Journal of Medical Microbiology (2013), **62**, 761–765 DOI 10.1099/jmm.0.053140-0

Multilocus sequence typing of DNA from faecal specimens for the analysis of intra-familial transmission of *Helicobacter pylori*

Takako Osaki,[1] Masumi Okuda,[2] Junko Ueda,[3] Mutsuko Konno,[4] Hideo Yonezawa,[1] Fuhito Hojo,[1] Kiyoko Yagyu,[3] Yingsong Lin,[3] Yoshihiro Fukuda,[2] Shogo Kikuchi[3] and Shigeru Kamiya[1]

Correspondence
Takako Osaki
osaki@ks.kyorin-u.ac.jp

[1]Department of Infectious Diseases, Kyorin University School of Medicine, Tokyo, Japan
[2]Department of General Medicine and Community Health Science, Hyogo College of Medicine, Hyogo, Japan
[3]Department of Public Health, Aichi Medical University School of Medicine, Aichi, Japan
[4]Department of Pediatrics, Sapporo Kosei General Hospital, Sapporo, Japan

Received 1 October 2012
Accepted 31 January 2013

This study used multilocus sequence typing (MLST) of total DNA extracted from faecal specimens to genotype *Helicobacter pylori* to analyse intra-familial transmission. Faecal DNA was extracted and amplified by nested PCR. The products were analysed by direct sequencing and the allele type was determined using an MLST website. Mother-to-child transmission was suspected in at least two of three families, and father-to-child transmission was suspected in one family.

INTRODUCTION

Infection by the Gram-negative microaerophilic rod *Helicobacter pylori* is associated with the development of chronic gastritis, peptic ulcers and gastric adenocarcinoma in humans (Kusters *et al.*, 2006). It is thought that one of the modes of transmission of *H. pylori* is between family members, and therefore the presence of infected family members is an important risk factor in children (Konno *et al.*, 2008). However, there have been few studies that have proven intra-familial infection using isolated family strains (Konno *et al.*, 2005; Nahar *et al.*, 2009). It is difficult to isolate *H. pylori* from the gastric mucosa of children, as endoscopic analysis is not often undertaken. Therefore, the genotypes of paediatric *H. pylori* strains have not been analysed fully in comparison with those of other adult family members.

Multilocus sequence typing (MLST) analysis has become the most common method for genetic analysis of bacterial strains. MLST has been applied previously for the root causal analysis of outbreaks (Chalmers *et al.*, 2008), hospital infections (Walker *et al.*, 2012) and intra-familial infections (Staples *et al.*, 2012). An *H. pylori* MLST database is available online, consisting of seven housekeeping genes (http://pubmlst.org/helicobacter/), with over 2000 alleles detected at each locus. The genetic identity of various pathogenic strains can be analysed by MLST, which is tied in with information on the geographical sources of *H. pylori*, exposing major events in the history of human settlement (Achtman *et al.*, 1999; Falush *et al.*, 2001, 2003; Linz *et al.*, 2007; Moodley *et al.*, 2009; Wirth *et al.*, 2004).

After *H. pylori* organisms reach the anaerobic environment of the intestine, the micro-organisms are unable to grow and change morphology to their coccoid forms, which are non-culturable (Shirai *et al.*, 2000). However, the DNA of *H. pylori* has been reported to be detected by PCR using faecal specimens from infected patients (Scaletsky *et al.*, 2011) and animals (Oshio *et al.*, 2009). Here, we compared the MLST of faecal DNA specimens for the detection of intra-familial *H. pylori* infection.

Abbreviations: MLST, multilocus sequence typing; ST, sequence type.

METHODS

Participants. Children aged 0–12 years attending seven elementary schools, three nursery schools and six kindergartens in Sasayama city, Hyogo, Japan, were recruited into an epidemiological study. The Sasayama Study, for *H. pylori* infection in children, was carried out from November 2010 to March 2011. Stool samples were collected from 783 children, and 15 samples gave positive results. Family members of the 15 stool antigen-positive children were asked to provide stool samples, and 35 people belonging to 12 families provided samples.

The Sasayama Study was undertaken in accordance with the Declaration of Helsinki with approval from the Ethics Committees of Kyorin University, Tokyo; Hyogo College of Medicine, Hyogo; and Aichi Medical University School of Medicine, Aichi. Informed consent was obtained from the parents of children and from participants.

Stool antigen test. The collected stool specimens were kept at −80 °C until use. A TestMate Pylori Antigen enzyme immunoassay

(Wakamoto) was used for selection of *H. pylori*-positive faeces according to the manufacturer's guidelines. Briefly, 30 mg faecal specimen was diluted with 1 ml diluent. Faecal solution (50 µl) was added to each well and mixed with the reagent. Absorbance at 450 nm/630 nm was measured using a spectrophotometer, and the cut-off value of the test was taken as 0.100.

DNA extraction. Total DNA of *H. pylori* antigen-positive faeces was extracted using a QIAamp Stool kit (Qiagen) according to the manufacturer's instructions. Briefly, 200 mg frozen faeces was used for each extraction, and 200 µl DNA solution in Buffer AE (Qiagen) was eluted at the final step. *H. pylori* 16S rRNA gene-targeted primers were used for detection of *H. pylori* DNA by real-time PCR (Osaki *et al.*, 2006) and confirmed the *H. pylori* antigen-positive faecal samples.

MLST. The primers used for MLST are shown in Table 1. Gene fragments containing the *efp*, *mutY*, *ppa* and *trpC* genes were amplified from *H. pylori*-positive specimens by nested PCR. For the first reaction, 10 µl 2× Ampdirect Plus buffer (Shimadzu), 0.1 µl BIOTAQ Hot Start DNA Polymerase (Bioline), 2 µl primer mix (10 pmol μl^{-1} each), 6.9 µl distilled water and 1 µl DNA sample made a reaction volume of 20 µl. The Ampdirect Plus buffer neutralizes inhibitory substances in biological samples, and, as a result, increases PCR detection. The reaction mixture was incubated in a TP600 thermal cycler (Takara). Ex-*Taq* (Takara) was used in the second PCR. The amplification program consisted of one cycle at 94 °C for 10 min (first PCR) or 1 min (second PCR) and followed by 40 cycles of 94 °C for 20 s, 50–58 °C for 45 s and 72 °C for 45 s, with a final cycle at 72 °C for 7 min. The PCR products were separated using a 2 % agarose gel, stained by ethidium bromide and visualized under UV light. If two bands were visualized on the gel, only the band identical in size to the control band was collected.

The products were analysed by direct sequencing. Sequencing reactions were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier thermal cycler using an ABI BigDye Terminator v3.1 Cycle Sequencing kit with AmpliTaq DNA polymerase (FS Enzyme; Applied Biosystems), according to the protocol supplied by the manufacturer. Single-pass sequencing was performed on each template using one of the second-PCR primers (forward or reverse, Table 1). The fluorescently labelled fragments were purified from the unincorporated terminator nucleotides by ethanol precipitation. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The direct sequencing results obtained were submitted to the MLST website, and the closest allele typing of each gene was determined.

Using the allelic profile of the four genes, the sequence type (ST) of each faecal sample was also determined through the MLST website by the nearest match.

RESULTS AND DISCUSSION

Fifteen stool antigen-positive children were found in the Sasayama Study. Family members of the 15 children were asked to provide stool samples, and 35 people from 12 families provided samples. We selected three families for MLST analysis according to the following; the proband child was diagnosed with *H. pylori* twice by a positive stool antigen test at 0 and 3 months, and the child had two stool antigen-positive family members. The remaining nine families did not match these conditions. All stool antigen-positive faeces were also positive for *H. pylori* DNA by PCR with no false-positive results.

MLST profiles of the *H. pylori* DNA extracted from faeces were determined in the three families in which there was an *H. pylori*-positive child and two family members (Table 2). In family A, *H. pylori* DNA and antigens were detected from the child and the parents but not from the sibling. The first faecal DNA sample of child A had four genes identical to its father. The second sample had the same *mutY* allele as its parents and the same *efp* allele as its mother.

In families B and C, *H. pylori* DNA and antigens were detected in the children, mothers and grandfathers but not

Table 1. Primers used in this study

Locus	PCR	Name	Primer*	Amplicon (bp)	Reference
efp	First	efp_for1	GGCAATTTGGATGAGCGAGCTC	558	MLST website
		efp_rev1	CTTCACCTTTTCAAGATACTC		MLST website
	Second	efp_for2	GGGCTTGAAAATTGAATTGGGCGG	500	MLST website
		efp_rev2	GTATTGACTTTAATGATCTCACCC		MLST website
mutY	First	mutY_for4	TTATGAAGTCTCTATATCAGCGAAGT	529	This study
		mutY_rev 4	TACCTAAACAATAAGGATTGAAAGG		This study
	Second	mutY_for 5	ATATCAGYGAAGTGATGAGC	516	This study
		mutY_rev 5	CCYAAACAATAAGGRTTKGAA		This study
ppa	First	ppa_for1-1	GAARTKAGCCATGACGCTRA	698	MLST website
		ppa_rev 4	GGGTTAARATCGTTAAATTGTAG		MLST website
	Second	ppa_for 1-2	AGCCATGACGCTRAKYCTTT	490	This study
		ppa_rev 1-2	CTCTTTGTTTTCAAACCCCTTG		This study
trpC	First	trpC_for8	AGCATCGCCCTCTAAAGGTT	618	This study
		trpC_rev 6	AAGCCCGCACACTTTATTTTC		This study
	Second	trpC_for 9	TCGCCCTCYAAAGGTTTRAT	564	This study
		trpC_rev 9	TCAAATCCTTTTCTTTCATYA		This study

*Y=C or T; K=G or T; R=A or G.

Table 2. MLST of faecal DNA in three families

Family	Family member*	Allele type for:			
		efp	*mutY*	*ppa*	*trpC*
A	Index child (1st)	1908†	703	1934	454
	Index child (2nd)	181	703	838	181
	Father	1908†	703	1934	454
	Mother	181	703	945	ND
	Sibling‡	–	–	–	–
B	Index child (1st)	1807	1540	502	1468
	Index child (2nd)	1807	1540	502	457
	Mother	1908	1540	502	1468
	Grandfather	1908	703	945	181
	Father‡	–	–	–	–
	Sibling‡	–	–	–	–
C	Index child (1st)	1908	2019	938	457
	Index child (2nd)	1908	703	1934	457
	Mother	1908	703	1934	1239
	Grandfather‡	–	–	–	–
	Father‡	–	–	–	–
	Sibling‡	–	–	–	–

ND, Not determined.

*1st and 2nd indicate the first and second samples taken, with an interval of 3 months between the first sample collection and the second.

†There were three differences from the 1908 allele sequence.

‡These family members were *H. pylori* negative.

from the faeces of siblings and fathers. The first faecal DNA sample from child B had identical allele types for the *mutY*, *ppa* and *trpC* loci but a different allele for the *efp* locus compared with those of the mother. The second sample from child B had identical *mutY* and *ppa* genes but different *trpC* and *efp* genes compared with its mother. The first faecal DNA sample from child C had the same *efp* gene only as its mother, but the second sample had identical alleles for *efp*, *mutY* and *ppa*.

The candidates for *H. pylori* sequence typing were defined from the database by combinations of MLST loci (Table 3). According to these data, in family A, the first sample of the child was shown to be same as that of its father and the second sample to be the same as its mother. In families B and C, the two isolates from the children were identified to be the same as those of their mothers.

We determined the source from whom the original strain was transmitted to the child. The implication was therefore that *H. pylori* was transmitted from mother to child in families B and C. In family A, *H. pylori* may have been transmitted either from the father and/or the mother. The results also implied that *H. pylori* strains from the grandfather were probably not the source of infection. In the Sasayama Study (from 2010 to 2011), no siblings of *H. pylori*-positive children were positive for faecal *H. pylori* antigen or the 16S rRNA gene. In this study, the infection rate was also very low. This may show that intra-familial transmission of *H. pylori* is rare.

Seven loci (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, *vacA* and *yphC*) of housekeeping genes are available for MLST analysis of *H. pylori* isolates. These are widely used markers for genomic diversity within *H. pylori* populations (Yamaoka, 2009). For the MLST analysis using faecal specimens, we used PCR to examine the above seven loci. It was difficult to obtain amplification products of *H. pylori* DNA >600 bp from faecal DNA due to the presence of either other bacterial DNA or substances inhibitory for PCR amplification. In addition, it may have been that the levels of *H. pylori* DNA were relatively low in the gut or that the DNA was damaged.

For the identification of allele types of *atpA*, *ureI* and *yphC*, fragments of ~600 bp (actually sizes of 627 bp, 535–585 bp and 504–631 bp, respectively) were needed to be amplified using available primers, so we instead selected the four shorter-length fragments (*efp*, *mutY*, *ppa* and *trpC*) for MLST using faecal samples.

In another study, we also showed that in one family the MLST profile of the child's *H. pylori* isolate from gastric mucus was identical to that of his mother's strain but not to that of the father's strain (data not shown). *H. pylori* MLST may therefore be useful as a tool for detection of the source of intra-familial infection.

Mother-to-child transmission occurs in early childhood and has been thought to be the most probable route of transmission of *H. pylori* in various countries including Bangladesh (Nahar *et al.*, 2009) and Japan (Konno *et al.*, 2008). In our study, mother-to-child transmission was suspected in two or three of the three cases analysed, whilst father-to-child transmission was suspected in one case. Furthermore, grandparent-to-child transmission was not detected. Our study indicated that parents can be a potential source of *H. pylori* infection in children.

The alleles of *trpC* belonged to different ST types in family C, and the *ppa* allele was different in two samples. One possible explanation is that multiple strains with different alleles had colonized the child or, less likely, that these genes had mutated in the 3-month study period.

It was reported by Raymond *et al.* (2004) that analysis of the isolates from family members indicated natural mixed infection in the family. Identical alleles were found in some strains isolated from the children and parents, demonstrating that strains had circulated within the family.

It is well known that high genetic diversity is a hallmark of *H. pylori*. Kennemann *et al.* (2011) reported very few mutations in an isolate cultured for 3 months after infection of a human volunteer, highlighting the importance of mixed infections for genetic diversification of *H. pylori* through recombination. As it has been reported that *H. pylori* strains exhibit diverse genotypes after long-term infection from childhood to adulthood (Kraft, *et al.*, 2006),

Table 3. Results of MLST

Family	Member*	Candidates for MLST (STs)†	Family member with similar STs
A	Child (1st)	**960/1660/2250/2265**	Father
	Child (2nd)	**181**	Mother
	Father	**960/1660/2250/2265**	
	Mother	**181**/664/960/975/978/1143/1145/1262/1264/1403/1445/1733	
B	Child (1st)	**489/1108/1346/1466/1565/1929**/2145	Mother
	Child (2nd)	**489**/669/**1108**/1290/**1466/1929**/2145	Mother
	Mother	**489/1108/1346/1466/1565/1929**/2265	
	Grandfather	181/960/1228/2265	
C	Child (1st)	669/870/1290/**2250/2265**	Mother
	Child (2nd)	669/1290/2207/**2265**	Mother
	Mother	960/1809/**2250/2265**	
	Grandfather	402/2269	

*1st and 2nd indicate the first and second samples taken, with an interval of 3 months between the first sample collection and the second.
†STs that were the same in each family are indicated in bold.

it is likely that intra-familial transmission of *H. pylori* can be determined by a molecular technique such as MLST.

In conclusion, these results demonstrated that MLST of faecal *H. pylori* DNA is a useful tool for the detection of intra-familial transmission.

ACKNOWLEDGEMENTS

This project was supported partially by Health and Labour Sciences Research Grants for Clinical Cancer Research from the Ministry of Health, Labor and Welfare, Japan, grants from the Japan Society for the Promotion of Science for Scientific Research (#22590613, #23590518 and #24593166), and the Waksman Foundation of Japan Inc. The authors would like to thank Mrs Tomoko Nozaki for her excellent technical assistance. This publication made use of the *Helicobacter pylori* MLST website (http://pubmlst.org/helicobacter/) developed by Keith Jolley and sited at the University of Oxford. The development of this site has been funded by the Wellcome Trust.

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Cancer Causes Control
DOI 10.1007/s10552-013-0214-0

ORIGINAL PAPER

A prospective cohort study of shift work and the risk of death from pancreatic cancer in Japanese men

Yingsong Lin · Junko Ueda · Kiyoko Yagyu · Michiko Kurosawa · Akiko Tamakoshi · Shogo Kikuchi

Received: 26 March 2013 / Accepted: 15 April 2013

Abstract
Purpose There is mounting evidence that shift work involving night work increases cancer risk. We examined the relationship between working rotating shifts and the risk of death from pancreatic cancer on the basis of data from the Japanese Collaborative Cohort Study (JACC Study).
Methods The present analysis was restricted to 22,224 men who were 40–65 years of age at baseline (1988–1990) and who reported working full time or were self-employed in the JACC Study. The subjects were followed through 31 December 2009. Information on occupation and lifestyle factors was collected using a self-administered questionnaire. The Cox proportional hazards model was used to estimate the relative risk (RR) and 95 % confidence interval (CI) for the risk of death from pancreatic cancer in relation to shift work.
Results During the follow-up period, 127 pancreatic cancer deaths were observed. Overall, we found no statistically significant increase in the risk of death from pancreatic cancer associated with rotating shift work. As compared to day-shift workers, the RRs were 0.83 (95 % CI 0.43–1.60) for rotating shift workers and 0.61 (95 % CI 0.22–1.60) for fixed night-shift workers, after adjustment for potential confounding factors. The multivariable-adjusted RR was 1.34 (95 % CI 0.66–2.75) among rotating shift workers in the analysis restricted to men who reported working full time at baseline.
Conclusions Our data did not support the hypothesis that shift work is significantly associated with the risk of death from pancreatic cancer in this cohort of Japanese men.

Keywords Pancreatic cancer · Shift work · Cohort study · Risk

Introduction

Pancreatic cancer is the fifth leading cause of cancer-related death in Japan, with 26,780 deaths in 2010. The etiology of pancreatic cancer remains largely unknown, although cigarette smoking and longstanding type-II diabetes have been shown to be associated with an increased risk [ ]. The lack of effective pancreatic cancer screening tools coupled with the dismal prognosis for this form of cancer make it crucial to identify modifiable risk factors that can be incorporated into a prevention strategy.

Approximately 15–20 % of the working population in industrialized countries engages in night-shift work, drawing significant interest in the effect of such work patterns on health, including on the formation of cancers. Exposure to light at night may suppress the production of melatonin, a hormone involved in circadian rhythms and sleep [ ]. Disruption of circadian rhythms has been shown to promote

This study was conducted for the JACC Study Group.

The members of the JACC Study Group are given in acknowledgments.

Y. Lin · J. Ueda · K. Yagyu · S. Kikuchi (✉)
Department of Public Health, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan
e-mail: kikuchis@aichi-med-u.ac.jp

M. Kurosawa
Department of Epidemiology and Environmental Medicine, Juntendo University School of Medicine, Tokyo, Japan

A. Tamakoshi
Department of Public Health, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Published online: 26 April 2013

carcinogenesis in animal studies [3–5]. Although the findings are not entirely consistent, epidemiologic studies have indicated that shift work is significantly associated with increased risks of breast, colorectal, and prostate cancers [6–13]. Furthermore, in the Nurses' Health Study, women who had worked 30 or more years on rotating night shifts had a 36 % elevated risk of breast cancer compared with women who had never worked rotating night shifts [9]. Recent studies have also indicated that women with morning preference who engage in nigh shifts may have a higher risk compared with women with evening preference [14, 15]. This finding suggests that extended periods of working rotating night shifts and chronotype may be important factors in determining cancer risk.

On the basis of sufficient evidence from animal studies and limited evidence from epidemiologic studies, the working group of IARC concluded in 2007 that "shift work that involves circadian disruption is probably carcinogenic to humans" [16]. There have been experimental data showing that disruption of circadian rhythms in mice is associated with accelerated growth of pancreatic cancer [5], but the association between shift work and pancreatic cancer risk in humans remains unclear. To test the hypothesis that rotating shift work might be associated with an increased risk of pancreatic cancer, we analyzed data from a prospective cohort study of middle-aged and elderly Japanese individuals.

Methods

Study cohort: the JACC Study

The JACC Study started in 1988, enrolling 110,585 people (46,395 men and 64,190 women) from 45 areas throughout Japan. Participants were 40–79 years of age at baseline. Informed consent was obtained by having the study subject sign the cover of the questionnaire, except in a few study areas where it was provided at the group level after the aim of the study and confidentiality of the data had been explained to community leaders. At enrollment, participants completed a self-administered questionnaire addressing demographic characteristics, family history of cancer, medical history, occupation, and lifestyle factors. Pre-coded options for response to occupation were employed, working part-time job, self-employed, housewife, no occupation, or others. The cohort participants were followed until 31 December 2009. Because of logistical problems, follow-ups were discontinued before 31 December 2009, in 10 areas. During the follow-up period, we verified the vital status of participants using resident-registry data from the municipalities. Mortality was ascertained from the causes of death recorded on death certificates. Pancreatic cancer was classified according to the 10th revision of the International Classification of Disease, malignant neoplasm of the pancreas (ICD10, C25). The ethics committee at the Aichi Medical University School of Medicine approved the JACC Study.

Subjects for the present analysis

Our analysis was restricted to men who were 40–65 years of age at baseline and who reported working full time or were self-employed at baseline. After excluding men with missing data on occupation and those who had a history of cancer at baseline, 22,224 men remained for inclusion in the present analysis.

Exposure data

We collected information on shift work based on the question: "Which form of work schedule have you engaged in for your longest occupation?" Men were asked to indicate the most regular schedule they had undertaken among three work schedules: fixed daytime work, fixed nighttime work, or rotating shift work.

We also collected information on covariates, including age, height, weight, medical history, family history of cancer, smoking (current smoker, former smoker, or nonsmoker), alcohol consumption (current drinker, former drinker, or nondrinker), job type (office work, manual work, or other), physical activity at work (sitting, alternate sitting and standing, or standing with/without moving), workplace (indoor, outdoor, or both), level of perceived stress (low, moderate, high, or very high), educational level, and marriage status. Body mass index (BMI) was calculated from height and weight reported by the subjects.

Statistical analysis

We computed person-years of follow-up for each cohort participant from baseline to 31 December 2009, or to the date of pancreatic cancer death, death from any cause, or loss to follow-up, whichever occurred first. The Cox proportional hazards model was used to estimate RRs and 95 % CIs for the association between shift work and the risk of death from pancreatic cancer. In the multivariable analyses, we adjusted for potential confounding factors, including age (continuous), BMI (<20, 20–22.4, 22.5–24.9, $\geqq 25.0$), history of diabetes (yes, no), alcohol drinking (never, past, current), cigarette smoking (never, past, current <20 cigarettes per day, current $\geqq 20$ cigarettes per day), sleep time (continuous), and perceived stress (low, moderate, high).

All analyses were conducted using SAS 9.1 (SAS Institute, Cary, NC, USA). p Values for statistical tests were two-tailed and considered to be statistically significant if they were <0.05.

Table 1 Baseline characteristics of the study subjects according to work schedule in the JACC Study

	Daytime work (n = 18,781)	Fixed nighttime work (n = 1,083)	Rotating shift work (n = 2,360)
Age (years)	52.2 ± 7.4	52.0 ± 7.2	50.4 ± 7.2
Body mass index (kg/m^2)	22.9 ± 3.7	23.2 ± 2.8	23.1 ± 2.7
History of diabetes (%)	4.9	3.5	4.3
Current smokers (%)	54.6	54.5	57.5
Current drinkers (%)	77.7	72.4	75.4
High perceived stress in daily life (%)	24.4	23.6	33.2
Job type (%)			
Office work	20.0	6.6	14.1
Manual work	52.0	64.4	48.9
Sleep time (h)	7.4 ± 1.0	7.3 ± 1.0	7.1 ± 1.0

Results

We recorded 127 pancreatic cancer deaths during an average follow-up period of 18 years. Daytime workers, fixed nighttime workers, and rotating shift workers accounted for 84.5, 4.9, and 10.6 % in the baseline cohort, respectively. Table 1 shows baseline characteristics of the cohort participants according to work schedule. Compared to daytime workers, rotating shift workers tended to be younger, were more likely to smoke, and were more likely to perceive high stress in daily life. The average sleep time tended to be shorter in rotating shift workers than in daytime workers.

Overall, we found no statistically significant increase in the risk of pancreatic cancer death associated with rotating shift work (Table 2). After adjustment for other potential confounding factors, the RR was 0.83 (95 % CI 0.43–1.60) among rotating shift workers in comparison with daytime workers. However, the multivariable-adjusted RR was 1.34 (95 % CI 0.66–2.75) among rotating shift workers in the analysis restricted to those who reported working full time at baseline.

We conducted an additional analysis that excluded all deaths within the first 2 years of follow-up to remove the potential effect of underlying diseases at baseline on the risk of death from pancreatic cancer. The risk estimation remained unchanged; the RR was 0.84 (95 % CI 0.44–1.62).

Discussion

In this cohort study of Japanese men, we evaluated the hypothesized association between shift work and the risk of death from pancreatic cancer. Overall, there was no significant association between rotating shift work and the risk of death from pancreatic cancer. The major strength of our study is that it is a prospective study, which precludes the recall bias that plagues case–control studies. Furthermore, we collected detailed information on lifestyle factors, allowing us to control for other confounding factors, such as cigarette smoking, history of diabetes, sleep time, and perceived stress level.

Epidemiologic studies are not entirely consistent in showing a positive association between shift work and cancer risk [6–13]. Several different mechanisms have been proposed in studies that observed a positive link. The principal mechanism involves a melatonin pathway. The biological function of melatonin is wide-ranging, and affects sleep, circadian rhythm, sexual maturation and reproduction, and aging [2]. As for cancer, melatonin has been shown to inhibit tumor growth through a direct antiproliferative effect, an enhancement of immune function, the scavenging of free radicals, and the modulation of the expression of tumor suppressor genes [2]. Longstanding exposure to light at night may be associated with a suppression of melatonin [17], which may in part contribute to the increased risk observed among shift workers. In addition to the melatonin pathway, metabolic disturbances, such as impairment of insulin sensitivity, may also explain some of the adverse late effects of prolonged shift work [18].

Although the mechanisms underlying the association between shift work and cancer risk are biologically plausible and are supported by experimental evidence, convincing data from epidemiologic studies are still lacking. To our knowledge, our study was the first prospective cohort study to address shift work and the risk of death from pancreatic cancer in Japanese individuals. The null finding should be interpreted in light of several limitations. First, given the small number of pancreatic cancer deaths observed in our study, especially in the category of those working fixed night or rotating shift schedules, we were limited to detecting statistically significant associations. Second, one possible explanation for the null finding is

Table 2 Association between rotating shift work and the risk of death from pancreatic cancer in the JACC Study

	Person-years	Deaths	RR1	95 % CI	RR2	95 % CI
Daytime work	322,341	111	1.00		1.00	
Fixed nighttime work	19,565	5	0.67	0.27–1.64	0.61	0.22–1.60
Rotating shift work	41,042	11	0.88	0.47–1.64	0.83	0.43–1.60

RR relative risk, *CI* confidence interval

RR1: adjusted for age (continuous)

RR2: adjusted for age (continuous), body mass index (<20, 20.0–22.4, 22.5–24.9, ≧25.0), history of diabetes (yes, no), alcohol drinking (never, past, current), cigarette smoking (never, past, current <20 cigarettes per day, current ≧20 cigarettes per day), perceived stress (low, moderate, high), and sleep time (continuous)

selection bias. However, we consider that the effect of the so-called health worker effect is minimal in our cohort study, because the prevalence of shift work at baseline was similar to that reported in a 1991 survey, and the mortality of cohort participants was also similar to that of the general Japanese populations. Third, the assessment of shift work was crude, with only one question to obtain exposure data. We lacked information on the duration of shift work, which is important to more accurately quantify the amount of exposure. Other epidemiologic studies had similar measurement problems in accurately defining and assessing relevant exposures. For example, the exact nature of work schedule/patterns is difficult to define; most studies did not differentiate between continuous and intermittent shifts. Fourth, although we adjusted for cigarette smoking, history of diabetes, sleep time, and perceived stress level, it is still possible that our risk estimates could have been affected by other unknown confounding factors, including hormone and stress levels. Unfortunately, these data were not available in the present study. Finally, the null result may be due, in part, to the variations in individuals' capacities to adjust to their irregular work schedules and their tolerance to disruptions of their normal circadian rhythms. Such variations could be accounted for by genetic variations in circadian genes [19]. A previous study has reported that the variant Per3 genotype was significantly associated with an increased risk of breast cancer among premenopausal women [20], suggesting the importance of interaction between genetic variants and environmental factors in cancer development.

In conclusion, our data did not support the hypothesis that shift work is significantly associated with the risk of death from pancreatic cancer in this cohort of Japanese men. Further studies are needed to address the role of shift work in the development of pancreatic cancer by improving exposure measurement and incorporating relevant biomarkers.

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (Monbusho), and Grants-in-Aid for Scientific Research on Priority Areas of Cancer, as well as Grants-in-Aid for Scientific Research on Priority Areas of Cancer Epidemiology from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Monbu-Kagaku-sho) (Nos. 61010076, 62010074, 63010074, 1010068, 2151065, 3151064, 4151063, 5151069, 6279102, 11181101, 17015022, 18014011, 20014026 and 20390156). We express our appreciation to Drs. Kunio Aoki and Yoshiyuki Ohno, Professors Emeritus of the Nagoya University School of Medicine and former chairpersons of the JACC Study. We are also greatly indebted to Dr. Haruo Sugano, former Director of the Cancer Institute, Tokyo, who greatly contributed to the initiation of the JACC Study, Dr. Tomoyuki Kitagawa, Director Emeritus of the Cancer Institute of the Japanese Foundation for Cancer Research and former chairman of the Grant-in-Aid for Scientific Research on Priority Area "Cancer" and to Dr. Kazao Tajima, Aichi Cancer Center and previous chairman of the Grant-in Aid for Scientific Research on Priority Area of Cancer Epidemiology, for their warm encouragement and support of this study. The present members of the JACC Study Group who co-authored this paper are: Dr. Akiko Tamakoshi (present chairperson of the study group), Hokkaido University Graduate School of Medicine; Drs. Mitsuru Mori and Fumio Sakauchi, Sapporo Medical University School of Medicine; Dr. Yutaka Motohashi, Akita University School of Medicine; Dr. Ichiro Tsuji, Tohoku University Graduate School of Medicine; Dr. Yosikazu Nakamura, Jichi Medical School; Dr. Hiroyasu Iso, Osaka University School of Medicine; Dr. Haruo Mikami, Chiba Cancer Center; Dr. Michiko Kurosawa, Juntendo University School of Medicine; Dr. Yoshiharu Hoshiyama, Yokohama Soei University; Dr. Naohito Tanabe, University of Niigata Prefecture; Dr. Koji Tamakoshi, Nagoya University Graduate School of Health Science; Dr. Kenji Wakai, Nagoya University Graduate School of Medicine; Dr. Shinkan Tokudome, National Institute of Health and Nutrition; Dr. Koji Suzuki, Fujita Health University School of Health Sciences; Dr. Shuji Hashimoto, Fujita Health University School of Medicine; Dr. Shogo Kikuchi, Aichi Medical University School of Medicine; Dr. Yasuhiko Wada, Faculty of Nutrition, University of Kochi; Dr. Takashi Kawamura, Kyoto University Center for Student Health; Dr. Yoshiyuki Watanabe, Kyoto Prefectural University of Medicine Graduate School of Medical Science; Dr. Kotaro Ozasa, Radiation Effects Research Foundation; Dr. Tsuneharu Miki, Kyoto Prefectural University of Medicine Graduate School of Medical Science; Dr. Chigusa Date, School of Human Science and Environment, University of Hyogo; Dr. Kiyomi Sakata, Iwate Medical University; Dr. Yoichi Kurozawa, Tottori University Faculty of Medicine; Drs. Takesumi Yoshimura & Yoshihisa Fujino, University of Occupational and Environmental Health; Dr. Akira Shibata, Kurume University; Dr. Naoyuki Okamoto, Kanagawa Cancer Center; and Dr. Hideo Shio, Moriyama Municipal Hospital.

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

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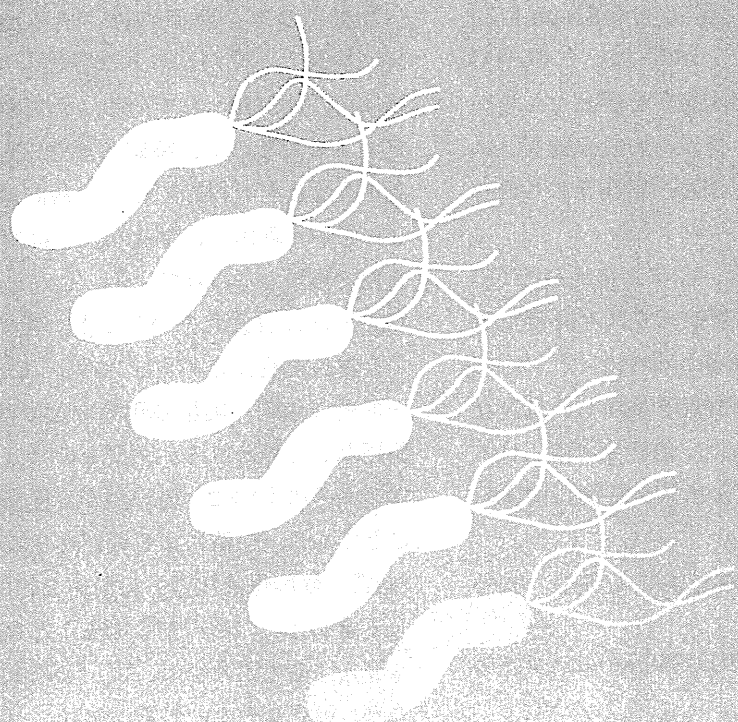
日本ヘリコバクター学会誌

Japanese Journal of *Helicobacter* Research

Vol.14 No.2
2013年1月15日



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胃がんリスク評価－ABC分類の問題点と対策

菊地 正悟

はじめに

所謂ABC分類は、*Helicobacter pylori*(以下*H. pylori*)抗体と血清pepsinogen(ペプシノゲン、ペプシノーゲンとも、以下PG)値とを用いて、個々人の胃がんリスクを評価する方法である[1]。*H. pylori*抗体による*H. pylori*感染の有無と、PG値による胃粘膜萎縮の有無とを組み合わせて2×2＝4つに分類する(**表1**)。*H. pylori*抗体価は能書記載(感染診断)のカット・オフ値が、PG値は三木らの基準(PG I≦70ng/mL and PG I / PG II≦3.0)[2]が用いられることが多いが、一部(PG I≦70ng/mL and PG I / PG II≦4.0)を萎縮としているところもある。この方法は、胃がんの将来的なリスクを評価するものであって、胃がんの存在そのものを推定するものではない。

日本ヘリコバクター学会では、今年から従来の「ペプシノーゲン検討委員会」が「胃がんリスク評価推進委員会」と改称され、血清PG検査だけでなく、胃がんリスク評価についても現在ある課題を検討し、有用な形でその普及を図ることとなった。他学会では、日本消化器がん検診学会に、「胃がんリスク評価に関する附置研究会」が設置されている。

ここでは、①ABC分類の名称に関する議論、②必要とされる背景、③リスク評価の精度(判定基準と精度の評価)に関する問題、④検診システムを構築する上でのデータ管理の問題、⑤除菌歴に関する問題、について検討する。日本ヘリコバクター学会会員諸兄には、釈迦に説法になってしまうところも多々あるかと思われるが、お許しをいただきたい。

表1 *H. pylori*抗体とPG値による胃がんのリスク評価分類(除菌歴のない人が対象)

	*H. pylori*抗体	PG値判定*	
A群	(−)	(−)	胃がんリスク低い
B群	(+)	(−)	胃がんリスク中等度
C群	(+)	(+)	胃がんリスク高い
D群	(−)	(+)	胃がんリスク高い

*PG I≦70ng/mLかつPG I /PG II≦3.0を陽性、PG I≦70ng/mLかつPG I /PG II≦4.0を陽性とするところも。C群とD群を合わせてC群とすることもある。

1) 胃がんリスク評価の名称

胃がんリスク評価には、「ABC検診」、「胃がんリスク検診」、「胃がんリスク分類」、「ABC分類」など、様々な呼称が使われている。将来の胃がんリスクを推定するものなので、特定の疾患(主にがん)の早期発見を目的とする「検診」という用語は必ずしも適切ではないという理由で、学会などでは用いないようにする方向で議論がなされている。しかし、「検診」という言葉が一般に普及していること、すでに「○○検診」という言葉を使って実施しているところもあることから、一般的な使用、実施の現場などでは「○○検診」でも問題ないと思われる。重要なのは、一般の人、特に受診者やその家族に、何がわかる検査なのかを周知することである。血清による胃がんリスク評価の実施主体(企業、健康保険組合、自治体など)、受託機関や関連する学会は、この周知を図ることも重要である。新しく導入するところでは、「○○分類」や「○○評価」の方が、説明しやすいと思われる。

2) リスク評価が必要とされる背景

これまでの研究で感染者と持続感染歴のない未感染者で20倍以上の胃がんリスクの違い(リスク比)があることが示されている[3,4]。*H. pylori*除菌後にも胃がんの発生は少なくなく、特に萎縮の進んだ例ではリスクが高いようである[5]。これまでの研究で示されてきた

愛知医科大学医学部公衆衛生学(日本ヘリコバクター学会胃がんリスク評価委員会委員長)

リスク比3～6倍という数字は、自然除菌による血清抗体価陰性化例が、非感染例として扱われることによる過小評価と考えられる。10年以上観察した前向き研究においても、採血時にすでに血清抗体が陰性化していた自然除菌例から発生した胃がんを未感染例からの発生と数えることによって過小評価されている。この影響は、病原性の強い東アジア型の*H. pylori*株の感染している地域で大きい[6]。

*H. pylori*の感染歴の有無で20倍以上胃がんのリスクが違うとすると、未感染者の胃がんリスクはかなり低いことになる。未感染者に関しては、現行の胃がん検診を実施することは、マイナス面が大きいことは明らかであり、実施すべきではない。

胃がん検診が開始された1960年頃は、がん年齢である40歳以上の人口のほとんどが、*H. pylori*感染者か自然除菌後であったと考えられる。胃がんのハイリスク者が人口のほとんどである状況のもとでは、現行の胃がん検診は理にかなった対策であった。当初、有効性の評価がなされないままに導入されてはいるが、後に症例対照研究で有効性が示されている。

図1に、最近のわが国の*H. pylori*感染有病率（prevalence）の推定値[7]を示す。胃がん検診の対象年齢の40歳以上の*H. pylori*陽性率は、40歳代で28％、50歳代で43％、60歳代で54％、70歳代で71％と推定される。このように、検診対象年齢で*H. pylori*陽性率が50％を割り込む状況においては、年齢区分した全対象に現行の胃がん検診を行うことは、非効率である。感染歴を有する者が50％である場合には、1人の胃がんを救命するのに必要な検診対象者数は100％であった時の2倍になる[8]。

すでに報告したように、わが国の胃がん罹患率、死亡率は、胃がん検診が始められた頃に比べ、年齢別にみるとかなり低下している（**表2**）[9]。これは、*H. pylori*陽性率が低下しているためである。

このような状況のもとでは、対象年齢の下限を引き上げて胃がん罹患率の高い年齢だけを対象にするか、*H. pylori*感染歴のない胃がん低リスク群と感染歴のある高リスク群に対象を篩い分けて高リスク群だけに胃がん対策を行う必要がある。

対象年齢を引き上げても、胃がん低リスク群が対象にかなりの割合で含まれてしまい、その割合は年々増加するので、あまり効率的な方法ではない。低リスクと高リスクの篩い分けの方が効率的である。尿素呼気試験（UBT）、便中抗原検査、血清/尿中抗体検査などの*H. pylori*の感染診断単独では、自然除菌例が低リスク群に入ってしまう。そこで、血清PG検査を同時に行って、実際に胃がんリスクの高い自然除菌例を高リスク群と判定するようにしたのが、胃がんリスク評価である。この方法では、*H. pylori*抗体陽性群の中でも、PG値によって胃がんリスクをある程度評価できる（**表1**のB群とC群の分類）ことが明らかになっている[1]。UBTなどと血清PG検査を組み合わせる方法もあるが、採血だけで済む血清抗体とPG検査の組み合わせが標準的な方法である。

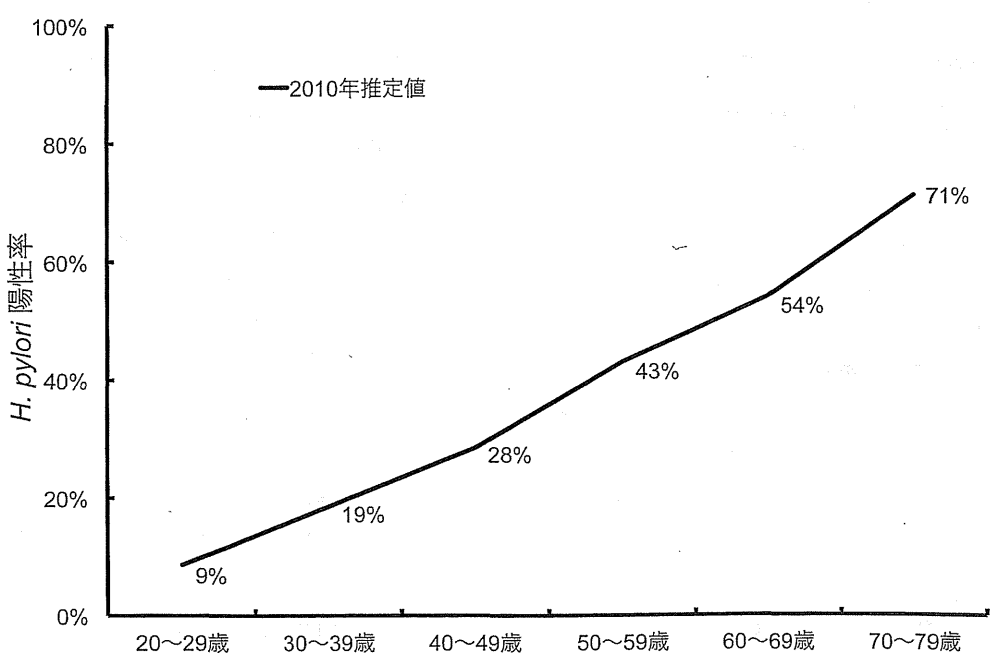

図1　2010年のわが国の*H. pylori*感染率の推定。文献7より引用（抜粋）

3) リスク評価（分類）の精度（判定基準と精度の評価）

a. 分類の判定基準（カット/オフ）の見直し

これまでの検討で、PG I≦70ng/mL and PG I / PG II≦3.0を4.0としているところがある。これは、3.0と4.0の間にも感染歴のある例がはいってしまうことがわかっているためである。三木らのPG I≦70ng/mL and PG I/PG II≦3.0という基準は、胃がんと診断された例と、非胃がん例を篩い分けることを考えた基準であり、*H. pylori*感染歴の有無を判定するための基準ではないので、感染歴の有無の篩い分けに用いるには再検討が必要である。また、*H. pylori*抗体価についても、感染歴のある例の見逃しが多いために、カット・オフ値を引き下げるべきだという意見が、第18回の日本ヘリコバクター学会学術集会（2012年6月岡山）でも出されている。

分類の判定基準については、主として検診機関の追跡調査のデータに依るものがほとんどである。このため、データの比較や統合が難しい。今後の方向性として、各検診機関が持っている*H. pylori*抗体とPG値の測定結果と、地域がん登録のデータを結合（record linkage）することにより、大きなデータ・セットを作成することが考えられる[9]。*H. pylori*抗体価、PG値のデータと、測定後5年間の胃がん罹患データがあれば、現在の基準だけでなく、様々な基準による分類についても、どれだけの精度で胃がんリスクの評価が可能かを明らかにできる。2つ以上の検査値を合わせた場合に、最適の判定基準を決める方法は確立されていない。しかし、多くの基準値の組み合わせによる分類を試行錯誤的にコンピュータで行うことと、部分的にROC曲線（receiver operating characteristics curve）を用いることで、最適の基準値と、その時の分類の精度を求めることが可能である。

これまで、**図2-1**に示すように、*H. pylori*抗体とPG値の判定基準は、他方の結果にかかわらず一定にする方法が採られてきた。しかし、どちらかの判定基準をまず決めて、その判定基準の結果によって他方の判定基準を変えるという判定方法も考慮すべきである。A群とBCD群の篩い分けが重要であるので、PG値による判定基準をまず固定する場合（**図2-2**）には、A群とB群の境界が重要であり、*H. pylori*抗体の判定基準を固定する場合（**図2-3**）には、A群とD群の境界が重要となる。**図2-2**のC群とD群の境界は、除菌治療を行うかという視点や、経過観察の頻度を変えるかという視点から決める必要があり、それぞれの目的で基準が異なることはむしろ当然である。**図2-3**のB群とC群の境界も同様である。なお、C群とD群は合わせてC群として扱うという考え方もある。

表2　1958年と2009年の胃がん死亡率（10万対）の比較

性別	年	30歳代	40歳代	50歳代
女	1958	14.12	36.53	80.30
	2009	2.11	5.57	14.05
男	1958	11.04	52.22	166.23
	2009	1.71	6.56	31.38

b. 精度の評価

胃がんリスク評価の精度は、**表1**、**図2**のA群の胃がん罹患率が0に近いほど、胃がん非罹患例がA群以外に少ないほどよいことになる。なお、精度にB群に比べてC群とD群で胃がん罹患率が高いという*H. pylori*感染歴があると推定される群の中でのリスク評価を加えることもある。

今のところ、*H. pylori*抗体とPG値の測定結果と、地域がん登録のデータを結合したものより大きなデータは考えられないので、リスク分類の精度の評価もこのデータで行う必要がある。なお、「*H. pylori*抗体とPG値による胃がんリスク評価の精度の評価について、この方法による胃がん対策全体の死亡率減少効果を確認すべきである」という議論が散見されるが、リスク評価（分類）の精度評価は、将来の胃がん罹患をどれだけ正しく推定するかという、A～D（C）群の胃がん罹患率の比較によるべきである。死亡率減少効果は、リスク分類に加えて除菌や内視鏡もしくはX線による経過観察の効果、その後の治療と合わせた胃がん対策全体の評価である。*H. pylori*陽性率が低下し、胃がん罹患率が低下しつつあるという状況のもとで、胃がんのリスク評価は重要である。まず、*H. pylori*抗体とPG値による胃がんリスク評価の精度を評価し、十分な精度であると判定されれば、直ちに一般に導入すべきである。

c. 境界領域と画像診断

A群とB群やA群とD群を区分する場合に、*H. pylori*感染歴のある群とない群が、境界領域で重なってしまう可能性がある。このような場合に、X線や内視鏡による画像診断によって、感染歴を判定すべきであ

ることが提案されている。PG値や*H. pylori*のように、判定基準が数値で決められるものと異なり、これらの画像診断は、経験やトレーニングによる高い技術水準が要求されると同時に、判定者によって結果が異なる場合がある。その結果として、当然1件あたりの費用は大きくなる。このような課題はあるが、PG値と*H. pylori*抗体によるリスク評価でA群とB群、A群とD群の境界領域の判定の精度が十分得られない場合の判定方法として検討すべきものである。

4) データ管理

胃がんリスク評価は、血清*H. pylori*抗体とPG検査を入口として、他の疾患の予防に重点を置くA群、除菌の効果が大きいとされるB群、胃がん罹患率が高く除菌の効果があまりないとされるC群、理論的には除菌の効果がないD群に分類し、除菌や定期的な画像診断を行うことになる。成人での除菌による胃がん罹患リスクの低下が確認され、胃がん対策は、理想的には除菌による罹患リスクの低下と定期的な画像診断による早期発見を組み合わせたハイブリッド型のものになる。画像診断についても、その間隔をリスクに応じて変えるという考え方が主流になりつつある。このように複雑な対象者管理を実施するためには、対象者のデータを一元管理して、適時に受診するように連絡する体制の整備が不可欠である。

しかし、このような管理を誰がどのように行うかは、実は非常に大きな課題である。X線による胃がん検診は、逐年受診を勧奨し、リスク別に検診間隔を変えることは行われてこなかった。胃がん検診はがんの有無を判定するもので、胃がんリスクを判定するという意識が実施側になかったことや、逐年検診でないと受診者が受診を忘れてしまうために実施側が逐年検診を勧めてきたことが原因である。このような事情から、実施主体である市区町村などに、検診の対象者の受診者別にリスクを記録するという体制はほとんどない。また、がん検診は市区町村（一部で雇用者）が実施する

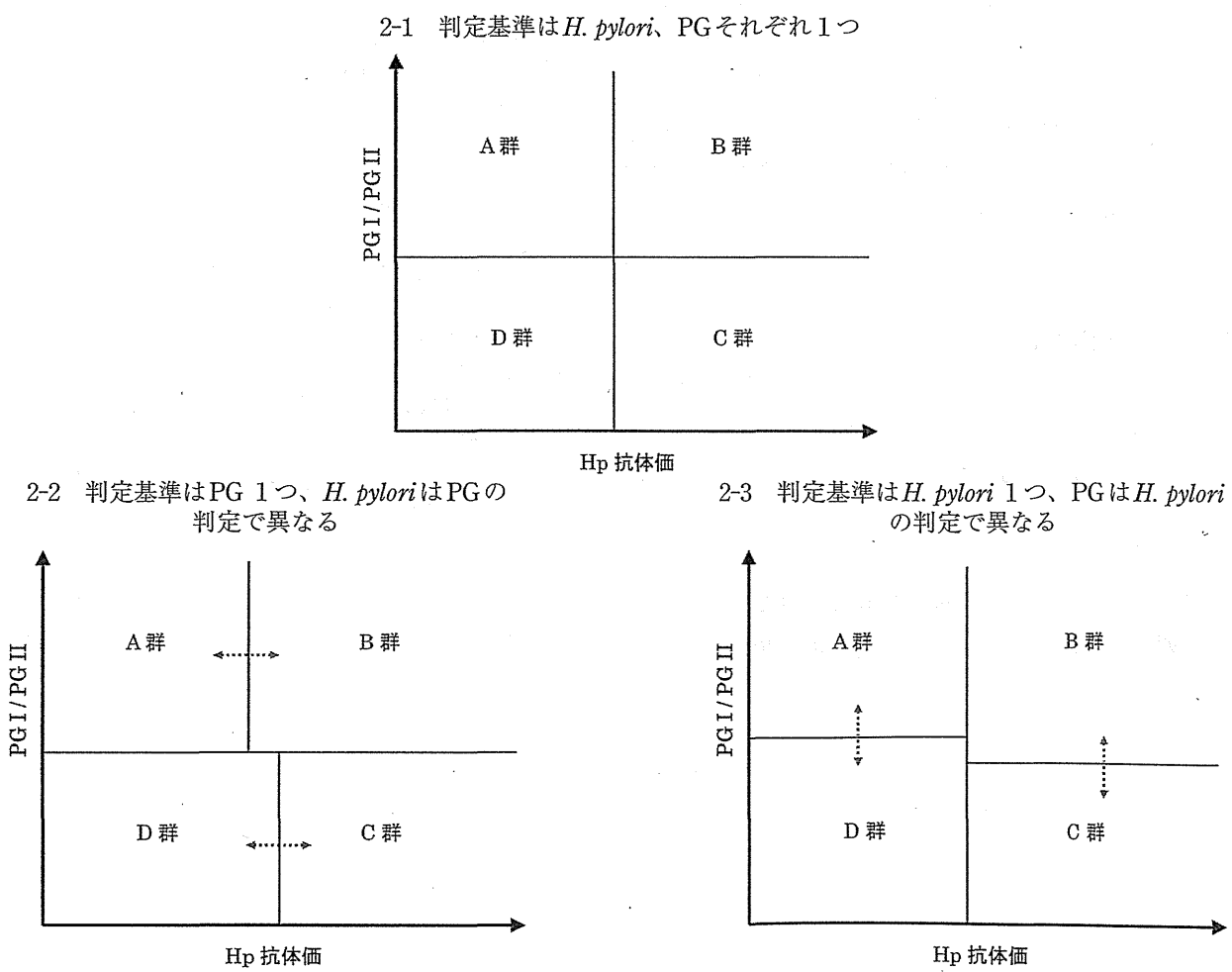

図2　従来の判定基準同士が独立した分類（2-1）以外に、PGの判定結果により*H. pylori*の判定基準を変える分類（2-2）や、*H. pylori*の判定結果によりPGの判定基準を変える分類（2-3）も考えられる。PGの判定基準として、PG I / PG IIが示されているが、2群に判定できる基準であればよい。

ことになっていて、転退職や転居があると、検診の実施主体は替わってしまう。胃がんリスク評価を生かしていくためには、対象者のデータを一元的に管理する体制の整備が不可欠である。

5) 除菌歴に関する問題

本誌ですでに指摘されている[10]ように、胃がんリスク評価方法は、除菌歴がない対象にしか適用できない。B群やC群を除菌すると、リスク評価の分類ではA群となる例が少なくない。しかし、胃がんリスクはB群やC群の除菌後の状態であるから感染歴がない場合に比べてかなり高く、定期的な検査が必要である。対象者の除菌歴がわかっていれば、胃がんリスクの誤評価は起こらないが、実際のところ本人が除菌歴を覚えていないことが結構あるようである。多くは、除菌治療を受けたが十分説明を受けていないために、本人が除菌歴を認識していないためと推測される。しかし、上気道感染の遷延や重傷化など、抗菌薬の投与が必要な疾患の治療によって除菌された例も含まれている可能性がある。この問題は、胃がんリスクの誤評価につながるという点で重大な問題である。除菌の実施にあたって十分な説明を行い、対象者が失念することのないようにする努力が必要である。また、他疾患の治療による意図しない除菌の実態を把握することも重要と思われる。

6) その他の問題

ここまで述べた以外にも、胃がんリスク評価にはいくつかの課題がある。別のところで検討している[8,9]ので、参照いただければ幸いである。

おわりに

胃がんリスク評価は、早急に普及させることが、胃がん死防止を図り、医療資源を効率的に使うという観点から求められている。しかし、判定基準を再検討してその精度を評価すること、胃がん予防をシステム的に管理する体制の整備、除菌歴の失念や意図せぬ除菌による誤評価など課題も少なくない。課題の解決のためには、適切な研究計画に基づいてデータを収集し、分析検討していく必要がある。

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昭和5年3月28日　第三種郵便物認可　2012年10月1日発行（毎月1回1日発行）

ISSN　0047-1852　Nippon Rinsho

Japanese Journal of Clinical Medicine

日本臨牀

2012　10

第70巻・第10号
（通巻第1029号）

特集　胃癌の予防と治療

―予防策と早期診断・治療―

株式会社 日本臨牀社

I. 総　論

疫学の視点から実施すべき2つの胃癌予防

菊地正悟

Two necessary strategies of gastric cancer prevention from the point of epidemiologic view

Shogo Kikuchi
Department of Public Health, Aichi Medical University School of Medicine

Abstract

H. pylori infection mainly occurs under five years of age, interruption of which may be an effective preventive strategy against gastric cancer. Infection to children can be interrupted if *H. pylori* harbored by persons around the children was eradicated. Prevention of gastric cancer death of adults with *H. pylori* infection may be another strategy. Risk evaluation of gastric cancer for each subject is inevitable because current Japanese population is a mixture of infected high risk subjects and low risk ones. Combined use of serum pepsinogens and *H. pylori* antibody test is a useful method of the risk evaluation. According to the evaluation, some subjects should undergo *H. pylori* eradication therapy and/or periodical examination, which may prevent gastric cancer deaths effectively.

Key words: *Helicobacter pylori*, interruption of infection to children, prevention of gastric cancer death, risk evaluation, serum pepsinogens

はじめに

Helicobacter pylori(*H. pylori*)と胃癌の関係が明らかになり，感染者と未感染者で20倍以上の胃癌リスクの違いがあることが明らかにされている[1,2]．また，一度分化型胃癌が発生した例では，内視鏡切除後に*H. pylori*を除菌することで，他部位の再発が1/3程度に減少することが示された[3,4]．内視鏡切除後の再発抑制は，内視鏡的に診断できる直前の状態まで成長した'癌の芽'が臨床癌に成長するのに*H. pylori*がプロモーターとして作用している過程を，除菌がある程度阻害することを示している．臨床癌になる最終の過程で，*H. pylori*のプロモーター作用を阻害することで'癌の芽'が臨床癌になる確率が1/3程度になることから，それより前の段階での除菌は，胃癌発生を予防する効果がより大きいと考えられる．感染者の胃癌リスクが未感染者(一度も感染したことのない人)の20倍以上であることと考え併せると，臨床胃癌の発生前に除菌を行えば，胃癌のリスクは，悪くても1/3程度には抑制されると考えられる．

このように，*H. pylori*感染が胃癌のリスクに極めて大きい影響を与えていることに加え，*H. pylori*除菌が胃癌のリスクをある程度減少させることが明らかになった．これらの知見によ

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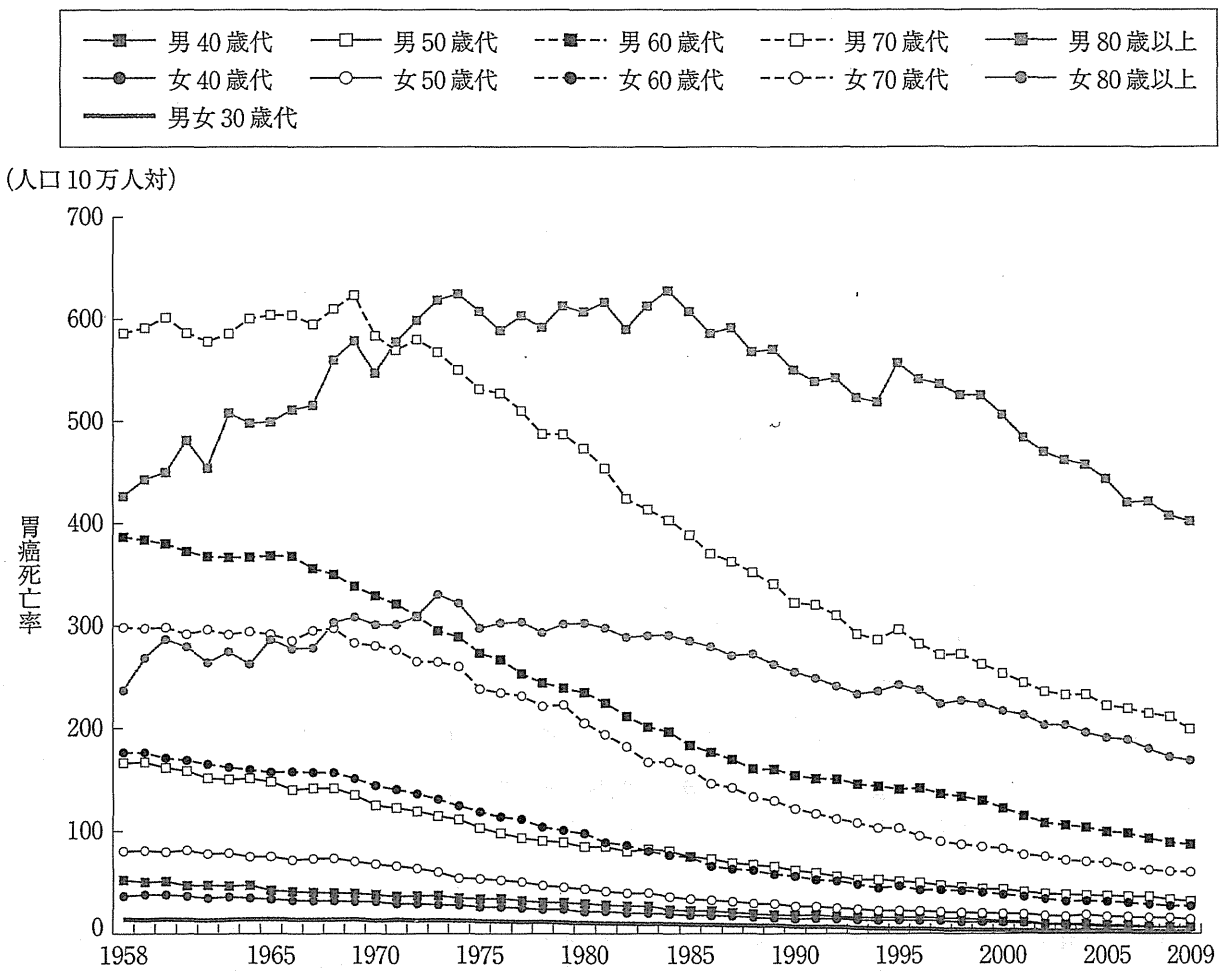

図1 我が国の胃癌死亡率の推移(人口10万人対)

り，胃癌対策はより効率的なものに進化すべきである．胃癌対策の将来像について検討した．

1. 我が国の胃癌死亡率と *H. pylori* 有病率

図1に我が国の1958-2009年の性別10歳階級の胃癌死亡率を示す[5]．70歳以下では1968年頃までは横ばいか緩やかな低下を示し，その後速度を上げて低下している．80歳以上では，女性では1973年頃まで，男性では1983年頃まで漸増～横ばいで，その後低下に転じている．

図2に年代ごとの *H. pylori* 感染有病率(以下有病率)を示す．1990年頃の実測値A[6]とB[7]から，有病率が年齢とともに漸増し，一定のところでプラトーに，より高齢で若干低下するというモデル[8]に合わせたものが1990年推定値である．1990年推定値を20年右へ平行移動して0-9歳を2%としたものが2010年推定値である．若い年齢では，*H. pylori* の自然除菌(胃粘膜萎縮の進行による)はまれと考えられので，陰性はほとんどが未感染者である．この群の胃癌リスクは，感染者の1/20以下であるので，この群の増加が，胃癌死亡率減少の主な原因と考えられる．

2. 我が国で実施すべき胃癌対策

このような状況の下で，我が国で実施すべき胃癌対策として，小児への感染防止と *H. pylori* 感染成人の胃癌死防止の2つが考えられる．

1) 小児への感染防止

H. pylori の主な感染時期は5歳までとされている．減少してはいるが，この時期に感染を受ける子どもは0ではない．全体として胃癌は急速に減少しているが，小児期に感染を受けた児

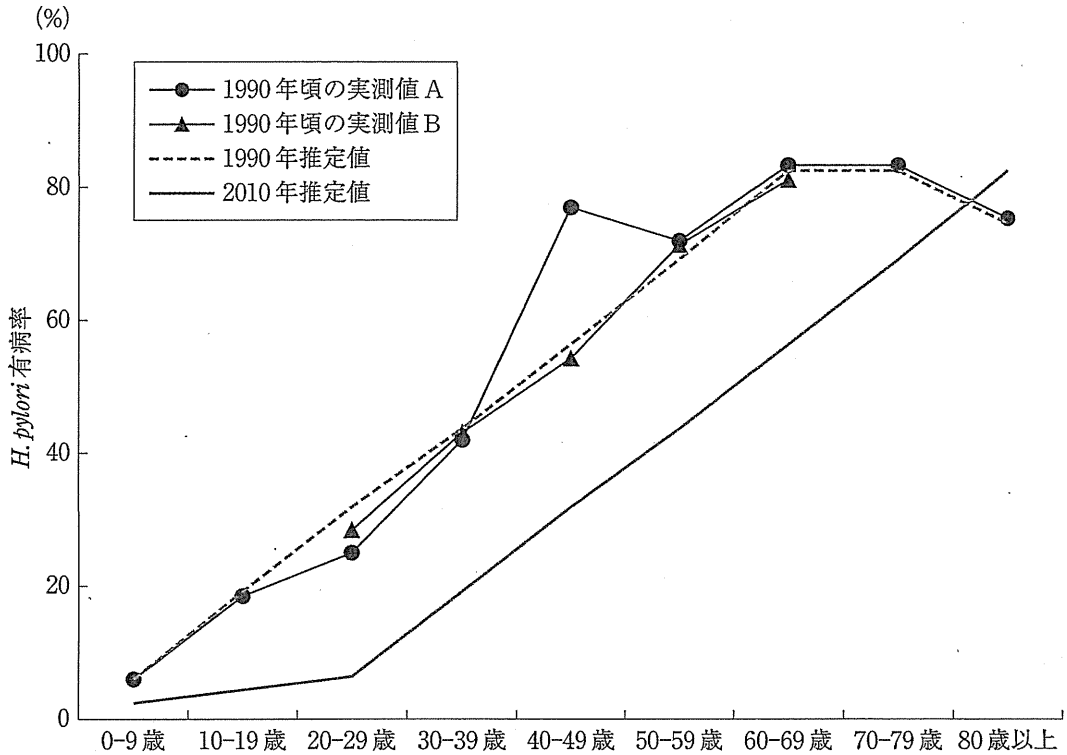

図2 *H. pylori* 年齢別有病率の推定値
過去の実測値AとBから最近の年齢ごとの有病率を推定した.

にとっては，生涯の胃癌リスクが高いことは，現在成人となっている感染者と変わらない．リスクの高い群(*H. pylori* 感染者)と低い群(未感染者)が混在する状態の下では，リスクを評価する感染診断を全員に実施する必要があり，対策にコストがかかる．5歳までの小児期の感染を防止することは，将来的に *H. pylori* 感染を0にすることにつながり，癌を含めた疾病対策のコストを大きく下げることになる．5歳以上での感染は極めてまれなので，小児期に感染を防止することの胃癌を含めた疾病予防効果は大きいと考えられる[9]．

課題として幾つかの事項が挙げられる．これまで多くの研究がなされたが，*H. pylori* がどのように感染するかについては明らかにされていない．最近は，関心は経路でなく感染源に移っている．感染源で明らかなのは感染しているヒトの胃である．除菌によって感染者＝感染源をなくすことで，感染防止が可能である．まず，家族内，保育園・幼稚園などの集団生活，それ以外という小児が生活の中で触れる範囲のどこで感染が起こっているのかを明らかにすることが重要である．特に，小児同士での感染が多いとすると，小児期での除菌が必要となる．感染防止に小児期での除菌が必要な場合，無症状の小児が対象となることが少なくない．症状のある対象に症状の軽快を目的として行う場合に比べ，無症状の対象に将来の疾病の予防を目的として行う場合，安全性はより厳しく要求されることになる．小児では成人の状況に近づく何歳以降に除菌すべきというように，年齢を考慮するのが一般的である．しかし，小児同士の感染を防ぐという視点からは早い方が望ましいというジレンマがある．また，何歳までに除菌すれば将来の胃癌リスクが無視できる程度のものになるかは明らかでない．この視点からも胃粘膜の変化が少ない早い時期の方が除菌の効果は大きいと考えられる．安全面との検討を含め，今後明らかにすべき課題である．

2) *H. pylori* 感染成人の胃癌死防止

これまで，主としてX線造影法による胃がん検診が担ってきた課題である．しかし，