Association between common genetic variant of *HRH2* and gastric cancer risk

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Abstract. Histamine plays important physiological roles in the upper gastrointestinal tract and acts via the H_2 receptor. The -1018 G>A (rs2067474) in an enhancer element of the promoter and non-synonymous rs79385261 (Asn46Thr) were identified in *HRH2*. We attempted to clarify the associations of these polymorphisms with gastric carcinogenesis. The study was performed in 321 patients with gastric cancer and 599 subjects with no evidence of gastric malignancies on upper gastroduodenal endoscopy. The genotypes were determined using a one-tube multiplex PCR-SSCP method. The degree of gastritis was assessed in 496 subjects and serum pepsinogen (PG) I/II levels were measured in 124 subjects without gastric cancer. The minor allele of Asn46Thr could not be detected. The frequencies of the -1018 A allele in the non-GC and GC groups were 13.5% and 8.26%, respectively (p=0.00077). Overall, -1018 GG homozygotes had an increased risk for developing gastric cancer (OR 1.68; 95% CI 1.17-2.42; p=0.0052), especially intestinal type cancer (OR 1.94; 95% CI 1.23-3.08; p=0.0047). In subjects aged >60 years, the adjusted risk for gastric cancer among individuals who were -1018 GG homozygotes was 1.87 (range 1.19-2.93; p=0.0065) compared with A carriers. In the gastric cancer cases located in the antrum and at comparative advanced stage, -1018 GG homozygosity was a significantly increased risk factor. In subjects >60 years, the metaplasia score was significantly higher in -1018 GG homozygotes than A carriers. Both atrophy and metaplasia scores were significantly increased with age only in -1018 GG homozygotes. The PG I/II ratio was significantly decreased in *H. pylori* positive GG homozygotes than negative GG homozygotes and positive A carriers. Our results suggest that -1018 GG homozygosity of *HRH2* may be associated with the severity of gastric mucosal atrophy. This genotype has an increased risk for the subsequent development of gastric cancer, especially intestinal type, at advanced age.

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Introduction

Gastric cancer remains a considerable public health problem worldwide. The incidence and mortality rates of gastric cancer have decreased gradually. Nevertheless, gastric cancer is second only to lung cancer as the leading cause of cancer death around the world (1,2). *Helicobacter pylori* (*H. pylori*) infection is now accepted as a crucial event in the development of gastric cancer, although the etiology of this tumor remains unclear. This infection first induces chronic superficial gastritis, which can progress to chronic atrophic gastritis, intestinal metaplasia, and dysplasia that leads toward gastric carcinoma (3-6). However, only a small number of infected patients actually develop gastric cancer. This suggests that host genetic factors, such as genes associated with inflammatory responses and acid secretion, may also play an important role in gastric carcinogenesis. Therefore, the associations between genetic polymorphisms and gastric carcinogenesis have been investigated in several studies (7-11). We have also revealed the significant association of polymorphisms in *TLR2* (12), *MIF* (13), *IL17A* (14) and pre-microRNAs (15) with the susceptibility to gastric carcinogenesis.

On the other hand, the stomach is exceedingly rich in the peptide hormone- or active amine-producing cells such as enterochromaffin-like (ECL) cells (histamine), D cells (somatostatin), EC cells (serotonin), and G cells (gastrin) (16). Histamine, one of the active amine released in response to a variety of physiological stimuli, is well known to be involved in the pathogenesis of gastro-duodenal ulceration and gastric inflammation (17). Although this bio-active amine modulates a variety of functions via interacting with specific receptors on the target cells, H_1, H_2 and H_3 receptors (18), H_2 receptor has a central role only in the regulation of acid secretion in stomach as confirmed by the widespread use of H_2 receptor blockers in the therapy of acid-related disorders (19,20). *H. pylori* infection as the main cause of gastric and duodenal ulcer heralded a new revolution in our understanding and treatment of acid-peptic disorders (21,22). Evidence was also provided that increased

gastric histamine contributed to the inflammatory changes and tissue damage associated with chronic *H. pylori* infection of the gastric mucosa (23,24). Thus, intra-gastric histamine plays an important role on the gastric inflammation acting via H_2 receptor, although *H. pylori* infection is one of the major contributing factors to the development of gastro-duodenal inflammation (25).

The association between genetic polymorphisms of histamine receptor genes and the susceptibility to psychological and neurological disorders has been investigated (26,27). The rs2607474 (-1018 G>A) focused in these studies is located in an enhancer element of the *HRH2* promoter, encoding histamine H_2 receptor (26). It is likely that the *HRH2* variant located in the promoter may induce changes in the expression of receptors. Although the investigators in the gastroenterological field have shown great interest in histamine H_2 receptors for a long time, there has been no report whether -1018 G>A polymorphism (rs2607474) affect on the development of and susceptibility to gastrointestinal disorders, including gastric cancer, or not. Furthermore, non-synonymous SNP (rs79385261, 137 A>C, Asn46Thr) was published in dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/snp). The distribution of this genotype in Japanese is still unknown.

This study is aimed to test the hypothesis that genetic alteration in *HRH2*, causing changes in the expression, may cause an increased risk for gastric carcinogenesis. We investigated the association between *HRH2* -1018 G>A (rs2607474) and gastric carcinogenesis. In addition, the influence of rs79385261 (Asn46Thr) was also investigated.

Materials and methods

Clinical samples. As a gastric cancer group, 321 patients with gastric cancer (GC group), who were enrolled at the Endoscopy Center of Fujita Health University Hospital or Kanazawa Medical University Hospital from January 2007 to December 2009, were selected. The diagnoses of all gastric cancers were done histologically at the Division of Pathology of our hospitals. As a control, 599 subjects without malignant neoplasm on endoscopic examination were randomly selected from our stocked DNA collected during the same period (non-GC group). Finally, the studied population comprised 920 subjects, whose polymorphisms could be clearly analyzed. The patients with severe systemic diseases, malignancies in other organs, and who had received non-steroidal anti-inflammatory drugs, antibiotics, and *H. pylori* eradication treatment were excluded.

All subjects underwent upper gastrointestinal endoscopy and, in some of them, biopsy specimens were taken from non-cancerous mucosa. Parts of each specimen were fixed in 10% buffered formalin and embedded in paraffin. Later, the degree of gastritis was evaluated. The genomic DNA was isolated from peripheral blood using FlexiGene DNA Kit (Qiagen GmbH, Hilden, Germany).

The Ethics Committees of Fujita Health University and Kanazawa Medical University approved the protocol, and written informed consent was obtained from all of the participating subjects.

Detection of H. pylori infection. *H. pylori* infection status was assessed by serology, histological examination, or the urea breath test. Patients were diagnosed as having infection when at least one of the diagnostic tests was positive.

Genotyping of polymorphisms. Sample stocked DNA isolated from peripheral blood was used. Polymorphisms were genotyped by the multiplex PCR-SSCP method as reported previously (14,28). To detect -1018 G>A and Asn46Thr (A>C) genotypes, using the primer pairs (-1018 forward: 5'-acctgaccc ttttctgaaaaagtttgtc-3' and -1018 reverse: 5'-ctactcctctgaagtgctg agaaccat-3' for -1018 G>A; and 46 forward: 5'-aatgtggtcgtctg tctggccgt-3' and 46 reverse: 5'-agagcatcacatccaggctggtg-3' for Asn46Thr; respectively), one-tube multiplex PCR was carried out in a volume of 20-μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 96˚C for 15 sec, 60˚C for 30 sec, and 72˚C for 30 sec, with final extension at 72˚C for 5 min. Thereafter, 2 μl of the PCR product was denatured with 10 μl of formamide (Sigma-Aldrich Co., St. Louis, MO, USA) at 95˚C for 5 min. SSCP was carried out at 6˚C using a GenePhor DNA separation system with GeneGel Excel 12.5/24 (GE Healthcare, USA), after which the denatured single strand DNA bands were detected using a DNA Silver Staining Kit (GE Healthcare).

Histological evaluation. In 496 of 599 control subjects, the severity of chronic gastritis was classified according to the updated Sydney system (29) by a pathologist who had no access to any clinical information.

Serological evaluation. The pepsinogen (PG) I/II ratio was calculated based on the data of the serum PG I and PG II levels measured by radioimmunoassay in 124 of 599 control subjects. A PG I/II ratio that showed a decrease in proportion to the severity of gastric mucosal atrophy was used as a marker of atrophic gastritis (30,31).

Statistical analysis. The data were expressed as mean ± SD. Mean ages between 2 groups was compared by Student's t-test. The ratios of *H. pylori* infection status and male/female were compared by Fisher's exact test. Allele and genotype frequencies were calculated by direct counting. The allele counts were also compared by a Fisher's exact test. The strength of association between allele frequencies and the disease was assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI) by logistic regression analysis. Adjusted ORs were calculated after adjustment for age, gender and *H. pylori* infection status. Each updated Sydney system scores and PG I/II ratio between 2 groups were compared by Mann-Whitney U-test. The relationship between age and updated Sydney system score was assessed by ANOVA. Concerning the power of study, when setting α=0.05, β-value was calculated. For all analyses, the level of significance was set at $p<0.05$.

Results

Characteristics of subjects and the frequencies of genotypes. Single strand DNAs of *HRH2* genotypes were clearly separated by SSCP (Fig. 1). The minor allele of rs79385261 (Asn46Thr) could not be detected in any subject. The distribution of -1018 G>A genotype in control subjects was 447GG, 142GA and 10AA (Table I). It was in the Hardy-Weinberg equilibrium (p=0.86).

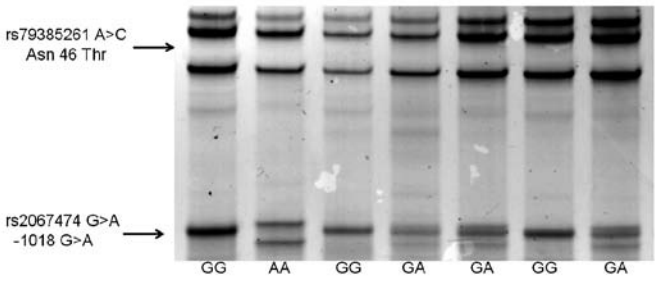

Figure 1. The images of PCR-SSCP. Single strand DNAs of -1018 G>A genotype were clearly separated by SSCP.

The characteristics of subjects in this study are summarized in Table I. The mean age, male/female ratio and *H. pylori* positive ratio were significantly higher in GC group than non-GC group. The distribution of -1018 G>A genotype in GC group was 269GG, 51GA and 1AA (HWE, p=0.71). The -1018 G>A minor allele frequencies in GC and non-GC groups were 8.26% and 13.5%, respectively (p=0.00077 and β=0.93). In addition, the frequency of -1018 GG homozygote was significantly different among GC and non-GC groups (p=0.00078 and β=0.91).

Association between HRH2 -1018 G>A and gastric carcinogenesis. Overall, -1018 GG homozygote had a significantly increased risk for gastric carcinogenesis by logistic regression analysis after adjustment for age, gender and *H. pylori* infection status (OR 1.68; 95% CI 1.17-2.42; p=0.0052; Table II). When assessed by subtypes of gastric cancer, -1018 GG homozygote had a more increased risk for the development of intestinal type of cancer (OR 1.94; 95% CI 1.23-3.08; p=0.0047, Table II), whereas no significant association was found between this genotype and diffuse type of cancer.

In the subjects aged <60 years, *HRH2* -1018 G>A was not associated with gastric carcinogenesis (Table III). On the other hand, in the subjects >60 years, -1018 GG homozygote had an increased risk for the development of gastric cancer (OR 1.87; 95% CI 1.19-2.93; p=0.0065).

Association between HRH2 polymorphism (-1018 G>A) and clinicopathological features of gastric cancer. We investigated the influences of genetic polymorphisms on the progression of gastric cancer using various parameters of clinicopathological features. The *HRH2* -1018 GG homozygote was significantly associated with the increased risk for the development of gastric cancer located at lower third of stomach (OR 2.26; 95% CI 1.28-3.99; p=0.0050, Table IV). When assessed by tumor stages, -1018 GG homozygote was associated with the increased risk for the cases invaded beyond musclaris propria (OR 1.96; 95% CI 1.19-3.23; p=0.0078). Regarding as lymph node metastasis, this genotype had an increased risk for the cases both with and without lymph node metastasis (OR 1.99; 95% CI 1.16-3.43; p=0.013 and OR 1.63; 95% CI 1.05-2.54; p=0.031, respectively).

Histological evaluations of gastritis among genotypes of HRH2 (-1018 G>A). In 496 control subjects evaluated for histological gastritis, the distribution of genotype was 397GG, 96GA and 3AA. Overall, each updated Sydney system score was not different among -1018 GG homozygote and A carrier (Fig. 2). However, in the subjects aged >60 years, metaplasia score was significantly higher in -1018 GG homozygote than A carrier (p=0.043).

Table I. Characteristics of the subjects and frequencies of genotypes.

	Non-GC group	GC group	p-value[a]
No. of subjects	599	321	
Mean age ± SD	61.7±13.2	65.4±11.0	<0.0001
Male:female	345:254	224:97	0.00028
H. pylori positive ratio	61.2%	86.0%	<0.0001
HRH2 genotype			
G/G	447	269	0.00078[b]
G/A	142	51	
A/A	10	1	
A allele frequency	13.5%	8.26%	0.00077[c]

[a]Non-GC group vs. GC group; [b]The frequency of GG genotype; [c]The minor allele frequency.

Table II. The risk of *HRH2* polymorphism (-1018 G>A) for gastric carcinogenesis.

	GG	GA	AA	GG vs. A carrier; OR (95% CI)	p-value
Non-GC	447	142	10	reference	-
Overall GC	269	51	1	1.68 (1.17-2.42)	0.0052
Intestinal	163	27	0	1.94 (1.23-3.08)	0.0047
Diffuse	104	23	1	1.36 (0.838-2.20)	0.21
(Unknown)	2	1	0	-	-

By logistic regression analysis after adjustment for age, gender and *H. pylori* infection status.

Table III. The risk of *HRH2* gene polymorphism (-1018 G>A) for gastric carcinogenesis in the subjects aged <60 years or >60 years.

	No. of subjects	GG	GA	AA	GG vs. A carrier; OR (95% CI)	p-value
<60						
Non-GC	219	164	51	4	reference	-
GC	100	81	19	0	1.60 (0.842-3.04)	0.15
≥60						
Non-GC	379	282	91	6	reference	-
GC	221	188	32	1	1.87 (1.19-2.93)	0.0065

By logistic regression analysis after adjustment for age, gender and *H. pylori* infection status.

Table IV. Association between *HRH2* -1018 G>A and clinicopathological features of gastric cancer.

	No. of subjects	GG	GA	AA	GG vs. A carrier; OR (95% CI)	p-value
Non-GC	599	447	142	10	reference	
Location						
Upper third	18	13	5	0	0.862 (0.297-2.50)	0.78
Middle third	164	134	29	1	1.44 (0.917-2.26)	0.11
Lower third	127	111	16	0	2.26 (1.28-3.99)	0.0050
Stage						
≤T1	159	131	27	1	1.53 (0.961-2.43)	0.073
≥T2	156	134	22	0	1.96 (1.19-3.23)	0.0078
Lymph node metastasis						
n (-)	183	152	30	1	1.63 (1.05-2.54)	0.031
n (+)	131	113	18	0	1.99 (1.16-3.43)	0.013

By logistic regression analysis after adjustment for age, gender and *H. pylori* infection status.

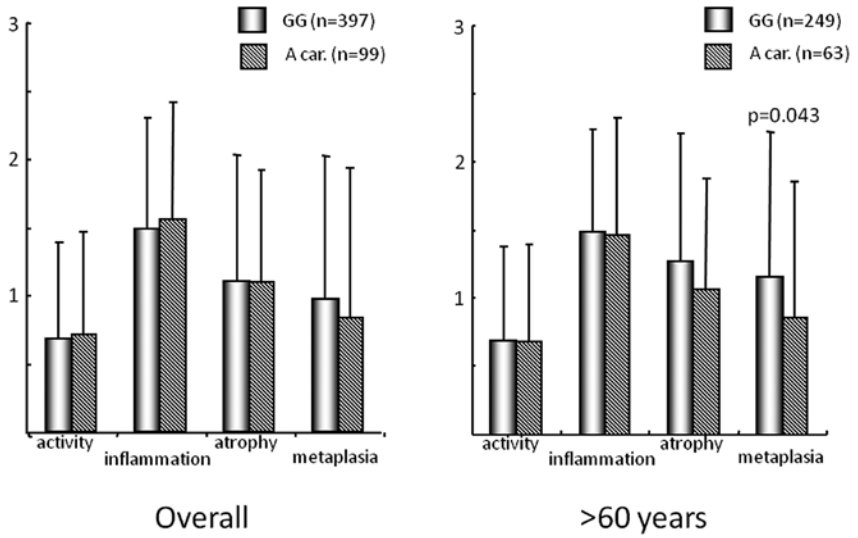

Figure 2. Comparison of each updated Sydney system score among -1018 GG homozygote and A carrier. Overall, each updated Sydney system score was not different among -1018 GG homozygote and A carrier. In subjects aged >60 years, metaplasia score was significantly higher in -1018 GG homozygote than A carrier.

In addition, in -1018 GG homozygote, both atrophy and metaplasia scores were significantly increased with age (both p-values by ANOVA: p<0.0001, Fig. 3). In A carrier, however, neither atrophy nor metaplasia score was significantly related to age.

Serum pepsinogen levels between -1018 GG homozygote and A carrier. In 124 control subjects measured for serum PG levels, 79 were *H. pylori* positive and 45 were negative. The distribution of genotype in *H. pylori* positive was 64GG, 14GA and 1AA, whereas the distribution in *H. pylori* negative was 32GG, 12GA and 1AA. In -1018 GG homozygote, PG I/II ratio was significantly decreased under influence of *H. pylori* infection (p<0.0001), whereas there was no significant difference among *H. pylori* positive and negative subjects in A carrier (Fig. 4). In *H. pylori* positive subjects, PG I/II ratio was significantly lower in GG homozygote than A carrier (p=0.036).

Discussion

In the present study, we investigated the association between polymorphisms of *HRH2*, encoding histamine H_2 receptor, and gastric carcinogenesis. A minor allele of Asn46Thr (rs79385261 A>C) could not be detected in our subjects. So, only -1018 G>A (rs2607474), genotype of which was in the Hardy-Weinberg equilibrium, was investigated. We found a strongly increased association between -1018 GG homozygote and gastric carcinogenesis, especially intestinal type of cancer. This strong association was found in the cases with tumor located at antrum and at the comparatively advanced stage. In addition, it was also

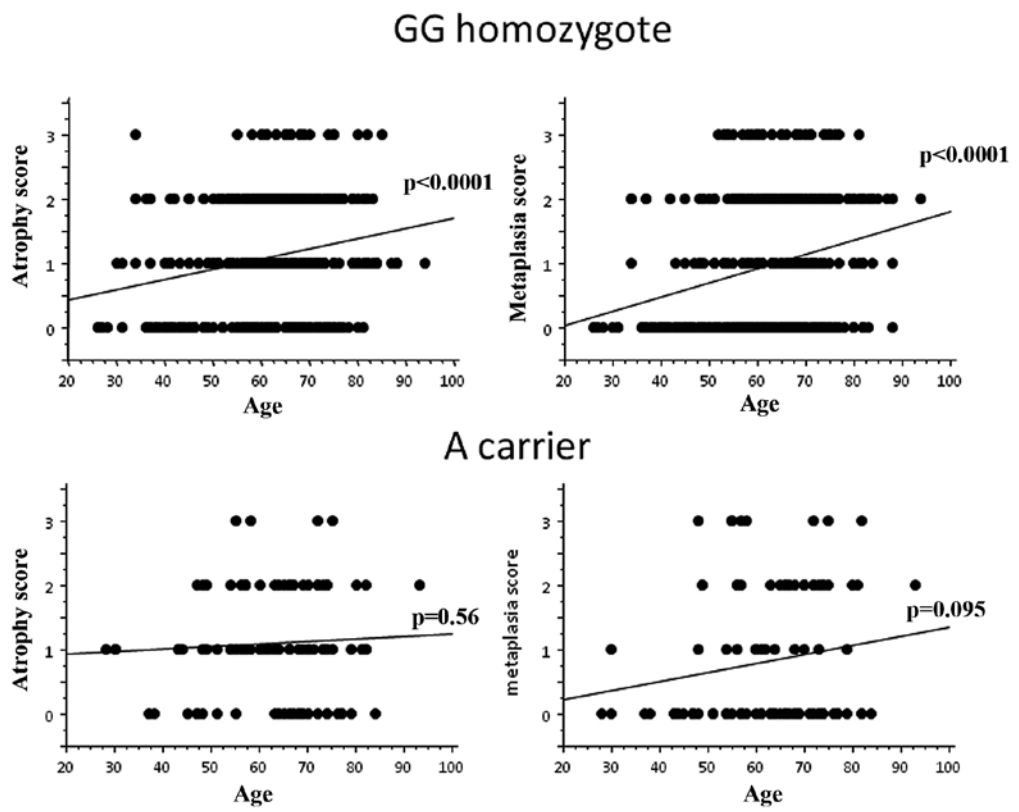

Figure 3. Relationship of age to atrophy and metaplasia scores. Both atrophy and metaplasia scores were significantly increased with age only in -1018 GG homozygotes.

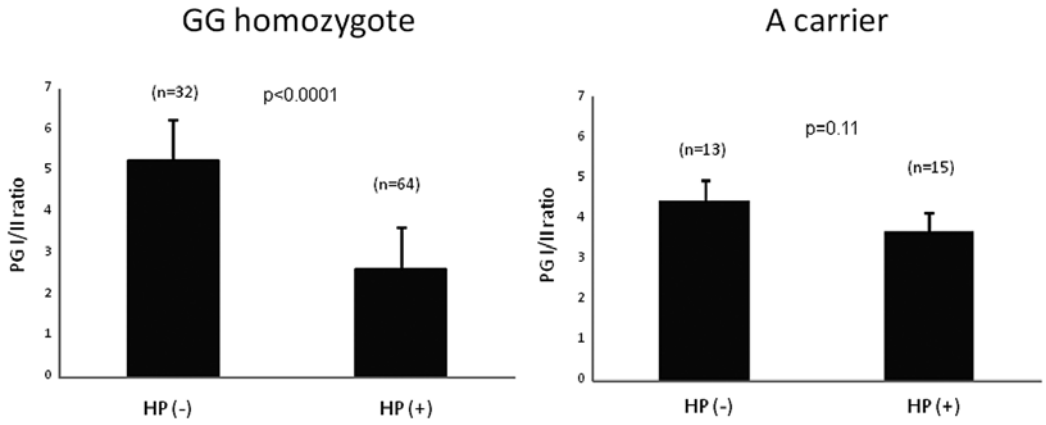

Figure 4. The serum PG I/II ratio and *HRH2* -1018 G>A genotype. In -1018 GG homozygote, PG I/II ratio was significantly lower in *H. pylori* positive than negative subjects. In *H. pylori* positive subjects, PG I/II ratio was significantly lower in GG homozygote than A carrier.

shown that gastric mucosal atrophy was more rapidly progressed with age and under influence of *H. pylori* infection in -1018 GG homozygote than A carrier. The *HRH2* -1018 A allele frequency in non-GC group was 13.5%. This frequency was slightly higher than that reported in Hap-Map JPT and slightly lower than that reported in Japanese by Ito *et al* (32), although it was lower in Caucasians (26,27). One limitation of this study was that our subjects (both cases and controls) came to our hospitals in order to have endoscopic examination for the complaint of abdominal discomfort, or for complete check up of gastric cancer following barium X-ray examination in the health check, not completely healthy subjects. Therefore, minor allele frequency might be comparatively high in our study. Another limitation was that mean age, *H. pylori* infection ratio and male/female ratio were higher in GC group than non-GC group. However, the adjustment for age, gender and *H. pylori* infection status was performed in genotype analysis using logistic regression.

There have been few reports that investigated the influence of polymorphisms of *HRH2* in the risk for human disorders. Most of such studies revealed no association between *HRH2* -1018 G>A polymorphism and psychological or neurological disorder (26,27,32). On the other hand, there is no report on

the association between this polymorphism and gastric carcinogenesis. Our results provided the first evidence that *HRH2* -1018 G>A polymorphism was significantly associated with the gastric carcinogenesis.

It has been well known that *H. pylori* infection has a major role on the progression of gastric mucosal atrophy, subsequently the development of gastric cancer. The factors promoting *H. pylori*-mediated gastric atrophy have been somewhat more controversial. *H. pylori* infection results in an elevation in serum gastrin level in the early stage of infection and precedes the development of atrophic gastritis. Gastrin acting on ECL cell leads to increased histamine release, which stimulate acid secretion through histamine H_2 receptors on parietal cells. A hypergastrinemic mouse at the age of 5 months later shows a marked decline in acid secretion with the spontaneous development of gastric atrophy, metaplasia, and invasive cancer that can be markedly accelerated by concurrent *Helicobacter* infection (33,34). In addition, the majority of clinical studies have accepted that proton pump inhibitors (PPIs), which induce achlorhydria and hypergastrinemia, accelerate the onset of atrophic gastritis in *H. pylori*-positive patients (35-37). The above suggests that hypergastrinemia and/or insufficient acid secretion may promote the gastric mucosal atrophy under influence of *H. pylori* infection. However, Takaishi *et al* have demonstrated that the gastrin-histamine axis contributes to the development of gastric atrophy and neoplasia in a mouse model (38). In contrast to the effects of hypergastrinemia seen in gastrin transgenic mice, long-term treatment of rats and mice with loxtidine, one of potent histamine H_2 receptor antagonists and inducing the ECL cells hyperplasia after long treatment as well as omeprazole (39), did not result in loss of parietal cells but instead appeared to result in increased parietal cells (40,41). Histidine decarboxylase knockout (HDC-/-) mice kept on a low-histamine diet showed an expanded parietal cell pool despite exhibiting marked hypergastrinemia (42). In addition, histamine has been shown to be important in modulating parietal cell maturation through H_2 receptors (43,44). These observations suggest that not only the influence of hypergastrinemia but up-regulated action of histamine with hypergastrinemia may contribute to the gradual down-regulation of parietal cell number, gastric atrophy.

There is no report whether *HRH2* -1018 G>A polymorphism affect on the expression and function of histamine H_2 receptors or not, although understanding the effect of this polymorphism on the histamine signal via H_2 receptor is informative. It is likely that the *HRH2* genome variant located promoter may induce changes in the expression of receptors. In our current study, age-related gastric mucosal atrophy gradually and rapidly progress in *HRH2* -1018 GG homozygote than -1018 A carrier. In addition, PG I/II ratio was significantly decreased in only -1018 GG homozygotes under the influence of *H. pylori* infection. These findings suggested that the action of histamine may be up-regulated in -1018 GG homozygote and A allele may be a loss of function allele.

According to the Lauren classification (45), there are two histologically distinct types of gastric cancer. The intestinal type develops in stomachs affected by chronic inflammation with passing through the intermediate steps of atrophic gastritis or intestinal metaplasia (46). On the other hand, the severity of mucosal inflammation and various host features may directly induce mutagenetic events that ultimately lead to the onset of the diffuse type. Therefore, intestinal type of cancer tends to arise at antrum, because more severe gastric atrophy and metaplasia develop in the early stage of *H. pylori* infection and rapidly progress at antrum. In our results, -1018 GG homozygote was associated with intestinal type of gastric cancer, with the cases in comparative older subjects and located at antrum. These findings suggest that -1018 GG homozygote may have an increased risk of which gastric mucosal atrophy progress more rapidly with age and intestinal type of gastric cancer occur as a result.

Our data showed that -1018 GG homozygote was more closely associated with the cases at comparatively advanced stage, invaded beyond muscularis propria and with lymph node metastasis. This result suggests that -1018 GG homozygote may be associated with the gastric cancer progression, as well as development. Previous reports suggested that cimetidine, one of H_2 receptor antagonists, might be considered as an anti-cancer agent. In 1988, it was firstly reported that post-operative treatment with cimetidine improved survival in gastric cancer patients of all stages (47). This effect of cimetidine is considered to be mediated by H_2 receptor blockade of suppressor T-lymphocytes, leading to their functional inhibition and stimulation of natural killer cell activity (48,49) and antagonism of histamine-stimulated growth (50). Thus, histamine seemed to promote the tumor growth by actions other than stimulation of gastric acid secretion, followed by gastric mucosal atrophy. These actions of histamine may more rapidly progress the gastric cancer in -1018 GG homozygotes.

In conclusion, the current findings indicate that the *HRH2* -1018 G>A polymorphism (rs2607474) may be associated with the susceptibility to gastric carcinogenesis in Japanese population. The -1018 GG homozygote may have an increased risk for the rapid progression of severe gastric mucosal atrophy and the subsequent development of intestinal type gastric cancer.

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