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Infrequent Overexpression of p53 Protein in Epstein-Barr Virus-associated Gastric Carcinomas

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Epstein-Barr virus (EBV) infection was studied in a total of 412 patients with poorly differentiated gastric adenocarcinoma by *in situ* hybridization for EBV-encoded small RNA. EBV-specific RNA was detected in tumor cell nuclei of 83 (20.1%) of 412 gastric carcinomas, of which 60 were histologically subclassified as gastric carcinoma with lymphoid stroma (GCLS). All EBV-positive gastric carcinomas as well as 90 randomly selected EBV-negative gastric carcinomas were further studied for p53 protein expression by immunohistochemistry. The overexpression of p53 protein was demonstrated in only 7 (8.4%) of 83 EBV-positive gastric carcinomas. This was in marked contrast to the frequency of 34.4% in EBV-negative gastric carcinomas. In addition, a few p53-positive nuclei were characteristically scattered in the tumors of many EBV-positive GCLS, but this was not regarded as p53 overexpression arising from mutation of the gene. Our findings suggested that EBV-associated gastric carcinomas may arise through a different mechanism from other types of gastric carcinomas without EBV infection.

Key words: Epstein-Barr virus — *In situ* hybridization — p53 protein — Gastric carcinoma with lymphoid stroma

The Epstein-Barr virus (EBV) is directly implicated in the pathogenesis of various lymphoproliferative disorders, including infectious mononucleosis, Burkitt's lymphoma, Hodgkin's disease, T-cell lymphoma and various B-cell lymphomas, especially in patients with human immunodeficiency virus infection and pyothorax.[1–5] Furthermore, EBV is a causative agent of nasopharyngeal carcinoma (NPC) and of a rare lymphoepithelioma-like carcinoma arising in the salivary gland, thymus and lung.[1, 2, 6]

EBV-DNA or EBV-encoded small RNA (EBER-1) has been demonstrated mostly in gastric cancers with a special type of morphology similar to that of nasopharyngeal carcinoma, most of them having an intense lymphoid infiltration.[7–25] EBV-infected gastric cancers are monoclonal and EBV infection is thought to play an important role in the early stage of gastric carcinogenesis.[11, 13, 17, 22] In human gastric carcinogenesis, however, various genetic changes accumulate following various morphological changes.[26] Among them, abnormalities of the p53 tumor suppressor gene are the most important and frequent changes in human gastric carcinogenesis.[26–28] We therefore investigated the presence of p53 abnormalities and the role of the *p53* gene in the development of EBV-associated gastric cancers.

MATERIALS AND METHODS

Specimens A total of 412 poorly differentiated gastric adenocarcinomas were collected from the pathological files of the First Department of Surgery, Gunma University Hospital and its affiliated hospitals from 1984 to 1994. The male-to-female ratio was 1.5 : 1 and the age distribution was from 36 to 85 years (mean age: 62.8 years). The histologic subtypes of gastric carcinoma were re-evaluated according to the criteria proposed by the Japanese Research Society for Gastric Cancer.[29] Clinical information was obtained from the pathological files and medical records of the patients.

Detection of EBV To detect EBV by *in situ* hybridization (ISH), 3-μm-thick paraffin sections cut from routinely 15% formalin-fixed and paraffin-embedded tissues containing representative primary gastric cancers and lymph node metastases were placed on silane-coated glass slides (Matsunami Co., Ltd., Tokyo). ISH was conducted according to Tokunaga *et al.* with slight modifications.[9] Briefly, paraffin sections were digested with 20 μg of proteinase K per milliliter for 30 min at 37°C after standard deparaffinization and rehydration. The sections were hybridized overnight at 37°C with a digoxigenin-labeled oligonucleotide probe (5′-ACA CAC GTC TCC TCC CTA GCA AAA CCT CTA -3′), that was complementary to EBER-1. The oligonucleotide probe was labeled with deoxythymidine uridine 5′-triphosphate (dUTP)-digoxigen using an oligonucleotide tailing kit (Boehringer GmbH, Mannheim, Germany). After hybridization, the sections were washed twice for 15 min at high stringency. An alkaline phosphatase-labeled monoclonal antibody against digoxigenin (Nichirei Corp., Tokyo) was then applied to the sections for 30 min at room temperature. After coloration with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate,

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the sections were examined by means of light microscopy.

Immunohistochemistry for p53 protein After ISH, both EBV-positive and 90 randomly selected EBV-negative cancers were immunohistochemically studied for p53. Paraffin sections were dewaxed with xylene and rehydrated with an alcohol series. After the abolition of endogenous peroxidase activity with H_2O_2, sections were microwave-irradiated for 9 min in citric acid buffer to retrieve antigens as described by Shi *et al.*[30] A primary antibody recognizing both wild and mutant p53 proteins (DO-7, Dako Japan Co., Ltd., Kyoto) was applied and the avidin-biotin-peroxidase complex procedure was followed. Finally the sections were visualized by means of the benzidine reaction.

To evaluate the p53 immunostain, the profile was classified into 4 types (negative, sporadic, focal and diffuse) as described before.[31] Overexpression or positivity was defined as diffuse and focal staining of more than 10% of p53-positive cells.

Statistical analysis The statistical significance of differences was evaluated by use of the χ^2 test. A P-value of less than 0.05 was considered statistically significant.

RESULTS

EBV was detected in 83 of 412 (20.1%) samples of gastric poorly differentiated adenocarcinoma. The hybridization signal for EBV was diffusely localized in the nuclei of carcinoma cells, but not in normal gastric mucosa or infiltrating reactive lymphocytes. EBV-positive carcinomas tended to be present in the cardia and body of the stomach as compared with the antrum. Histologically, 65 of 412 gastric cancers (15.8%) were diagnosed as gastric cancer with lymphoid stroma (GCLS) (Fig. 1) and a hybridization signal for EBER1 was detected in 60 of 65 (92.3%) GCLSs by ISH (Fig. 2). The average age of these EBV-positive patients was

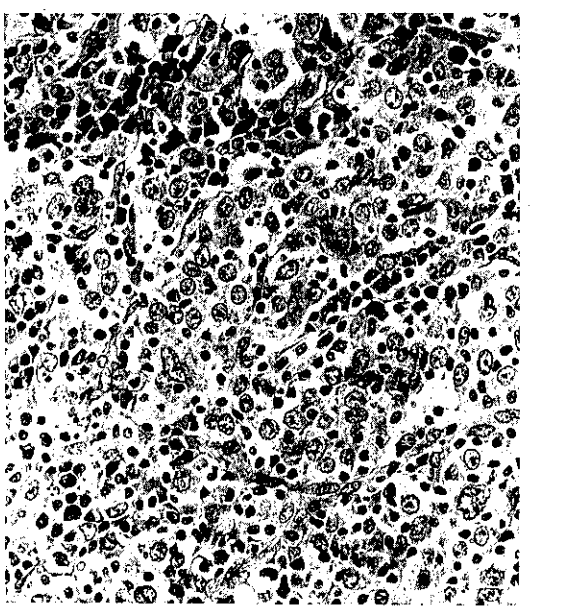

Fig. 1. Histology of gastric carcinoma with lymphoid stroma (GCLS). GCLS is composed of large epithelial cancer cells with prominent nucleoli and a large number of small lymphocytes, with histology very similar to that of nasopharyngeal lymphoepithelioma. Hematoxylin and eosin stain.

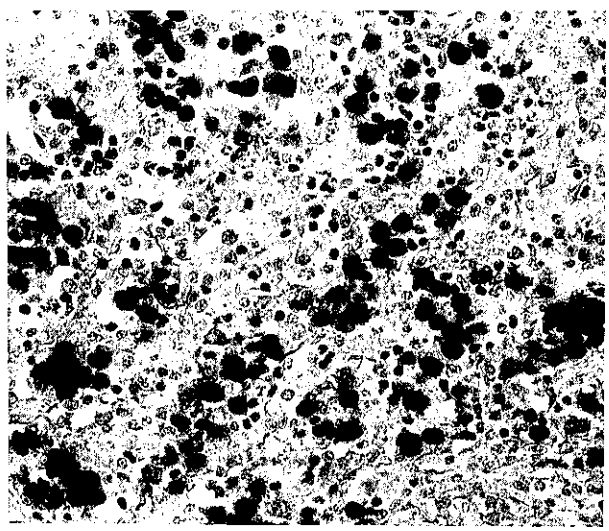

Fig. 2. *In situ* hybridization study for EBER-1 in GCLS. Hybridization signals are located in almost all nuclei of cancer cells. GCLS, gastric carcinoma with lymphoid stroma; EBER-1, Epstein-Barr virus-encoded small RNA.

Table I. p53 Expression Pattern in EBV-positive and -negative Poorly Differentiated Gastric Carcinomas

Type of carcinoma	Negative	Sporadic	Focal	Diffuse
EBV-positive (83 cases)	53	23*	1	6
EBV-negative (90 cases)	55	4	1	30*

EBV, Epstein-Barr virus. * $P<0.01$.

Table II. p53 Expression Pattern in EBV-positive Poorly Differentiated Gastric Carcinomas

Type of carcinoma	Negative	Sporadic	Focal	Diffuse
Non-GCLS (23 cases)	18	0	1	4
GCLS (60 cases)	35	23*	0	2

EBV, Epstein-Barr virus; GCLS, gastric carcinoma with lymphoid stroma. * $P<0.01$

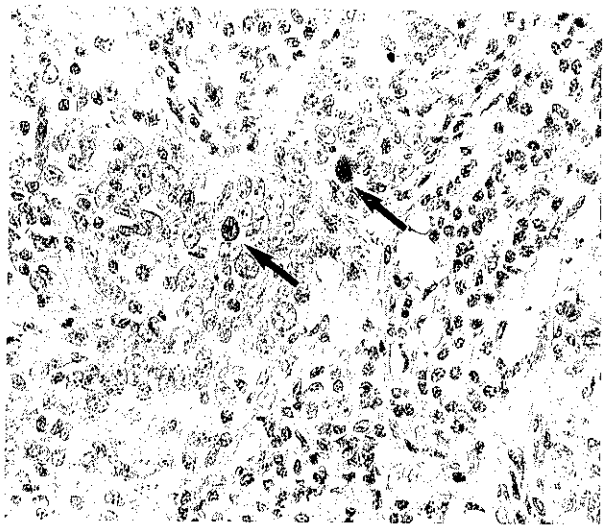

Fig. 3. Immunohistochemical demonstration of p53 protein in EBV-associated GCLS. p53-positive nuclei (arrows) are sporadically seen among cancer cells. EBV, Epstein-Barr virus. GCLS, gastric carcinoma with lymphoid stroma.

65.8 years for males and 58.9 years for females. The male-to-female ratio was 2.3 to 1.

Table I shows that sporadic and diffuse staining profiles of p53 were characteristic of EBV-positive and -negative gastric carcinomas, respectively ($P<0.01$). The frequencies of p53 overexpression in EBV-positive and -negative carcinomas were 8.4% (7 of 83 samples) and 34.4% (31 of 90 samples), respectively. EBV-positive carcinomas were histologically subclassified as GCLS and non-GCLS types and the p53 staining profiles of these subtypes were compared (Table II). The sporadic pattern (Fig. 3) was characteristic of GCLS-type carcinomas ($P<0.01$). In addition, only two GCLS-type carcinomas showed diffuse staining of p53 overexpression.

DISCUSSION

In 1965, Lauren first proposed two main histological types of gastric carcinoma, namely diffuse and intestinal.[32] These two types differ not only morphologically, but also clinically and epidemiologically.[33, 34] Intestinal-type gastric carcinomas are thought to be the end result of multistep carcinogenesis from chronic gastritis through intestinal metaplasia and gastric adenoma.[26, 27, 31] However, diffuse-type gastric cancers are thought to arise from intact gastric mucosa without any precursor lesions.[35] Here, we studied specimens of poorly differentiated gastric adenocarcinoma, which might have included a large number of diffuse-type gastric carcinomas.

Molecular biological studies have demonstrated common and unique genetic changes among diffuse and intestinal types of gastric carcinomas.[26] Abnormalities of c-met, p53 and TGF-α are common in both, while the expression of *cripto* and the Ki-*ras* gene mutation are restricted to the intestinal type. Abnormalities of the *p53* gene are most frequent among gastric carcinomas and the frequency of immunohistochemical p53 overexpression is reportedly from 39% to 65.6%.[27, 36, 37] Based on histological results, about 70% of intestinal-type or well and moderately differentiated gastric carcinomas were p53 positive.[26] Further, the ratio in diffuse type or poorly differentiated gastric carcinoma was around 50%, suggesting that the *p53* gene alteration is important in gastric carcinogenesis, especially of the intestinal type.[27, 37]

We are the first to demonstrate that p53 overexpression is infrequent among EBV-associated poorly differentiated gastric carcinomas. Only 8.4% of EBV-associated gastric carcinoma was associated with p53 overexpression, which is in marked contrast to the EBV-negative poorly differentiated adenocarcinoma used as the control in this study. However, Gulley *et al.* have recently reported that the prevalence of p53 accumulation in EBV-associated gastric cancer is not different from that of EBV-negative cancers, in contrast to our findings.[38] The difference may be due to the small number of EBV-associated gastric cancers that they investigated. Most EBV-associated gastric carcinomas have characteristic histological features similar to those of nasopharyngeal lymphoepithelioma, usually called GCLS, which is regarded as a special histological type distinct from both diffuse and intestinal types.[39] Therefore, EBV-associated gastric carcinomas or GCLS are thought to arise through a different route of carcinogenesis from that of the intestinal and diffuse types.

In gastric carcinogenesis, p53 protein accumulation, usually reflecting a missense *p53* gene mutation, seems to be an important change occurring at a late stage.[26, 27, 31] It remains unclear whether *p53* gene alteration plays an important role in the genesis of GCLS in cooperation with EBV infection. DNA viral oncoproteins such as adenovirus E1b, human papillomavirus (HPV) E6 and simian virus large T antigens interact with wild-type p53 protein and cause a loss of tumor suppressor function of the p53 protein.[40] There is an inverse relationship between HPV infection and *p53* gene mutation in human cervical cancers, which supports this notion.[41] In gastric carcinogenesis, especially of GCLS, EBV infection is thought to be an early event and to play an important role in developing cancers.[11, 13, 17, 22, 38] Until now, only 11 gene products of EBV, including EBV-encoded latent membrane antigen 1 (LMP1) and EBV-determined nuclear antigens, have been considered to be responsible for the immortalization process of tumor cells.[42] However,

LMP1 expression is rare in EBV-associated gastric carcinomas.[13] An EBV-encoded nuclear antigen was reported to bind to the retinoblastoma and p53 proteins, and could act as a negative growth regulator of the cell.[43] Further studies are necessary.

This study has revealed that a sporadic immunohistochemical profile of p53 staining is characteristic of EBV-positive gastric cancers, especially of GCLS. We previously showed that sporadic p53 immunostaining is not associated with *p53* gene mutation.[31] These data suggest that wild-type p53 protein is dominant in EBV-associated gastric carcinomas. Functionally, the wild type of p53 protein can be overexpressed after DNA damage to arrest the cell cycle at the G1 phase, to initiate DNA repair or apoptosis.[44, 45] We speculate that sporadic p53 staining might reflect a specific type of DNA damage caused by EBV replication or virus-host interaction in cancer cells.

In conclusion, p53 abnormality is infrequent in EBV-associated gastric carcinomas, suggesting that EBV-associated gastric carcinomas arise through a different mechanism from other gastric carcinomas without EBV infection.

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