Diagnosis of gallstones

The presence of gallstones in the gallbladder and the bile duct were diagnosed by abdominal ultrasonography. It was judged from the characteristic findings: (1) highly reflective echo from the anterior surface of the stone, (2) mobility of the stone by repositioning the patient, and (3) marked posterior acoustic shadowing. In our analysis, biliary sludge was not included in "gallstones", since sludge often disappears, as opposed to gallstones.

Evaluation of serum anti-*HP* antibody and serum pepsinogen levels

Serum anti-*HP* antibody was measured using a commercial enzyme immunoassay (EIA) kit (E-plate "EIKEN" *H. pylori* antibody, EIKEN Chemical Co Ltd, Tokyo, Japan). According to the manufacture's instruction, the antibody titer above 10 U/ml was considered as *H. pylori*-positive. Serum pepsinogen I and II were measured using a commercial latex agglutination reaction (LAR) kit (LZ test "EIKEN" pepsinogen I and pepsinogen II, EIKEN Chemical Co Ltd).

Questionnaires

A detailed questionnaire including inquiries about upper gastrointestinal tract-related symptoms, medical history, family history, lifestyle factors, etc., was given to all the participants. Answers filled in by the participants were carefully checked by the nursing staff before being recorded into our study database. To evaluate the factors associated with gallstones as simply as possible, we excluded subjects with regular intake of proton pump inhibitors (PPI) due to its modification of *HP* infectious status [24, 25], subjects taking antidiabetic drugs due to its influence upon insulin resistance [26, 27], and subjects taking anti-cholesterol drugs due to its possible preventive effect upon gallstone formation [28–30]. Accordingly, our questionnaire included 6 yes–no questions regarding regular intake of antidiabetics, anticholesterol drugs and PPI, and history of gastrectomy, cholecystectomy or *HP* eradication. In addition, we graded alcohol intake and smoking on a 5-grade scale (never, seldom, sometimes, often, and always), and further categorized alcohol intake and smoking into 2 groups: lower alcohol intake (never, seldom) vs. higher alcohol intake (sometimes, often, always), and current or past habitual smoker vs. nonsmoker.

Statistical analysis

We used JMP9 software or SAS 9.1.3 (SAS Institute Inc. Cray, NC, USA) for statistical analyses and matching process. In univariate analyses, odds ratios and 95 % confidence intervals were calculated by using Wilcoxon's rank-sum test and Fisher's exact test. Logistic regression analysis was conducted to assess the odds ratio for the presence of gallstones using the following covariates: age, gender, height, weight, body mass index (BMI), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), serum gamma glutamyltransferase (γ-GTP), serum high density lipoprotein cholesterol (HDL-Chol), serum low density lipoprotein cholesterol (LDL-Chol), serum triglyceride (TG), serum total protein (TP), serum albumin (Alb), hemoglobin concentration (Hb), mean cell volume of red blood cells (MCV), glycated hemoglobin (HbA1c), serum pepsinogen I (PG I), serum pepsinogen II (PG II), ratio of pepsinogen I/pepsinogen II (PG I/II), *HP* IgG antibody, habit of alcohol intake, and habit of smoking. In the final steps, standardized coefficient of each variable was calculated using multiple logistic regression analysis.

In the analysis of the effect of *HP* eradication on gallstone prevalence, Cochran–Armitage test was applied. In the matched pair analysis between the "*HP*-positive" subjects and "*HP*-eradicated" subjects, McNemar's test was used. Matching criteria consisted of gender, age (±3), BMI (±2), serum γ-GTP (±10), PG I/II ratio (±1), and habit of alcohol intake (lower alcohol intake or higher alcohol intake).

Results

Characteristics of study subjects

Of the 20,773 subjects who participated in this study, subjects over 20 years of age, who had undergone sufficient physical examination and abdominal ultrasonography, completely answered the questionnaires, and having sufficient blood test data including anti-*HP* IgG antibody were included. Of the 19,583 subjects who met these inclusion criteria (Fig. 1), we then excluded subjects with a history of gastrectomy or cholecystectomy, and regular intake of PPI or antidiabetic drugs or anticholesterol drugs. Regardless of the present *HP* infection status, all subjects with a history of *HP* eradication were assessed separately.

The primary study population of 15,551 subjects comprised of 8,625 men and 6,926 women with a mean age of 48.9 ± 9.1 years (range 20–87 years, Fig. 1). Among them, gallstones were detected in 694 subjects (4.47 %), which comprised of 409 men (4.74 %) and 285 women (4.11 %) with a mean age of 52.9 ± 8.2 years. Among the 1,333 subjects with a history of *HP* eradication, 276 were still positive for anti-*HP* antibody (Fig. 1). The residual 1,057 subjects, whose anti-*HP* antibody had converted to negative, were considered as "*HP*-eradicated" subjects who had succeeded in *HP* eradication.

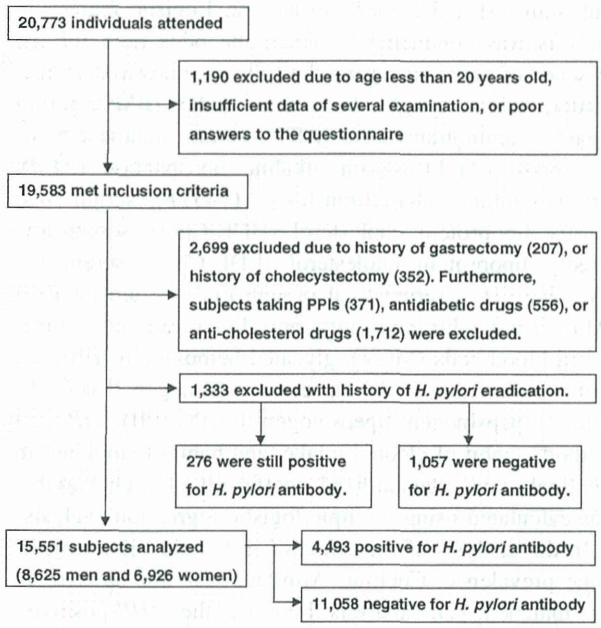

Fig. 1 Study recruitment flowchart. Of the 20,773 healthy adults attended, 15,551 subjects were mainly analyzed in our present study. The subcategory of 1,057 subjects who certainly succeeded in *Helicobacter pylori* eradication was also analyzed

Univariately correlated factors for gallstones

Focusing on the 21 continuous variables and 4 categorized variables mentioned above, their associations with the existence of gallstones were univariately analyzed (Tables 1, 2). Among the 25 examined factors, age, *HP* status, habit of alcohol intake, weight, BMI, AST, ALT, ALP, γ-GTP, T-Chol, HDL-Chol, LDL-Chol, TG, TP, Hb, HbA1c, PG I, PG II, and PG I/II displayed statistical significance. On the other hand, gender, height, smoking habit, Alb, T-Bil and MCV displayed no statistically significant association: all of them but gender were therefore excluded from the next step of multivariate analysis. We further excluded weight, AST, and T-Chol, because each coefficient of correlation was high; 0.840 between weight and BMI, 0.808 between AST and ALT, and 0.884 between T-Chol and LDL-Chol. Consequently, we focused on the remnant 17 variables for multivariate analysis.

Multivariate analysis evaluating background factors for gallstones

As shown in Table 3, multivariate analysis using the 17 factors demonstrated that eight factors showed statistical significance in association with the presence of gallstones. We next selected factors that showed significant association with $p < 0.01$ in order to construct the more accurate predictive model, and consequently five factors were selected: age, BMI, γ-GTP, alcohol intake, and *HP* status. We added two more variables used in the final-step multivariate analysis: gender as a basic factor and PG I/II ratio as a sensitive marker reflecting the intragastric environment [20, 21, 31].

Table 1 Characteristics of the 15,551 subjects with or without gallstones, focusing on correlation with 21 continuous variables

Variables	Presence of gallstones ($n = 694$)	Absence of gallstones ($n = 14,857$)	p value
Age (years)	52.9 ± 8.2	48.7 ± 9.1	<0.001*
Height (cm)	164.8 ± 8.7	165.0 ± 8.6	0.531
Weight (kg)	64.64 ± 11.97	62.02 ± 11.84	<0.001*
BMI (kg/m^2)	23.70 ± 3.39	22.67 ± 3.24	<0.001*
AST (IU/l)	22.8 ± 12.2	21.6 ± 12.0	0.022*
ALT (IU/l)	25.5 ± 30.3	23.2 ± 17.5	0.004*
ALP (IU/l)	205.1 ± 67.7	192.6 ± 57.0	<0.001*
γ-GTP (IU/l)	46.2 ± 64.9	35.8 ± 40.6	<0.001*
T-Bil (mg/dl)	0.86 ± 0.38	0.82 ± 0.32	0.114
T-Chol (mg/dl)	205.4 ± 33.3	201.6 ± 32.6	0.004*
HDL-Chol (mg/dl)	62.3 ± 16.9	65.4 ± 16.8	<0.001*
LDL-Chol (mg/dl)	127.6 ± 30.7	123.2 ± 31.0	<0.001*
TG (mg/dl)	121.2 ± 121.2	103.1 ± 72.2	<0.001*
TP (g/dl)	7.12 ± 0.38	7.07 ± 0.37	<0.001*
Alb (g/dl)	4.31 ± 0.23	4.32 ± 0.23	0.2124
Hb (g/dl)	14.28 ± 1.41	14.07 ± 1.50	<0.001*
MCV (fl)	92.18 ± 4.99	92.07 ± 5.16	0.992
HbA1c (%)	5.47 ± 0.58	5.38 ± 0.47	<0.001*
PG I (ng/ml)	53.82 ± 26.30	51.14 ± 23.09	0.002*
PG II (ng/ml)	12.93 ± 9.38	11.53 ± 8.92	<0.001*
PG I/II ratio	5.13 ± 2.09	5.46 ± 2.02	<0.001*

Data show mean ± SD (standard deviation) of each variable. By applying the Wilcoxon analysis, p values were calculated

* The level of significance is set below 0.05

Finally, odds ratios (OR) and standardized coefficients (β) of these seven factors were calculated (Table 4). The multiple logistic regression analysis revealed that positively associated factors for gallstones were older age (OR = 1.57, $\beta = 0.450$), higher BMI (OR = 1.30, $\beta = 0.263$), positivity of anti-*HP* antibody (OR = 1.49, $\beta = 0.200$), lower alcohol intake (OR = 1.33, $\beta = 0.141$), higher γ-GTP level (OR = 1.15, $\beta = 0.138$), and higher PG I/II ratio (OR = 1.13, $\beta = 0.112$).

Effect of *HP* eradication on gallstones

The multivariate analysis indicated that *HP* infection shows statistically significant positive association with the presence of gallstones (Tables 3, 4). We therefore speculated that *HP* eradication might lead to a state of fewer gallstones. To validate this speculation, the

Table 2 Characteristics of the 15,551 subjects with or without gallstones, focusing on correlation with four categorized variables

Variables	Presence of gallstones (%)	Absence of gallstones (%)	*p* value	OR	95 % CI
HP			<0.001*	1.63	1.40–1.91
Positive	273 (6.1)	4,220 (93.9)			
Negative	421 (3.8)	10,637 (96.2)			
Alcohol drinking			0.023*	0.83	0.71–0.97
Lower intake	396 (4.2)	9,129 (95.8)			
Higher intake	298 (4.9)	5,728 (95.1)			
Smoking			0.508	1.05	0.90–1.23
Smoker	331 (4.6)	6,895 (95.4)			
Nonsmoker	363 (4.4)	7,962 (95.6)			
Gender			0.061	1.16	0.99–1.35
Men	409 (4.7)	8,216 (95.3)			
Women	285 (4.1)	6,641 (95.9)			
Total	694 (4.5)	14,857 (95.5)			

By applying the Fisher's exact test, *p* values were calculated

OR odds ratio, *CI* confidential interval

* The level of significance is set below 0.05

Table 3 Multivariate analysis evaluating correlation between gallstone formation and 17 selected variables

Variables	Coefficient	Standard error	*p* value
Age	0.048	0.005	<0.001*
Gender (men)	−0.134	0.061	0.028*
BMI	0.072	0.013	<0.001*
ALT	−0.003	0.003	0.250
ALP	0.0003	0.0007	0.639
γ-GTP	0.003	0.0008	<0.001*
HDL-Chol	−0.004	0.003	0.168
LDL-Chol	−0.003	0.001	0.035*
TG	0.006	0.0005	0.180
TP	0.252	0.111	0.024*
Hb	0.070	0.040	0.078
HbA1c	0.003	0.077	0.972
PG I	0.005	0.003	0.089
PG II	−0.015	0.010	0.142
PG I/II ratio	0.012	0.038	0.755
HP (positive)	0.200	0.062	0.001*
Higher alcohol-intake	−0.135	0.044	0.002*

By applying the logistic regression analysis, *p* values were calculated

* The level of significance is set below 0.05

Table 4 Positively correlated variables for gallstone formation in humans

Variables	Odds ratio (95 % confidence interval)	Standardized coefficient	*p* value
Age	1.57 (1.44–1.71)	0.450	<0.001*
BMI	1.30 (1.21–1.40)	0.263	<0.001*
HP	1.49 (1.19–1.87)	0.200	<0.001*
Lower alcohol-intake	1.33 (1.12–1.57)	0.141	0.001*
γ-GTP	1.15 (1.08–1.21)	0.138	<0.001*
PG I/II ratio	1.13 (1.01–1.26)	0.112	0.043*
Gender (women)	1.08 (0.90–1.29)	0.035	0.435

Using the logistic regression analysis, *p* values were calculated

* The level of significance is set below 0.05, and the seven variables are shown in descending order of significance

Table 5 Prevalence of gallstone in *HP*-positive, *HP*-eradicated, or *HP*-negative subjects

	Presence of gallstones (%)	Absence of gallstones (%)
HP positive (without eradication)	273 (6.08)	4,220 (93.92)
HP negative after eradication	50 (4.73)	1,007 (95.27)
HP negative (without eradication)	421 (3.81)	10,637 (96.19)

Cochran–Armitage test; $p < 0.0001$

above-mentioned 1,057 subjects who had already succeeded in *HP* eradication were compared to the 4,493 subjects positive for anti-*HP* antibody and 11,058 subjects negative for anti-*HP* antibody (Fig.).

As shown in Table , gallstones were detected in 6.08 % of the 4,493 *HP*-positive subjects (who did not have a history of *HP* eradication therapy), 4.73 % of the 1,057 *HP*-eradicated subjects, and 3.81 % of the 11,058 *HP*-negative subjects (who did not have a history of *HP* eradication therapy). The Cochran–Armitage test showed a statistically significant trend in the reduction of gallstones among subjects who underwent *HP* eradication ($p < 0.0001$). Furthermore we compared gallstone prevalence between the *HP*-positive subjects and the *HP*-eradicated subjects by matched-pair analysis, using six factors derived from our multivariate analysis (Table). The 741 *HP*-positive subjects and 741 *HP*-eradicated subjects were matched according to age (±3), BMI (±2), γ-GTP (±10), PG I/II ratio (±1), drinking habit and gender. Among them, gallstones were detected in 6.74 % of the *HP*-positive

subjects (50/741) and 4.32 % of the *HP*-eradicated subjects (32/741): the McNemar's test showed a statistically significant difference ($p = 0.0415$).

Discussion

Prevalence and trend of gallstones in Japanese population

The prevalence of gallstone in our primary study population of 15,551 subjects was 4.7 % in men and 4.1 % in women (Table 2). It was significantly lower in comparison with previous large-scale studies, such as 7.9 % (men) and 16.6 % (women) in USA [7], 9.5 % (men) and 18.9 % (women) in Italy [4], 29.5 % (men) and 64.1 % (women) in American Indians [3], etc. Additionally, most of the identified background factors (Tables 3, 4) showed trends similar to those in previous reports [1, 4, 7, 17], whereas gender presented a very distinctive feature in our analysis. Many reports from Europe and North America have demonstrated that the female gender is an evident risk for gallstones [2, 4, 7, 9], but in our cohort, the prevalence of gallstone in women is even lower than that in men (Table 2). Our results are consistent with the generally accepted view that gallstone prevalence rate of both men and women are low in East Asia, which was recently confirmed by the report analyzing 3,333 Chinese adults (gallstone prevalence was 4.6 % of men and 5.4 % of women) [2, 11, 32]. Based on these, we speculate that gender is not a risk factor of gallstones for East Asians.

Risk factors for gallstones in Japan

Many risk factors for gallstones have been reported [1], based on epidemiological studies. Consistent with many previous reports [3–7, 9], our study of the Japanese population also demonstrated that age was one of the strongest risk factors for gallstones and low alcohol consumption was associated with an elevated risk of gallstones, whereas smoking seemed to have no influence on them (Tables 2, 3, 4).

BMI has also been reported as an obvious risk factor for gallstones [6, 7, 9]. Our results likewise demonstrated significant association between higher BMI and the presence of gallstones (Tables 3, 4). As more than half of gallstones are thought to be cholesterol stones [1, 2], dyslipidemia associated with higher BMI would be a likely cause for gallstone disease. However, quite a few reports have claimed that there is no relation between dyslipidemia and the presence of gallstones [32, 33]. In our analysis, serum LDL level showed negative association with the presence of gallstones, whereas the serum TG and HDL levels denoted no significant association (Table 3). Taking all into consideration, we must say that the relation between dyslipidemia and gallstones is still controversial, as was reported in the recent review [2].

Influence of *HP* infection on gallstone

HP is well known as a cause of many gastroduodenal diseases such as atrophic gastritis, gastric ulcer, duodenal ulcer, gastric carcinoma, and MALT lymphoma [34]. Although the effect of chronic *HP* infection upon organs other than the stomach or duodenum have been seldom evaluated, several studies using human samples suggested the possible correlation between *HP* infection and gallstones; RNA, DNA, antibodies, and antigens specific for *HP* were repeatedly detected in bile, gallbladder, and gallstones [35–41]. In addition, other *Helicobacter* species (*H.* spp) such as *H. hepaticus* have often been detected in bile samples of cholelithiasis patients [42]. These reports accordingly mentioned the possible role of the *H.* spp including *HP* in gallstone formation, though there have been conflicting results for differences between the gallstone patients and gallstone-free subjects [37, 40]. In an animal study using highly gallstone-susceptible C57L/J mice, it was clearly demonstrated that *H.* spp promoted gallstone formation [43]. Various mechanisms have been consequently speculated: *H.* spp as the nuclei for gallstones, affecting systemic immune responses, modulation of enterohepatic cycling of conjugated bile acids, and so forth [43]. However, the exact role of *H.* spp on gallstone formation is still a matter of speculation, and it is also obscure whether this finding in gallstone-prone mice can be applied to man with chronic *HP* infection [44].

By analysis of the large-scale cohort of more than 15,000 adults, our study for the first time showed positive association between *HP* infection and presence of gallstones in human (Table 4). Detailed analysis of subjects who have succeeded in *HP* eradication also supported our finding of "gallstone-prone" tendency in a state of chronic *HP* infection (Table 5 and the matched-pair analysis). Based on these results, we conclude that *HP* infection is a risk factor of gallstone formation in humans, although its precise mechanism should be elucidated in the future.

Future prospects and current limitation

For upcoming prospective analyses, we are going to continue monitoring the present large-scale cohort comprised of a total of 19,583 subjects (Fig. 1); surveillance of bile duct and gallbladder by ultrasonography will be continued, along with evaluation of all the 25 factors (Table 1). Results of the present cross-sectional analyses will be validated in these prospective cohort studies; in particular,

longtime follow-up of the *HP*-positive group, *HP*-eradicated group, and *HP*-negative group (Table) should verify our present conclusion that *HP* increases the risk of gallstones. In addition, we are also planning a randomized prospective study investigating the effect of *HP* eradication on cholelithiasis patients. Because both *HP* infection and gallstones are very common disorders worldwide, it is important to clarify whether *HP* eradication certainly has the potential to prevent gallstones.

One of the limitations of our study was the diagnostic method of gallstones. We might fail detecting some gallstones, since we did not perform cholecystectomy or lithotomy in diagnosis. Another limitations of our study was lacking the information of gallstone types. The mechanism and risks of gallstone formation are assumed to be different among gallstone types [,]. Therefore, by using operatively or endoscopically removed gallstones, an association between gallstone types and the status of *HP* infection should be further evaluated in the future. Based on accumulated evidences from epidemiological studies, precise physiological mechanism of *HP* infection on gallstone formation must be eventually elucidated.

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Conflict of interest The authors declare that they have no conflict of interest.

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TOPIC HIGHLIGHT

An updated review of gastric cancer in the next-generation sequencing era: Insights from bench to bedside and *vice versa*

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Abstract

Gastric cancer (GC) is one of the most common malignancies and remains the second leading cause of cancer-related death worldwide. There is an increasing understanding of the roles that genetic and epigenetic alterations play in GCs. Recent studies using next-generation sequencing (NGS) have revealed a number of potential cancer-driving genes in GC. Whole-exome sequencing of GC has identified recurrent somatic mutations in the chromatin remodeling gene *ARID1A* and alterations in the cell adhesion gene FAT4, a member of the cadherin gene family. Mutations in chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) have been found in 47% of GCs. Whole-genome sequencing and whole-transcriptome sequencing analyses have also discovered novel alterations in GC. Recent studies of cancer epigenetics have revealed widespread alterations in genes involved in the epigenetic machinery, such as DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs and microRNAs. Recent advances in molecular research on GC have resulted in the introduction of new diagnostic and therapeutic strategies into clinical settings. The anti-human epidermal growth receptor 2 (HER2) antibody trastuzumab has led to an era of personalized therapy in GC. In addition, ramucirumab, a monoclonal antibody targeting vascular endothelial growth factor receptor (VEGFR)-2, is the first biological treatment that showed survival benefits as a single-agent therapy in patients with advanced GC who progressed after first-line chemotherapy. Using NGS to systematically identify gene alterations in GC is a promising approach with remarkable potential for investigating the pathogenesis of GC and identifying novel therapeutic targets, as well as useful biomarkers. In this review, we will summarize the recent advances in the understanding of the molecular pathogenesis of GC, focusing on the potential use of these genetic and epigenetic alterations as diagnostic biomarkers and novel therapeutic targets.

Key words: Next-generation sequencing; Microsatellite instability; MicroRNA; Epigenetic field defect; Gastric washes; Insulin-like growth factor 1 receptor

Core tip: The genetic and epigenetic alterations in gastric cancers (GC) have biological and clinical implications. Recent advances in the molecular research of GC have introduced new diagnostic and therapeutic strategies to clinical settings. In this review, we summarize the key findings of past reports pertaining to the genetics and epigenetics of GC and their relationship to and future applications in next-generation sequencing (NGS). We also describe the recurrently mutated genes and alterations in GC identified by NGS technology and discuss the basic framework for future investigations, including the challenges of using NGS as a tool for biomarker and therapeutic target discovery.

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INTRODUCTION

Gastric cancer (GC) is the second highest cause of global cancer mortality. GC is a heterogeneous disease with multiple environmental etiologies and alternative pathways of carcinogenesis[1,2]. One of the major etiologic risk factors for GC is *Helicobacter pylori* (*H. pylori*) infection, but only a small proportion of individuals infected with *H. pylori* develop GC[3,4]. There is an increasing understanding of the roles that genetic and epigenetic alterations play in GCs (Figure 1). Consequently, the development of appropriate biomarkers that reflect an individual's cancer risk is essential to reduce the mortality from GC[5,6]. Recent advances in molecular research of GC have brought new diagnostic and therapeutic strategies into clinical settings.

Next-generation sequencing (NGS) is a technology that involves the parallel sequencing of enormous amounts of short DNA strands from randomly fragmented copies of a genome[7,8]. NGS methods used for genome[9], exome[10], epigenome[11] and transcriptome[12] sequencing have the potential to provide novel avenues towards achieving a comprehensive understanding of diseases, including cancer[13,14]. Such advances have also shown puzzling tumor heterogeneity with limited somatic alterations shared between tumors of the same histopathologic subtype[15-17]. Although NGS techniques are just beginning to expand our abilities to detect genome-wide alterations in GC, several NGS studies in GC have recently been published[18].

In this review, we summarize the key findings of past reports pertaining to the genetics and epigenetics of GC and their relationship to and future application in NGS. We also describe the recurrently mutated genes and alterations in GC identified by NGS technology and discuss the basic framework for future investigations, including the challenges of using NGS as a tool for biomarker and therapeutic target discovery.

MICROSATELLITE INSTABILITY

A type of genetic instability characterized by alterations in length within simple repeat microsatellite sequences, termed microsatellite instability (MSI), occurs in approximately 15% of sporadic GCs, mainly as a result of epigenetic changes[19-22]. Genetic and epigenetic inactivation of DNA mismatch repair (MMR) genes leads to the mutator phenotype, mutations in cancer-related genes and cancer development (Figure 2). MSI underlies a distinctive carcinogenic pathway because MSI-positive (MSI^+) GCs exhibit many differences in clinical, pathological and molecular characteristics compared with MSI-negative (MSI^-) GCs[19-22]. The differences in genotype occur because defective MMR results in a strong mutator phenotype with a very specific mutation spectrum. MSI mainly accumulates frameshift mutations in the repeated sequences located in the coding regions of a target tumor suppressor or other tumor-related genes[23-26]. The atypical genotype of MSI^+ GCs also includes specific patterns of gene dysregulation. MSI^+ GCs often show epigenetic alterations, such as hypermethylation of various genes, including the key MMR gene *MLH1*. The differences in genotype and phenotype between MSI^+ and MSI^- GCs are likely linked to their differences in biological and clinical features. Recent findings from NGS analysis, such as the frequent mutation of the AT-rich interactive domain 1A (ARID1A) in MSI^+ GCs, support this notion[27,28].

The clinicopathological, genetic, epigenetic, prognostic and therapeutic characteristics of MSI^+ GCs are becoming clearer, but further research is still required. Because molecular targeting therapeutics are being used in clinical settings and trials, the differential regulation of molecular target genes in MSI^+ and MSI^- GCs[29,30] needs to be clarified. Diagnostic characterization of the MSI status of GCs thus has important implications for basic and clinical oncology.

Frequent inactivating mutations of ARID1A in molecular subtypes of GC identified by exome sequencing

Holbrook *et al*[31] analyzed 50 GC samples with targeted deep sequencing of the DNA of 384 genes. In addition to the previously reported mutations in genes belonging to various pathways, the authors found tractable target genes, such as the genes for the thyrotropin receptor and the Rho-associated coiled-coil containing protein kinases ROCK1 and ROCK2. Wang *et al*[27] performed exome

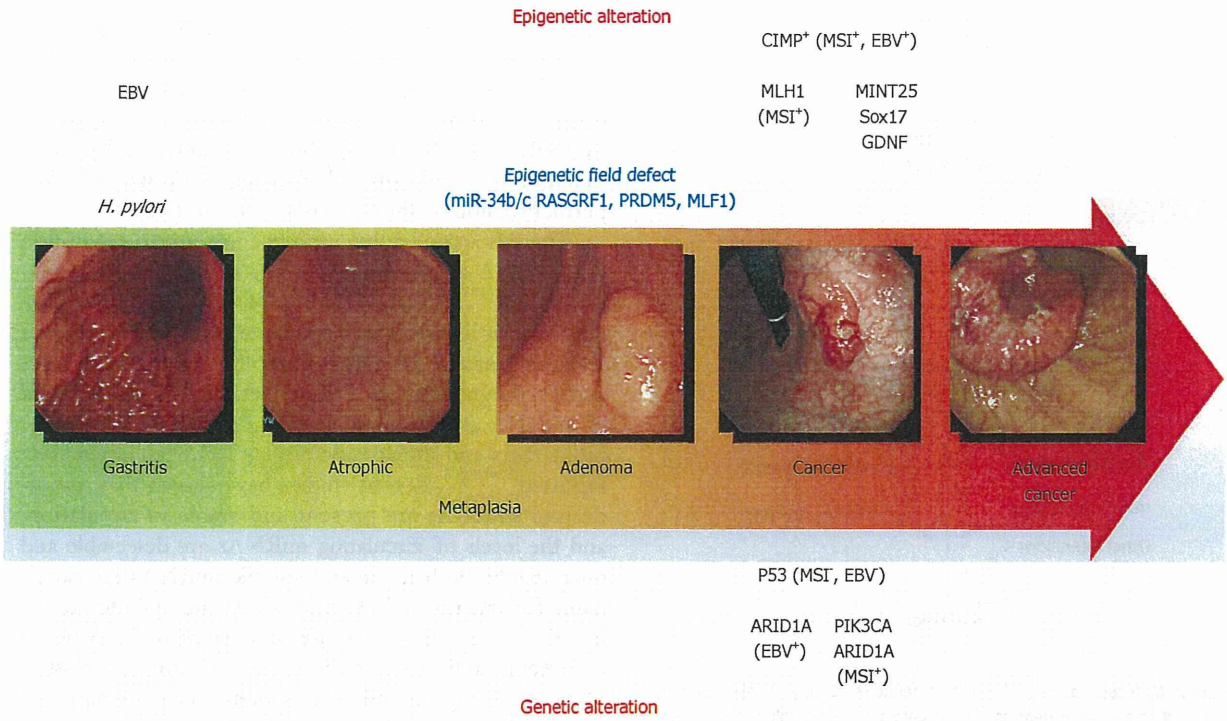

Figure 1 Genetic and epigenetic alterations in gastric carcinogenesis. The model for gastric carcinogenesis is presented based on genetic and epigenetic alterations. Methylation of the genes in blue appears to be involved in an epigenetic field defect. *H. pylori*: *Helicobacter pylori*; MSI: Microsatellite instability; EBV: Epstein-Barr virus; CIMP: CpG island methylator phenotype.

sequencing of 22 GC samples and found novel mutated genes and pathway alterations involved in chromatin modification. A validation study confirmed frequent inactivating mutations or protein loss of the ARID1A gene, which encodes one of the subunits in the Switch/Sucrose Nonfermentable (SWI-SNF) chromatin remodeling complex. The mutation spectrum for ARID1A differed among molecular subtypes of GC; mutations were detected in 83% of GCs with MSI, 73% of GCs with EBV infection and 11% of GCs without EBV and MSI. Moreover, ARID1A mutations were negatively associated with TP53 mutations. ARID1A alterations were associated with better prognosis in a stage-independent manner. These results suggest the importance of altered chromatin remodeling in the pathogenesis of GC.

Recurrent somatic mutations in cell adhesion and chromatin remodeling genes identified by exome sequencing

Zang *et al*[28] also analyzed a spectrum of somatic alterations in GC by sequencing the exomes of 15 GC specimens, including 11 intestinal-type, 1-mixed-type, and 3 diffuse-type adenocarcinomas and their matched normal DNAs. TP53 (11/15 tumors), PIK3CA (3/15) and ARID1A (3/15) were frequently mutated. Among the frequently mutated genes, cell adhesion was the most significant biological pathway affected. A prevalence screening confirmed mutations in FAT4, a member of the cadherin gene family, in 5% of GCs (6/110) and FAT4 genomic deletions in 4% (3/83) of GCs. Mutations in chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) were also found in 47% of GCs. ARID1A mutations were detected in 8% of GCs (9/110) and were associated with concurrent PIK3CA mutations and MSI. Both FAT4 and ARID1A showed tumor-suppressor activity in functional assays. Somatic inactivation of FAT4 and ARID1A may thus be key tumorigenic events in a subset of GCs. Because PI3K inhibitors are currently in clinical testing as treatment for GC[32], it will be interesting to evaluate whether the tumor responses to these compounds are affected by the genomic status of ARID1A.

Frequent loss of ARID1A expression in GC with EBV infection or MSI

Mutations of ARID1A lead to a loss of protein expression in GC and are particularly associated with EBV infection or MSI. Abe *et al*[33] investigated the significance of the loss of ARID1A in 857 GC cases, including 67 EBV^+ and 136 MLH1-lost MSI^+ GCs. Loss of ARID1A expression was significantly more frequent in cases of EBV^+ (23/67; 34%) and MSI^+ (40/136; 29%) GCs than in cases of EBV/MSI^- (32/657; 5%) GCs. Loss of ARID1A was correlated with larger tumor size, deeper depth of invasion, lymph node metastasis and poorer prognosis in cases of EBV/MSI^- GC. A correlation with tumor size and diffuse-type histology was found only in the MSI^+ GC; no correlation was observed in EBV^+ GC. Loss of ARID1A expression in EBV^+ GC was frequent in the early stage of GC, but EBV infection did not cause loss of ARID1A in GC cell lines. Thus, loss of ARID1A may be an early event in EBV^+ GC and may precede EBV infection in gastric epithelial cells. On the other hand,

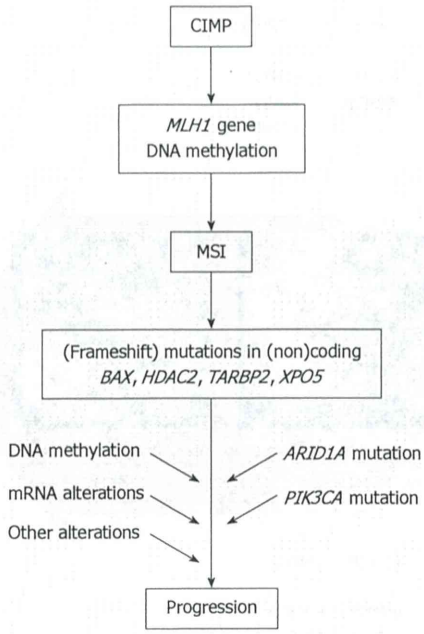

Figure 2 Molecular pathway for microsatellite instability+ gastric cancer. The model for the carcinogenesis of microsatellite instability (MSI)$^+$ gastric cancer is presented. CIMP: CpG island methylator phenotype.

loss of ARID1A may be involved in the progression of EBV/MSI$^-$ GCs. Thus, loss of ARID1A appears to have different, pathway-dependent roles in GC.

WHOLE-GENOME SEQUENCING ANALYSIS OF GC

To explore the complete list of somatic alterations in GC, Nagarajan *et al*[34] combined massively parallel short read and DNA paired-end tag sequencing for the first whole-genome analysis of two GCs, one with CIN and the other with MSI. Integrative analysis and de novo assemblies revealed the architecture of a wild-type KRAS amplification, a common driver event in GC[35]. Three distinct mutational signatures were discovered against a genome-wide backdrop of oxidative and MSI-associated mutational signatures. Combining sequencing data from 40 complete GC exomes and targeted screening of an additional 94 independent GCs led to the discovery of ACVR2A, RPL22 and LMAN1 as recurrently mutated genes in MSI$^+$ GC and the identification of PAPPA as a recurrently mutated gene in TP53 wild-type GC. These results highlight how whole-genome sequencing analysis can provide relevant information about tissue-specific carcinogenesis that would otherwise be missed in exome-sequencing data. WGS of more GCs will uncover more recurrently altered genes.

miRNA alterations

A microRNA (miRNA) is a small noncoding RNA that regulates gene expression at the posttranscriptional level and is critical in many biological processes and cellular pathways[36-40]. The causes of aberrant miRNA expression patterns in cancer include DNA copy number amplification or deletion, inappropriate transactivation, transcriptional repression by oncogenic and other factors, failure of miRNA post-transcriptional regulation and genetic mutation or transcriptional silencing associated with hypermethylation of the CpG island promoters.

There is accumulating evidence to support the notion that miRNA alterations play a key role in the pathogenesis of GC[41-44]. A large number of miRNAs with different biological functions have been found to be altered and correlated with clinicopathological characteristics and/or prognosis in GC. Moreover, the clinical potential of miRNA alterations as minimally invasive diagnostic biomarkers and therapeutic targets has been extensively reported[37,40,42,44]. Recent studies have shown that tumor-derived miRNAs are present and stable in circulation, and the levels of circulating miRNAs are detectable and quantifiable. Both tissue and soluble miRNAs are candidates for diagnostic biomarkers and therapeutic targets in GCs[44]. The basic strategy of current miRNA-based treatment studies is to either antagonize the expression of target oncogenic miRNAs with antisense therapy and other technology or to restore the function of impaired tumor suppressor miRNAs[42].

The inclusion of different isoforms of miRNA (isomiRs) that are natural variants of mature miRNAs will form a detailed miRnome. Because expression of isomiRs can be estimated by NGS, NGS platforms provide the most effective method of miRNA profiling, leading to the identification of the miRNA alterations with clinical applications. Li *et al*[45] sequenced small RNAs from one pair of GC and noncancerous tissue and found that isomiR patterns are significantly different between these tissues. Moreover, these authors found that the 5p arm and 3p arm miRNAs derived from the same pre-miRNAs have different tissue preferences in GC and noncancerous tissue, suggesting a novel mechanism regulating mature miRNA selection.

WHOLE-TRANSCRIPTOME SEQUENCING OF GC

The first comprehensive RNA-seq study in GC has been recently published. Kim *et al*[46] applied a whole-transcriptome sequencing approach to 24 GC samples and six noncancerous tissue specimens. Importantly, these authors developed a multilayered integrative analysis to identify various types of transcriptional aberrations, such as differentially expressed mRNAs and miRNAs, as well as recurrently mutated genes. A central metabolic regulator gene, AMPKa2 (PRKAA2), was identified as a potential functional target in GC. Six key miRNAs (miR-548d-3p, miR-20b, miR-135b, miR-140-3p, miR-93 and miR-19a) in GC were also identified.

Epigenetic alterations

Epigenetic regulation is essential for the normal develop-