Correlation Between Expression of the Cancer/Testis Antigen KK-LC-1 and *Helicobacter pylori* Infection in Gastric Cancer

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Abstract. *Background/Aim:* Our previous study indicated that Kita-kyushu lung cancer antigen-1 (KK-LC-1) is a cancer/testis antigen (CTA) expressed in 82% of gastric cancer cases. Here, we investigated the relationship between KK-LC-1 expression and *Helicobacter pylori* infection in Japanese patients with gastric cancer. *Patients and Methods:* We examined CTA expression in 25 surgical gastric cancer specimens and anti-*H. pylori* IgGs in the serum of each patient. *Results:* KK-LC-1 was expressed in 80% of tumor samples, markedly higher than melanoma antigen gene (MAGE)-A1, MAGE-A3, MAGE-A4, synovial sarcoma, X breakpoint 4 (SSX4) and New York esophageal squamous cell carcinoma-1 (NY-ESO-1). Anti-*H. pylori* IgG titers from the KK-LC-1-positive patients were significantly higher (67.5 ± 7.6) than those from KK-LC-1-negative patients (15.8 ± 7.5 , $p < 0.01$) although there were no significant differences between patients positive and negative for MAGE-A1, -A3 and -A4, SSX4 and NY-ESO-1. *Conclusion:* As far as we are aware, this is the first report of a correlation between a carcinogen and CTA expression in clinical samples. KK-LC-1 was frequently expressed in gastric cancer caused by *H. pylori* infection. The risk diagnosis for gastric cancer might be more accurate if KK-LC-1 expression status were also considered.

Several tumor-associated antigens have been identified in various human cancer types (1-3). These antigens are classified into four categories, excluding extrinsic viral antigens, as follows: cancer/testis antigens (CTAs), differentiation antigens, amplification or overexpression antigens, and tumor-specific mutated antigens. CTAs are especially attractive targets for immunotherapy because they are not, or are only minimally expressed in normal tissues except for testis but are aberrantly expressed in a range of human cancer types (4). Therefore, immunotargeting of these antigens is thought to have negligible adverse side-effects. Moreover, they could also be advantageous molecules for systemic diagnosis of cancer because of their specific expression pattern. However, data on the frequency of individual CTA expression is not currently sufficient for diagnostic applications.

We previously identified Kita-kyushu lung cancer antigen-1 (KK-LC-1) as a new CTA that has an epitope peptide recognized by cytotoxic T-lymphocytes (CTLs). KK-LC-1 maps to chromosome Xq22, is not expressed in normal tissues except for testis, and is expressed in 33% of non-small cell lung cancer (5, 6). Furthermore, we found that KK-LC-1 was expressed in 82% of gastric cancer (7). According to these data, KK-LC-1 could be categorized as a CTA, but its frequency in gastric cancer seems to depart from the traditional concept of CTAs.

In Japan, gastric cancer has the second highest incidence rate of all cancer, and more than 80% of cases are caused by *Helicobacter pylori* infection (8). We reported that *H. pylori* infection can induce expression of specific CTAs in addition to causing malignant transformation of host cells (9). These findings suggest that specific CTA expression might correlate with the initial cancer-causing event; therefore, CTAs could contribute as new candidates for immunotherapy and diagnosis of gastric cancer.

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Key Words: KK-LC-1, cancer/testis antigen, gastric cancer, *Helicobacter pylori*, carcinogen.

In this study, we investigated the relationship between expression of KK-LC-1 and *H. pylori* infection in gastric cancer.

Patients and Methods

Human rights statement and informed consent. This study involved human participants. All procedures followed were in accordance with the ethical standards of Kitasato University Medical Center (approval no. 23-8) and with the Declaration of Helsinki. All patients, or their legal guardians, contributed in this study provided their written informed consent before this study.

Patients. A total of 46 patients underwent surgical resection for gastric cancer at the Department of Surgery, Kitasato University Medical Center, Kitamoto, Japan between August 2011 and August 2012. We obtained 25 sets of tumor samples and serum from these patients. The clinicopathological findings were classified according to the Japanese Classification of Gastric Carcinoma [14th edition (10)]. There is no bias in the background characteristics among the patients who participated in this study (Table I).

Tissue specimens. Each sample from resected tumor tissues was immediately preserved at 4°C overnight in RNAlater (Life Technologies, Carlsbad, CA, USA), and then stored at -80°C until used.

Expression of CTAs. Total RNA extraction from tumor samples, cDNA synthesis, the assay of gene expression for β -actin and each CTA was described elsewhere by us (7). The gene expression of β -actin; melanoma antigen gene (MAGE)-A1, -A3 and -A4; New York esophageal squamous cell carcinoma-1 (NY-ESO-1); and synovial sarcoma, X breakpoint 4 (SSX4) were measured with TaqMan Gene Expression Assays ID Hs99999903_m1, Hs00607097_m1, H200366532_m1, Hs00365979_m1, Hs00265824_m1 and Hs02341532_m1, respectively, using a 7900HT Fast Real-Time PCR system (all Life Technologies, Carlsbad, CA, USA). For cDNAs for which expression [represented by threshold cycle number (Ct)] of the β -actin (*ACTB*) gene yielded a Ct of <28, the expression of KK-LC-1 was examined using end-point reverse transcription polymerase chain reaction (RT-PCR) rather than a probe-based assay, since an appropriate probe for detecting KK-LC-1 mRNA has not been established. For RT-PCR of KK-LC-1, the oligonucleotides 5'-ATGAACTTCT ATTTACTCCTAGCGAGC-3' and 5'-TTAGGTGGATTTCGGTGAGG-3' (Sigma-Aldrich Japan, Shinagawa-ku, Japan) were used as specific primers, and annealing was performed at 67°C for 40 cycles, yielding a 342-bp product.

Evaluation of *H. pylori*-infection. There are many reports showing that *H. pylori* infection can be accurately measured by detecting anti-*H. pylori* antibodies in serum (11). We evaluated *H. pylori* infection using serum from the 25 gastric patients with cancer. Serum anti-*H. pylori* IgG in was measured using a quantitative anti-IgG *H. pylori*, ELISA Kit, (Phoenix Pharmaceuticals, Burlingame, CA, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analyses of anti-*H. pylori* IgG titers between positive and negative expression groups for each CTA were performed using Wilcoxon's rank-sum test. *p*-Values of less than 0.01 were considered significant; JMP8.0 (SAS institute Japan, Minato-ku, Japan) was used for the analysis.

Table I. Characteristics of 25 patients with gastric cancer.

	Value
Mean age (range), years	69.5 (37-82)
Gender, n (%)	
Male	16 (64.0%)
Female	9 (36.0%)
Depth of invasion, n (%)	
T1	7 (28.0%)
T2	6 (24.0%)
T3	5 (20.0%)
T4	7 (28.0%)
Lymph node metastasis, n (%)	
N0	11 (44.0%)
N1	6 (24.0%)
N2	4 (16.0%)
N3	4 (16.0%)
Histological type, n (%)	
Papillary adenocarcinoma	1 (4.0%)
Tubular adenocarcinoma	11 (44.0%)
Mucinous adenocarcinoma	1 (4.0%)
Poorly differentiated adenocarcinoma	11 (44.0%)
Signet ring cell carcinoma	1 (4.0%)
Stage of disease, n (%)	
I	9 (36.0%)
II	10 (40.0%)
III	3 (12.0%)
IV	3 (12.0%)

Results

Expression of each CTA. We used reverse transcription polymerase chain reaction (RT-PCR) to determine the fraction of tumor samples from 25 patients that expressed specific CTAs. The expression profile of each CTA is shown in Table II. The frequency of expression of KK-LC-1 (80%) was the highest among CTAs tested.

Correlation between CTA expression and *H. pylori* infection. We next divided the patients into positive- and negative-expression groups for each CTA and examined the difference in anti-*H. pylori* IgG titer for each group. When patients were divided for KK-LC-1 expression, the IgG titer was significantly higher in the positive group (61.6±7.4 U/ml) than in the negative one (15.7±14.8 U/ml, *p*<0.01, Table III and Figure 1A). Four out of the eight patients with IgG titer below 20 U/ml also expressed KK-LC-1, while in the group with IgG titer above 20U/ml, 16 out of 17 (94%) expressed KK-LC-1. No significant differences in anti-*H. pylori* IgG titer were found when the patients were grouped for expression of any other CTA (Table III and Figure 1B-F).

Table II. The cancer/testis antigen (CTA) expression in 25 patients with gastric cancer.

CTA	Negative, n	Positive, n	Frequency of positivity (%)
KK-LC-1	5	20	80.0
MAGE-A1	14	11	44.0
MAGE-A3	13	12	48.0
MAGE-A4	20	5	20.0
NY-ESO-1	19	6	24.0
SSX4	21	4	16.0

KK-LC-1, Kitakyushu lung cancer antigen-1; MAGE, melanoma antigen gene; NY-ESO-1, New York esophageal squamous cell carcinoma-1; SSX4, synovial sarcoma, X breakpoint 4.

Table III. Anti-*Helicobacter pylori* IgG titer in each cancer/testis antigen (CTA) expression group.

CTA	IgG titer (average±SE)		p-Value
	Negative expression	Positive expression	
KK-LC-1	15.7±14.8	61.6±7.4	0.0098
MAGE-A1	59.6±10.0	43.3±11.2	0.2503
MAGE-A3	60.9±10.3	43.3±10.7	0.2109
MAGE-A4	48.3±8.3	69.1±16.7	0.3415
NY-ESO-1	56.8±8.6	38.8±15.3	0.2794
SSX4	49.5±8.2	68.1±18.8	0.4149

KK-LC-1, Kitakyushu lung cancer antigen-1; MAGE, melanoma antigen gene; NY-ESO-1, New York esophageal squamous cell carcinoma-1; SSX4, synovial sarcoma, X breakpoint 4.

Discussion

CTAs are expressed in about half of all tumors from a wide range of different organs such as melanoma, lung, esophagus, gastric, colon and breast carcinoma, but not in normal tissues except for germline tissues. KK-LC-1 is a CTA by this definition, because it is not expressed in normal tissues except for testis and cancer of multiple organs; however, it is expressed in 80% of gastric cancer cases, which is exceedingly more frequent than has been observed for other CTAs in other cancer types (7). To resolve this aberration, we confirmed the correlation of KK-LC-1 expression and *H. pylori* infection, which is the major carcinogenic factor for gastric cancer in Japan. There are a few reports that suggest that tumorigenic factors induced or enhanced the expression of specific CTAs (9, 12). However, as far as we are aware, this is the first report linking

Figure 1. Relationship between anti-*Helicobacter pylori* IgG and cancer/testis antigen (CTA) expression in gastric cancer. Patients were divided into positive or negative groups for expression of each CTA: A: Kitakyushu lung cancer antigen-1 (KK-LC-1), B: melanoma antigen gene (MAGE)-A1, C: MAGE-A3, D: MAGE-A4, E: New York esophageal squamous cell carcinoma-1 (NY-ESO-1) and F: synovial sarcoma, X breakpoint 4 (SSX4). * $p < 0.01$ using Wilcoxon rank-sum test.

carcinogen exposure to CTA expression in a clinical setting. In this study, we found that *H. pylori* infection was correlated with KK-LC-1-expression. These data might indicate that each CTA is induced by a specific individual or group of carcinogens. Additionally, it might be possible that the cause of cancerization could be clarified by analyzing CTA expression.

We assessed *H. pylori* infection using anti-*H. pylori* IgG in serum because this assay gives an accurate reflection of the infection status of the patient (11). Following the manufacture's protocol, a reading below 20 U/ml was considered negative for *H. pylori* infection. From our patients, four out of eight (50%) cases where the value was below 20 U/ml expressed KK-LC-1. The frequency of KK-LC-1-expression in this group was similar to that of other CTAs (Table III) (13, 14). In contrast, 16 out of 17 (94%) patients with titers over 20 U/ml were positive for KK-LC-1 expression. These results suggest that *H. pylori* infection might induce tumor cells to express KK-LC-1. In only one case was KK-LC-1 not expressed despite a high IgG titer against *H. pylori*. This might be due to the 10% inaccuracy rate of ELISA (11) or to the diversity of *H. pylori*; 90% of the *H. pylori* carried in the Japanese population is the East Asia type which has a strong potential for carcinogenesis (15).

CTAs are attractive candidates for diagnosing cancer even if their application for cancer immunotherapy is limited. KK-LC-1 may be a good candidate to help diagnose gastric cancer because of its high frequency of expression and specificity for tumors involving *H. pylori* infection. There is currently a risk of gastric cancer designated as the ABC(D) classification, which stratifies patients into three or four risk groups using *H. pylori* infection and atrophic gastritis by measuring anti-*H. pylori* IgG and pepsinogen I/II in serum (16). However, it is difficult to detect patients with gastric cancer even in the high-risk group (high *H. pylori* titer and low pepsinogen, and high or low *H. pylori* titer and high pepsinogen) because fewer than 3% of the tumors are detected (17). Mining KK-LC-1 might enable the high-risk group to be narrowed-down and the detection rate increased. We detected KK-LC-1 by gene expression using gastric cancer specimens. Therefore, this diagnostic assay needs to be accompanied by an invasive operation. Our future work hopes to establish a less invasive system for detect KK-LC-1. Adding KK-LC-1 detection to *H. pylori* and pepsinogen measurements might establish a highly accurate diagnosis of gastric cancer.

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