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Latent-lytic switch of Epstein-Barr virus infection in gastric carcinoma

Key Messages

1. Epstein-Barr virus (EBV) causes gastric cancer and was almost always latent in infected tumour cells. Tumour cells infected with the latent stage of EBV do not respond to the antiviral drug ganciclovir. Zinc finger E-box binding factor (ZEB1) is the transcriptional repressor pivotal for silencing the BZLF1 promoter (Zp). BZLF1 is sufficient to convert EBV from the latent to lytic form. However, the mechanism of ZEB1 regulating latent-lytic switch of the EBV life cycle in EBV-associated gastric cancer and the virus's role in gastric carcinogenesis remain unknown.
2. We investigated the effect of ZEB1 on latent-lytic switch of EBV infection in gastric cancer cell lines. Loss or gain of ZEB1 biological function indicated its potential as a novel molecular target for the intervention in EBV-associated gastric cancer.
3. In addition, TaqMan real-time PCR was performed to examine the existence of EBV in primary gastric cancer and premalignant lesions. The association between EBV and patient characteristics was assessed.
4. Our results suggest that ZEB1 is a key mediator of the latent-lytic switch of EBV-associated gastric cancer. Inhibition of ZEB1 may be a potential means of therapy.

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Introduction

Epstein-Barr virus (EBV) is an infective agent causing gastric cancer.[1] It is almost always latent in infected tumour cells. Tumour cells infected with the latent form of virus do not respond to the antiviral drug ganciclovir (GCV). The intermediate-early gene BZLF1 is a transcriptional activator of viral genes essential for lytic replication.[2] Zinc finger E-box binding factor (ZEB1) is the transcriptional repressor pivotal for the silencing of the BZLF1 promoter (Zp),[3] indicating that the aberrant regulation of ZEB1 expression in tumour cells may have an important influence on EBV dormancy and persistence. However, the mechanism by which ZEB1 regulates the latent-lytic switch of the EBV life cycle in EBV-associated gastric cancer and its role in gastric carcinogenesis in the Chinese remain unknown. In this study, we evaluated the effect of ZEB1 in modulating the latent-lytic switch of EBV infection in gastric cancer cells, and the potential of ZEB1 as a novel molecular target for the intervention in EBV-associated gastric cancer. We also addressed the clinical importance of EBV infection in gastric carcinogenesis in a large-scale cohort of Chinese patients.

Methods

This study was conducted from November 2008 to October 2010. Loss or gain of ZEB1 function was obtained by ZEB1 siRNA knockdown and ZEB1 overexpression in EBV-infected gastric cancer cell lines. Cell growth was evaluated by cell viability and a colony formation assay. The cell cycle distribution was determined by flow cytometry. The activity of Zp was examined after ZEB1 overexpression in AGS-EBV cells using a luciferase reporter activity assay.

Gastric cancer tissues were obtained from 711 primary gastric cancer patients in the First Affiliated Hospital of Sun Yat-sen University, Guangzhou from January 1999 to December 2006. In addition, 97 gastric tissues with precancerous lesions (intestinal metaplasia and/or atrophic gastritis) and 24 normal gastric tissues were collected. All patients and controls gave consent for participation, and the study protocol was approved by the Clinical Research Ethics Committee of the Sun Yat-sen University of Medical Sciences.

Genomic DNA was extracted from gastric tissue and EBV was detected using quantitative PCR and in situ hybridisation. ZEB1 expression level was examined by immunohistochemistry.

Results

Knockdown of ZEB1 markedly enhanced expression of the lytic gene BZLF1 in YCC10 cells, compared to cells treated with the control siRNA. A well-known marker for latent EBV infection, EBNA1 expression was significantly inhibited by ZEB1 knockdown. ZEB1 knockdown caused about 20% inhibition in cell numbers, compared to control siRNA transfected YCC10 cells ($P<0.01$). Fluorescence-activated cell sorting (FACS) analysis revealed a significant decrease in the number of cells in the S phase in YCC10 cells with ZEB1 knockdown compared to control cells ($P<0.01$). In addition to this inhibition of cell proliferation, there was a significant increase in the number of cells accumulating in the G2/M phase following ZEB1 knockdown with YCC10 cells

(P<0.01). We examined whether ZEB1 depletion could increase the sensitivity of gastric cancer cells to GCV. Following ZEB1 knockdown, GCV treatment demonstrated a significantly more additive effect on cell growth with 55% inhibition in cell viability (P<0.001), compared with YCC10 transfected with ZEB1-siRNA alone. Cellular apoptotic rate was determined using annexin-V-FITC/propidium iodide double staining. The number of early apoptotic cells 72 hours following ZEB1-siRNA transfection was substantially increased, as compared to control-siRNA transfected cells (P<0.05). Induction of apoptosis was further confirmed by analysis of two crucial apoptosis-related mediators of caspase-3 and PARP by Western blot. Enhanced expression of active forms of caspase-3 and PARP were demonstrated in YCC10 cells treated with ZEB1-siRNA. These results suggested that apoptosis concomitant with G2/M cell cycle arrest induced by down-regulation of ZEB1 was a plausible cause leading to the growth inhibition in ZEB1-depleted gastric cancer cells.

Overexpression of ZEB1 led to a significant inhibition of the EBV lytic gene (BZLF1) expression in AGS-EBV cells. The activity of Zp after ZEB1 overexpression in AGS-EBV cells was examined using the luciferase reporter activity assay. Our results indicated that the activity of Zp was significantly inhibited by ZEB1 re-expression (P<0.001). This suggests that ZEB1 inhibited BZLF1 transcription through reducing the activity of the BZLF1 promoter Zp. Ectopic expression of ZEB1 in AGS-EBV cells caused a significant increase of viable cells (P<0.01). The colony formation assay also confirmed that the colonies formed in ZEB1-transfected cells were significantly greater in number and larger in size than in empty vector-transfected cells (up to 100% of vector control, P<0.001). Moreover, FACS analysis of ZEB1-transfected AGS-EBV cells revealed a significant induction in the number of S-phase cells compared to vector-transfected cells (P<0.01). Overexpression of ZEB1 downregulated protein expression of cleaved caspase-3, cleaved caspase-9, and cleaved-PARP compared with vector-transfected AGS-EBV cells, indicating reduced cell apoptosis.

The presence of EBV in gastric tissue specimens was determined with two EBV DNA fragments targeting the BamHI-W region and EBNA-1 regions. Using both the BamHI-W PCR and the EBNA-1 PCR, EBV DNA was detected in 80 (11.3%) of 711 gastric cancers, 4 (4.1%) of 97 precancerous lesions, but none from tissues of the 24 healthy controls. The proportion of EBV DNA-positive cases among these groups was significantly different (χ^2=7.57, P<0.05). EBV DNA-positive cases were significantly more frequent in patients with gastric cancer than in those with precancerous lesions (χ^2=4.66, P<0.05).

The association between clinicopathologic features and EBV infection in human gastric cancers is listed in the Table. The presence of EBV was associated with age (P<0.05), male gender (P=0.0002), intestinal histological type (P=0.05), and marginally associated with well or moderate differentiated gastric cancer (P=0.08). However, there was no correlation between the EBV and the tumour location, *Helicobacter pylori* infection, and survival of gastric cancer patients.

We evaluated ZEB1 expression in EBV-positive and EBV-negative primary gastric cancer tissues by immunohistochemistry. ZEB1 was more frequently detected in EBV-positive gastric cancers (80%, 12/15) than in EBV-negative gastric cancers (10%, 5/50) (P<0.0001).

Table. Clinicopathologic features of gastric cancer patients with presence of Epstein-Barr virus (EBV)

Variable	No. (%) of patients		P value
	EBV-positive (n=68)	EBV-negative (n=487)	
Mean±SD age (years)	53.66±13.08	57.00±12.60	
Gender			0.0002
Male	60 (15.9)	318 (84.1)	
Female	8 (4.5)	169 (95.5)	
Location			>0.05
Proximal	16 (11.1)	127 (88.9)	
Distal	45 (11.9)	333 (88.1)	
Lauren histologic subtype			0.05
Intestinal	60 (13.9)	371 (86.1)	
Diffuse	8 (6.7)	111 (93.3)	
Differentiation			0.0845
Poor	51 (14.6)	298 (85.4)	
Well or moderate	10 (8.1)	113 (91.9)	
Tumour node metastasis stage			0.6398
I	7 (9.6)	66 (90.4)	
II	6 (8.5)	65 (91.6)	
III	22 (11.5)	170 (88.5)	
IV	26 (14.0)	160 (86.0)	
Helicobacter pylori infection			0.110
Positive	18 (15.8)	96 (84.2)	
Negative	16 (8.4)	175 (91.6)	

Discussion

Downregulation of ZEB1 in YCC10 causes upregulation of BZLF1 expression and downregulation of latent gene EBNA1 expression, thus promoting the latent-lytic switch of EBV infection. BZLF1 regulates the switch from latent infection to virus replication in EBV-infected cells and thus acts as a key mediator of reactivation from latency to the viral productive infection of EBV.[4] Expression of the BZLF1 gene is necessary and sufficient to disrupt EBV latency. EBNA1 is essential for maintenance of viral latent replication and persistence.[5] Thus, loss of ZEB1 may lead to reactivation into lytic replication due to the enhanced expression of BZLF1 and reduced expression of EBNA1. To better define the effect of ZEB1 in latent-lytic switch in gastric cancer, we examined its functional consequences by knocking down in the human gastric cancer cell line, YCC10. Decreased ZEB1 expression in YCC10 led to the inhibition of cell growth and S-phase cells, induction of apoptosis and caused cell cycle arrest in the G2/M phase. Induction of apoptosis was further confirmed by increased expression of activated form of caspase-3 and PARP, which leading to impairment of DNA repair and apoptosis. Thus, heightened ZEB1 depletion may diminish EBV-positive gastric cancer cell growth by upregulating apoptotic cell death pathways. Collectively, knocking down ZEB1 by itself was sufficient to induce EBV lytic replication in latently infected gastric cancer cell. We found that GCV alone was barely effective in controlling the YCC10, whereas induction of lytic EBV infection in YCC10 induced by re-expression of an immediate-early gene BZLF1 through knocking down ZEB1 enabled killing of the cells by GCV, because the host cells were in the lytic stage rather than the latent stage. EBV infection expressed virally encoded kinases to phosphorylate the prodrug GCV and changed to its cytotoxic form. As EBV-positive tumour cells are primarily in the latent form of EBV infection, induction of the latent-to-lytic switch of the EBV life cycle by ZEB1 inhibition could improve the clinical efficacy of GCV by specifically killing EBV-positive tumour cells and represents a new therapeutic option for EBV-associated gastric cancer.

We further investigated the role of the ZEB1 as a transcriptional repressor of BZLF1 and thus regulating the latent-to-lytic switch of the EBV life cycle in gastric cancer through again-of-function assay. Ectopic overexpression of ZEB1 in AGS-EBV led to downregulation of BZFL1. We further showed that this suppressive effect of ZEB1 on BZFL1 expression was specifically mediated through binding to a specific site of the BZLF1 promoter (Zp). This was supported by recent reports that ZEB1 can directly bind Zp via the ZV element, repressing transcription of BZLF1 initiated from Zp and therefore contribute to regulation of the switch between latency and lytic replication of EBV.[3] In addition, ectopic expression of ZEB1 in AGS-EBV cells showed a marked promoting effect on cell growth and cell proliferation. Moreover, ectopic expression of ZEB1 in AGS-EBV cells reduced expression of pro-apoptotic genes including cleaved caspase-3, caspase-9, and PARP. These results inferred that over-expression of ZEB1 is sufficient to inhibit lytic reactivation by inhibiting transcription of BZLF1, and that ZEB1 indeed plays a central role in maintenance of EBV latency in gastric cancer cells.

The association between EBV infection and gastric cancer has not been well documented in Chinese. Existence of EBV in gastric cancer tissues was determined by two real-time quantitative PCR tests targeting different part of the EBV genome, BamHI-W and EBNA-1, respectively, and validated by EBER assay. In our cohort, we observed that EBV-positive gastric cancer comprises 11.3% (80/711) of cases. This is similar with the EBV prevalence detected in gastric cancer in other countries. The EBV-carrying tumours are observed more often in males ($P<0.001$) and in younger patients ($P<0.05$) [Table]. The trends toward male predominance and younger age have been observed in Japanese and Dutch gastric cancer patients. EBV infection was also detected in precancerous lesions (atrophy and intestinal metaplasia), although its frequency was distinctly lower in these lesions than in the tumours ($P<0.05$). The infection was not detected in normal gastric tissues. This indicated that EBV enters the gastric epithelium at an early stage of the multistep process of gastric carcinogenesis. This is in line with observations that EBV is the precursor lesion in precancerous and carcinoma cells. Thus, it is likely that EBV might infect a dysplastic gastric epithelial cell, transforming it into a carcinoma cell as an additional mechanism contributing to gastric malignant progression. ZEB1 was more frequently detected in EBV-positive gastric cancers than in EBV-negative gastric cancers ($P<0.0001$), consistent with ZEB1 being essential for maintenance of EBV latent replication and persistence in gastric cancer. In addition, the overall survival of the EBV-carrying gastric cancer patients showed no difference to those with the EBV-negative tumour. These results suggest that EBV plays a distinct role in gastric carcinogenesis in Chinese patients.

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