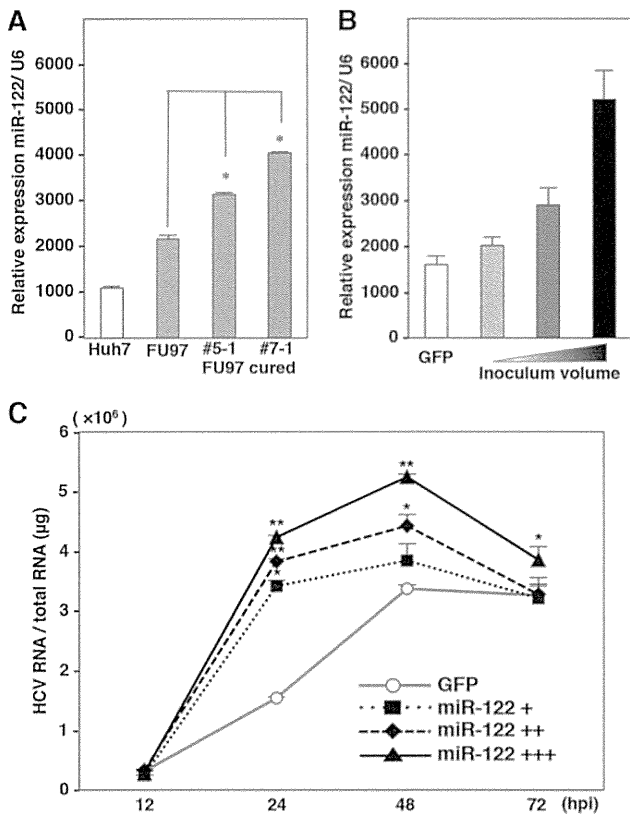


FIG 5 Innate immune response in cured FU97 cells. (A) Huh7, parental, and cured FU97 cells (clone 7-1) were stimulated with 100 IU/ml of IFN- $\alpha$  or infected with VSV. The expression of mRNA of ISG15 at 4, 8, 12, and 24 h posttreatment (hpt) was determined by qPCR and standardized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B) Huh7, parental FU97, and cured FU97 (clone 7-1) cells cotransfected with pIFN- $\beta$ -Luc and pRL-SV40 were infected with VSV at an MOI of 1 at 24 h posttransfection (left). Cells cotransfected with pISRE-Luc and pRL-SV40 were infected with VSV at an MOI of 1 or stimulated with 100 IU/ml of IFN- $\alpha$  at 24 h posttransfection (right). Luciferase activities were determined at 24 h posttreatment. (C) Huh7, parental FU97, and cured FU97 (clone 7-1) cells were infected with VSV at an MOI of 1 or stimulated with 100 IU/ml of IFN- $\alpha$ , fixed with 4% PFA at 18 h posttreatment, and subjected to immunofluorescence assay using anti-IRF3 and -STAT2 antibodies. Cell nuclei were stained by DAPI. Asterisks indicate significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ) from the results for control cells.

(2a) (34), and S310 strain (3a) (63) were established, the construction of infectious clones of other genotypes has not succeeded yet.

Because permissive cell lines for HCVcc infection *in vitro* had been limited to Huh7 cells due to cell tropism and the narrow host range (13, 14), the establishment of a novel cell culture system supporting HCV propagation is needed for further HCV analyses. Previous reports have demonstrated that HepG2, Hep3B, and HEK293 cells permit HCVcc propagation (16, 17, 64). However, exogenous expression of host factors is necessary for complete propagation of HCVcc in these cell lines. In HepG2 and Hep3B

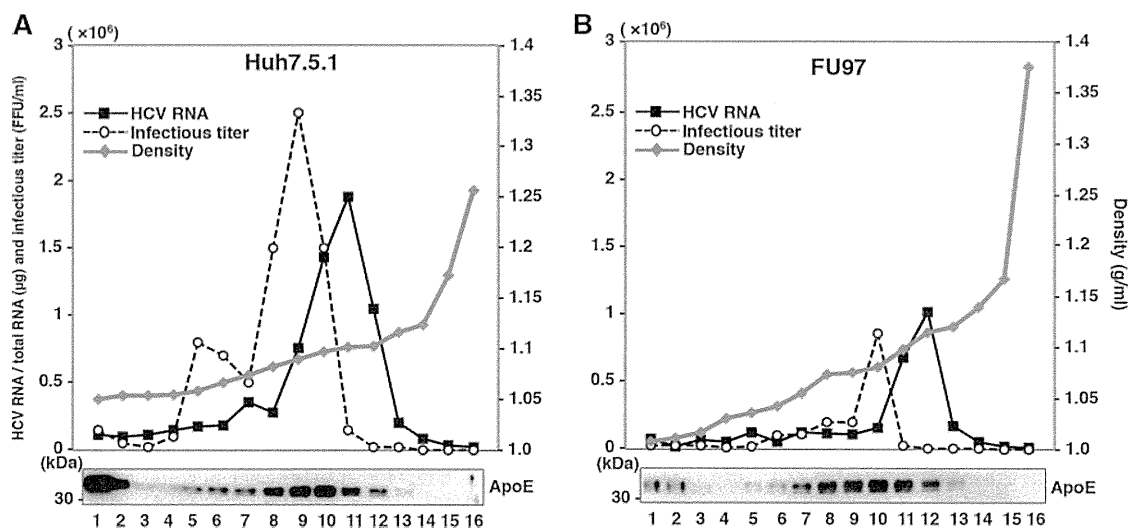
cells, overexpression of miR-122 is essential for efficient replication of HCV RNA (16, 17). In HEK293 cells, the exogenous expression of CLDN1, miR-122, and ApoE was required for infectious particle formation upon infection with HCVcc (64). On the other hand, JHH-4 and FU97 cells permit complete propagation of HCVcc without any exogenous expression of the host factors required for propagation of HCVcc. JHH-4 cells grown in a three-dimensional radial-flow bioreactor were successfully infected following inoculation with plasma from an HCV carrier and transfection of HCV RNA transcribed from full-length cDNA (44). In



**FIG 6** Expression of miR-122 is one of the determinants for HCV RNA abundances. (A) Total RNA was extracted from Huh7 and parental and cured FU97 (clones 5-1 and 7-1) cells, and the relative expression of miR-122 was determined by qPCR. U6 snRNA was used as an internal control. (B) Establishment of FU97 cell lines stably expressing various concentration of miR-122 by infection with a lentiviral vector. FU97 cells infected with lentiviral vector to express GFP were used as a control. (C) FU97 cell lines expressing various concentrations of miR-122 were infected with HCVcc at an MOI of 1, and HCV RNA abundances were determined at 12, 24, 48, and 72 h postinfection (hpi) by qRT-PCR. Asterisks indicate significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ) versus the results for control cells.

addition, JHH-4 cells were suggested to possess some host factors involved in the enhanced translation of HCV RNA (64, 65). Furthermore, high susceptibility of FU97 cells to HCVcc/JFH-2 infection compared to Huh7 cells raises the possibility of using FU97 cells for the propagation of HCVcc derived from other genotypes, including the H77, TN, and S310 strains.

AFP-producing gastric cancer (AFPGC) cell lines, FU97 and Takigawa cells (66), which were identified by using a cDNA array database, were shown to express high levels of liver-specific factors. AFPGC is a rare case and exhibits a worse prognosis and the characteristics of early hepatic metastasis (67). It is hypothesized that production of AFP, which is suppressed in mature hepatocytes, is induced in HCC by the dedifferentiation of cancer cells or the increase in oval cells in the oncogenic pathway (68). Oval cells are believed to be capable of producing AFP, are candidates for hepatic stem cells, have bipotentiality to differentiate into hepatocytes and bile duct epithelial cells, and play an important role in liver regeneration (69, 70). These hypotheses suggest that cancer cells acquired a new function, such as the ability to produce AFP through an alteration in differentiation status. Although the mechanism of AFP production in gastric cancer remains unknown, hepatic dedifferentiation might be induced in gastric cancer. Furthermore, previous reports have proposed the concept of "hepatoid adenocarcinoma" based on the differentiation of AFPGC into hepatocyte-like cells (71, 72), suggesting that FU97 and Takigawa cells obtained the hepatocyte-like characteristics required for HCV propagation through dedifferentiation during the oncogenic process. In addition, recent studies demonstrated that hepatocyte-like cells derived from induced pluripotent stem cells (iPS cells/iPSCs) express high levels of miR-122 and VLDL-associated proteins and support propagation of HCVcc and HCV derived from patient serum (28–30). These results suggest that hepatic differentiation required for hepatic functions plays crucial roles in HCV propagation. In accord with these observations, our data suggest that cancer cell lines differentiated into hepatocyte-



**FIG 7** HCV particles produced in FU97 cells exhibit similar characteristics to those in hepatic cells. HCV particles in the culture supernatants of Huh7.5.1 and FU97 cells were harvested at 72 h postinfection with HCVcc and analyzed by using iodixanol density gradient centrifugation. HCV RNA and infectious titers of each fraction were determined by qRT-PCR and focus-forming assay, respectively. Buoyant density was plotted for each fraction (upper panels). Expression of ApoE in each fraction was detected by immunoblotting using anti-ApoE antibody (lower panels).

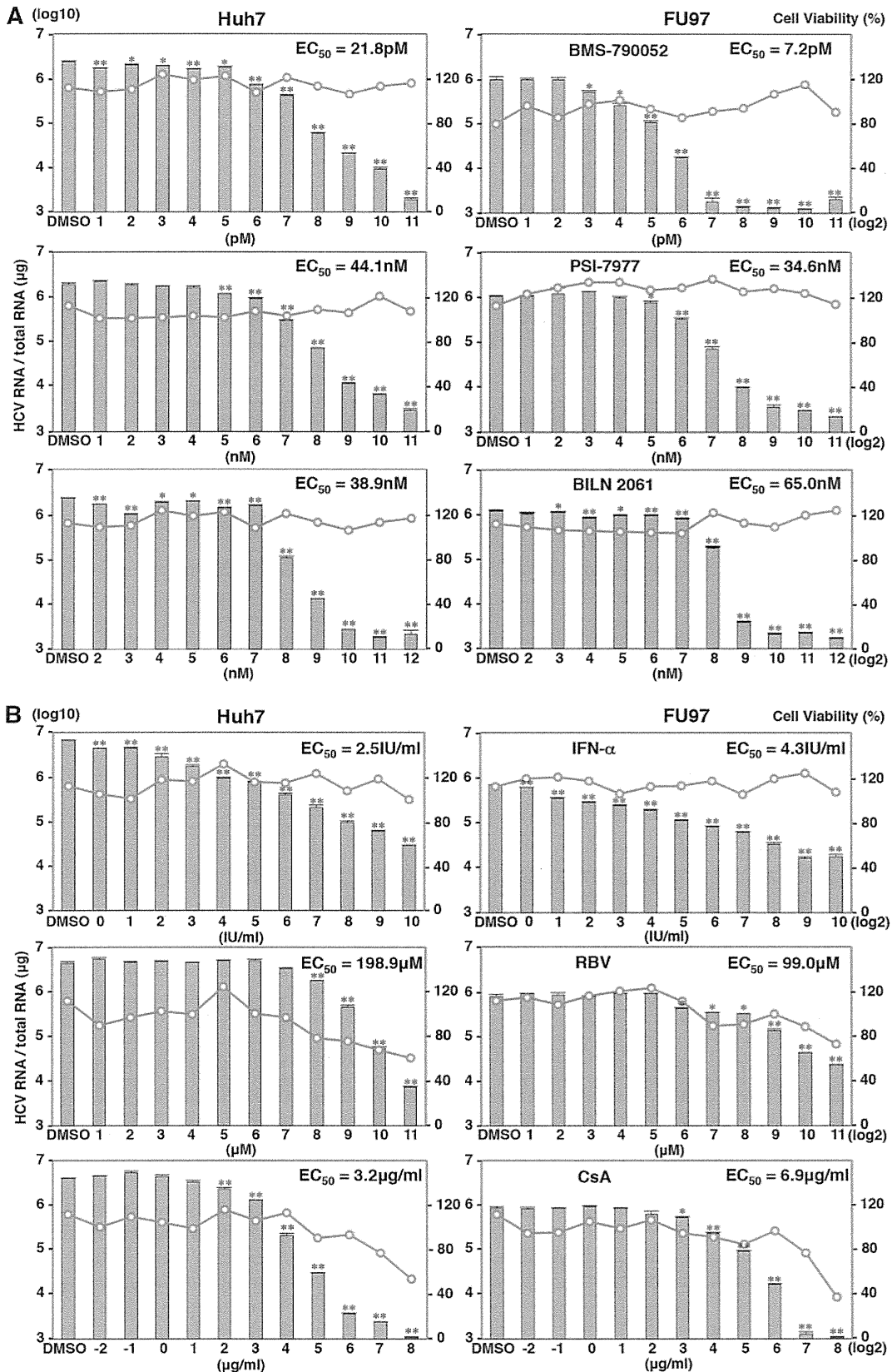
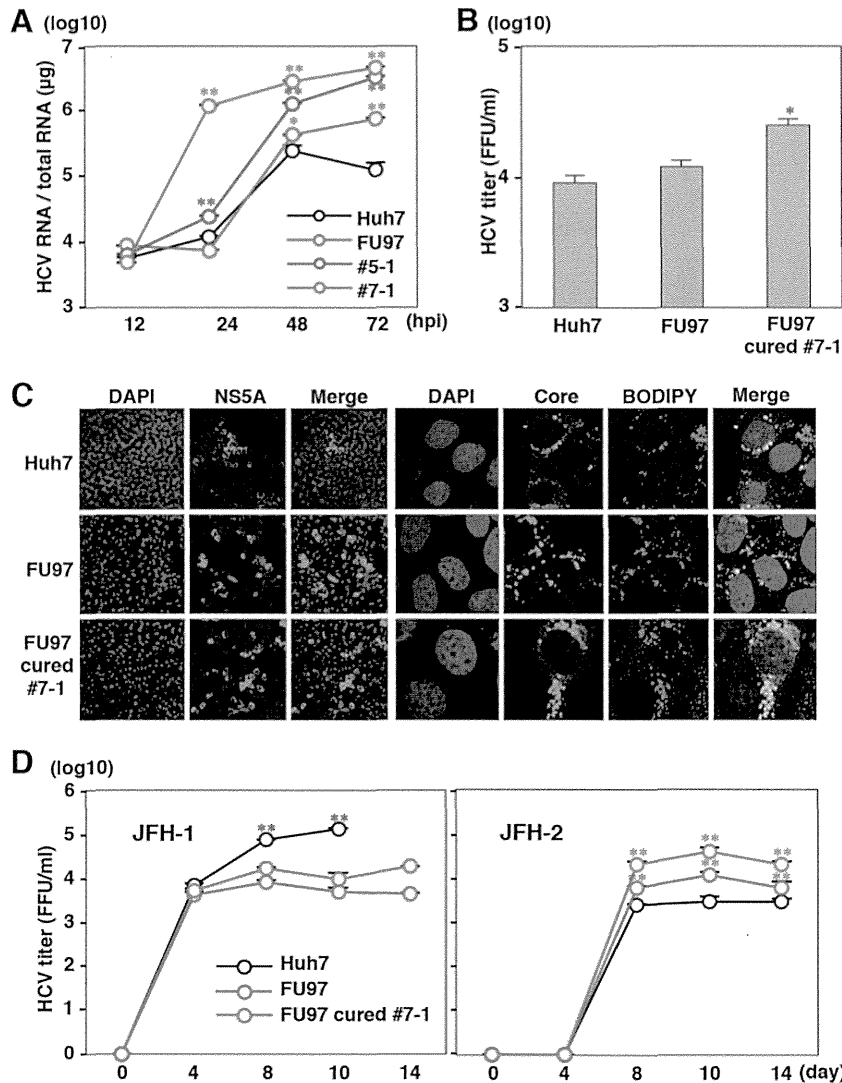


FIG 8 Effects of anti-HCV drugs on the propagation of HCVcc in FU97 cells. (A) Effect of DAAs on the propagation of HCVcc in Huh7 and FU97 cells. Cells infected with HCVcc at an MOI of 1 were treated with BMS-790052, PSI-7977, and BILN 2061 at 3 h postinfection (identifications in right-hand panels). (B) Effect of HCV inhibitors targeting host factors on the propagation of HCVcc in Huh7 and FU97 cells. Cells infected with HCVcc at an MOI of 1 were treated with IFN- $\alpha$ , RBV (middle), and cyclosporine (CsA) at 3 h postinfection (identifications in right-hand panels). Intracellular HCV RNA levels were determined by qRT-PCR at 48 h posttreatment (bar graphs), and cell viability was determined as a percentage of the viability of cells treated with 0.1% dimethyl sulfoxide (DMSO) at 48 h posttreatment (line graphs). From the assay results, the 50% effective concentration ( $EC_{50}$ ) of each reagent was determined. Asterisks indicate significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ) versus the results for control cells.



**FIG 9** Propagation of HCVcc/JFH-2 in FU97 cells. (A) Huh7, FU97 parental, FU97 cured 5-1, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, and the intracellular HCV RNA level was determined by qRT-PCR at 12, 24, 48, and 72 h postinfection. (B) Huh7, FU97, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, and infectious titers in the culture supernatants were determined by focus-forming assay. (C) Huh7, FU97, and FU97 cured 7-1 cells were infected with HCVcc/JFH-2 at an MOI of 1, fixed with 4% PFA at 72 h postinfection, and subjected to immunofluorescence assay using antibodies against NS5A or core. Lipid droplets and cell nuclei were stained with BODIPY and DAPI, respectively. (D) *In vitro*-transcribed JFH-1 and JFH-2 RNAs were electroporated into Huh7, FU97, and FU97 cured 7-1 cells. The infectious titers of JFH-1 and JFH-2 in the culture supernatants from these cells were determined by focus-forming assay up to 14 days posttransduction. Asterisks indicate significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ) versus the results for control cells.

like cells to gain hepatic functions could permit complete propagation of HCVcc.

Treatment with DAAs including BMS-790052 (NS5A inhibitor) (73), PSI-7977 (NS5B polymerase inhibitor) (74), and BILN 2061 (NS3/4A protease inhibitor) (75) inhibited propagation of HCV in both Huh7 and FU97 cells infected with HCVcc without any cell toxicity. Antiviral effects of BMS-790052 and BILN 2061 were significantly different between Huh7 and FU97 cells, suggesting that efficacies of DAAs are varied, depending on cell lines. Although anti-HCV drugs targeting host factors including IFN- $\alpha$ , RBV, and cyclosporine also inhibited propagation of HCVcc in a dose-dependent manner in both Huh7 and FU97 cells, treatment with RBV and cyclosporine produced cell toxicity at higher concentrations than treatment with DAAs. Although the antiviral

mechanism of RBV against HCV has not been well elucidated yet (53), inhibitory effects of RBV against HCV infection were significantly higher in Li23 cells than those in Huh7 cells (76, 77), and RBV also exhibited a low inhibitory effect upon infection with HCVcc in Huh7 cells compared to that in FU97 cells. Although adenosine kinase (ADK) was shown to be a determinant for the sensitivity of RBV (78), the expression levels of ADK in Huh7 and FU97 cell lines were comparable (data not shown).

The *IL28B* genotype is associated with the sensitivity of IFN treatment for chronic hepatitis C patients (79–81), and patients with the minor *IL28B* genotype exhibit lower susceptibility to the treatment than those with major genotypes. Although FU97 cells showed lower sensitivity to the IFN- $\alpha$  treatment than Huh7 cells, FU97 and Huh7 cells possess major and minor *IL28B* genotypes

(data not shown), respectively. Furthermore, induction of ISG15 by treatment with IFN- $\alpha$  was almost comparable between Huh7 and FU97 cells (Fig. 5A), and expression levels of IFN- $\alpha$  receptor in the cell lines were the same (data not shown), suggesting the involvement of other factors in the difference in the IFN responses between FU97 and Huh7 cells.

Cyclophilins possess peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and are involved in protein folding and assembly. Cyclophilin A (CypA), the most abundant cyclophilin, localizes in the cytoplasm and interacts with the immunosuppressive drug cyclosporine (82). In addition, CypA has been shown to be involved in the propagation of human immunodeficiency virus (83, 84), hepatitis B virus (85, 86), influenza A virus (87), and HCV (88). Replication of HCV RNA was inhibited by suppression of the PPIase activity of CypA by treatments with cyclosporine, mutation in the active site of CypA, and knockdown of CypA (55, 89–91). The same level of CypA expression in Huh7 and FU97 cells (data not shown) suggests that the difference in inhibitory effect of cyclosporine in the cell lines may be attributable to other reasons, such as a difference in PPIase activity of CypA in these cell lines. The differences in the efficacy of anti-HCV drugs between Huh7 and FU97 cells were small; however, FU97 cells have the possibility to possess antiviral activity different from that of Huh7 cells.

In summary, we identified novel permissive cell lines for complete propagation of HCVcc without any artificial manipulation. In particular, gastric cancer-derived FU97 cells exhibited a much higher susceptibility to HCVcc/JFH-2 infection than observed in Huh7 cells, suggesting that FU97 cells would be useful for further investigation of the HCV life cycle, as well as the development of therapeutic agents for chronic hepatitis C.

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Review

## Epstein-Barr Virus in Gastric Carcinoma

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**Abstract:** The Epstein-Barr virus (EBV) is detected in about 10% of gastric carcinoma cases throughout the world. In EBV-associated gastric carcinoma, all tumor cells harbor the clonal EBV genome. Gastric carcinoma associated with EBV has distinct clinicopathological features, occurs predominately in men and in younger-aged individuals, and presents a generally diffuse histological type. Most cases of EBV-associated gastric carcinoma exhibit a histology rich in lymphocyte infiltration. The immunological reactivity in the host may



represent a relatively preferable prognosis in EBV-positive cases. This fact highlights the important role of EBV in the development of EBV-associated gastric carcinoma. We have clearly proved direct infection of human gastric epithelial cells by EBV. The infection was achieved by using a recombinant EBV. Promotion of growth by EBV infection was observed in the cells. Considerable data suggest that EBV may directly contribute to the development of EBV-associated GC. This tumor-promoting effect seems to involve multiple mechanisms, because EBV affects several host proteins and pathways that normally promote apoptosis and regulate cell proliferation.

**Keywords:** Epstein-Barr virus; gastric carcinoma; DNA methylation

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## 1. Introduction

The Epstein-Barr virus (EBV) is associated with a variety of tumors derived from B cells, T cells, natural killer (NK) cells, and epithelial cells. Burkitt lymphoma [1], post-transplant lymphoproliferative disease [2], and Hodgkin's disease [3] are B-cell tumors. Peripheral T-cell lymphomas [3] and NK/T-cell lymphomas are T-cell tumors and NK-cell tumors, respectively. Nasopharyngeal carcinoma [1] and gastric carcinoma (GC) [3] are epithelial tumors.

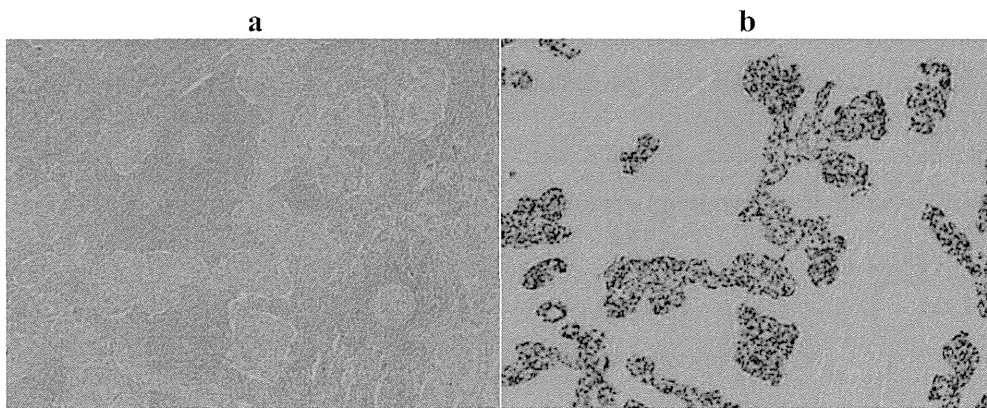
Existence of the EBV genome in GCs was first detected in 1990 by Burke *et al.* using the polymerase chain reaction (PCR) technique [4]. Since then, about 10% of GCs have been identified as EBV positive. In each EBV-positive case of GC, almost all carcinoma cells are infected with the virus [5,6], and tumor cells exist as a monoclonal proliferation of EBV-infected cells [7,8]. These facts suggest the significance of EBV in the development of GCs.

Gastric cancer is the second leading cause of cancer-related deaths globally, and 60% of these deaths occur in East Asia, which includes Japan [9]. The worldwide occurrence of EBV-associated GC is estimated at more than 50,000 cases per year [10]; therefore, EBV-associated GC is the most common cancer among EBV-related malignancies.

## 2. Definition

In addition to the detection of the EBV genome in GCs using PCR [4], EBV-encoded small RNA 1 (EBER1) was also detected using *in situ* hybridization (ISH). Various studies in the early 1990s indicated that EBV-associated GC comprises about 10% of all GCs worldwide [5–8]. EBER1 is highly abundant (10 million copies per cell) in individual infected cells. Typically, EBER1 can be detected in the nuclei of tumor cells; however, the EBER1 signal is negative in reactive lymphoid infiltrate cells or normal gastric mucosa cells (Figure 1). To make the diagnosis of EBV-associated GC before treatment, EBER1-ISH should be applied to gastric mucosal biopsy samples from patients who have undergone upper gastrointestinal endoscopy. Patients with EBV-associated GC have elevated levels of serum antibodies against EBV early antigen and EBV capsid antigen. However, EBV nuclear antigen (EBNA) 1 antibody titers do not show significant difference between patients and healthy counterparts [7].

**Figure 1.** Lymphoepithelioma-like subtype of Epstein-Barr virus (EBV)-associated gastric carcinoma. (a) H & E staining; (b) EBV-encoded small ribonucleic acid 1 (EBER1) *in situ* hybridization demonstrates positive nuclei in the carcinoma cells, which are surrounded by infiltrating lymphocytes.



### 3. Epidemiology

GC is one of the most common malignancies in Japan. Among the various histological types from Japanese gastric cancer cases, the incidence of EBV-positive cases was 7.0% in 1994 [7]. In contrast to Burkitt lymphoma and nasopharyngeal carcinoma, which are distributed endemically in equatorial Africa and Southeast Asia, respectively, EBV-associated GC is distributed worldwide in a similar proportion [10]. Regional difference in the incidence of EBV-associated GC is also reported. The incidence of EBV-associated GC in all cases of gastric cancer ranges from a high of 16%–18% in the USA and Germany to a low of 4.3% in China. The regional difference in the incidence of EBV-positive cases in gastric cancers indicates that the prevalence EBV-associated GC is inversely related to the incidence of GC [11].

EBV-associated GC has distinct clinicopathological features, is present predominately in men and in younger-aged individuals, and presents a generally diffuse histological type [12,13]. Most studies have not shown evident age dependence in the frequency of EBV-associated GC. Almost all of the studies showed male predominance of EBV-associated GC, suggesting that risks related to lifestyle or occupational factors may exist among males [14]. An interview study in Japan showed that salty food intake and exposure to wood dust and/or iron filings, which may induce mechanical injury to the gastric epithelia, are related to a higher risk of EBV-associated GC [15]. Camargo *et al.* recently showed that the association of smoking with gastric cancer is stronger for EBV-positive than EBV-negative tumors [16].

### 4. Pathology

EBV-associated GC has definite histological relevance to GC with lymphoid stroma (GCLS) [17–19], which was originally described by Watanabe *et al.* as a subtype of the carcinoma [20]. GCLS is a poorly differentiated adenocarcinoma with diffuse and intense lymphocyte infiltration similar to EBV-associated nasopharyngeal lymphoepithelioma. More than 80% of lymphoepithelioma-like GC is infected with EBV [17–19] (Figure 1), whereas ordinary-type GC, comprising 5%–10% of all cases of GC, shows features of moderately or poorly differentiated adenocarcinoma with various degrees of lymphocytic infiltration.

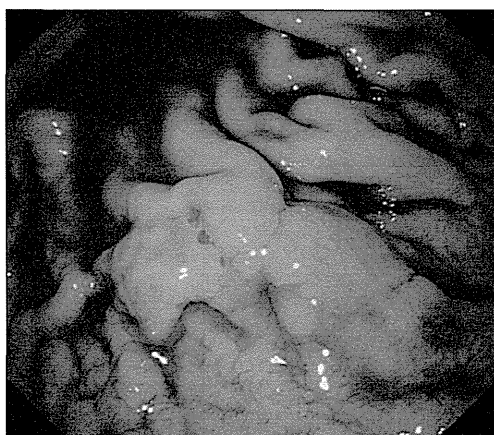
Further infiltration of the carcinoma (tumor cells) into the submucosa is occasionally accompanied by EBV-associated GC generally exhibiting a characteristic histology referred to as GCLS [21].

EBV-associated GC has a null or gastric phenotype as determined by the expression pattern of the mucin molecules MUC5AC and MUC6 [22,23] and is characterized by a relative lack of intestinal phenotypic expression, including Cdx2 [24]. According to these findings, the targets of EBV infection and their subsequent transformation are seemingly the precursor cells possessing intrinsic differentiation potential toward the gastric cell type.

## 5. Clinical Features

The most useful modality for the diagnosis of GC is endoscopy. In one analysis, 124 GCs from 117 patients were examined by EBER1-ISH. Of the 124 tumors, 12 (9.7%) were identified as EBV-associated tumors [25]. It is of note that EBV-associated GC predominantly localizes in the non-antrum part of the stomach (Figure 2) and appears as superficial depressed or ulcerated lesions. A histological feature of EBV-associated GC is a diffuse-type carcinoma accompanied by abundant lymphocyte infiltration (*i.e.*, GCLS). In some patients, endoscopic ultrasound reveals a hypoechoic mass in the third hyperechoic layer reflecting submucosal nodules of lymphoid stroma, which is composed of carcinoma cells and infiltrating lymphocytes [26].

**Figure 2.** Endoscopic image of an Epstein-Barr virus-associated gastric carcinoma in the upper gastric body. The tumor shows a protruded shape probably because of the abundant lymphocyte infiltration.



It is known that *Helicobacter pylori* is strongly related to cancer and is an etiological agent of chronic gastritis and intestinal metaplasia. It is distinctive that *H. pylori*-related gastritis frequently initiates in the antrum. In the case of EBV-associated GC, tumors are frequently located near the mucosal atrophic border, where mild to moderate atrophy is common [27]. We have frequently detected both EBV and *H. pylori* in the mucosa of patients with moderate chronic atrophic gastritis, where inflammatory cell infiltration is abundant, and not in the mucosa with marked atrophic gastritis, where inflammatory cell infiltration is scarce [28].

Gastric remnant cancer arises after distal gastrectomy for benign disease, which includes refractory gastric or duodenal ulcer disease and recurrent ulcer with gastric outlet obstruction. The incidence of gastric remnant cancer ranges from 1% to 7% of all GCs and is still increasing [29]. Gastric remnant

carcinoma is frequently (25% to 41.2%) associated with EBV infection. It is considered that the reflux of bile and pancreatic juice causes regenerative atypia and cell proliferation in epithelial cells [30]. Atrophic change of remnant gastritis in Billroth-II anastomoses is frequently accompanied by EBV-positive gastric remnant carcinoma [31]. Gastritis cystica polyposa, frequently observed in the remnant stomach, is a suspected precursor lesion of EBV-associated GC, but no direct evidence of EBV infection in these lesions has been found [32].

## 6. Treatment and Prognosis of EBV-Associated GC

The current therapy for EBV-associated GC does not use any special methods. Because undifferentiated-type cancer is prevalent in EBV-associated GC, most of these tumors are removed by surgical resection. Early EBV-associated GC has a low frequency of lymph node metastasis. Endoscopic treatment can be applied in such cases. The authors experienced a case of early EBV-associated GC with submucosal invasion in which palliative endoscopic treatment was performed. No recurrence was observed in the patient for more than 4 years [33]. A clinicopathological study in The Netherlands showed that EBV-associated GC has a significantly low frequency of lymph node metastasis compared with EBV-negative stomach cancer, resulting in a better prognosis than that with the EBV-negative cases [34]. A recent meta-analysis revealed that EBV-associated GC showed an infrequent tendency toward lymph node metastasis. After adjustment for TNM stage and other prognostic indicators, EBV positivity was associated with lower mortality [35]. Further studies are needed to identify the mechanisms underlying this prognostic association.

## 7. Growth-Promoting Effects of EBV in Epithelial Cells

### 7.1. Models of EBV Infection of Gastric Epithelial Cells

EBV infects both B lymphocytes and epithelial cells because the virus has been discovered in Burkitt lymphoma cells, Hodgkin cells, nasopharyngeal carcinoma cells, and GC cells. Experimental EBV infection of B cells is very efficient because EBV uses CD21, a high-affinity receptor, for its entry into the cell [36,37]. However, epithelial cells are CD21 negative, and infection of epithelial cells could not be achieved for a long time, not until CD21 expression was overcome by gene transfer [38,39]. Infection of EBV with human gastric epithelial cells was experimentally proved by our group [40], and EBV-infected gastric cells (AGS) have been established by Marquitz *et al.* [41]. A recombinant EBV with a neomycin resistance gene [42,43] was used for epithelial infection, and thus, epithelial cells, which do not express a CD21 EBV receptor, could be infected with EBV. This infection of CD21-negative epithelial cells was not blocked by anti-CD21 monoclonal antibody [40]. Next, EBV was efficiently transferred to epithelial cells by mixing epithelial cells with recombinant EBV-producing B cells [44]. There are several epithelial cell lines, such as CNE1 and HONE1, which can achieve experimental infection with EBV. Instead of these cell lines, SNU-719 cells [45], NCC24 cells [46], and KT cells [47] are a few of the cell types that retain the same clonal EBV genome and the pattern of EBV gene expression (type I EBV) as that in the original tumor biopsy. The KT cell is a good *in vivo* model of EBV-associated GC and expresses high IL-1 $\beta$  compared with EBV-negative gastric tumor cells [48].

## 7.2. Growth-Promoting Effects of EBV

EBV immortalizes B cells *in vitro*. EBNA 2 and latent membrane protein 1 (LMP1) appear to play the most important roles in the immortalization of lymphocytes. However, they are not expressed in EBV-associated GC, raising doubts about the importance of the presence of EBV. We attempted to infect gastric primary culture cells with EBV [49]. Primary gastric epithelial cells from healthy gastric mucosal biopsies were infected with recombinant EBV carrying a neomycin resistance gene, and infected cells were selected for using G418. As a result, we repeatedly separated cell clones that could be maintained for at least 300 generations. The selected EBV-infected cells expressed Qp-driven EBNA 1, EBER, BARTs, and latent membrane protein 2A (LMP2A). The pattern of latent gene expression was similar to EBV-associated GC. The EBV-infected clones had higher proliferation rates and at least twice the cell saturation density compared with non-infected clones into which the neomycin resistance gene had been introduced as a control, and the malignant phenotype was confirmed by colony formation in soft agar and tumorigenicity in SCID mice. EBV infection also promoted growth of gastric cancer cell lines NU-GC-3 and AGS [41,49].

## 8. Virus and Host Interactions at the Molecular Level

### 8.1. Genetic Alterations in EBV-Associated GC

In EBV-associated GC, studies of genetic alteration are limited. Van Rees *et al.* [50] and Chong *et al.* [51] reported that chromosomal losses were extremely rare in EBV-associated GC in contrast to the high frequency in EBV-negative GC. Chromosomal aberrations in EBV-associated GCs were globally tested by comparative genomic hybridization. Zur Hausen *et al.* showed that loss of chromosomes 4p, 11p, and 18q was distinct in EBV-associated GCs [52]. 18q harbors the DCC and SMAD4 genes, which are known tumor-suppressor genes. Chan *et al.* reported that gains in chromosome 11 copy numbers are common in EBV-associated malignancy including EBV-positive GC, lung cancer, and lymphoma [53]. As well, microsatellite instability is not common in EBV-associated GC [54]. Similarly, p53 mutation and overexpression are not frequent in EBV-associated GCs [55,56]. These findings indicate that genetic abnormality is not the major pathway to the development of EBV-associated GC.

### 8.2. DNA Hypermethylation in EBV and Host Genomes

Methylation of the tumor suppressor gene is a key abnormality in EBV-associated GC [57–59]. In tumor cells of EBV-associated GC, CpG island methylation is frequently observed at promoters of various tumor-related genes, which must take important parts in the development and progression of gastric cancer [60]. Methylation frequencies of several tumor suppressor genes, APC, PTEN, and RASSF1A, and cell adhesion molecules, THBS1 and E-cadherin, were reported to be significantly higher in EBV-associated GC [61–63]. Because demethylation agents induce lytic EBV infection in latently EBV-infected cells followed by apoptotic cell death, the therapeutic application of demethylating agents may lead to the lysis of cancer cells [64]. These facts strongly support possible application of demethylating agents to the medical treatment of EBV-associated GC.

We compared methylation status between EBV-associated GCs and EBV-negative controls whose age, sex, histology, depth of invasion, and stage were matched. EBV-associated GCs showed higher methylation frequencies in 12 of 16 tumor-related genes compared with EBV-negative controls. The frequency of methylation at 6 specific loci (MINT2, MINT31, p14, p16, p73, and RUNX3) was significantly higher in EBV-associated GCs than in EBV-negative controls [65]. Moreover, the DNA methylation status in the naturally derived EBV-positive gastric adenocarcinoma cell line SNU-719 was also examined by the method of methylated CpG island recovery on chip assay [66]. Expression of several genes was regulated by DNA methylation in EBV-associated GC. The methylation frequencies of p73, BLU, FSD1, BCL7A, MARK1, SCRNI, and NKX3.1 were significantly higher in EBV-associated GC than in EBV-negative GC [66].

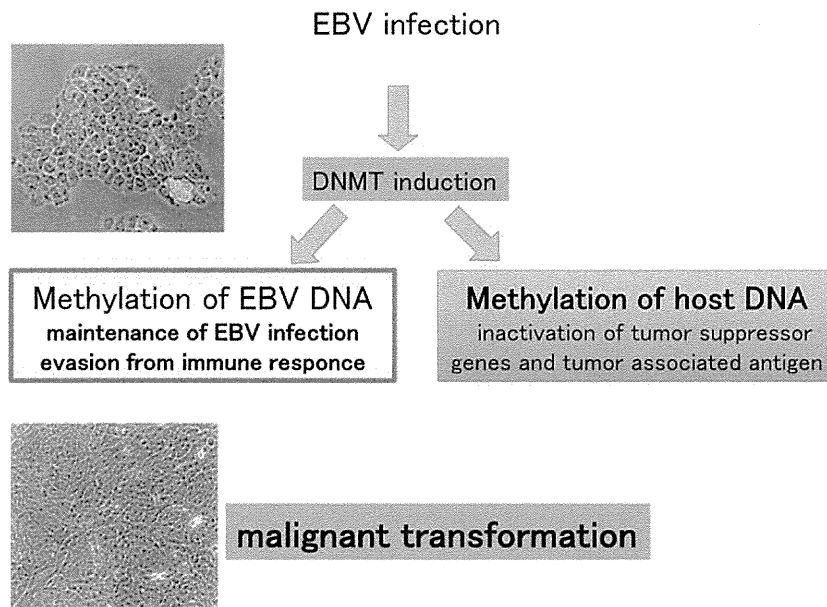
The precise molecular mechanism that induces host DNA methylation during the early stage of EBV infection of the gastric epithelium is not fully understood. LMP2A is reported to induce the phosphorylation of STAT3, which activates DNA methyltransferase 1 (DNMT1) transcription and causes loss of PTEN expression through CpG island methylation of the PTEN promoter in EBV-associated GC [67]. However, LMP2A is not expressed in every case of EBV-associated GC [68], and EBV-associated GC patients are usually negative for LMP2A antibody [69]. LMP1 can also induce aberrant DNA methylation by activating DNMT1 through the JNK signaling pathway [70] and inducing DNA methylation of host cells [71]. However, LMP1 is scarcely expressed, and LMP1 protein is generally absent in EBV-associated GC [72]. Methylation of similar genes has been reported in hepatitis B and C [73,74], suggesting that there must be a common mechanism underlying the formation of infection-associated cancers.

The status of DNA methylation in the EBV genome was intensively investigated [75]. The expression of EBV latent genes is strictly regulated through viral DNA methylation in EBV-infected cells. The Cp/Wp EBNA promoters are known to transcribe all EBNA. However, in Burkitt lymphoma and nasopharyngeal carcinoma, the Cp/Wp promoters are methylated and the only EBNA1 promoter, Qp, is used instead [76,77]. Moreover, in EBV-positive nasopharyngeal carcinoma, LMP1 expression is down-regulated by methylation in its promoter region [78]. The pattern of latent gene expression in EBV-positive GCs is similar to that of Burkitt lymphoma, in which only Qp is actively used [7]. These results indicate that the methylation status of the EBV genome regulates the pattern of latent gene expression in EBV-positive tumor cells. Because methylation occurs on viral DNA in EBV-associated GC cells, methylation of host cell DNA may also occur, for example, on tumor suppressor genes that regulate the cell cycle and apoptosis. Aberrant DNA methylation might occur in EBV-positive cells, thus promoting the development and progression of EBV-associated GC (Figure 3).

### 8.3. EBV Latent Genes and Host Interaction

Iwakiri *et al.* reported that EBV infection promoted growth of gastric cancer cells by increased production of insulin-like growth factor (IGF)-1 as an autocrine growth factor. It was also revealed that EBERs are responsible for the induction of IGF-1 [79]. The oncogenic role of EBERs has been reported for inhibition of apoptosis in the human epithelial cell line Intestine 407 [80]. EBER was found to bind double-stranded RNA-dependent protein kinase R, an interferon-inducible serine/threonine kinase, and abrogate its kinase activity. These results indicate that EBER contributes to the maintenance of epitheloid malignancy.

**Figure 3.** Aberrant DNA methylation might lead to the development and progression of Epstein-Barr virus (EBV)-associated gastric carcinoma. DNMT, DNA methyltransferase.



The oncogenic role of other genes such as BARF1 (BamHI A rightward open reading frame 1) [81] and LMP2A [67,82] has also been reported. The expression of the EBV-encoded oncogene BARF1 has been reported in EBV-associated GC. Wiech *et al.* reported that cyclin D1 is induced in BARF1-transfected epithelial cells and is overexpressed in EBV-associated GC [83]. LMP2A is reported to inhibit transforming growth factor- $\beta$ -induced apoptosis in a GC cell line [84]. Recently, it was demonstrated that LMP2A upregulated cellular survivin gene expression through the nuclear factor- $\kappa$ B pathway in GC cell lines with EBV infection [82]. In addition, LMP2A upregulates cellular DNMT1 in EBV-associated GC through the phosphorylation of STAT3, causing promoter hypermethylation of a tumor suppressor gene, PTEN [67].

#### 8.4. EBV microRNA and Gastric Cancer

A microRNA (miRNA) is a small (20 to 25 nucleotides) non-coding RNA derived from double-stranded RNAs, which functions in RNA silencing and post-transcriptional regulation of gene expression. miRNA is incorporated into the RNA-induced silencing complex (RISC) in cytosol, binds to the 3' un-translated region (UTR) of mRNA, and then silences translation by destabilizing mRNA through shortening of its poly A tail [85]. miRNA is found in plants, animals, and some viruses. EBV is one of the first viruses reported to contain viral miRNA, the genome of which codes 25 miRNA precursors and produces 44 kinds of different miRNAs [86,87]. A number of mRNA targets by EBV miRNAs have been reported mainly in B lymphocytes via the bioinformatics approach [88,89]. Recent results of EBV miRNA targets using gastric epithelial cells are introduced in this section.

Choy *et al.* reported on the regulation of p53 up-regulated modulator of apoptosis (PUMA) by an EBV miRNA, miR-BART5-5p, which is abundantly expressed in nasopharyngeal carcinoma and EBV-associated GC cells [90]. Marquitz *et al.* showed that in vitro infection of an AGS cell line with EBV alters the growth properties of the cells and induces growth in soft agar in accordance with high levels of expression of the BamHI A rightward transcript (BART) miRNAs [41]. They showed



downregulation of a tumor suppressor gene, PTEN, cellular adhesion proteins, integrin alpha 5 and alpha V, and signal transducer STAT6. These results suggested that the expression of EBV miRNA highly influences the genesis of EBV-associated GC. Choi *et al.* also investigated an AGS cell line and reported that the 3' untranslated region of baculovirus inhibitor of apoptosis repeat-containing ubiquitin-conjugating enzyme (BRUCE) was affected by EBV miR-BART15-3p [91]. miR-BART miRNAs target many other anti-apoptotic genes; however, the precise roles of each gene for tumor formation are still not well understood.

Many research groups reported expression of EBV miRNAs in gastric cancer cells and histological samples from gastric cancers [92,93]. It is of note that YCCEL1 and SNU-719 cell lines are derived from a gastric cancer patient, respectively, and maintain viral episomes. Seemingly, this is the reason why these cell lines show expression profiles of EBV miRNAs similar to samples from gastric cancer patients [94–96]. These two cell lines are expected to become important tools for the study of EBV miRNA.

## 9. Summary

Considerable data suggest that EBV can increase cell proliferation and survival; and through these effects; EBV may directly contribute to the development of EBV-associated GC. This tumor-promoting effect seems to involve multiple mechanisms; because EBV affects several host proteins and pathways that normally promote apoptosis and regulate cell proliferation.

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## Author Contributions

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## Conflicts of Interest

The authors declare no conflict of interest.

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