Systemic and local immune responses against *Helicobacter pylori* urease in patients with chronic gastritis: distinct IgA and IgG productive sites

S Futagami, H Takahashi, Y Norose, M Kobayashi

**Abstract**

**Background**—*Helicobacter pylori* urease is a major target for immune responses among various bacterial components in *H pylori* infected patients.

**Aims**—To analyse the relation between systemic and local humoral immune responses to *H pylori* urease and grades of chronic gastritis.

**Patients**—Seventy five patients with chronic gastritis associated with *H pylori* infection were classified into three groups (grade I, superficial gastritis; II, atrophic gastritis, quiescent; or III, atrophic gastritis, active).

**Methods**—Anti-*H pylori* urease specific antibodies in the serum, gastric juice, and biopsy specimens were determined by ELISA or western blotting analysis. The sites for *H pylori* urease and its specific antibody producing B lymphocytes were confirmed by immunohistochemical analysis.

**Results**—In the sera of patients with grade I gastritis, weak IgG but relatively strong IgA responses to *H pylori* urease were observed; dominant strong IgG responses were detected in grade II gastritis. In grade III gastritis, significant IgG and IgA responses were obtained. A similar pattern of IgA and IgG responses was detected in gastric juice and tissue. *H pylori* urease specific, antibody producing B cells were not found in the gastric mucosa of patients with grade I gastritis despite the presence of such B cells in the duodenal bulb. Specific B cells were observed in the gastric mucosa of patients with grade II and III gastritis with atrophy.

**Conclusions**—Purified *H pylori* urease, together with localisation of its specific antibody producing B cells, are useful for serological testing and histopathological analysis for determining the stage of chronic gastritis and studying the pathogenesis of *H pylori* infection.

Keywords: *Helicobacter pylori* urease; chronic gastritis; B lymphocytes; antibody production; local immunity

**Gut** 1998;43:168–175

**Accepted for publication**

18 February 1998
**Table 1** Characteristics of *H pylori* infected patient groups and histopathological grading according to the Sydney system

<table>
<thead>
<tr>
<th>Classification of gastritis</th>
<th>Number of patients *</th>
<th>Average age (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>25 (7)</td>
<td>26.3</td>
</tr>
<tr>
<td>Grade II</td>
<td>25 (14)</td>
<td>46.9</td>
</tr>
<tr>
<td>Grade III</td>
<td>25 (17)</td>
<td>45.8</td>
</tr>
<tr>
<td>Disease controls</td>
<td>25 (12)</td>
<td>44.1</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>25 (12)</td>
<td>20.3</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are men.

**Methods**

**PATIENTS**

Patients were tested by diagnostic upper gastrointestinal endoscopy for dyspeptic symptoms at the Digestive Endoscopic Centre of Nippon Medical School. A total of 75 patients with chronic gastritis who had a positive rapid urease test (CLOtest) and/or histological identification of *H pylori*, was selected as *H pylori* infected cases. Disease controls were 25 patients with drug induced gastritis due to long term use of non-steroidal anti-inflammatory drugs (such as aspirin and diclofenac sodium); an equal number of healthy volunteers (medical students from Nippon Medical School) with a mean age of around 20 years were selected as healthy controls. The disease control group was confirmed as *H pylori* negative with a negative CLOtest, urea breath test (UBT), and negative histology; the healthy control group was negative by UBT. Table 1 summarises the sex and age distribution of the various groups. The protocol was approved by the Ethical Review Committee of Nippon Medical School Hospital, and all patients gave written informed consent at the beginning of the study.

**CLOtest and HISTOLOGICAL EXAMINATION**

Six antral and three corpus biopsy specimens were obtained from every patient at the time of gastroscopy. One antral and one gastric body specimen were used for the CLOtest (Delta West, Bentley, Australia). Two antral and two corpus biopsy specimens were submitted for histological examination. The remaining three antral specimens were used as a source of local tissue samples. The samples of duodenum for immunohistochemistry were obtained from the duodenal bulb. *H pylori* associated gastritis was determined by either the presence of *H pylori* in histological specimens, a positive CLOtest, or both. The presence of *H pylori* was detected histologically using Giemsa staining. Biopsy specimens were stained with haematoxylin and eosin and graded for gastritis by the same experienced pathologist according to the Sydney system. The histological variables were scored on a four point scale: 0, absent; 1, mild; 2, moderate; and 3, severe. Inflammation was examined for the presence and density of mononuclear cells in the lamina propria; atrophy for the loss of gastric glands. *H pylori* associated gastritis was classified into three groups (grade I, II, and III; 25 patients each) based on the sum of inflammation (1–3) and atrophy (1–3) scores of antral biopsy specimens as defined in the Sydney system: grade I gastritis was shown as score 1–2, grade II as 3–4, and grade III as 5–6. As every patient with atrophy had also moderate (2) or severe (3) inflammation, grade I (1–2) corresponded precisely to superficial gastritis without atrophy as shown by Whitehead et al. Grades II and III corresponded closely to atrophic gastritis, quiescent (24/25; 96%), and atrophic gastritis, active (24/25; 96%), respectively. Other elements of scores in the Sydney system, such as intestinal metaplasia and activity, were not used to grade the gastritis.

**ELISA test for H pylori**

The purified *H pylori* urease antigen was diluted in 0.1 mol/l carbonate buffer (pH 9.6) to yield the optimal protein concentration of 10 µg/ml. A 50 µl aliquot of the antigen solution was added to each well of flat bottomed Immulon 2 plates (Dynatech Laboratories Inc., Alexandria, Virginia, USA) and incubated for 60 minutes at 37°C. After overnight blocking with 25% Block-Ace (Dainihon Seiyaku, Osaka, Japan), a 50 µl aliquot of appropriate dilution of the sample sera (sequentially diluted 1/10 to 1/80) and the positive reference serum (diluted 1/80) was plated for 60 minutes at 37°C. After washing, a 50 µl aliquot of an appropriately diluted class specific (IgA or IgG) alkaline phosphatase conjugated goat antihuman immunoglobulin (Cappel, Durham, North Carolina, USA) was added for another 60 minutes at 37°C; the amount of alkaline phosphatase bound to the well was determined by measuring the hydrolysis of ρ-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Missouri, USA) to the yellow

**Antigen (H pylori urease) preparation**

The antigen (*H pylori* urease) used in the following enzyme linked immunosorbent assay (ELISA) test was prepared from a mixture of *H pylori* NCTC 11637 and clinical isolates cultured on Skirrow plates containing 7% (vol/vol) defibrinated horse blood in an atmosphere consisting of 5% O₂, 15% CO₂, and 80% N₂ at 37°C for four to five days. A bacterial suspension was harvested by centrifugation (5000 g for 20 minutes at 4°C), washed twice with sodium phosphate, and stored as a cell pellet at −20°C. The cell pellet was resuspended in distilled water, vortexed for 45 to 60 seconds, and centrifuged (10 000 g for 20 minutes at 4°C). The supernatant was subjected to ion exchange chromatography (PRODUC-TIVE Column; BPS Separation Ltd, Spennymoor, Durham, UK) by a stepwise method (0, 0.2, 0.35, and 0.5 mol/l sodium phosphate buffer). The fractions showing maximum urease activity were further purified by size exclusion chromatography with a Sephacryl S-300 column (Pharmacia LKB Biotechnolog, Bjorkgatan, Uppsala, Sweden) and used as a purified *H pylori* urease antigen. The purified urease had a molecular weight of 350 kDa and was composed of six complexes of the small subunit of 30 kDa (UreA) and large subunit of 62 kDa (UreB) as determined by the use of specific monoclonal antibodies on western blotting analysis (not shown).
product, p-nitrophenolate, which was quantified by absorbance at 405 nm with a microplate reader (Model 3550; Bio-Rad, Tokyo, Japan). To standardize the ELISA, the cut off points for both IgA (0.28) and IgG (0.32) were determined using sera from 25 control patients not infected with *H pylori*; a titre was considered positive if the optical density (OD) exceeded the mean + 3 SD of the control serum samples. Based on these cut off points, reference IgA and IgG sera were made by pooling for each reference standard, five sera positive for either IgA or IgG specific to *H pylori* urease. The mean (SD) absorbance of the reference serum was 0.54 (0.14) for IgA and 0.97 (0.13) for IgG. These values were used to correct the absorbance reading when patient serum was tested. The results were expressed as the absorbance index (AI) obtained using the following formula:

\[
\text{Absorbance index} = \frac{(\text{mean OD reading (n=2) of patient's serum} - \text{mean OD of blank reading})}{(\text{mean OD reading (n=2) of reference serum} - \text{mean OD of blank reading})}
\]

where the blank OD represents the value of the *H pylori* urease uncoated control well. The intra-assay and interassay variations were less than 5%, as estimated with positive and negative control sera.

**DETECTION OF H PYLORI UREASE SPECIFIC ANTIBODIES IN GASTRIC JUICE**

At the beginning of endoscopic diagnosis, samples of fasting gastric juice from patients with chronic gastritis were aspirated into tubes containing protease inhibitors such as EDTA (0.65 mg/ml) and aprotinin (500 KIU) to prevent further degradation. The gastric juice samples were neutralised to pH 7.4 with 0.1 M NaOH before western blotting. Anti-*H pylori* urease specific IgG, IgA, and secretory component (SC) responses was determined by western blotting analysis. In brief, purified *H pylori* urease complex and standard molecular masses (Sigma) were further separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Gel; SPU-15S, Page; Atto Corporation, Tokyo, Japan) as described by Laemmli, and transferred onto nitrocellulose membranes by electroblotting. After blocking with 25% Block-Ace, the blots were incubated at room temperature with the sample gastric juice for 120 minutes. After washing to remove free sample antibody, the blot-sample complexes were incubated further with alkaline phosphatase conjugated goat antihuman IgA or IgG (diluted 1/300, 1/500 with 10% Block-Ace, respectively) or peroxidase conjugated goat antihuman SC specific antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) (diluted 1/200 with 10% Block-Ace) for 60 minutes at 37°C. After being washed four times, the bound conjugate was detected with 200 µl of substrate colour indicator.

**MEASUREMENT OF H PYLORI UREASE SPECIFIC ANTIBODIES IN GASTRIC TISSUE**

Three antral biopsy specimens (total weight of about 30 mg) were taken from the gastric mucosa of each patient listed in table 1 by endoscopic biopsy and homogenised. After adding 1 ml phosphate buffered saline (PBS) on ice, the samples were centrifuged at 16 000 g for 10 minutes. The protein volume of each supernatant was assayed for total protein using a modified Lowry method and the mean protein concentration was approximately 0.65 mg/ml. The supernatant was then adjusted to 0.5 mg/ml and harvested to measure the tissue IgA, IgG, and SC antibodies against *H pylori* urease by ELISA as described above. The cut off points for tissue IgA (0.22), IgG (0.19), and SC (0.18) were determined using gastric tissue from 25 *H pylori* uninfected disease control patients. Based on these cut off points, reference samples were made by pooling for each reference standard, five gastric samples positive for either IgA, IgG, or SC specific to *H pylori* urease. The mean (SD) absorbance of the reference sample was 0.38 (0.12) for IgA, 0.66 (0.13) for IgG, and 0.32 (0.10) for SC. Also, secretory component
conjugated IgA (SC IgA) in each supernatant was measured by an ELISA kit (MBL, Tokyo, Japan).

Results were expressed quantitatively as ng SC IgA per mg wet weight of tissue.

**IMMUNOHISTOCHEMICAL STUDIES**

The localisation of *H. pylori* urease specific antibody producing B lymphocytes inside the tissue was determined by the following procedure. In brief, cryostat sections (8 µm) were cut from the fresh frozen stomach specimens, air dried, and incubated with purified *H. pylori* urease (100 µl of 40 µg/ml) for two hours at room temperature. After being washed with PBS and fixed in cold acetone for 10 minutes, non-specific staining was blocked with PBS containing 5% normal goat serum and 1% bovine serum albumin. Endogenous peroxidase activity was blocked by incubating for 30 minutes in 0.6% H2O2 in methanol. Tissue sections were then incubated with a mouse anti-*H. pylori* urease specific monoclonal antibody19 (1/300 dilution) for 90 minutes at room temperature. After further washing, peroxidase conjugated goat antimouse immunoglobulin (Dako, Glostrup, Denmark) (1/100 dilution) was added. The immuno-peroxidase reaction was visualised with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) solution containing 0.03% H2O2. Meyer's haematoxylin, methyl green, and/or Alcian blue at pH 2.5 for goblet cells was used. Negative controls were made without adding the first antibody. The localisation of *H. pylori* urease was determined by the same staining procedure except for the step of incubation with purified *H. pylori* urease. The distribution of IgA producing lymphocytes was observed by direct staining with peroxidase conjugated rabbit antihuman IgA (Dako) (1/100 dilution).

**STATISTICAL ANALYSIS**

Results are expressed as the mean (SEM). Data were analysed by the Mann-Whitney U test and considered significant if any p value was less than 5%.

**Results**

**H PYLORI UREASE SPECIFIC IgA AND IgG RESPONSES IN THE SERA**

As indicated in fig 1, the mean absorbance index (AI) of *H. pylori* urease specific IgA responses in the sera of patients with grade I gastritis (0.63 (0.31)) was significantly higher than the mean in grade II gastritis (0.13 (0.08); p<0.05), the value of which was very close to the basal level of the uninfected disease controls (0.12 (0.06)) and uninfected healthy volunteer controls (0.10 (0.03)), both of which should not have any IgA antibody response against *H. pylori* urease. In contrast, the mean AI of *H. pylori* urease specific IgG responses in grade II gastritis patients (1.02 (0.25)) was significantly higher than in grade I gastritis (0.09 (0.03); p<0.05), the value of which was almost equal to the basal level of the uninfected disease controls (0.10 (0.05)) and uninfected healthy volunteer controls (0.10 (0.04)), both of which should not have any IgG antibody response to *H. pylori* urease. In grade III gastritis, the mean AIs of *H. pylori* urease specific IgA responses (1.04 (0.30)) and IgG responses (1.24 (0.25)) were both significantly higher than in the uninfected disease controls or healthy volunteer controls. We then calculated the IgA/IgG ratio in each patient based on the AI values and confirmed the mean IgA/IgG ratio of each gastritis group as 7.48 (3.13), 0.13 (0.07), and 0.91 (0.52) in grades I, II, and III gastritis, respectively. Therefore, the relative ratio of IgA:IgG in each case seems to be useful in classifying the type of gastritis associated with *H. pylori* infection; in particular, a high score (greater than 4.35: mean −SD) indicated grade I gastritis and low score (less than 0.20: mean +SD), grade II gastritis. As far as we have investigated, *H. pylori* urease specific IgM responses were mostly very weak in each group compared with control values (data not shown). Only IgA and IgG responses to purified *H. pylori* urease will therefore be measured for further study.

**H PYLORI UREASE SPECIFIC IgA SECRETION FROM GASTRIC MUCOSA**

As *H. pylori* urease specific IgA antibody was observed in the sera of grade III gastritis patients with mucosal damage, it is worth investigating such *H. pylori* urease specific functional IgA antibodies associated with SC in the gastric juice of these patients to determine whether local immunity to *H. pylori* urease exists in the gastric mucosa. Figure 2 shows clearly that IgG, IgA, and SC associated...
antibody (major component should be IgA) against \textit{H pylori} urease can be detected in the gastric juice of grade III gastritis patients with haemorrhagic erosions and severe atrophy (lanes D, E, F). Lane G shows \textit{H pylori} positive duodenal ulcer (active stage) as a positive control. Only an \textit{H pylori} urease specific IgG response was observed in grade II gastritis without mucosal change (atrophic gastritis, quiescent; lanes A, B, C) by western blotting analysis. We have analysed by the same procedure another six cases of grade II and equal numbers of grade III gastritis with a similar pattern and confirmed these findings.

\textbf{\textit{H pylori} urease specific antibody production in gastric tissue}

In order to investigate the local immune response to \textit{H pylori} urease in gastric tissue, \textit{H pylori} urease specific IgA, IgG, and SC antibodies in gastric tissue of the same patients with \textit{H pylori} associated chronic gastritis were measured by modified ELISA methods. As fig 3 shows, a tendency similar to that found in the sera could also be observed in the tissue. The mean AI of \textit{H pylori} urease specific IgA and SC antibody responses in grade I gastritis (0.84 (0.20), 0.77 (0.16)) was significantly higher than in grade II gastritis (0.30 (0.14), 0.41 (0.09); p<0.05); the mean AI value of \textit{H pylori} urease specific IgG in grade II gastritis (0.47 (0.14)) was significantly higher than in grade I gastritis (0.09 (0.03); p<0.05). In grade III gastritis, high values of the mean AI of \textit{H pylori} urease specific IgG (0.94 (0.32)), IgA (1.42 (0.42)), and SC (0.86 (0.26)) antibody responses were observed. Also, by measuring the amount of SC-IgA in the biopsy homogenate from each gastritis patient, we confirmed the existence of a comparable SC-IgA concentration: 173 (11.2) ng/mg tissue weight in grade I, 178 (10.3) ng/mg in grade II, and 188 (13.0) ng/mg in grade III gastritis.

\textbf{Distribution of \textit{H pylori} urease specific antibody producing B cells in gastric tissue}

As \textit{H pylori} urease specific humoral antibodies can be observed in gastric juice and tissue as well as in sera from chronic gastritis patients, it is important to investigate the distribution of antigenic \textit{H pylori} urease and its specific antibody producing B cells in the gastric tissue of various patients infected with \textit{H pylori}. In grade III gastritis with severe atrophy and haemorrhagic erosions, \textit{H pylori} urease can only be seen on the surface of the mucosal membrane in association with a cluster of \textit{H pylori} (fig 4A); \textit{H pylori} urease specific mature B cells were observed in most parts of the gastric mucosal tissue and within the lamina propria mucosae except lymphoid follicles (fig 4B) when compared with stained control (fig 4C). Figure 4B also indicates that \textit{H pylori} urease specific antibody producing mature B lymphocytes do not associate with neighbouring lymphoid follicles which contain many immature B lymphocytes.

In grade II gastritis bearing intestinal metaplasia, \textit{H pylori} and its specific urease could be seen, even in the neighbourhood of the metaplastic epithelium in the lumen of gastric mucosa associated with goblet cells, but not in the lamina propria as indicated by arrows in the magnified box (fig 4D). In addition, small numbers of \textit{H pylori} urease specific antibody producing mature B lymphocytes could also be seen in this quiescent type of
Figure 4  Immunohistochemical analysis of H pylori urease specific humoral responses. (A) Localisation of H pylori and H pylori urease in the stomach of a patient with grade III gastritis (enzyme immunohistochemistry with monoclonal H pylori urease specific antibody; Meyer’s haematoxylin, original magnification ×160; a higher magnification (×800) of the boxed area indicated in the left upper corner is shown in the left lower corner. (B) Distribution of H pylori urease specific antibody producing B lymphocytes (plasma cells) in the same tissue (enzyme immunohistochemistry with purified H pylori urease and monoclonal H pylori urease specific antibody; methyl green, original magnification ×150; LF, lymphoid follicle. (C) Localisation of mononuclear cells in the same tissue (haematoxylin and eosin, original magnification ×150). (D) Localisation of H pylori and H pylori urease indicated by arrows in the stomach of a patient with grade II gastritis (enzyme immunohistochemistry with monoclonal H pylori urease specific antibody; Alcian blue,
atrophic gastritis (fig 4E). In contrast, *H pylori* urease specific mature B lymphocytes could not be detected in the gastric mucosal tissue of grade I superficial gastritis patients (fig 4F) despite the evidence of strong production of *H pylori* urease specific IgA antibody. Surprisingly, we could see a number of such urease specific mature B lymphocytes which may produce *H pylori* urease specific IgA in the duodenal bulb of patients with superficial gastritis (fig 4G). Similar findings could be obtained in five cases of grade I gastritis (superficial gastritis) and it is interesting to note that all five cases also had duodenitis.

We also studied the distribution of IgA producing B cells in gastric tissue of the patients with grade I superficial gastritis and found such IgA positive B cells infiltrating the upper parts of the lamina propria (fig 4H). Thus, some other antigen specific IgA producing B cell may be activated in the gastric tissue of grade I gastritis patients. Similar findings but with more infiltration of such specific B cells were observed in grade III gastritis patients with severe atrophy and erosions.

**Discussion**

The major pathological feature of *H pylori* associated chronic gastritis is mononuclear cells, particularly plasma cells, infiltrating the lamina propria. Plasma cells are sparse or absent from the normal uninfected stomach. Such plasma cells emerging in association with *H pylori* infection may produce specific antibodies in an attempt to eliminate the infection. Thus, some of the humoral immune responses against *H pylori* components should correlate with inflammatory changes in the gastric mucosa and with the progression of gastritis.

Based on the sum of the inflammation and atrophy scores of antral biopsy specimens as defined in the Sydney system, we showed that *H pylori* associated chronic gastritis can be classified into three grades—I, II, and III; the relation between various types of *H pylori* associated chronic gastritis and humoral immune responses to *H pylori* can be graded. Under this classification scheme, we analysed IgA and IgG responses in the serum, gastric juice, and gastric tissue from patients with different grades of gastritis against purified *H pylori* urease, which we have identified as an antigenic molecule. Previous studies have reported that antigenic molecules which inhibit the entrance of external foreign antigens may induce systemic immunity. In addition, patients with surface mucosal changes together with chronic atrophy in the gastric mucosa of grade III gastritis (atrophic gastritis, active) produce both *H pylori* urease specific IgA and IgG responses. When we followed up some of these cases, such specific IgA responses diminished as the superficial changes disappeared, despite the continuous production of IgG against *H pylori* urease (data not shown). Thus, as speculated above, *H pylori* urease specific IgA responses might reflect superficial changes and its specific IgG responses may be associated with gastric atrophy. The reason for the existence of *H pylori* urease specific immune responses in gastric juice of such patients remains to be investigated.

To investigate further the biological meanings of such local immune responses to *H pylori* urease, we analysed the distribution of antigenic *H pylori* urease and urease specific antibody producing B cells in the gastric tissue of these patients. Although *H pylori* urease could be observed only in restricted sites (fig 4A,D), *H pylori* urease specific mature B cells were widely distributed in grade II and III gastritis with atrophy (fig 4B,E). In contrast, such *H pylori* urease specific mature B lymphocytes could not be observed in the gastric tissue of grade I gastritis (fig 4F) in which IgA producing B cell infiltrates were seen in the upper parts of the lamina propria (fig 4H). Unexpectedly, a number of such *H pylori* urease specific mature B lymphocytes could be observed in the duodenal bulb of the same patients with...
grade I gastritis (fig 4G). These findings showed that most IgA producing B cells in the upper parts of the lamina propria of patients with grade I superficial gastritis were not specific for *H pylori* urease and that some other part of the gastrointestinal tract, including the duodenum, may be a site for generating *H pylori* urease specific IgA producing B cells that secrete urease specific IgA both in serum and gastric juice. The above results appear to reflect the fact that a number of cases of superficial gastritis are accompanied by duodenitis and are compatible with the speculation of Bienenstock and Befus that synthesis and secretion of IgA may result mostly from local plasma cells in the lamina propria of the intestine. The reason why the major *H pylori* urease specific IgA antibody producing site appeared to shift from the gastric mucosa in grade I superficial gastritis patients remains to be studied further. Furthermore, if we reconsider the findings of Whitehead et al., it would be interesting to investigate the types of infiltrating lymphocytes and their specific antigens as observed in the stomach during superficial gastritis.

Taken together, our results suggest that *H pylori* urease specific IgG produced beneath the gastric mucosa may be associated with chronic atrophic changes within the gastric mucosa whereas *H pylori* urease specific IgA produced under the duodenal mucosa and circulated systemically may relate to superficial changes in the gastric mucosa. The findings shown here will be a useful tool for determining the actual stage of chronic gastritis and analysing the pathogenesis of *H pylori* infection.

We thank Drs Yu Fukuda, Kumiko Nagata, Taku Tsukui, and Graham R Leggatt for helpful suggestions and critical reading of the manuscript, Dr Kumiko Nagata for the kind gift of *H pylori* urease specific mouse monoclonal antibodies, and Drs Masaru Hosone, Asumi Tatsuzuki, Kaoru Hirota, Miss Miyuki Takanori, and Mr Hironori Katayama for expert technical assistance in immunohistochemical studies. This work was supported in part by grants from the Ministry of Education, Culture, and Science, from the Ministry of Health, and CREST, JST, Japan.