Table 3. Effect modification of *ADH1B*, *ADH1C* and *ALDH2* polymorphisms on gastric cancer risk associated with alcohol consumption

	ADH1B (rs1229984)						
	AA			AG+GG			
	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[b]	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[b]	P for interaction[b]
Alcohol consumption							
0 to <150 g/week	167/189	1.00 (reference)	1.00 (reference)	141/144	1.09 (0.79–1.49)	1.01 (0.72–1.42)	0.76
≥150 g /week	85/65	1.57 (1.04–2.38)	1.37 (0.86–2.19)	64/59	1.30 (0.84–2.02)	1.25 (0.77–2.05)	
	ADH1C (rs698)						
	AA			AG+GG			
	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[b]	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[b]	P for interaction[b]
Alcohol consumption							
0 to <150 g/week	270/277	1.00 (reference)	1.00 (reference)	38/56	0.68 (0.43–1.07)	0.66 (0.41–1.08)	0.02
≥150 g/week	126/114	1.23 (0.87–1.72)	1.16 (0.78–1.71)	23/10	2.49 (1.14–5.42)	2.54 (1.05–6.17)	
	ALDH2 (rs671)						
	GG			GA+AA			
	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[c]	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[c]	P for interaction[c]
Alcohol consumption							
0 to <150 g/week	177/185	1.00 (reference)	1.00 (reference)	131/148	0.93 (0.68–1.28)	0.98 (0.69–1.38)	0.08
≥150 g/week	110/107	1.16 (0.79–1.69)	1.09 (0.72–1.67)	39/17	2.51 (1.34–4.72)	2.08 (1.05–4.12)	

Based on unconditional logistic regression model.

[a]Adjusted for age (±3 years), sex, area, blood donation date (±2 months) and fasting time at blood donation (±5 h).

[b]Further adjusted for smoking status, BMI, total calorie, salt intake, family history of gastric cancer, *H.pylori* infection status, atrophy, history of DM and *ALDH2* polymorphism.

[c]Further adjusted for smoking status, BMI, total calorie, salt intake, family history of gastric cancer, *H.pylori* infection status, atrophy, history of DM and ADH1B and ADH1C polymorphisms.

Table 4. Effect modification of the combination of *ADH1B*, *ADH1C* and *ALDH2* polymorphisms on gastric cancer risk associated with alcohol consumption

		ALDH2 (rs671)						
		GG			GA+AA			
		Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[b]	Cases (*n*)/controls (*n*)	OR1 (95% CI)[a]	OR2 (95% CI)[b]	P for interaction[b]
ADH1B (rs1229984)	Alcohol consumption							
AA	0 to <150 g/week	103/105	1.00 (reference)	1.00 (reference)	64/84	0.77 (0.50–1.19)	0.75 (0.47–1.19)	0.40
	≥150 g/week	64/58	1.20 (0.74–1.94)	0.99 (0.58–1.68)	21/7	3.09 (1.23–7.76)	2.16 (0.83–5.63)	
AG+GG	0 to <150 g/week	74/80	0.91 (0.60–1.39)	0.79 (0.50–1.24)	67/64	1.07 (0.68–1.66)	1.06 (0.65–1.71)	
	≥150 g/week	46/49	1.01 (0.60–1.69)	0.94 (0.53–1.65)	18/10	1.93 (0.83–4.46)	1.66 (0.66–4.16)	
ADH1C (rs698)	Alcohol consumption							
AA	0 to <150 g/week	152/161	1.00 (reference)	1.00 (reference)	118/116	1.07 (0.76–1.51)	1.13 (0.78–1.63)	0.13
	≥150 g/week	92/98	1.07 (0.72–1.59)	1.01 (0.65–1.57)	34/16	2.34 (1.21–4.51)	1.92 (0.95–3.87)	
AG+GG	0 to <150 g/week	25/24	1.03 (0.56–1.90)	1.03 (0.54–1.99)	13/32	0.44 (0.22–0.87)	0.43 (0.21–0.91)	
	≥150 g/week	18/9	2.20 (0.94–5.14)	2.14 (0.83–5.52)	5/1	5.63 (0.64–49.22)	8.95 (0.62–129.25)	

Based on unconditional logistic regression model.
[a]Adjusted for age (±3 years), sex, area, blood donation date (±2 months) and fasting time at blood donation (±5 h).
[b]Further adjusted for smoking status, BMI, total calorie, salt intake, family history of gastric cancer, *H.pylori* infection status, atrophy and history of DM.

(rs1229984), *ADH1C* (rs698) and *ALDH2* (rs671) polymorphisms and gastric cancer risk. However, statistically significant interactions between inactive *ADH1C* and alcohol consumption and nonsignificant interactions between inactive *ALDH2* alleles and alcohol consumption were shown for gastric cancer risk.

To date, one prospective study in Europe (20) and several case–control studies (21–25) have reported an association between alcohol consumption and *ADH1B* (rs1229984), *ADH1C* (rs698) and *ALDH2* (rs671) polymorphisms and gastric cancer risk. For *ADH1B* (rs1229984) and *ADH1C* (rs698) polymorphisms, two previous studies reported that the inactive *ADH1B* allele was not associated with gastric cancer risk among drinkers (20,23). In one case–control study performed in the United States, the active *ADH1C* genotype was associated with an increased risk among drinkers and nondrinkers (24). However, the sample size of this case–control study was small, and caution may be needed in interpreting the results. For the *ALDH2* (rs671) polymorphism, two recent, large, Japanese and Korean case–control studies reported that the interaction between the inactive *ALDH2* allele and alcohol consumption regarding gastric cancer risk was statistically significant (21,22). Another case–control study conducted in China also indicated that inactive *ALDH2* allele carriers with larger cumulative amount of alcohol consumption had a marginally increased risk of gastric cancer compared with active *ALDH2* allele carries with smaller cumulative amount of alcohol consumption (23).

As shown in our study (Tables 3 and 4), the association between alcohol consumption, *ADH1B* polymorphism and gastric cancer risk was similar to that in previous studies. In contrast, a positive association between inactive *ADH1C* G allele and alcohol consumption regarding gastric cancer risk was found, opposite to that found in previous studies (24). However, the number of study subjects in our population is small for some *ADH1C* genotypes because *ADH1C* GG genotype is rare in Asians (8). In addition, evidence is lacking on any difference between *ADH1B* and *ADH1C* polymorphisms in the ability to metabolize ethanol. Caution is necessary when interpreting the results for *ADH1C* polymorphisms in our study. Inactive *ALDH2* A allele carriers who drink ≥150 g/week have an increased risk of gastric cancer, similar to that in previous studies, which may be attributable to accumulation of acetaldehyde. When subjects with an inactive *ALDH2* allele did not drink alcohol, the risk of gastric cancer did not increase. We suggest that accumulation of acetaldehyde modified by *ALDH2* (rs671), rather than *ADH1B* (rs1229984) and *ADH1C* (rs698) polymorphisms, may play an important role in gastric carcinogenesis.

It has been reported that the carcinogenic mechanisms of acetaldehyde are complicated and are not completely understood. Acetaldehyde reacts with the exocyclic amino group of deoxyguanosine to form DNA adducts, called N (2)-ethylidened-eoxyguanosine [N (2)- ethylidene-dGuo]. The DNA adducts are involved in metagenesis (26,27). The other kinds of acetaldehyde-related adducts are the 1,N (2)-propano-2′-deoxyguanosine [1, N (2)-propanodGuo] and 1,N (2)-etheno-dGuo (27). Other candidate mechanisms may be DNA hypomethylation by DNA methyltransferase, direct adduction of histone, and inhibition of the activity of O6-methylguanine-DNA methyltranseferase (MGMT), which protect against alkylation of DNA (27).

In our study, we conducted a stratified analysis of gastric atrophy. Interestingly, among the subjects with gastric atrophy, *ALDH2* A allele carriers who drink ≥150 g/week had an increased risk of gastric cancer compared with GG genotype carriers who drink 0 to <150 g/week; statistically significant interaction was also found. This phenomenon was not found among the

subjects without gastric atrophy. Our result suggests that acetaldehyde may induce gastric carcinogenesis with gastric atrophy, which is caused by chronic inflammation with *H.pylori* infection. In a previous study, although statistical interaction was not significant, similar results were found (21). Further studies are needed to clarify the contribution of acetaldehyde to gastric carcinogenesis.

This study has several strengths. First, this is a population-based prospective study, which is more reliable than case–control studies. Detailed information regarding the potential confounding factors including alcohol consumption was recorded before diagnosis of gastric cancer, thus confirming our results. A validated FFQ was used. Also, we were able to control potential confounding factors, as compared with a previous European study that was only adjusted for age, sex and country (20).

Our study does have some weakness. First, among 97 644 eligible subjects of the JPHC study, only 36 745 (37.6%) subjects provided blood samples. The participants in the health check-up survey relative to nonparticipants had a favorable lifestyle with less smoking and alcohol consumption, as reported previously (28). Second, we were not able to assess the other genes of ethanol-metabolizing enzymes such as *CYP2E1*. Third, we analyzed the gastric cancer risk only using the lifestyle information at baseline. Lifestyle habits of study subjects might change during the follow-up period. However, this change may not be different between cases and controls and likely would have led to the underestimation of results. Finally, sample size was not necessarily enough for evaluating the association among some anatomic sites.

In conclusion, to find the relation between alcohol consumption and gastric cancer risk, it is important to consider both alcohol consumption level and *ADH1C* and *ALDH2* polymorphisms. However, caution is needed to interpret the results associated with the ADH1C polymorphism because some genotypes of the *ADH1C* polymorphism occurred in only a small number of subjects.

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Plasma insulin, C-peptide and blood glucose and the risk of gastric cancer: The Japan Public Health Center-based prospective study

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To date, the association between diabetes mellitus (DM) and gastric cancer has been controversial, including the underlying mechanism. We investigated the association between plasma diabetic biomarkers (insulin, C-peptide, and blood glucose) and gastric cancer risk. In addition, homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of β-cell function (HOMA-β) were calculated. A total of 36,745 subjects aged 40–69 years in the Japan Public Health Center-based prospective study (JPHC) who returned the baseline questionnaire and provided blood samples were followed from 1990 to 2004. In the present analysis, 477 cases and 477 matched controls were used. The odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) for developing gastric cancer were calculated using conditional logistic regression models. Plasma insulin was positively associated with increased risk of gastric cancer; compared to tertile 1, ORs were 1.69 (95% CI = 1.11–2.59) and 2.01 (1.19–3.38) for tertiles 2 and 3, respectively (p for trend = 0.009). In men, C-peptide was also positively associated with a significant risk; corresponding ORs were 1.42 (0.85–2.38) and 1.91 (1.03–3.54), respectively (p for trend = 0.04). These findings were confirmed for blood samples from the fasting group (≥8 hr after a meal). Higher HOMA-IR was also associated with increased risk, whereas no association was observed for blood glucose. Our findings suggest that Japanese population with higher insulin and C-peptide levels derived from insulin resistance have an elevated risk of gastric cancer.

Epidemiology

Gastric cancer is the second leading cause of death and the fourth most common cancer in the world.[1] Although *Helicobacter pylori* (*H. pylori*) infection is well known as a major risk factor for gastric cancer, only some of the people infected with *H. pylori* will develop gastric cancer. Therefore, other risk factors might affect the association between *H. pylori* and gastric cancer occurrence.

Diabetes mellitus (DM) is associated with many types of cancer, including colorectal, liver, breast, and pancreatic cancer.[2] However, the association between DM and gastric cancer remains to be clarified. Some prospective studies reported that DM determined by questionnaire or medical records is positively associated with gastric cancer,[3–6] but others found a null association.[7–12] However, DM can be easily misclassified when based on self-report of disease in questionnaire survey or medical records. To overcome this problem, several studies were directly based on diabetic biomarkers, such as hemoglobin A1c (HbA1c) and blood glucose, but the associations were also inconsistent in these prospective studies.[13–16]

Key words: gastric cancer risk, plasma insulin, plasma C-peptide, plasma blood glucose, prospective study

Abbreviations: BMI: body mass index; CagA: cytotoxin associated gene A; CI: confidence interval; DM: diabetes mellitus; HbA1c: hemoglobin A1c; HOMA-IR: homeostasis model assessment of insulin resistance; HOMA-β: homeostasis model assessment of β-cell function; ICD-O: international classification of diseases for oncology; IGF: insulin-like growth factor; JPHC: Japan public health center–based prospective study; OR: odds ratio; PHC: public health center; SD: standard deviation

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What's new?
The idea that diabetes mellitus may play a role in some instances of gastric carcinogenesis is intriguing but controversial. Here, a positive association was identified for gastric cancer risk and plasma insulin levels, based on investigation of plasma biomarkers in a Japanese study population. The association was evident for measures of homeostasis model assessment of insulin resistance (HOMA-IR). By contrast, no association was found for blood glucose levels. The results suggest that hyperinsulinemia derived from insulin resistance, rather than hyperglycemia, is important in gastric carcinogenesis.

Another possible candidate biomarker is insulin, which may be involved in the biological mechanisms of carcinogenesis that underlie the association between DM and gastric cancer. To date, several *in vivo* and *in vitro* studies have reported a positive association between insulin and carcinogenesis including gastric mucosa.[17,18] To our knowledge, no prospective study has evaluated the association between insulin and the risk of gastric cancer.

In this study, we investigated the association between plasma insulin, C-peptide, and blood glucose and gastric cancer risk in a case-control study nested within a large-scale population-based study. C-peptide is a metabolic product of insulin and is more stable than insulin in blood. In addition, we calculated homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of β-cell function (HOMA-β) to evaluate the extent of insulin resistance and pancreatic β-cell function,[19] respectively.

Material and Methods

Study population

The Japan Public Health Center-based prospective study (JPHC) was established in 1990 for cohort I (subject age range 40–59 years) and in 1993 for cohort II (40–69 years), as described previously.[20] The JPHC consisted of 11 public health centers (PHCs) in Japan and included 140,420 subjects (68,722 men and 71,698 women). The subjects from one PHC (Tokyo) in cohort I were excluded from this study because the data on cancer incidence were not available. In addition, one subgroup of cohort II (Osaka) was excluded because the selection of subjects differed from that of other cohort subjects, which left 123,576 subjects (61,009 men and 62,567 women). This study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

Baseline survey

In the baseline survey, a self-administered questionnaire was used in each cohort. The study subjects were asked about various lifestyle factors, such as sociodemographic characteristics, personal medical history, family history, smoking and drinking habits, dietary habits and physical activity. A total of 99,808 subjects (47,525 men and 52,283 women) responded (response rate: 80.8%).

We asked each subject to provide a 10-ml blood sample at the time of the health checkup. After exclusion of subjects who self-reported cancer at baseline ($n = 2136$), who were non-Japanese ($n = 18$), and who did not live in the area at the baseline ($n = 11$), 97,644 subjects (46,803 men and 50,841 women) remained eligible. (One subject both self-reported cancer at baseline and was non-Japanese.) Among the eligible subjects, 36,745 subjects (13,467 men and 23,278 women) provided blood samples at baseline. Plasma levels of blood glucose were measured at each PHC area at the time of the baseline health check-up and the values were used for the present analysis. One PHC (Niigata) in cohort II and two PHCs (Akita and Iwate) in cohort I did not routinely measure glucose ($n = 174$). According to the Osaka Medical Center for Health Science and Promotion, the accuracy of plasma blood glucose measurements in all the laboratories was found to be satisfactory.[21] The plasma and buffy coat were divided into four tubes, each holding 1.0 ml (three tubes for plasma and one for the buffy coat), and then preserved at −80°C until analysis.

The blood samples were collected from 1990 to 1992 in cohort I and from 1993 to 1995 in cohort II. Following the standard protocol, we requested that subjects avoid having a meal after 21:00 on the day before the health checkup, and recorded the approximate last time of caloric intake, including a meal and/or drinking.

Follow-up

Subjects were observed from 1 January 1990 to 31 December 2004 for cohort I and from 1 January 1993 to 31 December 2004 for cohort II. Residence status, survival, and death were identified annually through residential registries in each PHC area. In Japan, residence and death registration are required by law, and the registries are believed to be complete. Among the 36,745 subjects, 1,423 (3.9%) moved outside the study area, 1,610 (4.4%) died, and 11 (0.03%) were lost to follow-up during the study period.

Cancer registry for the JPHC

Incidence data on gastric cancer cases were collected for the JPHC cancer registry from two sources: local major hospitals and population-based cancer registries (usually prefecture-wide). Death certificate information was also used. In our cancer registry system, information for 7.6% of gastric cancer cases was based on the case first identified *via* a death certificate and 2.1% were registered based on information from the death certificate alone.

Selection of cases and controls

Over the entire study period from 1990 to 2004, 1681 new gastric cancer cases with a histologically proven diagnosis

Table 1. Baseline characteristics of cases and controls

Characteristics	Cases	Controls	*p* value[1]
N	477	477	
Age, mean (SD)	57.2 (7.19)	57.2 (7.21)	Matching value
Men (%)	319 (66.9)	319 (66.9)	Matching value
Smoking status			
Never smoker (%)	218 (45.7)	237 (49.7)	
Past smoker (%)	88 (18.5)	93 (19.5)	
Current ≤20 cigarettes/day (%)	132 (27.7)	106 (22.2)	
Current ≥21 cigarettes/day (%)	39 (8.1)	41 (8.6)	0.28
Alcohol consumption			
Never or occasional (%)	229 (48.0)	236 (49.5)	
≥1 day, <300 g/week (%)	185 (38.8)	194 (40.7)	
≥1 day, ≥300 g/week (%)	63 (13.2)	47 (9.8)	0.27
BMI (kg m^{-2})[2]			
BMI < 22 (%)	169 (35.7)	158 (33.3)	
22 ≤ BMI < 25 (%)	207 (43.8)	198 (41.7)	
25 ≤ BMI (%)	97 (20.5)	119 (25.0)	0.25
Family history of gastric cancer (%)	58 (12.2)	39 (8.2)	0.04
Past history of DM (%)	44 (9.2)	21 (4.4)	0.003
Drug treatment for DM (%)	15 (3.1)	8 (1.7)	0.14
Helicobacter pylori positive (%)[3]	449 (94.1)	357 (74.8)	<0.001
CagA positive (%)	359 (75.3)	335 (70.2)	0.08
Atrophy (%)[4]	390 (81.8)	278 (58.3)	<0.001

[1]Based on chi-square test or Student's *t* test.
[2]Subjects for whom we were unable to calculate body mass index due to missing height or weight data (four cases and two controls) were deleted.
[3]Based on immunoglobulin G antibody.
[4]Atrophy: positive if pepsinogen I ≤ 70 ng ml^{-1} and pepsinogen I/pepsinogen II ratio ≤3.
Abbreviations: BMI: body mass index; CagA: cytotoxin associated gene A; DM: diabetes mellitus; SD: standard deviation.

were observed in the two cohorts. Among these cases, blood samples and questionnaire responses at baseline had been obtained from 512 cases. The anatomic subsite of each case was coded on the basis of the International Classification of Diseases for Oncology (ICD-O), 3rd edition.[22] Tumor located in the upper third of the stomach was referred to as proximal gastric cancer (cardia subsite) (ICD-O code C16.0 and 16.1), and that in the lower portion of the stomach was classified as distal gastric cancer (non-cardia subsite) (ICD-O code C16.2–16.7). The remaining cases were tumors that could not be classified because of overlapping lesions (ICD-O code C16.8) or no information (ICD-O code C16.9). The subdivisions by histological type were based on the Lauren classification.[23] For each case, we selected one control subject from those who were not diagnosed with gastric cancer during the follow-up period when the case was diagnosed. We matched case and control for gender, age (±3 years), study area, fasting time at blood donation (±5 hr), and blood donation date (±2 months). Among the 512 new gastric cancer cases, 1 case was excluded due to a technical error in the measurement of *H. pylori* and 34 cases were excluded due to no volume left for the present measurement. The final analysis included 477 matched sets of cases and controls.

Laboratory assays for insulin and C-peptide

Plasma levels of insulin and C-peptide were measured at GeneticLab, Hokkaido, Japan. All laboratory personnel were blinded about case and control status. Plasma diabetic biomarkers were simultaneously assayed using a Human Endocrine Milliplex Kit (#HEND-65K; Millipore Company, 6 Research Park Drive, St. Charles, MO). The kit used polystyrene bead–based assays to measure the markers in 25-μl samples across panels. Based on the measurement of eight median fluorescent intensities, a standard curve of the biomarker was used to convert optical density values into concentrations, with limits of assay detection of 5.8 pg ml^{-1} (1 pmol l^{-1}) for insulin and 3.6 pg ml^{-1} (1 pmol l^{-1}) for C-peptide. Using the curve-fit measurements for each standard, technicians also estimated coefficients of variation, which were calculated as the ratio of the observed and expected concentrations. The average coefficients of variation for plasma levels of insulin and C-peptide were 7.2 and 4.2%, respectively. Some plasma samples could not be measured because of insufficient volume: 27 for insulin and 2 for C-peptide.

Statistical analysis

Tertiles of plasma diabetic biomarkers and HOMA-β were based on levels in control subjects. The chi-square test and Student's *t* test were used to compare background characteristics between cases and controls. Matched odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were calculated using conditional logistic regression models. OR1 was matched for age (±3 years), gender, PHC area, blood donation date (±2 months), and fasting time at blood donation (±5 hr). OR2 was calculated by multivariate conditional logistic regression analysis adjusting for potential confounding factors such as smoking status, alcohol consumption, total calorie intake, salt intake, body mass index (BMI), family history of gastric cancer, *H. pylori* infection status, and atrophy. OR3 was further adjusted for past history of DM and drug treatment for DM.

Smoking status was divided into four groups: never smoker, past smoker, current smoker with ≤20 cigarettes per day, and current smoker with ≥21 cigarettes per day. Alcohol consumption was divided into four groups: never drinker, occasional drinker, current drinker who intakes <300 g of ethanol per week, and current drinker who intakes ≥300 g of

Table 2. ORs and 95% CIs for the association between plasma levels of diabetic biomarkers and gastric cancer risk

		Cases (*n*)/ controls (*n*)	OR1 (95%CI)[1]	OR2 (95% CI)[2]	OR3 (95% CI)[3]
Insulin (pg ml^{-1})	Tertile 1 (10.7–228.7)	137/152	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (233.1–468.7)	163/153	1.25 (0.87–1.80)	1.63 (1.08–2.47)	1.68 (1.10–2.56)
	Tertile 3 (471.0–7933.3)	157/152	1.36 (0.88–2.11)	1.91 (1.15–3.18)	2.03 (1.21–3.41)
	p for trend		0.17	0.01	0.007
C-peptide (pg ml^{-1})	Tertile 1 (130.5–653.6)	160/158	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (659.7–1292.8)	160/159	0.99 (0.70–1.40)	1.15 (0.77–1.71)	1.15 (0.77–1.72)
	Tertile 3 (1303.0–8739.4)	155/158	1.02 (0.68–1.55)	1.31 (0.82–2.11)	1.30 (0.81–2.10)
	p for trend		0.92	0.26	0.28
Blood glucose (mg dl^{-1})	Tertile 1 (72.0–92.0)	138/124	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (93.0–106.0)	114/124	0.81 (0.55–1.18)	1.01 (0.66–1.55)	0.98 (0.63–1.50)
	Tertile 3 (107.0–406.0)	121/125	0.85 (0.57–1.29)	0.96 (0.61–1.53)	0.84 (0.52–1.36)
	p for trend		0.41	0.88	0.50

[1]Matched for age (±3 years), gender, public health center area, blood donation date (±2 months), and fasting time at blood donation (±5 hr).
[2]Adjusted for smoking, alcohol consumption, body mass index, total calories, salt intake, family history of gastric cancer, *Helicobacter pylori* infection status, and atrophy.
[3]Further adjusted for past history of diabetes mellitus and drug treatment for diabetes mellitus.
Abbreviations: CI: confidence interval; OR: odds ratio.

ethanol per week. Total calorie and salt intakes were treated as continuous variables. BMI was divided into three classes: BMI <22 kg m^{-2}, $22 \leq$ BMI <25, and $25 \leq$ BMI. Subjects who were missing value for BMI ($n=6$), total calorie ($n=1$), and salt intakes ($n=1$) were excluded when adjusting for these confounding factors. Family history of gastric cancer was considered positive if at least one parent or sibling had gastric cancer. The *H. pylori* infection status was regarded as positive if subjects had either *H. pylori* antibody $\geq$10 U ml^{-1} or cytotoxin associated gene A (CagA) antibody $>$10. Atrophy was regarded as positive if pepsinogen I was $\leq$70 ng ml^{-1} and the pepsinogen I/pepsinogen II ratio was $\leq$3.[24] Because we do not have any data from upper gastrointestinal endoscopies and biopsies, the pepsinogen data were used. Urita et al. reported that the pepsinogen I/pepsinogen II ratio $\leq$3 identified gastric atrophy with a sensitively of 71.7% and a specificity of 66.7%.[25] We believe that the pepsinogen data could explain the level of atrophy, to some extent, if added to the model. Past history of DM and drug treatment for DM were considered positive if subjects were diagnosed with DM before and used a diabetic drug at the time of the baseline survey, respectively. Stratified analysis based on fasting status ($\geq$8 hr or $<$8 hr after a meal) was also conducted for each plasma diabetic biomarker. Furthermore, for the subjects who were in the fasting group ($\geq$8 hr after a meal) at blood donation and not under drug treatment for DM, we calculated HOMA-IR [fasting plasma insulin level (μU ml^{-1}) $\times$ fasting plasma glucose level (mg dl^{-1})/405] and HOMA-β [360 $\times$ fasting plasma insulin level (μU ml^{-1})/fasting plasma glucose level (mg dl^{-1}) − 63].[19] HOMA-IR $\geq$1.73 was defined as the presence of insulin resistance.[26] According to the manufacturer of the insulin measuring kit (Millipore), conversion of insulin units was based on the human insulin international reference preparation of WHO (1 μIU ml^{-1} = 35 pg ml^{-1}).

Reported *p* values are two-sided, and $p<0.05$ was defined as statistically significant. All statistical analyses were performed with SAS software version 9.3 (SAS Institute, Cary, NC).

Results

Baseline characteristics of cases and controls are shown in Table 1. Family history of gastric cancer, past history of DM, *H. pylori* positivity, and atrophy were significantly more frequent among cases compared to controls. The distributions of other factors were similar in cases and controls. At baseline, 9.2% of cases and 4.4% of controls had past history of DM, and 3.1% of cases and 1.7% of controls had received drug treatment for DM.

Table 2 shows ORs and 95% CIs for the associations between plasma levels of diabetic biomarkers and gastric cancer risk using conditional logistic regression models. We found that plasma insulin was dose-dependently associated with an increased risk of gastric cancer. Compared to tertile 1, OR2 (adjusted for smoking, alcohol consumption, BMI, total calories, salt intake, family history of gastric cancer, *H. pylori* infection status, and atrophy) for tertiles 2 and 3 was 1.63 (95% CI = 1.08–2.47) and 1.91 (1.15–3.18), respectively (*p* for trend 0.01). When further adjusted for past history of DM and drug treatment for DM, corresponding values for OR3 were 1.68 (1.10–2.56) and 2.03 (1.21–3.41), respectively (*p* for trend 0.007). We found no association between the other diabetic biomarkers and risk of gastric cancer.

In Table 3, the associations between plasma levels of diabetic biomarkers and gastric cancer risk are shown for men

Table 3. ORs and 95% CIs for the association between plasma levels of diabetic biomarkers and gastric cancer risk in men and women

		Cases (*n*)/ controls (*n*)	OR1 (95% CI)[1]	OR2 (95% CI)[2]	OR3 (95% CI)[3]
Men					
Insulin (pg ml^{-1})	Tertile 1 (10.7–224.3)	92/102	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (226.4–491.0)	108/103	1.29 (0.82–2.03)	1.76 (1.00–3.09)	1.75 (0.99–3.10)
	Tertile 3 (495.9–7933.3)	107/102	1.50 (0.87–2.60)	2.43 (1.23–4.78)	2.49 (1.25–4.96)
	p for trend		0.15	0.01	0.01
C-peptide (pg ml^{-1})	Tertile 1 (130.5–643.1)	95/106	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (644.2–1380.9)	111/106	1.25 (0.82–1.90)	1.39 (0.83–2.30)	1.43 (0.86–2.40)
	Tertile 3 (1388.3–8739.4)	112/106	1.42 (0.85–2.38)	1.90 (1.04–3.48)	1.96 (1.06–3.64)
	p for trend		0.18	0.04	0.03
Blood glucose (mg dl^{-1})	Tertile 1 (73.0–94.0)	91/87	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (95.0–108.0)	70/81	0.81 (0.51–1.29)	0.91 (0.53–1.57)	0.92 (0.54–1.59)
	Tertile 3 (109.0–406.0)	89/82	1.07 (0.66–1.74)	1.18 (0.67–2.08)	1.02 (0.57–1.83)
	p for trend		0.85	0.59	0.98
Women					
Insulin (pg ml^{-1})	Tertile 1 (41.1–238.4)	49/50	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (239.8–429.1)	54/50	1.05 (0.57–1.93)	1.44 (0.71–2.94)	1.61 (0.77–3.37)
	Tertile 3 (430.1–5237.4)	47/50	0.91 (0.45–1.84)	1.08 (0.48–2.46)	1.27 (0.54–3.00)
	p for trend		0.79	0.81	0.56
C-peptide (pg ml^{-1})	Tertile 1 (158.2–679.1)	69/52	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (685.7–1181.6)	43/53	0.44 (0.22–0.88)	0.58 (0.27–1.26)	0.54 (0.25–1.20)
	Tertile 3 (1183.2–3496.9)	45/52	0.46 (0.22–0.97)	0.59 (0.25–1.39)	0.58 (0.25–1.38)
	p for trend		0.04	0.23	0.23
Blood glucose (mg dl^{-1})	Tertile 1 (72.0–90.0)	50/41	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (91.0–103.0)	37/42	0.69 (0.36–1.35)	0.89 (0.41–1.97)	0.88 (0.39–1.98)
	Tertile 3 (104.0–235.0)	36/40	0.69 (0.32–1.51)	0.59 (0.22–1.57)	0.48 (0.17–1.33)
	p for trend		0.29	0.32	0.19

[1]Matched for age (±3 years), public health center area, blood donation date (±2 months), and fasting time at blood donation (±5 hr).
[2]Adjusted for smoking, alcohol consumption, body mass index, total calories, salt intake, family history of gastric cancer, *Helicobacter pylori* infection status, and atrophy.
[3]Further adjusted for past history of diabetes mellitus and drug treatment for diabetes mellitus.
Abbreviations: CI: confidence interval; OR: odds ratio.

and women separately. In men, besides insulin, plasma C-peptide was also dose-dependently associated with gastric cancer risk; OR2 was 1.39 (0.83–2.30) and 1.90 (1.04–3.48) for tertiles 2 and 3, respectively (*p* for trend 0.04). Corresponding values for OR3 were 1.43 (0.86–2.40) and 1.96 (1.06–3.64), respectively (*p* for trend 0.03). In women, plasma C-peptide was inversely associated with gastric cancer risk (OR1), but it lost statistical significance after further adjustment (OR2 and OR3).

Participants who provided blood samples more than 8 hr after a meal were defined as the fasting group. Because plasma insulin and C-peptide showed positive associations with gastric cancer (Tables 2 and 3), further stratified analysis by fasting status (≥8 hr and <8 hr after a meal) was performed for these biomarkers, as well as HOMA-IR and HOMA-β. After excluding pairs with different fasting status, conditional logistic regression analysis was conducted (Table 4). The levels of these biomarkers differed by fasting status. We found that higher levels of plasma insulin and C-peptide were marginally associated with gastric cancer risk in the fasting group (≥8 hr after a meal). For the non-fasting group (<8 hr after a meal), whose biomarker levels may be strongly influenced by the meal, a weakly increased risk was also observed, but not significantly so. Moreover, a higher HOMA-IR was associated with increased risk of gastric cancer; OR2 for HOMA-IR ≥1.73 was 1.88 (1.03–3.45) compared to HOMA-IR <1.73. Corresponding values for OR3 were 1.97 (1.07–3.65). Higher HOMA-β also showed a trend toward a positive association.

We conducted stratified analyses by alcohol consumption, smoking status, menopausal status (menopausal or not menopausal), and atrophy, and no differences according to such

Table 4. ORs and 95% CIs by fasting status for the association between insulin, C-peptide, HOMA-IR, and HOMA-β and gastric cancer risk

		Cases (*n*)/ controls (*n*)	OR1 (95%CI)[1]	OR2 (95%CI)[2]	OR3 (95%CI)[3]
Non-fasting group[4]					
Insulin (pg ml^{-1})	Tertile 1 (92.3–366.5)	92/86	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (367.4–621.1)	81/87	0.84 (0.51–1.36)	1.07 (0.58–1.98)	1.03 (0.56–1.91)
	Tertile 3 (628.1–7933.3)	86/86	0.94 (0.56–1.59)	1.26 (0.66–2.42)	1.21 (0.63–2.32)
	p for trend		0.84	0.47	0.56
C-peptide (pg ml^{-1})	Tertile 1 (140.4–1012.2)	93/89	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (1022.3–1755.5)	87/89	0.94 (0.57–1.54)	1.29 (0.72–2.30)	1.26 (0.70–2.27)
	Tertile 3 (1762.0–8739.4)	87/89	0.96 (0.56–1.64)	1.52 (0.79–2.93)	1.54 (0.79–2.98)
	p for trend		0.89	0.21	0.20
Fasting group[4]					
Insulin (pg ml^{-1})	Tertile 1 (10.7–179.5)	51/62	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (180.3–283.3)	72/63	1.42 (0.84–2.41)	1.62 (0.89–2.93)	1.58 (0.87–2.88)
	Tertile 3 (286.0–4457.3)	65/63	1.35 (0.76–2.40)	1.84 (0.93–3.63)	1.89 (0.95–3.77)
	p for trend		0.31	0.08	0.07
C-peptide (pg ml^{-1})	Tertile 1 (130.5–493.6)	54/65	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (497.5–755.4)	78/66	1.39 (0.86–2.26)	1.68 (0.95–2.97)	1.80 (1.00–3.24)
	Tertile 3 (776.0–2717.4)	65/66	1.23 (0.72–2.08)	1.80 (0.92–3.53)	1.76 (0.89–3.47)
	p for trend		0.46	0.09	0.10
HOMA-IR[5]	<1.73	96/104	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	≥1.73	60/52	1.29 (0.79–2.11)	1.88 (1.03–3.45)	1.97 (1.07–3.65)
HOMA-β (%)[5]	Tertile 1 (17.6–52.7)	41/52	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
	Tertile 2 (53.3–89.0)	58/52	1.49 (0.82–2.69)	1.34 (0.67–2.67)	1.45 (0.71–2.93)
	Tertile 3 (89.3–1580.9)	57/52	1.47 (0.81–2.66)	1.60 (0.81–3.14)	1.94 (0.94–4.03)
	p for trend		0.23	0.17	0.08

[1]Matched for age (±3 years), gender, public health center area, and blood donation date (±2 months).
[2]Adjusted for smoking, alcohol consumption, body mass index, total calories, salt intake, family history of gastric cancer, *Helicobacter pylori* infection status, and atrophy.
[3]Further adjusted for past history of diabetes mellitus and drug treatment for diabetes mellitus.
[4]Fasting group: ≥8 hr after a meal; Non-fasting group: <8 hr after a meal.
[5]Subjects under drug treatment for diabetes mellitus were excluded, and OR3 was further adjusted for past history of diabetes mellitus only.
Abbreviations: HOMA-IR: homeostasis model assessment of insulin resistance; HOMA-β: homeostasis model assessment of β-cell function; CI: confidence interval; OR: odds ratio.

stratification were observed. Higher insulin and C-peptide levels were positively associated with the distal subsite and intestinal type of gastric cancer risk, but not significantly so. In addition, the cardia subsite and diffuse type of gastric cancer also showed a trend toward a positive association with insulin, but not with C-peptide, possibly due to the small number of subjects (data not shown). When we excluded the subjects with a past history of DM and drug treatment for DM, similar associations were observed between plasma insulin and C-peptide and gastric cancer risk. Higher HOMA-IR and HOMA-β values also showed similar associations when subjects with past history of DM were excluded (data not shown). Finally, when we excluded the subjects who developed gastric cancer within 2 years of blood donation and their matched controls, similar associations were observed (data not shown).

Discussion

In this case-control study nested within a large-scale population-based study, we observed an increased risk of gastric cancer according to higher insulin levels, C-peptide levels, and HOMA-IR, independent of several confounding factors. The positive association was also observed when excluding subjects who had past history of DM and drug treatment for DM. In contrast, plasma levels of blood glucose were not associated with gastric cancer risk. No association was observed for any of the diabetic biomarkers in women.

Several postulated DM-related mechanisms of carcinogenesis, including hyperglycemia itself and/or decreased bioactivity of insulin such as hyperinsulinemia or insulin resistance, have been controversial.[27,28] A meta-analysis of several prospective studies reported that not only higher levels of insulin and C-peptide but also higher levels of blood glucose

significantly increased the risk of pancreatic and colorectal cancers.[29] But this meta-analysis had a critical limitation, in that few studies took fasting status into account. In more recent reports of large population-based nested case-control studies of pancreatic and colorectal cancer, fasting group (≥8 hr after a meal) was considered. For the risk of pancreatic cancer, when HbA1c and insulin were adjusted, only a higher level of plasma proinsulin was found to increase the risk, whereas the proinsulin/insulin ratio, a marker of pancreatic β-cell function, was not.[30] For the risk of colorectal cancer, higher insulin level and HOMA-IR were associated with an increased risk, whereas no association was observed for blood glucose.[31] Therefore, the authors concluded that their results did not support the hypothesis that hyperglycemia is causally associated with increased risk of pancreatic and colorectal cancers. We observed that higher levels of insulin and C-peptide significantly increase the risk of gastric cancer, not blood glucose levels. This may suggest the importance of hyperinsulinemia, rather than hyperglycemia, in gastric carcinogenesis as well as other cancer sites, such as pancreatic and colorectal cancer.

Insulin is a well-known key regulator of carcinogenesis, including gastric cancer.[17,18,32] Insulin can enhance insulin-like growth factor (IGF)−1 bioavailability by inhibiting the production of IGF-binding proteins.[18,32] Insulin and bioavailable IGF-1 signal transduction occurs through insulin, IGF-1, and hybrid receptors in the cell membrane.[18] Inhibition of apoptosis and stimulation of cellular proliferation and carcinogenesis occurs because of the several downstream pathways activated by these receptors. The binding of insulin or bioavailable IGF-1 to the receptors activates phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and Ras/MAPK (mitogen-activated protein kinase) pathways.[18]

In our study, the positive associations between plasma insulin and C-peptide levels and gastric cancer occurrence were clearly observed in men, but not in women. One possible explanation is hormonal differences. A recent meta-analysis showed that women with longer exposure to estrogen by either ovarian (fertility) or exogenous origin (hormone replacement therapy) may be protected from gastric cancer,[33] and that the body mass of postmenopausal women correlates with blood estrogen levels.[34] The possible protective effect of estrogen might mask the risk of developing gastric cancer in women, although the analysis stratified by menopausal status (menopausal or not menopausal) did not show a clear difference between the two. Another explanation is that alcohol consumption[35] and smoking[36] may determine insulin resistance and hyperinsulinemia thereby resulting in gastric carcinogenesis. In our study, most alcohol drinkers and smokers were male. However, additional analysis did not show any clear interaction between smoking status or alcohol consumption and diabetic biomarkers.

In the fasting group (≥8 hr after a meal), we analyzed not only plasma insulin and C-peptide levels, but also HOMA-IR and HOMA-β. By calculating HOMA, we can estimate the background of hyperinsulinemia at fasting group such as insulin resistance (HOMA-IR) and/or greater functioning of pancreatic β-cell function (HOMA-β). We found that higher HOMA-IR was positively associated with gastric cancer risk. Therefore, our findings suggest that insulin resistance is the main mechanism underlying the positive association between hyperinsulinemia and gastric cancer risk. HOMA-β also showed a marginal association. One previous study showed an increasing pancreatic β-cell volume to compensate for insulin resistance,[37] which may result in increased β-cell function. A possible explanation for insulin resistance leading to hyperinsulinemia may be that it is a consequence of *H. pylori* infection. According to a recent systematic review, a positive trend toward an association between *H. pylori* infection and insulin resistance was found.[38] Several mechanisms underlying the relationship between *H. pylori* infection and insulin resistance suggest that reactive oxygen species, proatherogenic substances, and inflammatory substances are released by *H. pylori* infection. *H. pylori* infection also promotes the activation/aggregation of platelets and apoptosis.[39]

This is the first population-based prospective study to indicate a positive association between higher levels of insulin and C-peptide and gastric cancer risk. Based on the study design, the blood samples were collected before subjects were diagnosed with gastric cancer, which enabled us to investigate the factors associated with a subsequent risk of gastric cancer incidence. In addition, we have robust data on other factors including fasting status, history of DM, drug treatment for DM, lifestyle factors, atrophy, CagA, and *H. pylori* infection.

Our study did have some limitations. First, among the 97,644 eligible subjects who responded to a self-administered questionnaire in this study, only 36,745 (37.6%) subjects provided a blood sample. Those subjects who participated in the health checkup survey had a more favorable lifestyle, such as less smoking and alcohol consumption, as compared to those who did not participate. Therefore, generalizing the findings of this study to a large population needs to be performed carefully, as described previously.[40] Second, these diabetic biomarkers were measured only once at the baseline. We do not have information regarding the onset of DM in those with high-level diabetic biomarkers, so we cannot speculate regarding the length of suffering attributable to DM. Moreover, given that the follow-up of the subjects lasted for many years, it is possible that these levels might have changed over the course of the years. However, this is not different between cases and controls and likely would have led to underestimation of the results. Third, it is difficult to completely exclude undiagnosed gastric cancer at the baseline survey because past history of gastric cancer was based on self-administered questionnaire. However, when we excluded those subjects who developed gastric cancer within 2 years of blood donation based on the cancer registry, similar associations were obtained. Fourth, with regard to asking past history of DM, we did not distinguish between type 1 and type 2 DM in the questionnaire. However, because type 1 DM is far less frequent then type 2 DM, especially in the adult population, it would be

reasonable to suppose that most of the subjects had type 2 DM. Fifth, we did not have data regarding HbA1c or adequate samples to measure HbA1c. HbA1c levels reflect mean blood glucose over the preceding 3 months. Thus, it is possible that we might have missed subjects who were pre-diabetic or subjects with optimal blood glucose control. Sixth, the proportion of the subjects in the non-fasting group was much higher than that in the fasting group, which may have an effect on the validity of our observations. Therefore, caution should be used when interpreting the results. Finally, the number of subjects may not have been sufficient to identify the association in some anatomic sites or histological types. Therefore, additional large prospective studies are needed to confirm the association in cardia subsite and diffuse type gastric cancer.

In conclusion, our findings suggest that Japanese population with higher insulin and C-peptide levels derived from insulin resistance have an elevated risk of gastric cancer.

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Appendix

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ORIGINAL ARTICLE

Demonstration of the usefulness of epigenetic cancer risk prediction by a multicentre prospective cohort study

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ABSTRACT

Background Epigenetic alterations accumulate in normal-appearing tissues of patients with cancer, producing an epigenetic field defect. Cross-sectional studies show that the degree of the defect may be associated with risk in some types of cancer, especially cancers associated with chronic inflammation.

Objective To demonstrate, by a multicentre prospective cohort study, that the risk of metachronous gastric cancer after endoscopic resection (ER) can be predicted by assessment of the epigenetic field defect using methylation levels.

Design Patients with early gastric cancer, aged 40–80 years, who planned to have, or had undergone, ER, were enrolled at least 6 months after *Helicobacter pylori* infection discontinued. Methylation levels of three preselected genes (*miR-124a-3*, *EMX1* and *NKX6-1*) were measured by quantitative methylation-specific PCR. Patients were followed up annually by endoscopy, and the primary endpoint was defined as detection of a metachronous gastric cancer. Authentic metachronous gastric cancers were defined as cancers excluding those detected within 1 year after the enrolment.

Results Among 826 patients enrolled, 782 patients had at least one follow-up, with a median follow-up of 2.97 years. Authentic metachronous gastric cancers developed in 66 patients: 29, 16 and 21 patients at 1–2, 2–3 and ≥3 years after the enrolment, respectively. The highest quartile of the *miR-124a-3* methylation level had a significant univariate HR (95% CI) (2.17 (1.07 to 4.41); p=0.032) and a multivariate-adjusted HR (2.30 (1.03 to 5.10); p=0.042) of developing authentic metachronous gastric cancers. Similar trends were seen for *EMX1* and *NKX6-1*.

Conclusions Assessment of the degree of an epigenetic field defect is a promising cancer risk marker that takes account of life history.

Significance of this study

What is already known about this subject?

- Epigenetic alterations, represented by aberrant DNA methylation, accumulate in cancers and also in normal-appearing tissues surrounding cancers.
- Cross-sectional studies show that aberrant methylation levels in normal tissues may be associated with a risk of cancer in some types of cancer, especially chronic inflammation-associated cancers.
- An epigenetic cancer risk marker is considered to reflect past exposure to environmental factors and to differ from single nucleotide polymorphism cancer risk markers that do not reflect life history.

What are the new findings?

- By a multicentre prospective cohort study with 826 patients, the methylation level in non-cancerous gastric mucosae of patients with gastric cancer was shown to be significantly (p=0.042) associated with an increased risk of developing metachronous gastric cancers.
- *miR-124a-3* is an informative marker gene for predicting the risk of developing a metachronous gastric cancer.

How might it impact on clinical practice in the foreseeable future?

- This is the first time that the usefulness of an epigenetic cancer risk marker has been demonstrated in any type of cancer by a multicentre prospective cohort study. This new class of cancer risk marker has the potential to be expanded to cancers of other tissues.
- The intensity of surveillance for metachronous gastric cancer can be adjusted depending upon the risk predicted by the *miR-124a-3* methylation level.

INTRODUCTION

Epigenetic alterations, represented by aberrant DNA methylation, are deeply involved in carcinogenesis.[1 2] Aberrant methylation accumulates in cancers and also in normal-appearing tissues, especially those of chronic inflammation-associated cancers, such as gastric cancers,[3 4] hepatocellular carcinoma,[5 6] oesophageal adenocarcinoma,[7 8] colon cancers,[9] colitic cancers[10–12] and breast cancers.[13] In such normal-appearing tissues, both tumour-suppressor genes (driver genes), such as *CDKN2A* and *MLH1* and many other genes that have little

expression in normal tissues (passenger genes), such as *HAND1*, are methylated. Driver genes are methylated only at low levels—that is, in a minor fraction of cells, in normal-appearing tissues. Passenger genes, however, are methylated at high levels—that is, in a large fraction of cells, reflecting the degree of past exposure to inducers of aberrant methylation.[3 14]

The accumulation of such methylation was shown to be associated with a risk of cancer development by cross-sectional studies in the stomach,[14] the liver,[5 6] the urothelium,[15] the oesophagus[8 16] and the colon.[17] In the stomach, a quantitative methylation analysis of both driver and passenger genes showed that patients with gastric cancer had higher methylation levels in normal-appearing tissues than those in healthy individuals.[14 18] Furthermore, patients with multiple gastric cancers had significantly higher methylation levels than those with a single gastric cancer, whose methylation levels were higher than those in healthy individuals.[19] This correlation in the three groups, together with the associations in various types of cancers, strongly supports the suggestion that the accumulation of aberrant methylation in non-cancerous tissues produces an epigenetic field for cancerisation (field defect) and that the presence of such a field is associated with cancer development.[3]

As an inducer of an epigenetic field defect in the stomach, *Helicobacter pylori* (*H. pylori*) infection was associated with high methylation levels in gastric mucosae.[14] Chronic inflammation triggered by the infection was shown to be causally involved in methylation induction in Mongolian gerbils.[20] It is hypothesised that aberrant DNA methylation is induced in stem, progenitor and differentiated cells when active *H. pylori* infection is present[3] because eradication of *H. pylori* leads to a decrease of methylation.[21–23] Importantly, the methylation levels in gastric mucosae without active *H. pylori* infection, but not those in mucosae with active infection, were correlated with gastric cancer risk.[14 19 24] Therefore, measurement of methylation levels in individuals without active *H. pylori* infection is expected to enable us to predict the cancer risk of an individual by analysing the degree of epigenetic field defect.

For incorporation of such a new type of cancer risk marker into practice, demonstration of its usefulness by a multicentre prospective cohort study is requisite. In gastric cancer, a prospective cohort study can be conducted for risk prediction of primary gastric cancer among healthy individuals and for prediction of a metachronous gastric cancer after endoscopic resection (ER). The need for the latter is becoming greater because an increasing number of patients with gastric cancer are now treated by ER and the incidence of metachronous gastric cancer has reached as high as 2.5% a year,[25] although eradication of *H. pylori* decreases or delays its occurrence.[26] Nevertheless, almost all patients treated by ER have similar risk factors and their stratification for future cancer risk has so far been impossible. In addition, this high incidence allows a prospective cohort study with a relatively small number of patients.

Here, we aimed to demonstrate, by a multicentre prospective cohort study, that the risk of metachronous gastric cancer can be predicted by assessment of an epigenetic field defect. This will provide a proof-of-concept that cancer risk prediction can be achieved using the epigenetic field defect.

PATIENTS AND METHODS

Patients

Eligibility was assessed for 964 patients with early gastric cancer aged 40–80 years and who planned to have, or had undergone, endoscopic submucosal dissection, one of the procedures of ER, in one of three hospitals (National Cancer Center Hospital (NCC), Tokyo University Hospital (TYU) and Wakayama Medical University Hospital (WMU)) between 2008 and 2010. Patients were excluded if they had received additional gastrectomy, had cancer in other tissues, died of other diseases before a test for *H. pylori* infection or were receiving anticoagulant therapy and unable to suspend it, refused or had other complications. The remaining 850 patients were tested for *H. pylori* infection. When *H. pylori* infection was absent, they were enrolled at the assessment (n=388). When *H. pylori* infection was present (n=462), patients received eradication therapy and were enrolled at least 6 months after establishment of successful *H. pylori* eradication (n=438) (figure 1). A total of 826 patients were enrolled and at the time of enrolment, biopsy samples were taken from a fixed point in the antrum (the lesser curvature at 2 cm from the pyloric ring) for DNA methylation analysis. Written informed consent was obtained from all the patients and the study was approved by the institutional review boards at each hospital.

Detection of *H. pylori* infection, its eradication and the pepsinogen test

The presence of *H. pylori* infection was examined with the urea breath test (Otsuka, Tokushima, Japan), serum anti-*H. pylori* antibody (Eiken, Tokyo, Japan) and either the culture method or rapid urease test (Otsuka, Tokushima, Japan). To eradicate *H. pylori*, patients were treated with lansoprazole (30 mg), amoxicillin (750 mg) and clarithromycin (200 mg), each taken twice daily for 1 week. Successful eradication was established by negative results of the urea breath test 2 months or more after the eradication. When the eradication was unsuccessful, the patients received second-line eradication therapy. Implementation of third-line and further eradication therapy was left to the doctors in charge. Fasting blood samples were collected on the day of endoscopy at the enrolment and serum levels of pepsinogen I and II were measured by the LZ test (Eiken, Tokyo, Japan).

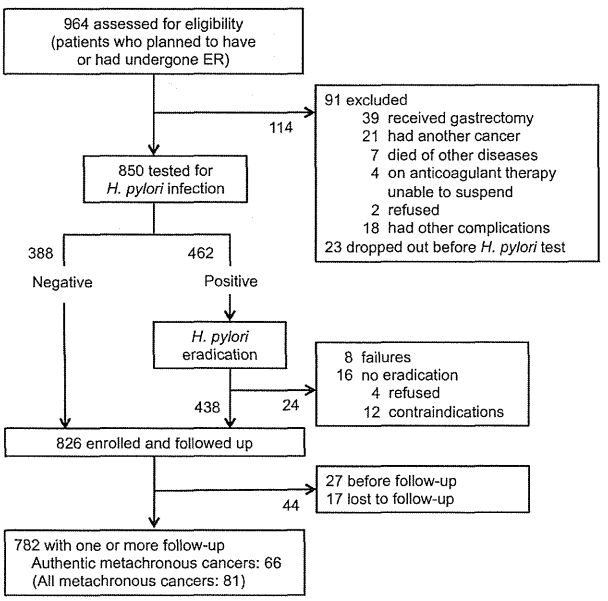

Figure 1 Study profile. ER, endoscopic resection.

Table 1 Baseline characteristics of patients according to the quartiles of DNA methylation levels of the three genes

	DNA methylation level								
	miR-124a-3			*EMX1*			*NKX6-1*		
Characteristic	Q1 (lowest)	Q4 (highest)	p Value	Q1 (lowest)	Q4 (highest)	p Value	Q1 (lowest)	Q4 (highest)	p Value
DNA methylation level, median (IQR)	14.4 (8.3–23.0)	89.8 (88.1–91.9)		11.0 (6.3–16.5)	89.7 (87.4–91.9)		37.5 (24.9–46.3)	87.3 (84.5–90.4)	
Mean age, years (SD)	65.8 (8.5)	68.6 (6.1)	**0.0007**	65.4 (8.4)	68.7 (6.1)	**0.0001**	66.0 (8.2)	68.2 (6.7)	**0.0247**
Male (%)	77.4	84.1	0.3667	79.0	84.1	0.3552	80.0	82.1	0.9434
H. pylori infection before enrolment (%)	40.5	62.1	**0.0004**	39.5	54.4	**0.0041**	39.5	59.0	**0.0008**
Serum pepsinogen index (%)									
(+)	5.1	8.7	**0.0010**	6.2	7.2	0.0556	4.6	8.7	**0.0072**
(2+)	13.9	20.0		14.4	21.5		19.0	18.5	
(3+)	24.1	33.3		21.0	27.7		18.5	30.3	
Past history of ER (%)									
Twice	8.2	7.7	0.6443	9.2	10.3	0.5077	9.2	8.7	0.9836
Three times	1.0	2.1		1.5	2.6		2.1	1.5	
Pack-years of smoking (%)									
1–39	40.3	40.7	**0.0175**	39.7	41.5	**0.0050**	39.1	38.7	0.6757
≥40	20.4	33.9		21.2	35.8		26.3		
Green vegetable intake (%)									
Almost daily	28.3	36.7	**0.0070**	28.7	33.5	**0.0426**	33.7	35.5	0.9862

p values <0.05 are shown in bold type.
*Based on the χ^2 test or the Fisher's exact test for percentage difference and the analysis of variance test for mean age difference.
ER, endoscopic resection.

Follow-up by endoscopy and definition of metachronous cancers

Patients were followed up endoscopically once a year after the enrolment and the primary endpoint was defined as detection of a metachronous gastric cancer. If a patient was lost to follow-up, the follow-up was censored at the time of their last endoscopic examination. Metachronous cancers were defined according to the criteria of Moertel *et al*[27]—namely, (i) each lesion is histopathologically malignant, (ii) each lesion is separated from another and (iii) each lesion is not the result of a local extension or metastasis of other lesions. In particular, 'authentic' metachronous gastric cancers were defined as those found after exclusion of cancers that developed within the first 1 year after the enrolment. This was because there is concern that a gastric cancer developing within the first 1 year after enrolment might have been a cancer undetected at the time of the emrolment[28–30] and might be influenced by the promoting effect of *H. pylori* infection.[26]

Marker genes and quantitative methylation analysis

Three marker genes (*miR-124a-3*, *EMX1* and *NKX6-1*) were preselected in our previous cross-sectional studies,[31 32] and no other marker genes were analysed to avoid multiple testing. *miR-124a-3* was previously identified as a gene whose methylation levels remain high, even in individuals with past *H. pylori* infection.[31] *EMX1* and *NKX6-1* were identified as genes highly informative for detecting patients with gastric cancer among patients with past *H. pylori* infection with ORs of 23.8 and 15.0, respectively.[32] The methylation levels of the three genes were analysed by quantitative methylation-specific PCR (qMSP), as previously described.[31 32] Briefly, bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA. qMSP was performed with primer sets specific to methylated and unmethylated sequences by real-time PCR using SYBR Green I (BioWhittaker Molecular Applications, Rockland, Maine, USA) and an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). To correct for the variation in the number of methylated and unmethylated DNA molecules, depending upon the dilution of the standard DNA, two specific batches of control DNA (fully methylated and unmethylated DNA) were analysed in each qMSP analysis. All the samples were measured twice and the correlation of methylation levels in the two analyses was high (correlation coefficient=0.94).

Statistical analysis

Our study question was whether, at any time, the methylation level in gastric mucosae could predict the risk of gastric cancer and we counted person-years since the enrolment (ie, since the time of biopsy of gastric mucosa).

To analyse the relationship between the methylation levels and a risk of metachronous gastric cancer, all the patients were categorised into quartiles (Q) according to the methylation level of one of the three genes (*miR-124a-3*, *EMX1* and *NKX6-1*), with the baseline characteristics of Q1 (lowest) and Q4 (highest) shown in table 1. Since we assumed that a higher methylation level would be associated with a risk of metachronous gastric cancer, we estimated the HR and 95% CI of Q4 compared with Q1 as the major goal.

Correlations between methylation levels of two genes were analysed using Spearman's rank correlation coefficient. Cox proportional hazard regression analysis was used to calculate univariate and multivariate-adjusted HRs (95% CIs) of metachronous gastric cancer incidence. Kaplan–Meier analysis was performed to compare development of metachronous gastric cancer in the four quartiles. The proportional hazard assumptions for the methylation level in comparing Q4 with Q1 were evaluated and met using graphical and time-dependent variable approaches. Analyses were conducted using the SAS statistical software, V.9.1 (SAS Institute Inc, Cary, North Carolina, USA). The results were considered significant when a p value <0.05 was obtained by two-sided tests.

In multivariate analysis, HRs were adjusted for potentially confounding factors (age (<50, 50–59, 60–69 or ≥70 years), gender, pepsinogen index ((−), pepsinogen I>70 or pepsinogen I/II ratio >3; (+), pepsinogen I≤70 and pepsinogen I/II ratio ≤3; (2+), pepsinogen I≤50 and pepsinogen I/II ratio ≤3; (3+), pepsinogen I≤30 and pepsinogen I/II ratio ≤2), smoking (0, 1–39 or ≥40 pack-years), green vegetable intake (≤2 days, 3–4 days or almost daily), hospital (NCC, TYU or WMU), *H. pylori* infection before the enrolment (positive or negative) and past history of ER (0, 1, 2 or 3 times)).

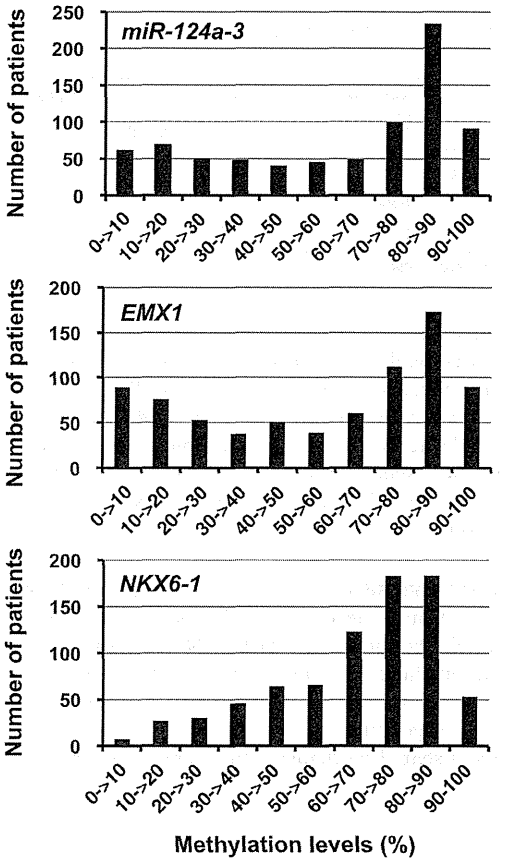

Figure 2 The distribution of methylation levels of *miR-124a-3*, *EMX1* and *NKX6-1* among the 782 patients. *miR-124a-3* and *EMX1* showed bimodal distributions, whereas *NKX6-1* showed a unimodal distribution.

RESULTS

Enrolment and follow-up for a metachronous gastric cancer

Among the 826 enrolled patients, 782 patients (617, 97 and 68 patients at NCC, TYU and WMU, respectively) had at least one follow-up endoscopic examination (figure 1). Among the 782 patients, 81 patients (59, 10 and 12 patients in NCC, TYU and WMU, respectively) developed a metachronous gastric cancer. Among the 81 patients, 15, 29, 16 and 21 patients developed a metachronous cancer in <1, 1–2, 2–3 and ≥3 years after the

Table 2 Correlation among methylation levels of *miR-124a-3*, *EMX1*, and NKX6-1

	miR-124a-3		*EMX1*		*NKX6-1*	
	r	p Value	r	p Value	r	p Value
miR-124a-3	–	–	0.89	10^{-6}	0.77	10^{-6}
EMX1	0.89	10^{-6}	–	–	0.70	10^{-6}
NKX6-1	0.77	10^{-6}	0.70	10^{-6}	–	–

r, correlation coefficient.

enrolment, respectively, and 66 patients (52, 6 and 8 patients in NCC, TYU and WMU, respectively) were considered to have authentic metachronous gastric cancers. The median follow-up period was 2.97 years (2.99, 2.51 and 2.49 years in NCC, TYU and WMU, respectively). The median period for development of all the metachronous cancers after the enrolment was 1.88 years (1.92, 1.97 and 1.32 years in NCC, TYU and WMU, respectively).

Univariate analysis of the effect of methylation levels

The methylation levels of *miR-124a-3* and *EMX1* showed bimodal distribution while that of *NKX6-1* showed a unimodal distribution (figure 2). The methylation levels of the three genes were highly correlated (correlation coefficients=0.70–0.89; table 2), suggesting that methylation of these three genes reflected a shared mechanism, the epigenomic damage in gastric stem cells.

A univariate analysis using the authentic metachronous gastric cancers showed that Q4 (highest) *miR-124a-3* methylation had a significantly higher HR than Q1 (lowest) (95% CI) (2.17 (1.07 to 4.41); p=0.032) (p for trend=0.041) (table 3). The presence of the same trend in Q2 and Q3 with a trend p of 0.041 supported the higher HR in the Q4. Although not significant, similar trends were seen for *EMX1* and *NKX6-1* (p=0.075 and 0.11, respectively). A univariate analysis using all the metachronous gastric cancers also showed that Q4 *miR-124a-3* methylation had a higher HR than Q1 (95% CI) (1.71 (0.90 to 3.22); p=0.10) (p for trend=0.094) (table 3).

The influence of known risk factors for gastric cancer (age, gender, pepsinogen index, smoking and green vegetable intake) and other potential risk factors (hospital, *H. pylori* infection before enrolment, past history of ER) was analysed using the authentic metachronous gastric cancers. Significant associations were seen for young age (HR (95% CI)=3.50 (1.23 to 10.0); p=0.019), being female (0.43 (0.19 to 1.00); p=0.049), past history of ER (5.66 (2.01 to 15.9); p=0.001) and smoking (2.24 (1.27 to 3.96); p=0.006) (table 4). For all the metachronous gastric cancers, in addition to these factors, different hospitals had significantly different HRs (2.26 (1.21 to 4.21); p=0.01).

Multivariate analysis and Kaplan–Meier analysis

A multivariate analysis was conducted by adjusting for hospital, gender, age, *H. pylori* infection before enrolment, pepsinogen index, past history of ER, smoking and green vegetable intake (table 5). Using the authentic metachronous gastric cancers, the

Table 3 Univariate HRs (95% CI) of quartiles of the three genes for a metachronous gastric cancer

	Quartile of DNA methylation level				
Variable	Q1 (lowest)	Q2	Q3	Q4 (highest)	p for trend
No. of patients	195	196	196	195	
Authentic metachronous gastric cancers					
miR-124a-3					
No. of events	12	17	15	22	
HR (95% CI)	1	1.26 (0.60 to 2.65)	1.17 (0.55 to 2.51)	2.17 (1.07 to 4.41)	**0.041**
p Value		0.53	0.68	**0.032**	
EMX1					
No. of events	12	17	16	21	
HR (95% CI)	1	1.47 (0.70 to 3.08)	1.30 (0.62 to 2.76)	2.03 (1.00 to 4.14)	0.075
p Value		0.31	0.49	0.051	
NKX6-1					
No. of events	14	11	20	21	
HR (95% CI)	1	0.70 (0.32 to 1.54)	1.31 (0.66 to 2.60)	1.43 (0.73 to 2.82)	0.11
p Value		0.37	0.44	0.30	
All metachronous gastric cancers					
miR-124a-3					
No. of events	16	20	21	24	
HR (95% CI)	1	1.13 (0.59 to 2.19)	1.24 (0.65 to 2.38)	1.71 (0.90 to 3.22)	0.094
p Value		0.71	0.52	0.10	
EMX1					
No. of events	16	20	19	26	
HR (95% CI)	1	1.28 (0.66 to 2.48)	1.16 (0.60 to 2.26)	1.83 (0.98 to 3.42)	0.080
p Value		0.46	0.66	0.058	
NKX6-1					
No. of events	18	16	24	23	
HR (95% CI)	1	0.81 (0.41 to 1.58)	1.24 (0.67 to 2.29)	1.23 (0.66 to 2.28)	0.29
p Value		0.53	0.49	0.51	

p values <0.05 are shown in bold type.

Table 4 Univariate HRs (95% CI) of known and potential risk factors for metachronous gastric cancer

	Authentic metachronous gastric cancers			All metachronous gastric cancers		
Variable	**No. of events**	**HR**	**p Value**	**No. of events**	**HR**	**p Value**
Hospital						
NCC	52	1		59	1	
TYU	6	1.03 (0.44 to 2.40)	0.95	10	1.43 (0.73 to 2.80)	0.30
WMU	8	1.77 (0.84 to 3.73)	0.14	12	2.26 (1.21 to 4.21)	**0.01**
Age						
≥70	27	1		34	1	
<50	4	3.50 (1.23 to 10.0)	**0.019**	4	2.76 (0.98 to 7.79)	0.055
50–59	6	0.58 (0.24 to 1.39)	0.22	9	0.71 (0.34 to 1.48)	0.36
60–69	29	0.91 (0.54 to 1.54)	0.72	34	0.86 (0.54 to 1.39)	0.55
Gender						
Male	60	1		74	1	
Female	6	0.43 (0.19 to 1.00)	**0.049**	7	0.41 (0.19 to 0.89)	**0.023**
Pepsinogen index						
(−)	28	1		35	1	
(+)	5	2.18 (0.83 to 5.68)	0.11	6	1.88 (0.79 to 4.50)	0.15
(2+)	16	1.65 (0.89 to 3.06)	0.11	18	1.44 (0.82 to 2.55)	0.21
(3+)	17	1.56 (0.85 to 2.87)	0.15	22	1.54 (0.90 to 2.63)	0.12
H. pylori infection before the enrolment						
Positive	41	1		31	1	
Negative	25	1.02 (0.61 to 1.71)	0.94	50	0.93 (0.58 to 1.47)	0.74
Past history of ER (times)						
0	13	1		16	1	
1	43	1.18 (0.63 to 2.19)	0.6	49	1.10 (0.63 to 1.93)	0.74
2	5	0.81 (0.29 to 2.29)	0.69	10	1.38 (0.62 to 3.04)	0.43
3	5	5.66 (2.01 to 15.9)	**0.001**	6	5.46 (2.13 to 14.0)	**0.0004**
Smoking (pack-years)						
0	15	1		18	1	
1–39	17	1.17 (0.57 to 2.40)	0.66	25	1.55 (0.85 to 2.83)	0.15
≥40	29	2.24 (1.27 to 3.96)	**0.006**	32	2.06 (1.21 to 3.50)	**0.007**
Green vegetable intake (per week)						
≤2 days	20	1		25	1	
3–4 days	25	0.67 (0.37 to 1.21)	0.18	32	0.69 (0.41 to 1.16)	0.16
Almost daily	18	0.65 (0.34 to 1.23)	0.18	20	0.57 (0.32 to 1.02)	0.06

p values <0.05 are shown in bold type.
ER, endoscopic resection; NCC, National Cancer Center Hospital; TYU, Tokyo University Hospital; WMU, Wakayama Medical University Hospital.

Q4 (highest) *miR-124a-3* methylation had a higher HR than Q1 methylation (95% CI) (2.30 (1.03 to 5.10); p=0.042) (p for trend=0.057). when all the metachronous gastric cancers were used, Q4 *miR-124a-3* methylation also had a higher HR (95%CI) (1.99 (0.97 to 4.09); p=0.061) (p for trend=0.072). We also conducted a multivariate analysis using the HR of the Q4 with the combined Q1–Q3 as a reference (see online supplementary table S1) and confirmed an association between the high *miR-124a-3* methylation level and the occurrence of authentic metachronous gastric cancers (HR (95% CI)=1.95 (1.11 to 3.43; p=0.021)).

Kaplan–Meier curves of the cumulative incidence rates of all the metachronous gastric cancers for patients were drawn for quartiles (Q1–Q4) of methylation levels for each of the three genes (*miR-124a-3*, *EMX1* and *NKX6-1*) (figure 3 and see online supplementary figure S1). Q4 methylation had higher incidences of a metachronous gastric cancer for all the three genes compared with Q1 methylation.

Analysis of potential confounding factors

The influence of *H. pylori* infection status before the enrolment did not affect the occurrence of metachronous gastric cancers (table 4). However, taking its potential influence on the predictive power of the methylation level, we conducted a stratified analysis by the *H. pylori* infection status before the enrolment. The median methylation levels of the three marker genes were lower in patients without *H. pylori* infection than those in patients with *H. pylori* infection (see online supplementary table S2). In the stratified analysis, the Q4 (highest) *miR-124a-3* methylation levels showed HRs (95% CI) of 1.32 (0.37 to 4.77) and 2.43 (0.81 to 7.32) for the authentic metachronous gastric cancers in the patients with and without *H. pylori* infection, respectively (see online supplementary table S3).

The influence of a past history of ER on the predictive power of the methylation level was also analysed by stratifying patients according to this past history. Again, we observed consistent results in each stratum, although the p values were not statistically significant owing to the decreased numbers of events (see online supplementary table S4). Also, we analysed the variability in the time between the first ER and enrolment in each quartile (see online supplementary table S5). There were no increasing or decreasing trends in the time according to the marker quartiles. Additionally, we conducted a stratified analysis according

Table 5 Multivariate-adjusted HRs (95% CI) for a metachronous gastric cancer according to DNA methylation levels of the three genes

	Quartile of DNA methylation level				
Variable	**Q1 (lowest)**	**Q2**	**Q3**	**Q4 (highest)**	**p for trend**
No. of patients	195	196	196	195	
Authentic metachronous gastric cancers					
miR-124a-3					
No. of events	12	17	15	22	
HR* (95% CI)	1	1.33 (0.60 to 2.93)	1.15 (0.51 to 2.61)	2.30 (1.03 to 5.10)	0.057
p Value		0.49	0.73	**0.042**	
EMX1					
No. of events	12	17	16	21	
HR* (95% CI)	1	1.56 (0.71 to 3.41)	1.21 (0.55 to 2.67)	1.88 (0.86 to 4.13)	0.20
p Value		0.27	0.64	0.11	
NKX6-1					
No. of events	14	11	20	21	
HR* (95% CI)	1	0.76 (0.33 to 1.74)	1.39 (0.66 to 2.96)	1.52 (0.72 to 3.21)	0.12
p Value		0.52	0.39	0.27	
All metachronous gastric cancers					
miR-124a-3					
No. of events	16	20	21	24	
HR* (95% CI)	1	1.30 (0.64 to 2.64)	1.31 (0.65 to 2.64)	1.99 (0.97 to 4.09)	0.072
p Value		0.47	0.45	0.061	
EMX1					
No. of events	16	20	19	26	
HR* (95% CI)	1	1.47 (0.74 to 2.95)	1.16 (0.58 to 2.34)	1.84 (0.92 to 3.67)	0.150
p Value		0.27	0.68	0.082	
NKX6-1					
No. of events	18	16	24	23	
HR* (95% CI)	1	0.93 (0.46 to 1.87)	1.41 (0.72 to 2.76)	1.42 (0.71 to 2.81)	0.19
p Value		0.83	0.32	0.32	

p values <0.05 are shown in bold type.
*Adjusted for hospital, gender and age (<50, 50–59, 60–69 or ≥70), *H. pylori* infection before the enrolment (positive or negative), pepsinogen index, past history of endoscopic resection (0, 1, 2 or 3 times), pack-years of smoking (0, 1–39 or ≥40), and green vegetable intake (≤2 days per week, 3–4 days per week, or almost daily).

to the time between the first ER and enrolment (>5 or ≤5 years) and observed a similar association in each stratum (see online supplementary table S4).

DISCUSSION

It was shown for the first time by a multicentre prospective cohort study that assessment of an epigenetic field defect using methylation levels can be used as a biomarker of cancer risk. This study warrants translation of previous retrospective cross-sectional studies showing that methylation levels accumulated in various tissues are correlated with cancer risk.[6 16 17 19 33 34] Thus, the intensity of surveillance for metachronous gastric cancer can be adjusted according to the risk predicted by the *miR-124a-3* methylation level. In addition, methylation accumulation is known to be caused by exposure to its inducers, such as chronic inflammation,[35] and cancer risk prediction using accumulated methylation is considered to take account of the life history of individuals. This point makes epigenetic cancer risk markers entirely different from genetic cancer risk markers, mostly single nucleotide polymorphisms, which cannot take account of the life history of individuals.

We defined the authentic metachronous gastric cancers because distinction of a metachronous cancer from that undetected at the time of the enrolment is difficult when it is detected at 1 year after the enrolment.[28–30] Also, metachronous gastric cancer developing within 1 year after enrolment might have been influenced by the promoting effect of *H. pylori* infection.[26] Analyses using both the authentic and all the metachronous gastric cancers were conducted and a clearer difference was seen using the authentic metachronous gastric cancers. For example, the methylation level of *miR-124a-3* was associated with development of an authentic metachronous gastric cancer with significant p values of 0.032 and 0.042 by univariate and multivariate analyses, respectively. This supported the hypothesis that the presence of an epigenetic field defect is associated with occurrence of independent multiple cancers.

The quality of this translational study was supported by multiple parameters. The follow-up rate of patients enrolled in this study was high (97.9%; 809/826). The median follow-up period reached 2.97 years and was uniform in the three hospitals. Accordingly, the bias introduced by incomplete follow-up is minimised. Also, all the patients were followed up with a consistent interval of 1 year in the three participating hospitals. This is reflected in the Kaplan–Meier curves, which show that the incidence of a metachronous gastric cancer increases every 1 year. Further, even after adjusting for multiple variables, the influence of methylation levels on the occurrence of authentic metachronous gastric cancer was significant. The fact that two variables (gender and smoking) known to be associated with gastric cancer risk[36] were confirmed as independent risk factors supported the statement that collection of lifestyle information was appropriately conducted in this study.