the 22 independent series represented in the 20 reports, population-specific seroprevalences ranged from 7% to 32%. The pooled seroprevalence of anti-p53 was 15% (95% CI, 13%–18%), with high between-study heterogeneity (\( I^2 = 61\% \); Fig. 2). Seroprevalence varied significantly by test method (\( P < 0.01 \)), with lower seroprevalence by Luminex-based multiplex and immunoassay as compared with ELISA.

**Discussion**

This multicountry case–case comparison found higher antibody levels against EBV-specific proteins in patients with EBV-positive gastric cancer as compared with EBV-negative cases. These serologic patterns are consistent with viral reactivation in the presence of EBV-positive tumors as three (anti-VCA, anti-EA-D, and anti-ZEBRA) of the four studied antibodies target proteins expressed during lytic replication. This pattern of viral reactivation could represent lytic phase infection in either EBV-positive gastric cancer cells or in benign lymphocytes related to reduced immune function.

Our findings are in agreement with higher antibodies to VCA, EA and EBNA in EBV-positive gastric cancer as previously reported in two cross-sectional comparisons and a prospective (nested case-control) study (Supplementary Table S2; refs. 20–22). A unique feature of our study was the evaluation of anti-ZEBRA. ZEBRA is encoded by the EBV immediate early gene BZLF1, and is a key mediator of reactivation from latency to the viral productive cycle. Elevated antibodies to ZEBRA have also been detected in patients with nasopharyngeal carcinoma and acquired immune deficiency syndrome (23–25).

Antibody reactivity to EBV has been variably linked to gastric cancer overall. In a case-control study from Spain increasing antibody reactivity against EBNA-1 and VCA-p18 was associated with gastric cancer (26). On the other hand, a nested case-control study from East Asia found no association (27). Notably, these studies may be relatively insensitive since EBV-positive cases comprise less than 10% of gastric cancer overall.

Gastric carcinogenesis is usually characterized by identifiable precursor lesions, and the presence of EBV prior to cancer is uncertain (28–31). In a longitudinal study of gastric atrophy by Schetter and colleagues (32), individuals with elevated baseline VCA IgG and EBNA IgG titers had a higher likelihood of progressing to more severe gastric lesions. Additional studies of neoplastic stages are needed to fully elucidate the role of EBV in gastric cancer development.

EBV is strongly associated with nasopharyngeal carcinoma and several studies have indicated that EBV antibody testing has diagnostic
utility (33–35). High antibody levels, particularly IgA antibodies directed against EBV structural proteins, precede the development of this neoplasia (36–38). For other EBV-associated malignancies, serological results are inconsistent (39). Given the rarity of EBV-positivity in gastric cancer, it may never be justifiable to use anti-EBV antibodies for general population cancer screening. Among patients diagnosed with gastric cancer, EBV-positive tumor status may be a useful predictor of response to immunotherapy (40). Anti-viral antibody tests thus have potential value for both noninvasive clinical management and research applications in settings where gastric tissue is unavailable for EBV assessment. Additional serologic discrimination of tumor EBV status could be achieved by combination with blood tests for other viral (e.g., EBV miRNAs) or host response factors (e.g., PD-L1; ref. 41).

The EBV genome encodes 85 genes (42). Previous serologic studies of EBV-positive gastric cancer have focused on a small number of EBV proteins, limiting our understanding of the role of the humoral immune response in cancer development. Among patients diagnosed with gastric cancer, EBV-positive tumor status may be a useful predictor of response to immunotherapy (40). Anti-viral antibody tests thus have potential value for both noninvasive clinical management and research applications in settings where gastric tissue is unavailable for EBV assessment. Additional serologic discrimination of tumor EBV status could be achieved by combination with blood tests for other viral (e.g., EBV miRNAs) or host response factors (e.g., PD-L1; ref. 41).

The EBV genome encodes 85 genes (42). Previous serologic studies of EBV-positive gastric cancer have focused on a small number of EBV proteins, limiting our understanding of the role of the humoral immune response in cancer development. Taking advantage of emerging technologies (43), future research efforts should investigate wider ranges of viral epitopes, antibody functional types, and immunoglobulin classes and subclasses. Longitudinal studies should also evaluate whether changes in EBV serology patterns over time are predictive of EBV-positive gastric cancer risk. Elevation of specific antibodies years before cancer onset may support a viral role in carcinogenesis, whereas

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**Figure 2.**

Estimated prevalences and 95% CIs of anti-p53 seropositivity among patients with gastric cancer. Study-specific prevalences are shown as squares, with the size of the symbol inversely proportional to the study-specific variance. Random-effects pooled prevalences are shown as diamonds, with the middle corresponding to the point estimate and the width representing the 95% CI. Heterogeneity between groups \( P < 0.01 \). Overall \( I^2 = 61\% (P < 0.01) \).
altered antibody patterns close to the time of diagnosis could reflect impaired immunity in individuals with EBV-positive gastric cancer (i.e., reverse causality).

Although rare in EBV-positive gastric cancer, mutation of the p53 tumor suppressor gene is found in about half of gastric carcinomas overall (6, 44). The protein product of a mutated p53 gene generally has a longer half-life than wild-type p53 protein (45), leading to accumulation of mutant protein and production of anti-p53 antibodies. The lower prevalence of anti-p53 in our patients with EBV-positive gastric cancer is consistent with the expected rarity of the corresponding mutation (6). Because p53 inhibition is a central carcinogenic pathway, EBV-positive gastric cancer may have an alternative mechanism to abrogate p53 activity. One possible mediator is EBV-miR-BART5-3p, which facilitates degradation of p53 proteins and also targets the 3'-UTR of TP53 to consequently downregulate CDKN1A, BAX, and FAS expression. The clinical implication of anti-p53 antibodies in patients with gastric cancer is largely unknown, although seropositivity has been associated with poor survival in some studies. Hot spot mutations in TP53 have been associated with worse survival (46) and tumor EBV-positivity with better survival (5). These findings are similar to the case of human papillomavirus-related head and neck carcinoma, in which viral presence is associated with both low prevalence of TP53 mutation and better survival (47).

In conclusion, patients with EBV-positive gastric tumors have elevated antibodies to EBV. Our results further implicate EBV in gastric carcinogenesis, potentially as an alternative pathway to p53 inhibition, and may provide a useful diagnostic marker for clinical and research applications.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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