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RAD51 G135C genetic polymorphism and their potential role in gastric cancer induced by *Helicobacter pylori* infection in Bhutan

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SUMMARY

In order to evaluate the role of the RAD51 G135C genetic polymorphism on the risk of gastric cancer induced by *Helicobacter pylori* infection, we determined allele frequency and genotype distribution of this polymorphism in Bhutan – a population documented with high prevalence of gastric cancer and extremely high prevalence of *H. pylori* infection. The status of RAD51 G135C was examined by restriction fragment length polymorphism analysis of PCR amplified fragments and sequencing. Histological scores were evaluated according to the updated Sydney system. G135C carriers showed significantly higher scores for intestinal metaplasia in the antrum than G135G carriers [mean (median) 0·33 (0) *vs.* 0·08 (0), $P = 0{\cdot}008$]. Higher scores for intestinal metaplasia of G135C carriers compared to those of G135G carriers were also observed in *H. pylori*-positive patients [0·3 (0) *vs.* 0·1 (0), $P = 0{\cdot}002$] and *H. pylori*-positive patients with gastritis [0·4 (0) *vs.* 0·1 (0), $P = 0{\cdot}002$] but were not found in *H. pylori*-negative patients. Our findings revealed that a combination of *H. pylori* infection and RAD51 G135C genotype of the host showed an increasing score for intestinal metaplasia. Therefore, RAD51 G135C might be the important predictor for gastric cancer of *H. pylori*-infected patients.

Key words: Gastric cancer, *Helicobacter pylori* infection, polymorphisms, RAD51.

INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium that colonizes the stomach of half of the human population throughout the world [1]. Although there is strong evidence that *H. pylori* infection increases the risk of gastric cancer (GC), the second most frequent cause of cancer-related death [2], the molecular mechanisms of *H. pylori*-associated gastric carcinogenesis remain undefined. Recently, a study provided evidence that *H. pylori* infection introduces DNA double-strand breaks (DSBs) in host cells and prolonged active infection leads to saturation of cellular repair capabilities,

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which may contribute to the genetic instability and frequent chromosomal aberration that are hallmarks of GC [3]. On the other hand, to date, more data suggest that severe *H. pylori*-mediated diseases are associated with not only persistent *H. pylori* infection but also genetic variants of the host [4–7].

Genetic variants could affect mRNA splicing, regulation of transcription, translation efficiency or mRNA stability, leading to altered polypeptide product levels, which could affect the function of proteins such as RAD51 [8–10], which may underlie the variation in clinical outcomes from *H. pylori* infection. RAD51 is a well-known repair protein; it controls DSBs via gene conversion. It has been observed that the RAD51 G135C polymorphism increased activity of RAD51 [10] and was associated with the development of GC [9].

In this study, to further explore whether the RAD51 G135C polymorphism was important in identifying patients who are at a higher risk of developing GC, we investigated the distribution of the genotypes and frequencies of alleles of this polymorphism in Bhutan, a country documented as having an extremely high prevalence of *H. pylori* infection (73·4%) and high prevalence of GC [11, 12]. The age-standardized incidence rate of GC was reported to be high in Bhutan with 17·2 cases/100 000 per year (accessible at http://globocan.iarc.fr/). Indeed, when we performed a survey using gastroduodenal endoscopy in Bhutan in 2010, we found five cases of GC in 372 volunteers [11, 12]. Therefore, we believe that the actual number of GC patients in Bhutan is higher than previously estimated.

SUBJECTS AND METHODS

Subjects

We recruited individuals with dyspeptic symptoms from Bhutanese volunteers. The surveys took place at the Jigme Dorji Wangchuk National Referral Hospital, Thimpu, Bhutan in December 2010. Written informed consent was obtained from all the participants. The protocol was approved by the ethics committee of Oita University Faculty of Medicine (Japan), and by the hospital where sample collection was performed.

During each endoscopy session, three gastric biopsy specimens were obtained from the antrum: one each for *H. pylori* culturing/DNA examination, rapid urease test, and histological examination. Clinical presentations included gastritis, duodenal ulcer (DU), gastric ulcer (GU), and GC. *H. pylori*-positive participants without peptic ulcers and/or gastric malignancy were defined as gastritis. We also collected blood from all volunteers on the same day of endoscopy to evaluate anti-*H. pylori* antibody in serum. Patients with a history of partial gastric resection were excluded. We also excluded patients with using proton pump inhibitors, but not other anti-reflux medication, during the last month. We further excluded patients with previous *H. pylori* eradication therapy (either successful or not).

H. pylori diagnosis

In this study, we selected *H. pylori*-positive cases from *H. pylori*-seropositive cases with additionally at least one more positive test result which included rapid urease test, histology or culture. Patients were considered to be *H. pylori*-negative when all the tests were negative as described in previous studies [11, 12] or when only serology was positive.

Histopathology

Biopsy specimens for histology were fixed in 10% buffered formalin for 24 h, then embedded in paraffin. Serial sections were stained with haematoxylin and eosin and Giemsa stains, followed by evaluation by a single pathologist blinded to the patient's clinical diagnosis or the characteristics of the *H. pylori* strains. Histological analyses of the gastric mucosa were evaluated according to the updated Sydney system [13]. The degree of inflammation, neutrophil activity, atrophy, intestinal metaplasia, and bacterial density were classified into four grades (0, normal; 1, mild; 2, moderate; 3, marked) [14, 15]. All histological analyses were performed by one co-author (T.U.) without knowledge of clinical data or the experimental protocol for the respective tissue.

Genotyping of the RAD51 polymorphism

Genomic DNA was isolated from biopsy specimens which was used for *H. pylori* culture using QIAamp DNA mini-kit (Qiagen, USA). The G135C single nucleotide polymorphisms (SNPs) of the RAD51 gene were determined by restriction fragment length polymorphism analysis of PCR amplified fragments (PCR-RFLP). RAD51 genotyping was analysed by PCR amplification of a 175-bp

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region around nucleotide 135 using the following primers: forward (5'-TGG GAA CTG CAA CTC ATC TGG-3') and reverse (5'-GCG CTC CTC TCT CCA GCAG-3') [16]. This region contained a single *Mva*I site that abolished the 135C allele; therefore only wild-type alleles were digested by *Mva*I resulting in 86-bp and 71-bp products. The digested fragments were separated and visualized by 15% acrylamide gel electrophoresis (Wako Pure Chemical Industries Ltd, Japan). Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM Big Dye™ Terminator v. 3.0 Cycle Sequencing Read Reaction kit (Applied Biosystems, USA). After purification, the sequencing products were visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems).

Statistical analysis

The individual genotype and allele frequencies were analysed using a 2×2 contingency table with χ^2 test to examine the association between each genotype and clinical outcome. All determinants with P values of <0·10 were entered together in the full model of logistic regression, and the model was reduced by excluding variables with P values >0·10. Spearman rank coefficients (r) were also determined to evaluate the association between the different genotypes of the strains. A P value <0·05 was accepted as statistically significant. The SPSS statistical software package v. 19.0 (SPSS Inc., USA) was used for all statistical analyses. Hardy–Weinberg equilibrium was calculated for the genetic polymorphisms by χ^2 test.

RESULTS

Subjects

We selected the first 150 volunteers (mean age 39·0 years, range 16–79 years) who matched our criteria for enrolment in the study, including the criteria for diagnosing *H. pylori* infection, from 372 volunteers examined in our previous study, which was a survey of *H. pylori* prevalence [17]. After exclusion of 12 subjects with failed genotype or undetermined *H. pylori* status (e.g. only seropositive subjects), we successfully identified RAD51 genotyping in 138 subjects (122 with gastritis, three with GU, seven with DU, two GC and four samples without diagnostic information). The characteristics of studying population are described in the Table 1. There was no difference in the male:female ratio in the study population.

Table 1. *Characteristics of the subjects*

Clinical and histological features	(N = 138)
Mean age, years	38·77 ± 13·67
Male, *n* (%)	62 (44·9)
No diagnosis, *n* (%)	4 (2·9)
Gastritis, *n* (%)	122 (88·4)
Gastric ulcer, *n* (%)	3 (2·2)
Duodenal ulcer, *n* (%)	7 (5·1)
Gastric cancer, *n* (%)	2 (1·4)
H. pylori infection, *n* (%)	77 (55·8)
Antrum	
Neutrophil	1·05 (1)
Monocyte	1·4 (1)
Atrophy	1·33 (1)
Intestinal metaplasia	0·13 (0)
H. pylori density	1·12 (0)
Corpus	
Neutrophil	0·68 (1)
Monocyte	1·01 (1)
Atrophy	0·51 (0)
Intestinal metaplasia	0·01 (0)
H. pylori density	1·16 (1)
G/G genotype, *n* (%)	105 (76·1)
G/C genotype, *n* (%)	32 (23·2)
C/C genotype, *n* (%)	1 (0·7)
G allele, *n* (%)	242 (87·8)
C allele, *n* (%)	34 (12·2)

For histological scores minimum to maximum (0–3) and mean (median) values are presented.

Allele frequencies of RAD51 G135C SNPs

All samples were divided into three genotypes of the RAD51 5'-UTR: G135C wild type (G/G), heterozygous (G/C) and mutant homozygous (C/C). The allelic distribution of the RAD51 gene G135C SNPs in Bhutanese subjects is given in Table 1. G135G genotype was more dominant than G135C, C135C genotype was rare and only one subject carried the genotype. Genotypic and allelic frequencies of RAD51 G135C were in agreement with Hardy–Weinberg equilibrium.

RAD51 G135C SNPs and clinical outcome

We examined the association between RAD51 genotypes and clinical outcome (Table 2). Probably due to the small number of DU, GU and GC subjects, the difference between clinical outcome and distribution of the RAD51 genotypes was not found in present study.

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Table 2. *Clinical and histological features of 137 subjects with the allelic distribution of RAD51 G135G and RAD51 G135C*

	Distribution of RAD51 G135G and RAD51 G135C		*P* value	
	G/G ($n = 105$)	G/C ($n = 32$)	*P*†	*P*‡
No diagnosis, *n* (%)	4 (3·8)	0 (0·0)		
Gastritis, *n* (%)	93 (88·6)	28 (87·5)		
Gastric ulcer, *n* (%)	2 (1·9)	1 (3·1)		
Duodenal ulcer, *n* (%)	5 (4·8)	2 (6·2)		
Gastric cancer, *n* (%)	1 (1·0)	1 (3·1)		
H. pylori positive, *n* (%)	56 (53·3)	22 (68·8)	n.s.	
H. pylori negative, *n* (%)	49 (46·7)	10 (31·2)		
Antrum				
Neutrophil	1·06 (1)	1·07 (1)		n.s.
Monocyte	1·44 (1)	1·27 (1)		n.s.
Atrophy	1·34 (1)	1·3 (1)		n.s.
Intestinal metaplasia	0·08 (0)	0·33 (0)		0·008*
H. pylori density	1·04 (0)	1·43 (1·5)		n.s.
Corpus				
Neutrophil	0·65 (1)	0·77 (1)		n.s.
Monocyte	0·99 (1)	1·1 (1)		n.s.
Atrophy	0·49 (0)	0·61 (0)		n.s.
Intestinal metaplasia	0·02 (0)	0 (0)		n.s.
H. pylori density	1·08 (0·5)	1·48 (2)		n.s.

n.s., Not significant ($P > 0·05$).
For histological scores minimum to maximum (0–3) and mean (median) values are presented.
† By Pearson's χ^2 test.
‡ By Mann–Whitney test.
* Significant at $P < 0·05$.

RAD51 G135C SNPs and histological findings

In all subjects, histological scores for activity, inflammation and atrophy were not different between G135G and G135C carriers both in the antrum and corpus (Table 2). However, G135C carriers showed significantly higher scores for intestinal metaplasia in the antrum than G135G carriers [mean (median) 0·33 (0) *vs.* 0·08 (0), $P = 0·008$] (Table 2). Interestingly, higher scores for intestinal metaplasia of G135C carriers compared to G135G carriers were also observed in *H. pylori*-positive volunteers [mean (median) 0·3 (0) *vs.* 0·1 (0), $P = 0·002$], and even limited to *H. pylori*-positive volunteers with gastritis [mean (median) 0·4 (0) *vs.* 0·1 (0), $P = 0·002$] (Table 3). By contrast, these differences were not observed in either *H. pylori*-negative volunteers or *H. pylori*-negative gastritis subjects. Regarding *H. pylori*-negative volunteers and *H. pylori*-negative gastritis volunteers, G135G genotype showed higher atrophy scores in the antrum compared to G135C genotype ($P = 0·02$ in both cases).

DISCUSSION

This is the first study to show severe *H. pylori*-mediated diseases being associated with the RAD51 G135C polymorphism. A previous study showed a strong association between RAD51 G135C polymorphism and the occurrence of GC in individuals with high levels of oxidative DNA damage or impaired repair of such damage [9]. However, that study did not examine *H. pylori* status, which is well known as a pathogen that can cause DNA damage in host gastric epithelial cells and a source of reactive oxygen species (ROS). In addition, the ethnicity of the subjects enrolled in that study were not described in detail. In the present study, all the enrolled subjects were selected from volunteers with a low number of GC cases, which is a limitation of this study; thus the association between GC and RAD51 G135C polymorphism was not found. However, the association between gastric intestinal metaplasia and adenocarcinoma of stomach is well known [18–20] and we found a strong association between RAD51 G135C polymorphism and higher intestinal metaplasia score in the antrum in *H. pylori*-positive subjects in Bhutan. The correlation coefficient of G135C genotype with high score of intestinal metaplasia in the antrum were highest in *H. pylori*-positive gastritis subjects ($r = 0·4$, $P = 0·001$) followed by *H. pylori*-positive subjects ($r = 0·3$, $P = 0·002$) and gastritis subjects ($r = 0·2$, $P = 0·007$). Interestingly, this association was not found in *H. pylori*-negative volunteers. While G135G genotype was evenly distributed in *H. pylori*-negative and *H. pylori*-positive subjects (47% *vs.* 53%), prevalence of G135C genotype in *H. pylori*-negative subjects was less than two-fold lower in *H. pylori*-positive subjects (31% *vs.* 69%). Compared to G135C carriers, G135G carriers only showed higher atrophy scores in the antrum in *H. pylori*-negative subjects with a very limited number of G135C carriers. Our findings reveal that a combination of *H. pylori* infection and RAD51 G135C genotype of the host showed an increasing intestinal metaplasia score, which might be useful for predicting the risk of GC.

The severity of *H. pylori*-induced gastric diseases are not only associated with the bacterium's persistence in the host but also with the adaptation of

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Table 3. *The correlation of RAD51 G135C SNPs and histological score*

		Antrum					Corpus				
	n	Neutro	Mono	Atrophy	IM	*H. pylori* density	Neutro	Mono	Atrophy	IM	*H. pylori* density
H. pylori (+)											
G/G	55	1·7 (2)	1·9 (2)	1·5 (1)	0·1 (0)	2 (2)	1 (1)	1·3 (1)	0·6 (0)	0 (0)	2 (2)
G/C	21	1·5 (2)	1·6 (1)	1·6 (2)	0·3 (0)	2·1 (3)	1·1 (1)	1·4 (1)	0·8 (1)	0 (0)	2·2 (3)
P value		n.s.	n.s.	n.s.	0·002*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
H. pylori (–)											
G/G	48	0·4 (0)	0·9 (1)	1·1 (1)	0·1 (0)	0 (0)	0·2 (0)	0·6 (1)	0·3 (0)	0 (0)	0 (0)
G/C	10	0·1 (0)	0·5 (1)	0·7 (0)	0·3 (0)	0 (0)	0·2 (0)	0·4 (0)	0·3 (0)	0 (0)	0 (0)
P value		n.s.	n.s.	0·02*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gastritis											
G/G	93	1 (1)	1·4 (1)	1·3 (1)	0·1 (0)	1 (0)	0·7 (1)	1 (1)	0·5 (0)	0 (0)	1·1 (0)
G/C	28	1 (1)	1·2 (1)	1·3 (2)	0·4 (0)	1·3 (0)	0·7 (1)	1·1 (1)	0·7 (1)	0 (0)	1·5 (2)
P value		n.s.	n.s.	n.s.	0·008*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gastritis with *H. pylori* (+)											
G/G	47	1·7 (2)	1·9 (2)	1·5 (2)	0·1 (0)	2 (2)	1·1 (1)	1·3 (1)	0·7 (1)	0 (0)	2·1 (2)
G/C	19	1·5 (2)	1·6 (1)	1·6 (2)	0·4 (0)	1·9 (2)	1·1 (1)	1·5 (2)	0·9 (1)	0 (0)	2·2 (3)
P value		n.s.	n.s.	n.s.	0·002*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gastritis with *H. pylori* (–)											
G/G	45	0·4 (0)	0·9 (1)	1·1 (1)	0·1 (0)	0 (0)	0·3 (0)	0·7 (1)	0·3 (0)	0 (0)	0 (0)
G/C	9	0·1 (0)	0·4 (0)	0·7 (0)	0·3 (0)	0 (0)	0 (0)	0·3 (0)	0·2 (0)	0 (0)	0 (0)
P value		n.s.	n.s.	0·02*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s., Not significant ($P > 0·05$); IM, intestinal metaplasia.
For histological scores minimum to maximum (0–3) and mean (median) values are presented.
P values by Mann–Whitney test.
* Significant at $P < 0·05$.

bacterium to host differences, which in turn is associated with the host's remarkable genetic variability. GC with *H. pylori* infection shows genetic instability, besides impairment of important DNA repair pathways [21–25]. A number of studies have demonstrated that *H. pylori* infection induces the synthesis of ROS and causes DNA damage [3, 22, 26] or decreases the activity of DNA repair pathways in the host [27–29], and that a lengthy period of infection will increase the risk for development of GC. In response to DNA damage, human RAD51 has been described as a well-known protein functioning in DNA repair. RAD51 has been shown to be involved in the repair of different kinds of DNA lesions during replication and it promotes genomic stability in eukaryotic cells [30, 31]. The increased DSB repair capacity might be followed by RAD51 up-regulation. However, the overexpression of RAD51 also affects other cellular processes influencing cell survival, cell cycler progression or promotion of apoptosis in the cells [32, 33]. The higher mRNA expression of RAD51 in tumour tissue of GC compared to normal tissue was observed in a previous study [34]. Moreover, the RAD51 G135C polymorphism was expected to result in increased activity of RAD5 [10]. These findings support for the hypothesis that the contribution of RAD51 G135C polymorphism and *H. pylori* infection might be responsible for the aberrant increase in RAD51 expression following increased risk of GC. In future work the importance of such polymorphisms for outcome of *H. pylori*-associated diseases should be studied in more detail using a larger number of *H. pylori* infection with GC.

In conclusion, our results reveal that RAD51 G135C SNPs might be an important predictor of GC in *H. pylori*-infected patients.

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DECLARATION OF INTEREST

None.

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