

Ezrin expression and its phosphorylation in gastric carcinoma with lymphoid stroma and Epstein-Barr virus infection

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Abstract. Gastric carcinoma with lymphoid stroma (GCLS) is a unique variant of gastric carcinoma that represents prominent lymphocytic infiltration and is correlated with Epstein-Barr virus (EBV) infection. Ezrin expression and activation are crucial in tumor metastasis and induce cell migration of EBV-related nasopharyngeal carcinomas. Using immunohistochemical methods, the expression of total and phosphorylated ezrin (p-ezrin), Thr567, was examined in 104 GCLS cases, including 78 EBV-positive and 26 EBV-negative cases, as well as 29 non-GCLS cases. Positive ezrin expression was detected to be at markedly higher levels in GCLS compared to non-GCLS ($P<0.0001$). Furthermore, ezrin expression was detected to be at higher levels in EBV-positive compared to EBV-negative GCLS ($P=0.0294$). High expression of p-ezrin in GCLS was associated with positive lymph node metastasis ($P=0.0187$). In summary, these results demonstrated that ezrin overexpression is correlated with the histologic characteristics of GCLS and EBV infection. Phosphorylation of ezrin may, therefore, contribute to lymph node metastasis in GCLS.

Introduction

Gastric carcinoma with lymphoid stroma (GCLS) is rare due to the unique histological features of gastric carcinoma with a favorable prognosis (1). GCLS demonstrates a well-defined tumor border and contains several lymphocytes, plasma cells and lymphoid follicles (1). Lymphoepithelioma-like carcinoma demonstrates prominent lymphoplasmacytic infiltration and is commonly associated with Epstein-Barr virus (EBV) infection (2), which is well-known to be present in GCLS cancer cells (3-6).

Ezrin is a member of the ezrin/radixin/moesin (ERM) family, and is characterized by small-sized molecules linking the plasma membrane and the actin cytoskeleton (7). Ezrin expression and phosphorylation are also crucial in the regulation of tumor metastasis (8-10). Ezrin expression is reduced in diffuse-type gastric carcinoma (11), while ezrin overexpression is known to be correlated with the progression and poor prognosis of gastric carcinomas (12,13). Recently, ezrin phosphorylation was found to be associated with EBV latent membrane protein 1 (LMP1) and to induce tumor migration in nasopharyngeal carcinomas (14).

Ezrin expression has been identified in ordinary gastric carcinomas, whereas ezrin expression in GCLS and its correlation with EBV infection have not been previously studied. Therefore, the present study aimed to investigate ezrin expression and its phosphorylated form in GCLS and non-GCLS.

Materials and methods

Tissue samples. GCLS or without lymphoid stroma (non-GCLS) samples were surgically resected and diagnosed at the Department of Anatomic Pathology of the Kyushu University (Fukuoka, Japan) between 1970 and 2008. The study protocol used was in compliance with the Ethical Guidelines of the 1975 Declaration of Helsinki. For strict privacy protection reasons, information identifying the samples was removed prior to analysis. The GCLS group included 74 males and 26 females with a median age of 62.0 years (range, 37-90), whereas the non-GCLS group included 16 males and 13 females with a median age of 58.4 years (range, 39-82). GCLS was defined as a well-circumscribed and expansive growth pattern adenocarcinoma (Fig. 1A) with a dense and diffuse lymphoid infiltration (Fig. 1B), and a lace-like or small-nested proliferative pattern of carcinoma cells with various degrees of cellular pleomorphism (Fig. 1C), according to previously published studies (1,3,4). Non-GCLS for the control cases was defined as medullary growth-type adenocarcinoma lacking frequent lymphoid infiltration or a glandular component. Subsequently, GCLS (n=104) were collected from 100 patients and non-GCLS (n=29) from 29 patients, based on the histopathological definition provided above.

In situ hybridization. To test for the presence of EBV, *in situ* hybridization (ISH) was performed on paraffin sections with

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Table I. Clinicopathological characteristics of patients.

| Characteristics | GCLS (n=104) | non-GCLS (n=29) | P-value |
|-------------------------------|-----------------|--------------------|---------|
| Age (years) | 62.0 | 58.4 | 0.1605 |
| Gender (M/F) | 78/26 | 16/13 | 0.0381 |
| Tumor size (cm ²) | 4.29 | 5.97 | 0.0084 |
| Location | | | |
| Upper/middle/low | 54/29/21 | 7/15/7 | 0.0194 |
| Histology W/M/P | 5/25/74 | 0/3/26 | 0.1083 |
| Depth of invasion | | | |
| M, SM/MP, SS | 48/56 | 18/11 | 0.4306 |
| Lymphatic invasion +/- | 1/103 | 2/27 | 0.1197 |
| Vessel invasion +/- | 22/82 | 11/18 | 0.0644 |
| Lymph node +/- | 10/94 | 7/22 | 0.0383 |
| EBER +/- | 78/26 | 0/29 | <0.0001 |

GCLS, gastric carcinoma with lymphoid stroma; non-GCLS, gastric carcinoma without lymphoid stroma; M, male; F, female; histology: W, well-differentiated; M, moderately-differentiated; P, poorly-differentiated; depth of invasion: M, mucosa; SM, submucosa; MP, muscularis propria; SS, subserosa.

an EBV-encoded RNA (EBER)-specific peptide nucleic acid (PNA) probe and a PNA ISH detection kit (DakoCytomation, Carpinteria, CA, USA), according to the manufacturer's instructions, as previously described (15). EBV-positive nasopharyngeal carcinoma (non-keratinizing undifferentiated carcinoma) was used as the positive control.

Immunohistochemical staining and evaluation. Tumor samples were fixed with 10% formaldehyde, embedded in paraffin, and sectioned into 4- μ m slices. Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method (Histofine[®] staining kit; Nichirei Co., Tokyo, Japan). The primary antibodies used in this study were rabbit polyclonal anti-ezrin (dilution, 1:200; Cell Signaling Technology, Inc., Beverly, MA, USA), and rabbit polyclonal anti-phospho-ezrin (dilution, 1:200; Cell Signaling Technology, Inc.). The phosphorylated form identified the endogenous levels of ezrin, radixin and moesin only when phosphorylated at Thr567 of ezrin, Thr564 of radixin or Thr558 of moesin, respectively. Following the inhibition of endogenous peroxidase in a 3% H₂O₂-methanol solution for 15 min and antigen retrieval microwave irradiation in citrate buffer (pH 6.0) for the two antibodies, the sections were exposed to each primary antibody at 37°C overnight. The sections were then reacted in 3,3'-diaminobenzidine, counterstained with hematoxylin and mounted. Scoring of the immunohistochemical results was performed by two pathologists (TT and SA), who were unaware of the clinical data. Immunohistochemical staining was evaluated in the carcinoma cell component. The proportion of positive cells was counted in >1,000 carcinoma cells and was recorded as a percentage.

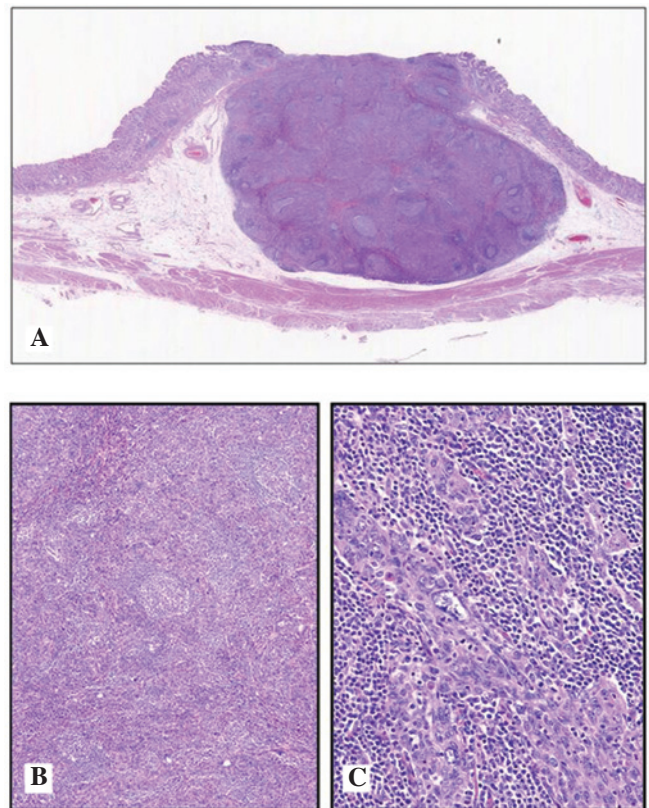


Figure 1. (A) Representative gastric carcinoma shows a well-circumscribed and expansive growth pattern. (B) Dense and diffuse lymphoid infiltration together with lymphoid follicles. (C) Lace-like or small-nested proliferative pattern of carcinoma cells with irregular nuclei.

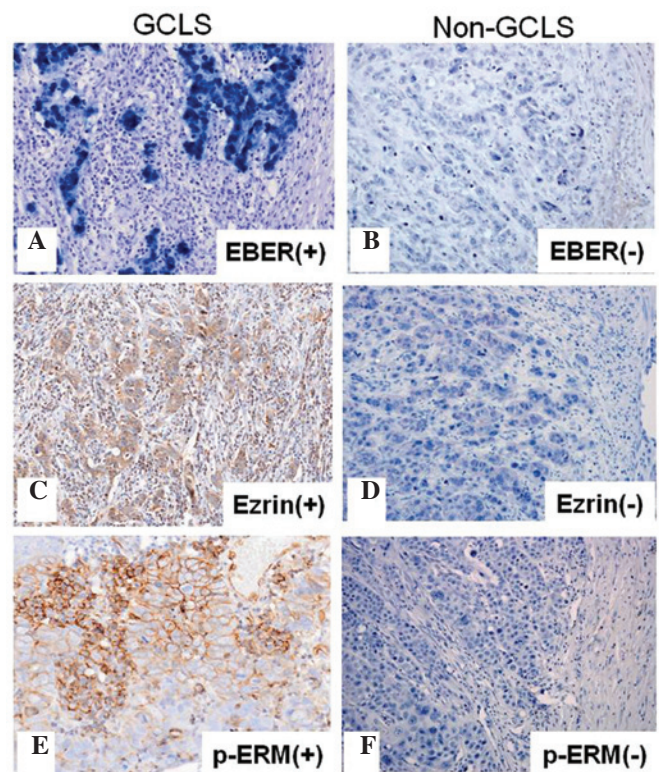


Figure 2. Expression of (A and B) EBER, (C and D) ezrin and (E and F) p-ERM. The expression is observed in (A, C and E) GCLS, but not in (B, D and F) non-GCLS. EBER, EBV-encoded RNA; p-ERM, phosphorylated-ezrin/radixin/moesin.

Table II. Correlation between ezrin or p-ERM expression and clinicopathological characteristics.

| Characteristics | Ezrin | | | p-ERM | | |
|-------------------------------|----------------|---------------|---------|----------------|----------------|---------|
| | >30% (n=95) | <30% (n=9) | P-value | >30% (n=45) | <30% (n=59) | P-value |
| Age (years) | 62.1 | 60.7 | 0.7238 | 60.7 | 62.9 | 0.3407 |
| Gender (M/F) | 70/25 | 8/1 | 0.4444 | 31/14 | 47/12 | 0.2088 |
| Tumor size (cm ²) | 4.446 | 2.656 | 0.0867 | 4.800 | 3.903 | 0.1313 |
| Location | | | | | | |
| Upper/middle/low | 47/29/19 | 6/0/3 | 0.1412 | 25/15/5 | 28/14/17 | 0.0840 |
| Histology W/M/P | 4/23/68 | 1/2/6 | 0.6519 | 4/10/31 | 1/15/43 | 0.2329 |
| Depth of invasion | | | | | | |
| M, SM/MP, SS | 42/53 | 6/3 | 0.2962 | 18/27 | 30/29 | 0.2716 |
| Lymphatic invasion +/- | 20/75 | 2/7 | 0.9346 | 9/36 | 13/46 | 0.8013 |
| Vessel invasion +/- | 0/95 | 1/8 | 0.0865 | 1/44 | 0/59 | 0.4327 |
| Lymph node +/- | 10/85 | 0/9 | 0.3059 | 8/37 | 2/57 | 0.0187 |

p-ERM, phosphorylated ezrin/radixin/moesin; M, male; F, female; histology: W, well-differentiated; M, moderately differentiated; P, poorly-differentiated; depth of invasion: M, mucosa; SM, submucosa; MP, muscularis propria; SS, subserosa.

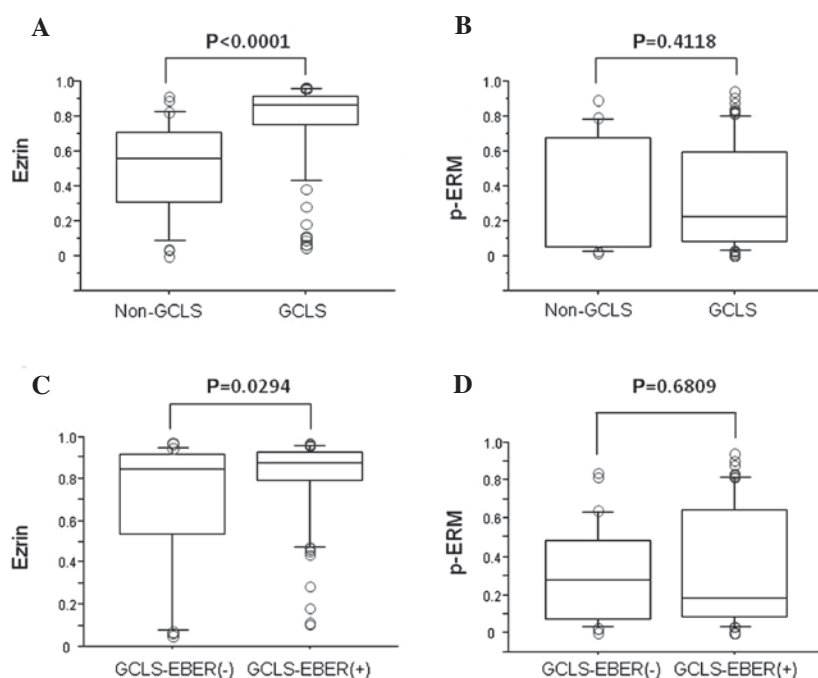


Figure 3. Comparison of (A) ezrin and (B) p-ezrin expression between non-GCLS and GCLS. Comparison of (C) ezrin and (D) p-ezrin expression between EBER-negative and EBER-positive GCLS. GCLS, gastric carcinoma with lymphoid stroma; non-GCLS, gastric carcinoma without lymphoid stroma; p-ERM, phosphorylated-ezrin/radixin/moesin; EBER, EBV-encoded RNA.

Statistical analysis. For the statistical analysis, the expression of ezrin or p-ezrin in carcinoma cells was identified as a high-expression group, when the cytoplasm or membrane staining showed >30% of carcinoma cells. Statistical analysis of group differences was carried out using the Chi-square, Fisher's exact and Student's-t tests. $P < 0.05$ was considered statistically significant.

Results

Clinicopathological characteristics of GCLS and non-GCLS patients. The clinicopathological characteristics of patients are summarized in Table I. The GCLS group included 74 males and 26 females with a median age of 62.0 years (range, 37-90), whereas the non-GCLS group included 16 males and 13 females

with a median age of 58.4 years (range, 39-82) ($P=0.0381$). The tumor size of GCLS was larger compared to that of non-GCLS ($P=0.0084$). There were 54, 29 and 21 GCLS cases from the upper, middle and lower stomach, as well as 7, 15 and 7 non-GCLS cases from the upper, middle and lower stomach, respectively ($P=0.0194$). Lymph node metastasis in GCLS was markedly infrequent compared to non-GCLS ($P=0.0383$).

GCLS cases were divided into EBER-positive ($n=76$) (Fig. 2A) and -negative cases ($n=28$) by *in situ* hybridization. By contrast, non-GCLS cases included no EBER-positive cases (Fig. 2B) ($P<0.0001$).

Ezrin and p-ezrin expression in GCLS and non-GCLS. Positive ezrin expression was diffusely observed in the cytoplasm and membrane of carcinoma cells (Fig. 2C), whereas p-ezrin was predominantly detected in the membrane of carcinoma cells alone (Fig. 2E). Ezrin expression was markedly higher in GCLS compared to non-GCLS ($P<0.0001$) (Fig. 3A), although there were no differences in the p-ezrin expression in GCLS and non-GCLS (Fig. 3B). Ezrin expression was higher in EBV-positive GCLS compared to -negative GCLS ($P=0.0294$) (Fig. 3C), although there was no difference in p-ezrin expression in EBV-positive and -negative GCLS (Fig. 3D).

Correlation between ezrin/p-ezrin expression and clinicopathological characteristics of GCLS patients. The correlation between the expression of ezrin or p-ezrin and the clinicopathological patient characteristics is summarized in Table II. High expression of ezrin and p-ezrin (>30% of positive carcinoma cells) of GCLS was observed in 95/104 (91%) and in 45/104 (43%) of cases, respectively. Forty five of p-ezrin-positive cases included 42 ezrin-positive and 3 -negative cases. No significant differences were observed between ezrin expression and the clinicopathological characteristics, however, the high expression of ezrin tended to correspond to a larger tumor size ($P=0.0867$). Only p-ezrin in GCLS was associated with positive lymph node metastasis ($P=0.0187$).

Discussion

EBV-associated gastric carcinoma accounts for ~10% of gastric carcinomas and histologically resembles GCLS (16). Consistent with a previous study, the GCLS cases of this study demonstrated a male predominance, a greater tendency towards being located in the fundic or upper gastric regions and a lower frequency of lymph node metastasis, compared to the control cases (17). Moreover, 75% of GCLS cases demonstrated EBER positivity. Taken together, these findings indicate that the cases examined in this study might be characterised as typical GCLS cases.

Molecular abnormalities of EBV-associated gastric carcinomas remain unknown, whereas the CpG island methylation in the promoter region of PTEN gene has been previously documented (18). Endo *et al* (14) have demonstrated EBV LMP1 to be associated with ezrin phosphorylation in nasopharyngeal carcinoma, whereas the present is the first study to indicate that ezrin expression is correlated with GCLS and EBER positivity.

Ezrin is widely expressed in malignant tumors, including gastric (12,13) and colon cancers (19), hepatocellular carcinoma (20), ovarian (21) as well as breast cancer (22), and is

associated with poor prognosis. Furthermore, ezrin expression is correlated with an early recurrence of hepatocellular carcinoma (22), invasion of pancreatic adenocarcinoma (23) and lymph node metastasis of prostate cancer (24) and nasopharyngeal carcinoma (25).

According to a comparison between ezrin or p-ezrin expression and clinicopathological characteristics, only lymph node metastasis is associated with p-ezrin. In the present study, anti-ezrin unphosphorylated type and anti-ERM [ezrin (Thr567)/radixin (Thr564)/moesin (Thr558), Cell Signaling Technology, Inc.] antibody was used as the anti-ezrin phosphorylated form at the Thr567 site, also used as the p-ezrin antibody at Thr567 in previously published studies (26,27). The phosphorylation of residual Thr567 in ezrin alters the protein to expose its binding sites (28) thus resulting in oncogene-induced transformation (29). The phosphorylation at Thr567 may not be necessary for osteosarcoma metastasis (27). In addition, phosphorylation at Tyr353, but not at Thr567, is associated with lymph node metastasis of pancreatic adenocarcinoma (30). However, Tang *et al* (27) have reported that ezrin phosphorylation at Thr567 may induce lymph node metastasis of nasopharyngeal carcinomas. The findings in the present study were consistent with the hypothesis that p-ezrin at Thr567 is crucial in lymph node metastasis. Notably, nasopharyngeal carcinoma and GCLS are commonly associated with EBV infection.

Based on recent studies of targeting for ezrin, reduction of the ezrin gene by RNA interference (RNAi) may inhibit the migration and invasion of human gastric cancer cells (31). Berberine also inhibits metastasis of nasopharyngeal carcinoma cells by targeting Rho kinase-mediated ezrin phosphorylation at Thr567 (27). These findings suggest that the molecular target therapy of ezrin and its phosphorylation should be considered to be EBV-associated GCLS.

In conclusion, ezrin expression is correlated with GCLS with EBV infection, while the phosphorylation of ezrin is essential in the lymph node metastasis of GCLS.

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