The Association of EBV and Gastric Cancer

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Abstract

The present study aimed to investigate the clinicopathological features of EBV positive and EBV negative gastric cancer patients and the expression levels of proliferative, apoptotic and cell signaling proteins in tissues samples from these patients. The biological role of EBV infection was assessed in gastric cancer.

Results: EBV was localized in the nuclei of gastric cancer cells (positive rate 6.86%). The infection rate of EBV in normal gastric mucosal cells, which were adjacent to cancer tissues, was 0. The difference noted was significant (P = 0.023). The expression levels of caspase-3 (P = 0.0423), FASL (P = 0.00297) and cyclin D1 (P = 0.0345) proteins were significantly different in EBV positive and negative gastric cancer tissues. When the parameters gender, age, Lauren classification, histological grade, early and advanced tumor stage, vascular and nerve invasion, TNM grade and survival status were compared, the maximum tumor diameter, number of lymph node metastasis, caspase-8, Ki67 and P53 protein expression did not reveal significant differences. Bcl-2 protein expression was positive in only one gastric cancer cell sample and negative in the other gastric cancer cell samples as well as in the corresponding normal gastric mucosal epithelial cells. However, significant differences were noted with regard to the positive expression of Bcl-2 in the immune cells of gastric cancer and adjacent tissues (P = 1.17749E-39). The expression levels of Bcl-2 in the immune cells of EBV positive and EBV negative gastric cancer tissues were not significantly different. The expression levels of caspase-8, caspase-3, FASL, Ki67, cyclin D1 and P53 proteins in gastric cancer cells were significantly different compared to those of normal gastric mucosal cells derived from adjacent tissues (P < 0.05). These findings were noted in both EBV positive and/or EBV negative gastric cancer cases (P < 0.05). The survival time of the patients with EBV positive gastric cancer was higher than that of the patients with EBV negative gastric cancer, whereas the differences were not significantly different. The aforementioned results suggested that the EBV virus may directly infect cancerous cells but not normal gastric mucosal cells. With the exception of caspase-3, the expression levels of the proteins FASL and cyclin D1 were closely associated with EBV-positive gastric cancer. EBV did not have a specific effect on the expression of the signaling molecules associated with proliferation and apoptosis of gastric cancer cells. Its effect on gastric cancer cells may be associated with other factors and requires further discussion. No significant differences were noted in the clinicopathological features of EBV positive compared to those of EBV negative gastric cancer patients. However, the prognosis of EBV positive gastric cancer patients was better than that of EBV negative gastric cancer patients. The mechanism of action associated with these processes requires further verification.

Main Body

The incidence and mortality rates of gastric cancer rank fifth and fourth, respectively among all malignant tumors in the world. Gastric cancer is mostly advanced and its exact pathogenetic mechanisms remain unclear. The pathogenesis of gastric cancer may be associated with Helicobacter pylori infection, exposure to the chemical carcinogen MNNG as well as genetic and environmental factors. At present, it has been suggested that various carcinogenic factors act on tumor stem cells,
which initially leads to chronic atrophic gastritis, intestinal metaplasia, atypical hyperplasia and finally early and advanced gastric cancers \(^2\). The continuous development of specific treatment methods has improved the survival rate of gastric cancer patients. However, the pathogenesis of gastric cancer remains unclear. A high number of gastric cancer patients have been identified with poor prognosis resulting in a high incidence rate of this disease. Further investigation of the specific pathogenesis and factors that may affect disease prognosis is required to provide theoretical and experimental basis for successful prevention and identification of more effective treatment methods for gastric cancer \(^2\).

In 1990, Burke et al. detected the expression of Epstein-Barr virus (EBV) in gastric adenocarcinoma tissues by PCR. Since then, several reports have been published indicating that EBV expression can be detected in gastric cancer tissues \(^3\text{-}^5\). The positive rate of EBV expression was basically stable and estimated at approximately 10\% \(^3\text{-}^5\). However, its role in gastric cancer and the identification of the specific signaling molecules involved in the development of this disease remain unclear. The positive rate of EBV infection in gastric cancer cases was estimated to approximately 10\%. Moreover, the analysis of large sample data indicates that the prognosis of EBV positive gastric cancer patients is better than that of EBV negative gastric cancer patients, whereas the specific mechanism associated with these effects remains unclear \(^3\text{-}^5\). Previous studies that have utilized a small sample size indicated no significant differences in the prognosis between EBV positive and EBV negative gastric cancer patients \(^5\). The low positive rate of EBV in gastric cancer reduces the sample size and limits further the validity of the statistical analysis. However, the factors that may improve the prognosis of EBV positive gastric cancer patients are yet to be identified and the results suggesting improvement in prognosis are contradictory \(^5\). The possible mechanism may involve the activation of lymphocytes following EBV infection and the elimination of gastric cancer cells. In EBV positive gastric cancer, tumor suppressor gene expression is upregulated and these cells are more sensitive to chemotherapy. However, controversial studies have been published examining this mechanism of action \(^5\).

The present study investigated whether EBV initially infected normal gastric mucosal epithelial cells and subsequently cancer cells, or whether it acted by directly infecting gastric cancer cells. Based on the assumption that EBV positive gastric cancer patients exhibit an optimal prognosis, we addressed whether this is caused by EBV infection of gastric cancer cells or whether it is associated with other factors, such as the proteins involved in proliferation, apoptosis and signaling of gastric cancer and peripheral immune cells as well as the clinicopathological features of the patients.

To address this hypothesis, the present experimental study was designed. We investigated whether EBV infected initially normal gastric mucosal epithelial cells or directly gastric cancer cells. Secondly, the present study investigated whether the expression of associated signaling proteins, such as those involved in proliferation, apoptosis and immunosuppression of EBV positive gastric cancer cells was different from that of adjacent cells and EBV negative gastric cancer cells. Finally, the correlation between clinicopathological diagnosis and prognosis of EBV positive and EBV negative gastric cancer patients was explored to provide theoretical and experimental basis for Meta big data analysis.
Materials And Methods

Statement: All methods were performed in accordance with the relevant guidelines.

Subjects: A total of 102 cases of gastric cancer with follow-up data were collected from January 1, 2016 to December 31, 2017 in the Department of Pathology, Affiliated Hospital of Medical College of Guilin. The inclusion criteria were the following: (1) Cases of gastric adenocarcinoma diagnosed by two experienced pathologists. The diagnostic criteria were based on the 2019 NCCN guidelines; (2) cases with incomplete follow-up data and clinicopathological data were excluded; (3) complete information of TNM staging and tumor samples was obtained from all cases. The patients were followed up until December 31, 2020. The present study was approved by the Medical Ethics Committee of the Affiliated Hospital of Medical College of Guilin and informed consent was obtained for all patients who were willing to participate in this study.

Experimental materials and key equipment: The antibodies against Bcl-2 (MAB-0711), P53 (MAB-0674), Ki67 (MAB-0672) and cyclin D1 (RAM-0541), the biotin-labeled sheep anti-mouse/rabbit IgG polymer (SP KIT-C1) and the DAB Kit (DAB-0031) were purchased from MXB Biotechnologies. The anti-CASP8 antibody (A00042) was purchased from BOSTER Biological Technology Co., Ltd., whereas the anti-cleaved-caspase-3 rabbit antibody (GB11532) and the anti-Fas ligand rabbit pAb (GB11090-1) were purchased from Servicebio Biotechnology Co., Ltd. The EBER in situ hybridization kit (ISH-7001) was purchased from Beijing ZSGB Biotechnology Co., Ltd. The digital pathological section scanner (KF-PRO-005) was purchased from Konfoong Biotech International Co., Ltd.

Preparation of paraffin-embedded tissue and tissue microarray samples and hematoxylin eosin staining (H&E staining): The tissue samples were fixed with 10% neutral formalin at room temperature for 24-48 h. Following sampling, conventional fixation, dehydration and paraffin embedding, wax blocks were made, sectioned continuously to a thickness of 4 μm, dewaxed to water and stained with H&E. Two experienced pathologists verified the selection of the typical lesion sites (cancer and normal gastric mucosal tissue more than 5 cm away from the cancer lesion). The core points were selected according to a diameter of 1.5 mm and the tissue chips were made (Fig. 1). Each chip was provided with two marking points. The tissue microarray was continuously sectioned with a thickness of 4 μm, dewaxed to water and stained with H&E. The sections were incubated with alcohol and xylene to produce the H&E sections.

Immunohistochemical experiment and data analysis: The tissue microarray was continuously sectioned for 4 μm and conventionally dewaxed to water. Following antigen thermal repair (100°C boiling water EDTA high temperature repair for 10 min), 1% hydrogen peroxide was added dropwise to block the samples at room temperature for 10 min. PBS was used for washing (5 min/3 times). Blocking was performed using 2% fetal bovine serum at room temperature for 10 min and the aforementioned primary antibodies (i.e. Bcl-2 antibody 1:1, P53 antibody 1:1, Ki67 antibody 1:1, cyclinD1 antibody 1:1, anti-CASP8 antibody 1:200, anti-cleaved-caspase-3 rabbit antibody 1:200 and anti-Fas Ligand rabbit pAb 1:200) were added. The samples were incubated at 37°C for 1.5 h and washed three times with PBS containing 1/1,000 Tween 20 for 5 min each. Moreover, biotin-labeled sheep anti-mouse/rabbit IgG polymer was
added to the samples, which were incubated at room temperature for 30 min and washed 3 times with PBS containing 1/1,000 Tween 20 for 5 min each. The DAB color developing solution was added dropwise and the color development was controlled under the microscope. Finally, the nucleus was stained with hematoxylin, dehydrated and sealed. The sections were prepared by a digital pathological section scanner (KF-PRO-005) and the immunohistochemical results were interpreted. The evaluation of the nuclear positive markers (Ki67, cyclin D1, P53) was performed using the hot spot counting method to select the site with the highest positive expression and the positive percentage was counted. The expression of the cytoplasmic and membrane positive markers (caspase-8, caspase-3, FASL, Bcl-2) was assessed based on a specific score according to the following methods: (1) Positive area: 0: positive area < 5%; 1 point: positive area 6-25%; 2 points: positive area 26-50%; 3 points: positive area 51-75%; 4 points: positive area 76-100%. (2) Positive intensity: 0 points: no expression or extremely weakly positive; 1 point: weakly positive; 2 points: moderate positive intensity; 3 points: strongly positive. The sum of the two was used as the final evaluation score of the immunohistochemical staining.

**Detection and evaluation methods of EBV infection:** The EBER (Epstein-Barr virus encoded RNAs) in situ hybridization staining was used to detect whether the tissues in the aforementioned microarray were infected with EBV. The tissue chip was continuously sectioned with a thickness of 4 μm, the paraffin section was placed in fresh xylene solution, soaked 3 times for 10 min each and then placed in absolute ethanol. Subsequently, the tissues were soaked 3 times for 3 min each and dried in air for 10 min. A total of 300 μl gastric enzyme working solution was added dropwise to the sections of the tissue microarray, which were incubated at 37°C for 10 min. The gastric enzyme solution was discarded and the tissues were dehydrated with gradient ethanol (75%, 95% and 100% for 2 min each) and finally dried with air. A total of 100 μl digoxigenin labeled probe or blank control reagent was added dropwise to the tissue, which was sealed with silicified cover glass and rubber cement. The samples were incubated overnight at 37°C (in wet box). The slides were immersed in PBS buffer for 10 min, rinsed 3 times with PBS buffer for 2 min each and rinsed further 2 times with deionized water for 2 min each. A total of 100 μl of freshly prepared DAB color solution was added to the slides, which were incubated at room temperature for 10 min, washed with water, re-stained with hematoxylin for 10 sec, differentiated and colored blue. Finally, the slices were dehydrated and sealed. The brown-yellow marker appeared in the nucleus of the target cell as positive. The margins of > 5% positive area and above medium positive were considered as reliable positive criteria. Each tissue chip was assessed with a positive control tissue.

**Clinicopathological analysis:** The aforementioned tissue samples were re-examined by two experienced pathologists according to the 2019 NCCN guidelines to confirm the basic data, such as histological classification, tumor classification and staging, nerve and vascular invasion and TNM staging. All cases were followed up by telephone and information, such as survival and time of death were retrieved.

**Statistical processing:** All data were statistically processed by the SPSS 23 software. The differences in the qualitative data were analyzed by the chi-square test (comparison of two independent sample rate composition ratio), whereas the differences in the unpaired quantitative data were compared using the T test (double sample equal variance test). The differences in the paired quantitative data were assessed
by the T test (paired two-sample analysis of mean value). Survival analysis was performed by Kaplan-Meier analysis and Life table analysis. P < 0.05 was considered to indicate statistically significant differences.

Results

1. Correlation of EBV status (positive or negative) in gastric cancer with clinicopathological parameters

In the present study, 102 wax specimens of gastric cancer and normal tissues (localized over 5 cm adjacent to the cancer tissue) were collected from the Department of Pathology, Affiliated Hospital of Medical College of Guilin from January 1, 2016 to December 31, 2017. All cases had follow-up data and the follow-up ended on December 31, 2020. Among them, 77 males and 25 females were included. The male:female ratio was 3.08; the age ranged from 37 to 78 years, with an average age of 59.27 ± 9.08 years (mean ± standard deviation); a total of 16 cases of early gastric cancer and 86 cases of advanced gastric cancer were noted, whereas 7 cases of EBV positive gastric cancer and 95 cases of EBV negative gastric cancer were included. The positive rate of EBV infection was 6.86%. The Chi-squared test was used for analysis and the data indicated no significant differences between EBV positive and EBV negative gastric cancer with regard to gender (P = 0.844), age (P = 0.9355), gross classification (P = 0.844), Lauren classification (P = 0.96), histological grade (P = 0.149), early and advanced gastric cancer (P = 0.665), vascular invasion (P = 0.426), nerve invasion (P = 0.802), T stage (P = 0.776), N stage (P = 0.499), M stage (P = 0.306), survival status (survival or death) (P = 0.843), maximum tumor diameter (P = 0.446) and number of lymph node metastasis (P = 0.466). However, significant differences were noted in the expression of EBER between cancer and adjacent tissues. EBV was mainly positive in cancer tissues and negative in normal gastric mucosal tissues (P = 0.023) (Tables 1, 2 and Fig. 2). The aforementioned results suggested that the EBV status (positive or negative) in gastric cancer did not correlate with the aforementioned factors. No significant differences were noted in specific gastric cancer subpopulations with regard to stage and histological type. However, the EBV virus was only present in gastric cancer cells and did not exist in normal gastric mucosal cells, suggesting that EBV may play a certain role in the development and progression of this disease. Normal gastric mucosal cells will not be infected by EBV and gastric cells may be more susceptible to EBV infection following carcinogenesis of the gastric mucosa.
| Category                        | EBV positive | EBV negative | Chi-square test | P-value |
|--------------------------------|--------------|--------------|-----------------|---------|
| Gender                         |              |              |                 |         |
| Male                           | 6            | 71           | 0.0386          | 0.844   |
| Female                         | 1            | 24           |                 |         |
| Age                            |              |              |                 |         |
| <=55                           | 3            | 32           | 0.0065          | 0.9355  |
| > 55                           | 4            | 63           |                 |         |
| General types                  |              |              |                 |         |
| 1 Ulcerative type              | 6            | 72           | 0.823           | 0.844   |
| 2 Uplift type                  | 0            | 7            |                 |         |
| 3 Flat type                    | 0            | 3            |                 |         |
| 4 Diffuse type                 | 1            | 13           |                 |         |
| Lauren type                    |              |              |                 |         |
| Intestinal type                | 4            | 46           | 0.003           | 0.96    |
| Diffuse type                   | 3            | 49           |                 |         |
| Histological grading           |              |              |                 |         |
| Highly differentiation         | 0            | 19           | 3.806           | 0.149   |
| Medium differentiation         | 3            | 16           |                 |         |
| Low differentiation            | 4            | 60           |                 |         |
| Early and advanced gastric cancer |          |              |                 |         |
| Early stage                    | 1            | 15           | 0.187           | 0.665   |
| Middle and late stage          | 6            | 80           |                 |         |
| Vessel invasion                |              |              |                 |         |
| Yes                            | 6            | 60           | 0.633           | 0.426   |
| None                           | 1            | 35           |                 |         |
| Nerve invasion                 |              |              |                 |         |
| Yes                            | 5            | 56           | 0.063           | 0.802   |
| None                           | 2            | 39           |                 |         |
| T stage                        |              |              |                 |         |
| T1                             | 1            | 15           | 1.11            | 0.776   |
| T2                             | 0            | 9            |                 |         |
| T3                             | 1            | 19           |                 |         |
| T4                             | 5            | 52           |                 |         |
| N stage                        |              |              |                 |         |
| N0                             | 2            | 38           | 2.37            | 0.499   |
Table 2
Clinicopathological Features of EBV Positive and EBV Negative Gastric Cancer Tissues

| Category | EBV positive (mean ± variance) | EBV negative (mean ± variance) | Two-sample equal variance analysis (T Stat) | P-value |
|----------|--------------------------------|--------------------------------|------------------------------------------|---------|
| N1       | 2                              | 17                             |                                          |         |
| N2       | 0                              | 15                             |                                          |         |
| N3       | 3                              | 25                             |                                          |         |
| M stage  |                                |                                |                                          |         |
| M0       | 6                              | 94                             | 1.05                                     | 0.306   |
| M1       | 1                              | 1                              |                                          |         |
| Survival status |                        |                                |                                          |         |
| Survival | 4                              | 65                             | 0.0338                                   | 0.843   |
| Death    | 3                              | 30                             |                                          |         |
| Paracancerous tissue |              |                                |                                          |         |
| EBV positive | 0                     | 0                               | 5.14                                     | 0.023   |
| EBV negative  | 7                     | 95                               |                                          |         |

Kaplan-Meier analysis and Life table analysis were used to statistically analyze the follow-up data of 102 gastric cancer cases. The survival time of EBV positive gastric cancer (169.43 ± 30.39 weeks) was longer than that of EBV negative gastric cancer (165.94 ± 8.09 weeks) and the prognosis was improved (Fig. 3). The difference was not statistically significant (Log Rank. P = 0.974; Breslow. P = 0.927; Tarone-Ware. P = 0.924), which may be associated with the small number of cases (Table 3).
Table 3
Comparison of the Survival Time Periods between EBV Positive and Negative Gastric Cancer cases

|                      | Survival time (mean ± standard deviation) | Log Rank (Mantel-Cox) | P-value | Breslow (Generalized Wilcoxon) | P-value | Tarone-Ware | P-value |
|----------------------|-------------------------------------------|------------------------|---------|-------------------------------|---------|-------------|---------|
| EBV positive gastric carcinoma | 169.43 ± 30.39                            | 0.001                  | 0.974   | 0.008                         | 0.927   | 0.009       | 0.924   |
| EBV negative gastric carcinoma | 165.94 ± 8.09                            |                        |         |                               |         |             |         |

2. The differences in the expression levels of signaling molecules associated with proliferation and apoptosis of EBV positive and EBV negative gastric cancer and normal gastric mucosal (adjacent to cancer tissues) tissues.

Immunohistochemical analysis was used to detect the differences in expression levels of cell proliferation- and apoptosis-associated signaling molecules, such as Bcl-2, caspase-8, caspase-3, FASL, Ki67, cyclin D1 and P53 in all 102 cases of gastric cancer and normal tissues (adjacent to the cancer epithelia). The results indicated that the expression levels of these molecules in gastric cancer specimens were significantly different than those noted in adjacent tissues. Among them, Bcl-2 (P = 1.17749E-39) (positive expression site in cytoplasm and cell membrane) was expressed at low levels in immune cells found in gastric cancer tissues and at high levels in immune cells found in adjacent tissues (Table 4) (Fig. 4). With the exception of a Bcl-2 positive gastric cancer sample, Bcl-2 was negatively expressed in 101 gastric cancer specimens and in all of 102 adjacent normal gastric mucosal epithelial samples. Caspase-3 (P = 5.42128E-26) (positive expression site in the cytoplasm), caspase-8 (P = 4.37322E-14) (positive expression site in the cytoplasm) and FASL (P = 3.64E-27) (positive expression site in the cell membrane) were expressed at low levels in gastric cancer tissues and at high levels in normal gastric mucosal tissues (adjacent to cancer tissue) (Fig. 5). The mutant P53 gene (P = 2.00994E-17) (positive expression site in nucleus) was highly expressed in gastric cancer tissues, whereas it was expressed at low levels in normal gastric mucosal tissues (adjacent to cancer tissues) (Fig. 6). However, Ki67 (P = 2.68778E-18) (positive expression site in the nucleus) and cyclin D1 (P = 6.21448E-23) (positive expression site in the nucleus) were highly expressed in gastric cancer tissues and expressed at low levels in normal gastric mucosal tissues (Table 4) (Fig. 6).
Table 4
Differences in the Expression of Proliferation- and Apoptosis-associated Signaling Molecules between all (EBV Positive and EBV Negative) Gastric Cancer and Adjacent Tissues

| All gastric cancer tissues | Cancer tissue (mean ± variance) | Paracancerous tissue (mean ± variance) | Paired T-test (T-Stat) | P-value       |
|---------------------------|--------------------------------|-------------------------------------|----------------------|--------------|
| bcl-2                     | 2.75 ± 1.75                    | 5.79 ± 0.56                        | -21.606              | 1.17749E-39 |
| Caspase-8                 | 5.28 ± 1.31                    | 6.55 ± 0.57                        | -8.778               | 4.37322E-14 |
| Caspase-3                 | 4.76 ± 2.02                    | 6.8 ± 0.18                         | -12.288              | 5.42128E-26 |
| FASL                      | 5.09 ± 1.33                    | 6.9 ± 0.13                         | -14.86               | 3.64E-27    |
| Ki67                      | 0.31 ± 0.043                   | 0.073 ± 0.005                      | 10.692               | 2.68778E-18 |
| CyclinD1                  | 0.305 ± 0.032                  | 0.066 ± 0.003                      | 12.83                | 6.21448E-23 |
| P53                       | 0.407 ± 0.129                  | 0.042 ± 0.001                      | 10.295               | 2.00994E-17 |

Following analysis of 7 EBV positive gastric cancer tissues and the corresponding adjacent tissues, no differences were noted in the expression levels of these signaling molecules. The results indicated that Bcl-2 was negative in all EBV positive gastric cancer cells and in the corresponding normal gastric mucosal epithelial cells, whereas the positive expression of Bcl-2 in immune cells of gastric cancer tissues was lower than that noted in normal adjacent tissues. The difference was statistically significant (P = 0.004061027). The expression levels of caspase-3 (P = 0.000212546), caspase-8 (P = 0.00096) and FASL (P = 9.06334E-05) in cancer tissues were lower than those noted in adjacent gastric mucosal tissues. The expression levels of Ki67 (P = 0.001600915), cyclin D1 (P = 0.00174) and P53 (P = 0.0127) were higher in cancer tissues than those noted in adjacent gastric mucosal tissues and the differences were statistically significant. The results suggested that in EBV positive gastric cancer, Bcl-2, caspase-8, caspase-3, FASL, Ki67, cyclin D1 and P53 and other cell proliferation and apoptosis-associated signaling molecules were all involved in the occurrence and development of this disease (Table 5).
Table 5
Difference in the Expression levels of Proliferation- and Apoptosis-associated Signaling Molecules between EBV Positive Gastric Cancer and Adjacent Tissues

| All gastric cancer tissues | Cancer tissue (mean ± variance) | Paracancerous tissue (mean ± variance) | Paired T-test (T-Stat) | P-value       |
|----------------------------|--------------------------------|--------------------------------------|----------------------|--------------|
| bcl-2                      | 2.86 ± 2.14                    | 5.71 ± 0.9                           | -4.51                | 0.004061027  |
| Caspase-8                  | 5.28 ± 0.57                    | 7 ± 0                                | -6                   | 0.00096      |
| Caspase-3                  | 3.71 ± 0.57                    | 6.71 ± 0.24                         | -7.94                | 0.000212546  |
| FASL                       | 3.86 ± 1.14                    | 7 ± 0.33                             | -9.242               | 9.06334E-05  |
| Ki67                       | 0.2857 ± 0.015                 | 0.131 ± 0.017                       | 5.44                 | 0.001600915  |
| CyclinD1                   | 0.4429 ± 0.036                 | 0.081 ± 0.0019                      | 5.353                | 0.00174      |
| P53                        | 0.4 ± 0.073                    | 0.047 ± 0.0008                      | 3.51                 | 0.0127       |

Further analysis demonstrated differences in the expression levels of these signaling molecules in 95 EBV negative gastric cancer tissues and in the corresponding adjacent tissues. The results indicated that Bcl-2 expression was positive in cancer cells, with the exception of 1 case of gastric cancer. Bcl-2 expression was negative in the remaining 94 gastric cancer cell specimens and in 95 normal gastric mucosal epithelial samples (adjacent to cancer tissues). The expression of this protein in immune cells was significantly lower in EBV negative gastric cancer tissues than that noted in normal tissues (P = 1.92083E-37). Caspase-8 (P = 2.4811E-12), caspase-3 (P = 1.65E-11) and FASL (P = 1.33E-24) were expressed at lower levels in gastric cancer tissues than in adjacent tissues, while Ki67 (P = 4.28609E-17), cyclin D1 (P = 1.04761E-20) and P53 (P = 5.65185E-16) were expressed at higher levels in gastric cancer tissues than in adjacent tissues. The results were significantly different (Table 6).
### Table 6

**Difference in the Expression levels of Proliferation- and Apoptosis-associated Signaling Molecules between EBV Negative Gastric Cancer Tissues and Adjacent Tissues**

| All gastric cancer tissues | Cancer tissue (mean ± variance) | Paracancerous tissue (mean ± variance) | Paired T-test (T-Stat) | P-value |
|---------------------------|---------------------------------|----------------------------------------|------------------------|---------|
| bcl-2                     | 2.75 ± 1.74                     | 5.8 ± 0.54                             | -21.11                 | 1.92083E-37 |
| Caspase-8                 | 5.28 ± 1.38                     | 6.52 ± 0.59                            | -8.052                 | 2.4811E-12 |
| Caspase-3                 | 4.84 ± 2.05                     | 6.81 ± 0.28                            | -13.256                | 3.08102E-23 |
| FASL                      | 5.18 ± 1.23                     | 6.89 ± 0.12                            | -13.938                | 1.33E-24 |
| Ki67                      | 0.312 ± 0.045                   | 0.069 ± 0.004                          | 10.298                 | 4.28609E-17 |
| CyclinD1                  | 0.294 ± 0.031                   | 0.065 ± 0.004                          | 12.018                 | 1.04761E-20 |
| P53                       | 0.407 ± 0.134                   | 0.041 ± 0.002                          | 9.77                   | 5.65185E-16 |

3. Difference in the expression levels of proliferation and apoptosis-associated signaling molecules between EBV positive and EBV negative gastric cancer tissues.

Immunohistochemical analysis was used to detect the expression levels of Bcl-2, caspase-8, caspase-3, FASL, Ki67, cyclin D1 P53 and other cell proliferation and apoptosis-associated signaling molecules in EBV positive and EBV negative gastric cancer tissues. The data were analyzed by the T and the chi-squared tests. The results indicated that the expression levels of the apoptosis-signaling proteins caspase-3 (P = 0.0423) and FASL (P = 0.00297) in EBV positive gastric cancer cells were lower than those in EBV negative gastric cancer samples. The expression levels of cyclin D1 (P = 0.0345) revealed the opposite results compared to those of caspase-3. The differences noted were significant. The expression levels of the other signaling molecules (Bcl-2, caspase-8, Ki67 and P53) in EBV positive and negative gastric cancer cases exhibited no significant differences, suggesting that caspase-3, FASL and cyclin D1 were more closely associated with EBV positive gastric cancer. However, the specific mechanism requires further experimental confirmation (Table 7).
Table 7
Differences in the Expression levels of Proliferation- and Apoptosis-associated Signaling Molecules between EBV Positive and EBV Negative Gastric Cancer Tissues

|                        | EBV positive group (mean ± variance) | EBV negative group (mean ± variance) | T-test (T-Stat) | P-value |
|------------------------|--------------------------------------|--------------------------------------|-----------------|---------|
| bcl-2                  | 2.86 ± 2.14                          | 2.75 ± 1.74                          | 0.211           | 0.833   |
| Caspase-8              | 5.28 ± 0.57                          | 5.28 ± 1.37                          | 0.003           | 0.99    |
| Caspase-3              | 3.71 ± 0.571                         | 4.84 ± 2.05                          | -2.057          | 0.0423  |
| FASL                   | 3.857 ± 1.14                         | 5.18 ± 1.23                          | -3.045          | 0.00297 |
| Ki67                   | 28.6% ± 1.47%                        | 31.18% ± 4.5%                        | -0.32           | 0.74    |
| CyclinD1               | 44.3% ± 3.6%                         | 29.4% ± 3.1%                         | 2.143           | 0.0345  |
| P53                    | 40% ± 7.33%                          | 40.7% ± 13.4%                        | -0.051          | 0.959   |

Discussion

Since 1990, Burke et al. reported that EBV virus was detected in gastric adenocarcinoma cells and a large number of studies have reported the association between EBV viral infection and gastric cancer \(^5\). The detection rate of EBV virus in gastric cancer ranges from 7 to 15%, but the majority of these patients are stable cases (approximately 10%). The results of the present study indicated that this index was 6.86%, which is close to the previously reported findings \(^6\)–\(^10\).

EBV is a double-stranded linear DNA virus, which mainly infects lymphocytes and specific epithelial cells. Its infection rate in humans can reach 90% or higher \(^5\),\(^8\),\(^10\)–\(^14\). At present, the mechanism of the EBV-mediated gastric mucosal epithelium infection has been described as follows: Initially, EBV infects B lymphocytes and oral epithelial cells; subsequently, EBV is reactivated in B lymphocytes in the stomach and released to infect epithelial cells \(^5\),\(^8\),\(^10\)–\(^14\). In the current study, EBV was not detected in normal gastric epithelial cells. However, the expression levels of EBV were detected only in less than 7% of gastric cancer cells, suggesting that it was unlikely for the virus to directly infect gastric mucosal cells. It is likely that gastric mucosal cells will exhibit an altered phenotype after their initial carcinogenic transformation, which facilitates EBV infection. However, this hypothesis requires further experimental confirmation. In the present study, we proposed a hypothesis for EBV-positive gastric cancer, suggesting that antibodies against EBV may play a therapeutic role with lower side effects. Previous meta-analysis data demonstrated that the prognosis of EBV positive gastric cancer patients is improved compared to that of EBV negative gastric cancer patients. Previous research studies with small sample sizes have revealed similar findings. However, no significant differences were observed \(^6\),\(^10\),\(^11\). The present study demonstrated that the survival time of EBV positive patients was longer than that of EBV negative patients. However, the difference was not statistically significant and additional samples have to be
collected for further analysis. The present study indicated that EBV positive and negative gastric cancer cases did not reveal significant differences with regard to gender (P = 0.844), age (P = 0.9355), gross classification (P = 0.844), Lauren classification (P = 0.96), histological grade (P = 0.149), early and advanced gastric cancer stage (P = 0.665), vascular invasion (P = 0.426), nerve invasion (P = 0.802), T stage (P = 0.776), N stage (P = 0.499), M stage (P = 0.306), survival status (survival or death) (P = 0.843), maximum tumor diameter (P = 0.446) and number of lymph node metastasis (P = 0.466). However, the EBV status was positive in gastric cancer cells and completely negative in normal tissues, which were adjacent to cancer tissues (P = 0.023), suggesting that EBV infection is an accompanying state rather than an inducing cause of the occurrence of gastric cancer. The association of the EBV status with the occurrence of gastric cancer should in theory result in the expression of the virus in normal tissues. It is important to note that the adjacent normal tissues were negative with regard to EBV infection. Therefore, we speculated that EBV only infected cancerous cells, but not normal gastric mucosal cells. The expression of signaling molecules in cancerous cells may aid the infection of these cells by EBV. Targeting of EBV positive gastric cancer cells with specific anticancer drugs may increase their cytotoxicity in order to produce the desired therapeutic effect.

Bcl-2, caspase-8, caspase-3 and FASL are apoptosis-associated signaling molecules. Bcl-2 is an important inhibitor of apoptosis, which forms dimers and promotes cell survival. The present study demonstrated that positive expression of Bcl-2 was only found in 1 case of gastric cancer cells. In the other cases, Bcl-2 was expressed in immune cells and the positive rate of its expression in immune cells derived from cancer tissues was lower than that noted in the immune cells of adjacent normal tissues. These findings suggested that gastric cancer cells may inhibit the expression of Bcl-2 in immune cells and promote induction of apoptosis. This will in turn facilitate immune escape. It is suggested that the increased expression levels of Bcl-2 in immune cells can exert certain anticancer effects. Caspase-8, caspase-3 and FASL are apoptosis-promoting factors. FASL binds to the ligand FAS and subsequently activates caspase-8 and caspase-3 resulting in the induction of apoptosis. It has been reported that the expression levels of caspase-8, caspase-3 and FASL in gastric cancer are lower than those in normal gastric mucosal tissues, which reduces the induction of apoptosis and promotes tumor cell proliferation. This phenomenon has been noted in all gastric cancer samples and EBV positive and negative samples. It is suggested that EBV positive gastric cancer also evades apoptosis and promotes proliferation through this signaling pathway. However, the expression levels of caspase-3 and FASL signaling molecules in EBV positive gastric cancer cases were slightly lower than those in EBV negative gastric cancer samples, suggesting that EBV positive gastric cancer cells may be able to escape apoptosis more efficiently. However, additional experiments are required to confirm these findings.

Ki67 is a protein that promotes proliferation. It is mainly located in the nucleus and is overexpressed in gastric cancer and various malignant tumors. The higher the positive rate of expression, the stronger the cell proliferative activity. Cyclin D1 is a regulator of the CDK4/6 enzymes that promote cell cycle progression. Its expression is often increased in malignant tumors and its function is to promote the G1 to S transition of the cell cycle. The present study indicated that the expression levels of Ki67 and...
cyclin D1 in cancer tissues were higher than those in normal tissues, suggesting that the proliferative activity of gastric cancer cells was higher than that of normal gastric mucosal cells. However, only the expression levels of cyclin D1 in EBV-positive gastric cancer and EBV-negative gastric cancer exhibited significant differences, suggesting that cyclin D1 was closely associated with EBV-positive gastric cancer and that the EBV virus may affect the expression of this protein in gastric cancer cells. Previous studies have reported that the p16 protein and the latent protein encoded by EBV may affect the expression of cyclin D1 in EBV positive gastric cancer.

P53 is a tumor suppressor gene. Wild-type P53 inhibits tumor progression. However, following P53 mutation, this transcription factor loses its ability to suppress cell proliferation. In the current study, mutant P53 expression was detected. The results indicated that the expression levels of mutant P53 in cancer tissues were higher than those in normal tissues adjacent to cancer, suggesting that gastric cancer cells had lost their intracellular self-regulation ability leading to abnormal proliferative activity compared to that noted in normal gastric mucosal cells (adjacent to the cancer tissues). However, no significant differences were noted in the expression levels of mutant P53 protein between EBV positive and EBV negative gastric cancer cases.

The present study was a retrospective study with a sample size of 102 cases of gastric cancer and their corresponding adjacent controls. The sample sources were limited, all of which originated from the inventory tissues of the Pathology Department of the Affiliated Hospital of the Medical College of Guilin. The follow-up time period was short. The shortest follow up time period was 38 months and the longest was 60 months. The mean ± standard deviation was 49.63 ± 6.18 months. The comparison of the aforementioned parameters with the majority of the indices used in the study led to non-significant comparisons. Additional EBV positive and negative gastric cancer cases have to be collected for further analysis in subsequent studies. However, the data in the present study may provide detailed information for the design of subsequent large sample meta-analyses.

In conclusion, the positive detection rate of EBV in gastric cancer tissues was 6.86%. All these cases were expressed in cancer cells; the expression levels of the apoptosis inhibitor Bcl-2 was almost absent in epithelial cells of cancer tissues and in normal samples derived from specimens adjacent to cancer tissues. However, Bcl-2 expression was positive in immune cells of cancer and normal tissues. Its expression levels in immune cells from cancer tissues was lower than that noted in adjacent tissues; the expression levels of apoptosis-associated proteins, such as caspase-3, caspase-8 and FASL in cancer tissues were lower than those noted in normal gastric mucosal cells adjacent to cancer tissues. However, the expression of the proliferation-associated proteins in cancer tissues was higher than that noted in the corresponding adjacent tissues, suggesting that gastric cancer cells exhibited higher proliferative activity and lower apoptotic rate than normal cells adjacent to cancer tissues. Moreover, the expression levels of caspase-3, FASL and cyclin D1 noted in EBV-positive gastric cancer tissues were significantly different compared to those of EBV-negative gastric cancer tissues. The follow-up data indicated that the survival time period of EBV-positive gastric cancer patients was longer, whereas the difference was not statistically significant and consequently additional data need to be collected for further analysis.
Declarations

Conflict of Interest Statement

None.

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Authors’ contributions

Xuming Wang and Tian Liu and Lijuan Yu were responsible for thesis writing and experimental design. Tian Liu purchased and supplied with the materials in the experiment. Lijuan Yu and Wenfa Mo and Longkuan Xu were responsible for data processing and performed the experiment. Xiang Qiu, Xinmei Zhong, Guangying Qi performed the data collection. All authors read and approved the final manuscript.

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Figures
Figure 1

H&E staining tissue microarray. (A and B) microarray H&E staining images of 102 cases derived from cancer and adjacent tissues. (C) In the interpretation of the tissue chip, some core points that are missing or poorly drawn are supplemented with materials to re-make the H&E staining map of the chip.
Figure 2

EBER in situ hybridization map. (A and B) EBER was positively expressed in the nuclei of gastric cancer cells. (C and D) EBER was negatively expressed in gastric mucosal cells adjacent to cancer tissues.
Life table analysis indicating that the survival time period of EBV positive gastric cancer is longer than that of EBV negative gastric cancer cases and that the disease prognosis is better (Fig. 3). However, the differences were not statistically significant.
Figure 4

Immunohistochemical staining of Bcl-2. (A and B) The Bcl-2 protein was mainly expressed in the immune cells, which were around gastric cancer cells, negatively expressed in gastric cancer cells and weakly positive in immune cells around gastric cancer cells. (C and D) The Bcl-2 protein was mainly expressed in immune cells around normal gastric mucosal cells, negatively expressed in gastric mucosal cells and strongly positive in immune cells around gastric mucosal cells.
Figure 5

Immunohistochemical staining of caspase-3, caspase-8 and FASL. (A and B) Caspase-3 expression was weakly positive in gastric cancer cells and strongly positive in normal gastric mucosal cells adjacent to cancer tissues. (C and D) Caspase-8 expression was weakly positive in gastric cancer cells and strongly positive in normal gastric mucosa adjacent to cancer tissues. (E and F) FASL was weakly positive in gastric cancer cells and strongly positive in normal gastric mucosal cells adjacent to cancer tissues.
Figure 6

Immunohistochemical staining of P53, Ki67 and cyclin D1. (A and B) P53 was highly expressed in gastric cancer cells and expressed at low levels in the corresponding normal tissues, which were adjacent to cancer. (C and D) Ki67 was highly expressed in gastric cancer cells and expressed at low levels in the corresponding normal tissues, which were adjacent to cancer tissues. (E and F) Cyclin D1 was highly
expressed in gastric cancer cells and expressed at low levels in the corresponding normal tissues
adjacent to cancer tissues