RISK FACTORS FOR GASTRIC CANCER AND RISK STRATIFICATION IN SINGAPORE CHINESE POPULATION

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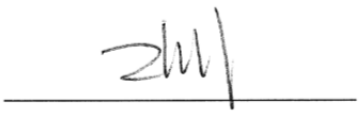
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

About one million people are diagnosed with gastric cancer each year worldwide, making gastric cancer the 4th most common cancer. Half of the global burden occurs in East Asia. Gastric cancer is also the second leading cause of cancer death worldwide accounting for 736,000 deaths in 2008.

The known risk factors for gastric cancer are *Helicobacter pylori* (HP) infection, diet with high intake of salt and preservatives, smoking, alcohol, family history of gastric cancer and pernicious anemia. The disease etiology is multi-factorial and genetic risk factors are not the main driver, however in association with environmental factors, gene polymorphism studies have identified specific genetic susceptibilities predisposing to development of gastric cancer (GC).

Primary prevention is theoretically possible for instance through attempting eradication of *Helicobacter pylori* infection. Secondary prevention by screening and surveillance is practised in Japan and Korea where the population incidence of GC incidence is high. Early detection by endoscopy screening and surveillance has been shown to reduce mortality rate in Korea. It is thought to be not cost-effective to screen the general population in countries with low to intermediate GC incidence rates such as Singapore.

Endoscopy is the only current technology that allows diagnosis of early GC. Early detection by endoscopy screening is the key to reducing mortality, however it is costly and invasive. Risk stratification to identify individuals at high risk of GC is a potential method to reduce the cost of screening and surveillance. The gastric carcinogenesis

sequence from superficial gastritis to subsequent development of atrophic gastritis, intestinal metaplasia, dysplasia and finally culminating in adenocarcinoma is well established. People with precursor lesions such as atrophic gastritis and intestinal metaplasia are believed to be at higher risk of developing GC.

This thesis sets out to describe the risk factors for GC in our local population and to explore the possibility of stratifying the Singapore Chinese population into those with low or high risk for GC based on genetic risk factor and precursor lesions of GC.

The first two studies (GCEP, described in Chapter III of this thesis) describes a cohort of Singapore Chinese subjects recruited for the GCEP study, which aimed to determine whether a local programme of systematic prospective endoscopic screening is able to detect early GC and/or high grade dysplasia in a high risk Singapore Chinese population. In this cohort we studied the clinical risk factors and the prevalence of premalignant lesions such as atrophic gastritis and intestinal metaplasia.

In the third study (Chapter IV), we investigate the presence of gene polymorphisms associated with GC risk in subjects with gastric intestinal metaplasia, regarded as a pre-neoplastic precursor for GC.

In the fourth study, we evaluate the use of blood markers for predicting GC risk. Since *Helicobacter pylori* (HP) is the most important single risk factor for GC and gastric atrophy is a well-established risk factor, we studied the combination of HP antibodies and serum pepsinogen (PG) in a serology panel and explored the use of this HP-PG panel for risk stratification purposes in our population.

The results from these studies suggest that it is possible to risk stratify the Singapore Chinese population based on genetic polymorphisms, HP infection and presence of atrophic gastritis and intestinal metaplasia. Five clinical risk factors were found to be significantly associated with the development of early gastric neoplasia. Three genetic polymorphisms in combination with HP infection were found to be useful to identify individuals who are more likely to develop IM and therefore GC. Individuals with both HP infection and atrophic gastritis as measured by PG index had 14-fold increased risk of GC

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ABBREVIATION

GC	Gastric Cancer
SEER	Surveillance, Epidemiology and End Results in U.S (http://seer.cancer.gov)
HP	*Helicobacter pylori*
RR	Relative risk
WHO	World Health Organization
HR	Hazard ratio
OR	Odds ratio
OGD	Oesophagogastroduodenoscopy, gastroscopy, endoscopy
UGIS	Upper gastrointestinal series (X-ray test)
CT	Computed Tomography
AJCC	American Joint Commission on Cancer
GCEP	Gastric cancer Epidemiology and Molecular Genetics Program
SCHS	Singapore Chinese Health Study
EGN	Early gastric neoplasia
ESD	Endoscopic submucosal dissection
EMR	Endoscopic mucosa resection
IM	Intestinal Metaplasia
PG	Pepsinogen
NGS	Next genome sequencing
GWAS	Genome wide association study

Chapter I

BACKGROUND

1.1 Epidemiology

Gastric cancer incidence

Despite falling gastric cancer (GC) incidence rates in most countries, there were still about one million new cases diagnosed in 2008, making it the 4th most common cancer in the world since 2002 (1, 2). A declining trend in incidences of GC had been observed in many countries, including Nordic countries, North American countries with low incidence rate and Eastern Asian countries with high incidence rate (Figure 1.1). GC is more common in the elderly and incidence increases with age (Figure 1.2). Males have 2 times higher risk than females.

As shown in Figure 1.3, Korea has the highest age-standardized incidence rate for males in Asia and in the world (62.2 per 100,000), followed by Mongolia (48.2 per 100,000) and Japan (46.8 per 100,000). China has an incidence rate of 41.3 per 100,000 for males. Given the size of its population (688 million males in 2011), this translates to an estimated 284,468 new male GC cases annually, exceeding that of any other Asian countries. The GC incidence rate among females is the highest in Korea, Mongolia, and China (24.6, 22.3 and 18.5 per 100,000 females, respectively). In comparison, incidence in the United States is much lower with the rate of 2.8 per 100,000 females (Figure 1.3) (3).

In Singapore, GC incidence rate has declined since 1975 but incidence increased sharply in people who were age 55 and above (Figure 1.4). Singaporeans of Chinese descent, which comprise 70% of the total population, have higher age standardized incidence rate than Malays and Indians, and account for 90% of new GC cases every year. The age-adjusted relative risk for male and female are all significantly lower in Malay and Indian than in Chinese (Table 1.1). The age standardized incidence rate of GC for the period 2006 to 2010 is 14.3 per 100,000 per year in Singapore Chinese males and 7.9 per 100,000 per year in Singapore Chinese females (Singapore Cancer registry). The risk of GC in Singapore Chinese is at intermediate levels, in contrast with countries with high GC incidence such as Korea and Japan and low GC incidence countries such as India and the United States (4).

Figure 1.1 Trends in Incidence of gastric cancer in selected countries

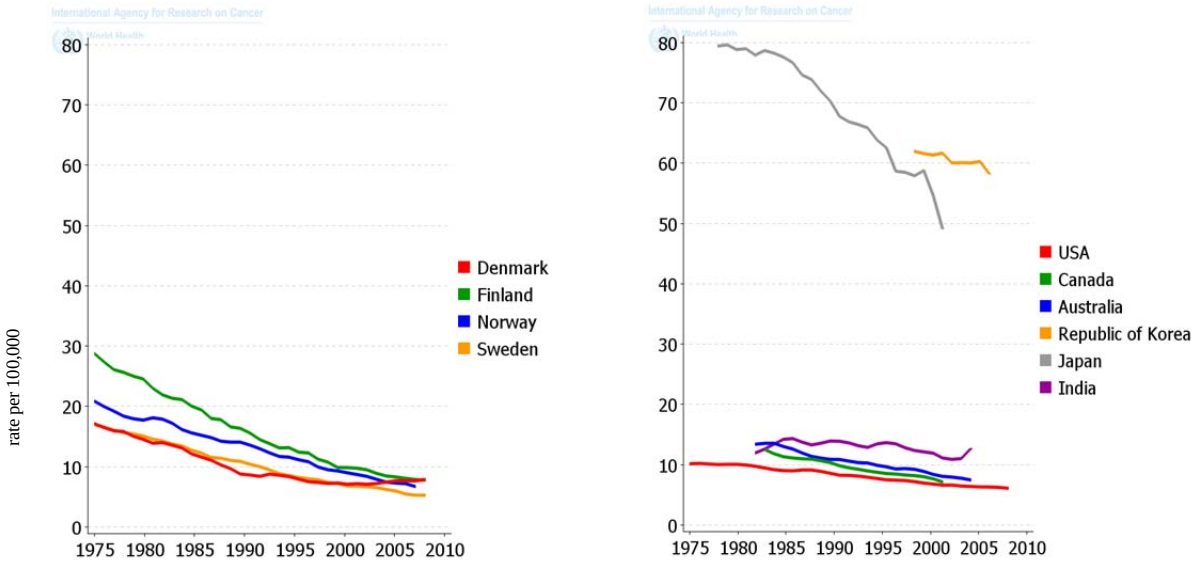


Figure 1.2 Age-specific Incidence of stomach cancer in the world and in Eastern Asia

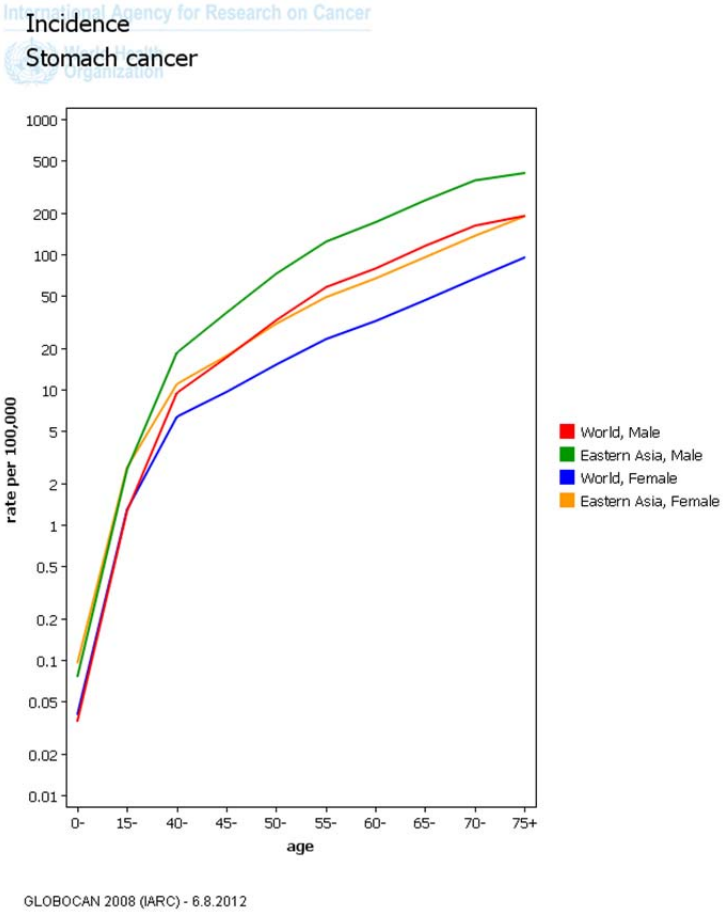


Figure 1.3 Estimated Gastric Cancer Incidence and mortality Worldwide in 2008 in male and female

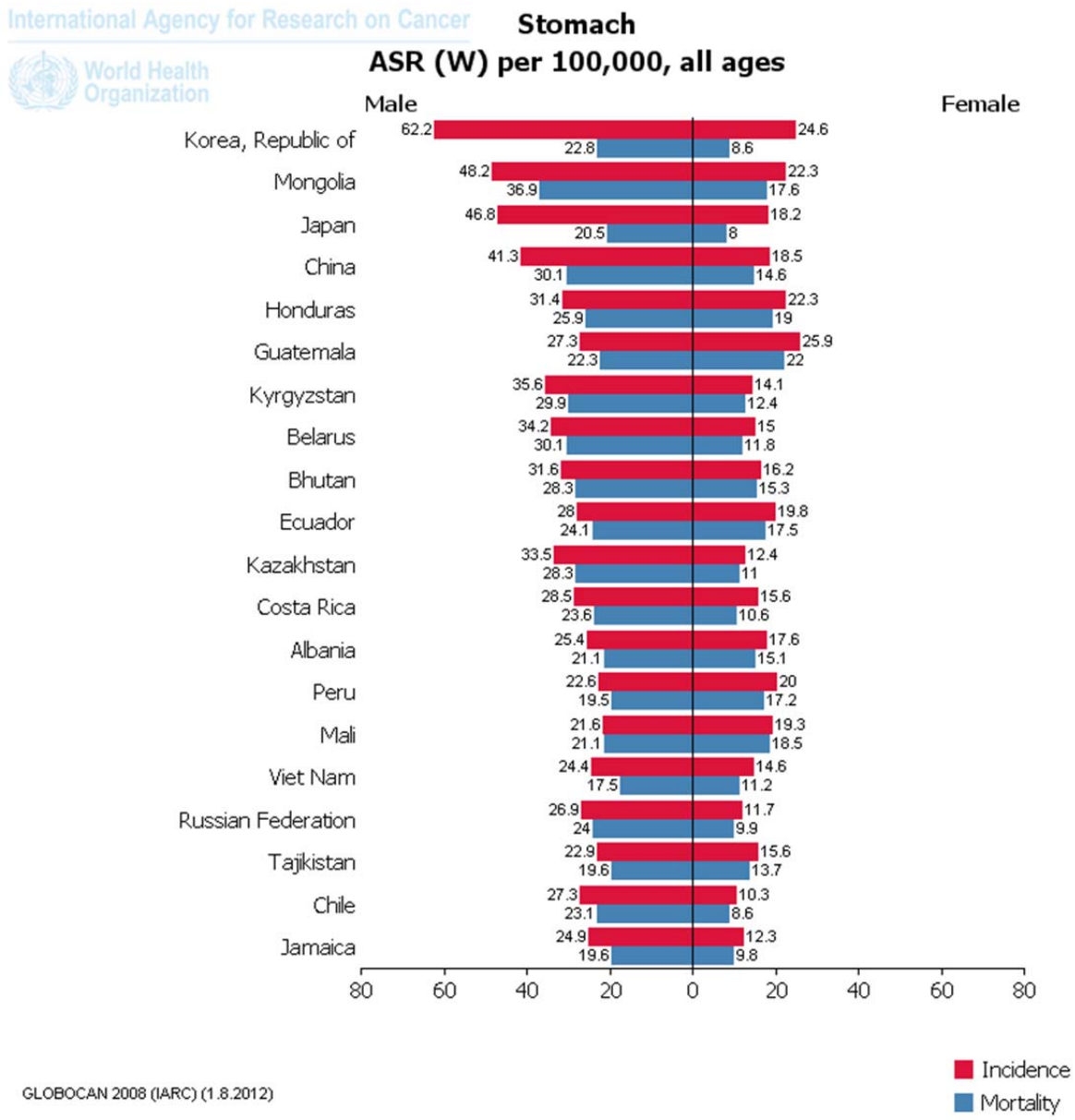
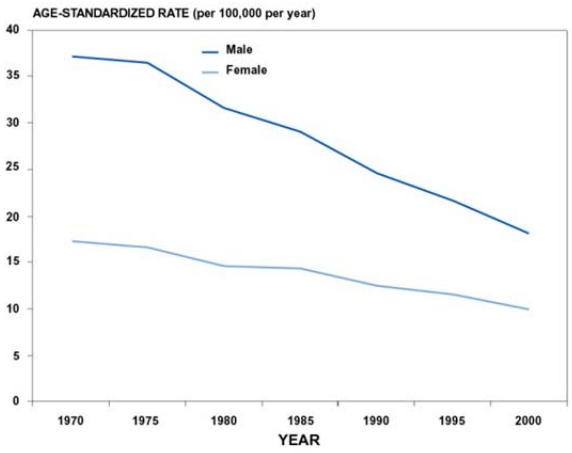
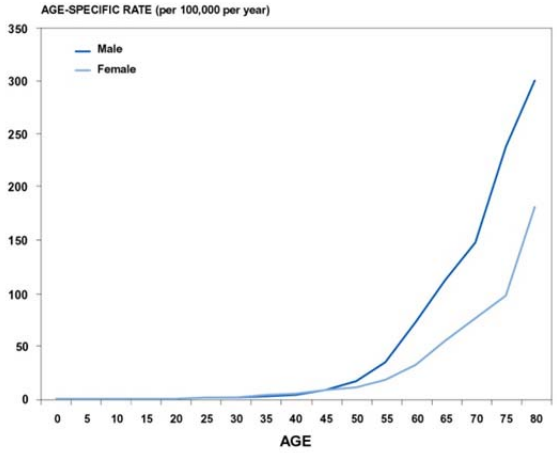


Figure 1.4 Trends in gastric cancer incidence in Singapore (1968-2002)



STOMACH: AGE-STANDARDIZED INCIDENCE BY SEX, 1968-2002



STOMACH: AGE-SPECIFIC INCIDENCE BY SEX, 1998-2002

Table 1.1 Incidence of Gastric Cancer in Singapore among Chinese, Malay and Indian (1998-2006)

Incidence data	Male			Female		
(1998-2002)	No.	ASR*	RR**	No.	ASR*	RR**
All Residents	1442	18	-	966	9.9	-
Chinese	1310	21.4	1	883	10.8	1
Malay	64	6.6	0.3	41	3.8	0.4
Indian	61	7.8	0.4	34	6.1	0.6
(2002-2006)						
All Residents	1340	14.4	-	863	7.6	-
Chinese	1200	17	1	798	8.5	1
Malay	56	5.7	0.3	-	-	-
Indian	72	7.7	0.5	27	4	0.5

*Age standardized incidence rate (to world population) per 100,000/year

**Age adjusted Relative risk

Gastric cancer mortality

GC is the second leading cause of cancer death in both genders worldwide, accounting for 736,000 deaths in 2008 (1). The highest mortality rates were seen in Eastern Asia (28.1 per 100,000 in men, 13.0 per 100,000 in women), the lowest in Northern America (2.8 and 1.5 respectively). Overall, females are more likely to survive GC than males. The survival rate is also related to stage distribution. In the United States, the survival rate during the period 2002-2008 was 62% in early stage and only 3.7% in metastatic GC (Table 1.2). The survival rate is higher in those countries with higher proportion of early gastric cases such as Japan (5). Among countries with high incidence rates, Japan has the highest survival for GC (Figure 1.3).

Table 1.2 Stage distribution and 5-year survival by stage at diagnosis for 2002-2008, based on U.S SEER data

Stage at Diagnosis	Stage Distribution (%)	5-year Survival (%)
Localized (confined to primary site)	24	62.3
Regional (spread to regional lymphnodes)	31	27.7
Distant (cancer has metastasized)	34	3.7
Unknown (unstaged)	11	17.9

1.2 Risk Factors

GC is a multi-factorial disease with genetic and environmental risk factors. The incidence of GC shows geographic variation, time trend and immigration effect. This suggests both environmental and lifestyle factors contribute significantly in the development of GC.

Helicobacter pylori infection

Helicobacter pylori (HP) was recognized in 1994 by the WHO as a class I carcinogen. It is one of the most significant environmental risk factors. The risk associated with HP was about 2 - 8 fold (6-9).

The prevalence of HP infection varies in different countries and population and it increases with age. In Asia, where the incidence of GC is high, the prevalence of HP is also very high. In China, prevalence had been shown to be as high as 92% in cohort study population (10). In Japan, it was shown to be around 80% in a few prospective cohort studies (11-13). Prevalence of HP in South Korea was reported as about 60% (14, 15).

There are broadly two divergent responses of long-standing HP infection. One is antral-dominant gastritis with little atrophy which causes duodenal ulcer and is reported to have little risk for GC. The other response is corpus-dominant gastritis with multi-focal atrophy which indicates high risk of GC. Research on GC carcinogenesis supported HP as carcinogen of GC and it was believed that HP triggered the sequential pathologic changes of the gastric mucosa through atrophic gastritis, intestinal metaplasia and dysplasia, then carcinoma (16-18). The susceptible

host, other environmental virulence factors and duration of HP infection also played important roles in HP induced GC.

A few mechanisms on how the HP infection induced the DNA molecular changes in the GC development had been proposed. The leading hypothesis is oxidative stress. The HP infection caused production of reactive oxygen and nitrogen species and suppresses the host antioxidant defense mechanisms, leading to oxidative DNA damage (19). The other mechanisms include 1) HP promotes the formation of mutagenic substances through inflammatory mediators (16). 2) HP infection induces mutations in the mitochondrial and nuclear and causes DNA damage (20). 3) Aberrant DNA methylation induced by HP infection affects the risk of GC (21, 22).

There are many kinds of HP strains and individuals infected with HP may have more than one strain (23-25). Past studies had shown that infection containing virulent cagA strains increased the risk of GC (26, 27).

Although about two thirds of the world population is infected with HP, only 1 in 1000 HP infected people will develop GC (28). This makes HP eradication not cost-effective in GC prevention. Studies had also suggested that HP eradication should be carried out early because it was only effective before the premalignant lesions developed (29-31).

Diet

It is well known that high salt intake increases the risk of GC while high consumption of vegetable and fruits protects people from getting GC. Based on a

recent meta-analysis of prospective studies, salt intake was shown to be directly associated with the risk of GC and the risk increased with increase in the level of consumption (32). Other possible dietary risk factors include intake of nitrate, nitrite and nitrosamine which are often found in either nature food such as cabbage, cauliflower, carrot, celery, radish, beets, and spinach or in preserved food. Based on a population study in Japan, isoflavones, which are found in soy food, have a protective effect in the development of GC (33).

Smoking and Alcohol

Smoking had been proven to be associated with the 1.5-2.5 fold increased risk of GC in both case-control and cohort studies in different populations. The risk increases with the frequency and duration of smoking. In a meta-analysis, the relative risk was 1.6 in men and 1.1 in women. Two studies (the European Prospective Investigation into Cancer and Nutrition study and the Multiethnic Cohort (MEC) Study, in Hawaii and Los Angeles) had shown higher hazard ratios (HR) of GC in cardia (HR 2.86-4.10) than in the distal part of the stomach (HR 1.52-1.94) (34, 35).

Unlike smoking, results on the association between alcohol and the risk of GC had been inconsistent. There are a few possible reasons. First, self-reporting of alcohol consumption is unreliable as the measurement of the quantity of alcohol consumption is not standardized. Secondly, recall bias in case-control study may affect the result significantly. Lastly, alcohol effect may be so small that the effect could not be shown in past studies. Recently, two prospective large cohort studies reported positive association between alcohol consumption and GC risk. In the European Prospective Investigation into Cancer and Nutrition study, heavy

consumption of alcohol (>60 g/day) was found to be positively associated with the risk of intestinal type non-cardia GC in men with HR (95%CI) 1.65 (1.06-2.58) (36). In a population based cohort study in Shanghai, China, heavy drinkers were found to have significantly increased risk of GC with HR (95% CI) of 1.46 (1.05-2.04) (37).

Family history of gastric cancer

People who had family history of GC had been well studied and were consistently reported to have increased risk of GC (OR 1.5-3.5) in case-control studies in Chinese, Japanese and Korean (38-43). The risk in those with family history of GC was reported to be higher in Turkey and India (44-46). It was also higher in those with more than one first-degree relative who had GC (43). The positive association between family history and GC development may be due to the genetic susceptibility and also exposure to the other same risk factors such as HP infection, specific diet and smoking. However, the specific genes which elevated the risk of GC in family members of GC subjects have not been identified. In order to estimate the real effect of familial risk, a large twin study including 44,788 pairs of twins in Scandinavia had been conducted (47). In dizygotic twins, the risk of the other twin getting GC when one partner has had GC was 6.6 times as high as twins with no partner having GC. The risk increased to 10 fold when the twins were monozygotic. The study further estimated that the inherited gene contribution was about 28% in the GC development, with another 10% contributed by shared environmental risk factors, and the remaining 62% contributed by non-shared environmental factors. The study confirmed that familial factors play a role in GC development but compared with genetic factors, environmental factors are major contributors.

1.3 Pathology

The stomach can be divided into four anatomic parts: cardia, fundus, body and antrum. The cardia is located just next to the esophagus. The fundus is the left upper area of the stomach. The antrum is the distal one third of the stomach, and extends to the pyloric canal which connects the stomach and the duodenum. The body, which is between the fundus and antrum, is the largest part of the stomach. While the incidence rate of GC is declining worldwide, the incidence of gastric cardia cancer was reported to be increasing in western countries (48-50). It is believed that cardia cancer has different epidemiologic characteristics and risk factors from cancers in other parts of the stomach (8, 51, 52). Based on past studies, there were two etiologies for cardia cancer (53, 54). In areas where prevalence of atrophic gastritis caused by HP is high, the majority of cardia cancers are related to HP infection, similar to the cancer in other parts of stomach (55, 56). However, in western countries where atrophic gastritis is rare but gastroesophageal reflux disease is common, cardia cancer is closely related to esophagus cancer which is strongly associated with Barrett's esophagus (57).

According to WHO classification, gastric adenocarcinoma has four main histologic types (58). Tubular adenocarcinoma is the most common one, followed by signet-ring cell carcinoma, papillary adenocarcinoma and mucinous adenocarcinoma (59). Based on morphological features of the tumors, Lauren classified the gastric adenocarcinoma into intestinal type, diffuse type and mixed type(58). Intestinal type adenocarcinoma is characterized by gland formation while the diffuse type has little or no gland formation. Intestinal type cancer is seen more often in the elder male population with premalignant lesions such as atrophic gastritis, intestinal metaplasia or dysplasia (60,

61). Diffuse type cancer usually arises from younger population without any histologically abnormal background in the stomach (62). It is well known that these two types of GC have two different pathways (63). Intestinal type is believed to follow the famous stepwise progression which starts from HP infection, progresses to atrophic gastritis, intestinal metaplasia, dysplasia and finally to carcinoma in the stomach (64). Diffuse type is also associated with HP but without any intermediate lesions (65). The studies had shown E-cadherin/CDH1 mutation was one of the causes of hereditary diffuse GC (66-68) and indicated that genetic risk factors may play an important role in diffuse type GC development.

1.4 Clinical Diagnosis

Oesophagogastroduodenoscopy (OGD, endoscopy) and biopsy

Performing an OGD is the only way to detect early GC. The diagnostic endoscopic procedure allows direct visualization and the biopsy of any suspected lesions in the upper part of the gastrointestinal tract up to the duodenum. The device is a flexible tube carrying a CCD video-chip in front which relays an image to the viewing screen. The sensitivity of OGD is 74-84% (69). New technologies such as chromoendoscopy with indigo carmine spray (70, 71), narrow band imaging (NBI) (72-74), autoflourescence imaging (AFI) (75) and confocal endomicroscopy (CE) (76, 77) help improve the detection rate of cancer or premalignant lesions . OGD has been used as the one of main screening tools in Korea since 1999 (78).

Double-contrast barium study (X-ray test, photofluorography, upper gastrointestinal series)

The double-contrast barium study has been widely used for population screening in Japan since 1960 (69). It was believed that the population screening has contributed to the reduction of the mortality rate of GC in Japan which has dropped significantly since the 1980s.

Computed Tomography

Computed Tomography (CT scan) is performed for the staging of GC by indicating the size of the tumor and location of the tumor (79). The thorax, abdomen and pelvis are included to assess distant metastasis and involvement of lymph nodes. However, CT scan was found to be not sensitive in detecting peritoneal metastasis (80).

1.5 Staging

Staging of GC is the objective evaluation of the stage of development and/or metastasis of the cancer (81) (82). The stage of the GC determines the treatment plan and the prognosis of the disease. A standardized staging system is also important in comparing the treatment outcome in clinical trials or between different centers or different countries. The AJCC system (American Joint Commission on Cancer) is widely used in western countries as well as some Asia countries (83). Japan has its own system in defining GC stages (84). The AJCC system describe 1) the size and depth of the tumor in stomach (T); 2) whether the cancer spreads to regional lymph nodes (N); 3) distant metastasis - whether the cancer spreads to the other organs (M) (82). The 7th edition of the AJCC staging system has grouped gastric into IA, IB, IIA, IIB, IIIA, IIIB, IIIC and IV based on the combination of tumor, lymph nodes and metastasis information (85). The AJCC TNM system has proved to be accurate in the prediction

of prognosis (86). Early stage GC was found associated with better survival rate compared with late stage GC (87).

1.6 Treatment

Surgery is the only curative treatment option for GC (88). The aim of the surgery is to remove the tumor and the affected lymph nodes completely. Gastrectomy is indicated for stage IB – III when the tumor is locally resectable and has no metastasis.

Chemotherapy in resectable gastric cancer

Post-operative (adjuvant) chemotherapy was not part of the standard of care in GC patients because most trials failed to show adjuvant chemotherapy improved the 5-year survival rate (89-91). However, one recent meta-analysis suggested there was some benefit from adjuvant chemotherapy (92). The Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial (93) compared subjects who were given both pre-operative chemotherapy and post-operative chemotherapy with those who had surgery alone and showed that subjects who had surgery alone had significant survival benefit with chemotherapy. Five-year survival rate was 36% in the chemotherapy group compared with 23% in the surgery only group. However, the study included 22% esophagus cancer patients. Out of 253 patients who underwent surgery alone, only 42% underwent D2 distal dissection or D2 total dissection. More randomized trials taking into consideration of types of GC, types of surgery (D1 or D2 dissection), the local recurrence and the combination of the drugs are needed.

Chemotherapy in advanced gastric cancer

In advanced GC, the main objective is to relieve the symptoms and improve the quality of life. Chemotherapy may be offered to extend the lifespan but should be balanced by the toxicity of drugs which could affect the patient's quality of life (94, 95).

Endoscopic submucosal dissection (ESD)

ESD is an endoscopic procedure for removing benign, malignant lesions in gastrointestinal tract and has been used widely for the treatment of early GC in Japan as the proportion of early GC detected was high(96) (97-99). It is less invasive and is able to cure the early GC without removing the stomach. The criteria in applying ESD for treatment of early GC are 1) well or moderately differentiate histopathology; 2) tumor size ≤3cm; 3) tumor invasion is limited to submucosal layer; 4) no lymphatic or vascular invasion (100). ESD was accepted as an efficient and easy to use method for the treatment of early GC (101, 102).

1.7 Prevention and Screening

GC is a common disease with high mortality rate (1). A high proportion of GC cases are detected in late stage because lack of specific early symptoms. Primary prevention and secondary prevention are important in reducing the GC incidence and mortality rate (103, 104). Primary prevention of GC includes eliminating the common risk factors for cancer such as low socioeconomic status, smoking, dietary factors (intake of salt and preserved food) (105) and specific risk factor for GC - HP infection (106, 107). An example of primary prevention is the improvement of socioeconomic status caused the decline in GC incidence rates in Singapore since 1970s (108). It was around the same period when the Singapore economy grew rapidly with GDP

increase between 5-15% every year during the period of 1965-1985 (109). At that time, the public housing was initialized and the water supply and sanitation system was improved significantly. The usage of refrigerators became popular and it helped keep food fresh and reduced the consumption of salted or preserved food. This may help explain the sharp drop in incidence rate since 1970s in Singapore.

After HP was recognized as group I carcinogen by WHO in 1994, a few randomized trials had been carried out to investigate the effectiveness of HP eradication in GC prevention (29, 110-112). The sample size of those studies was relatively small. The evidence of preventive effect of HP eradication was not significant in overall cancer incidence but all of the trials demonstrated the regression of premalignant lesions after successful eradication. One of the trials performed in China in 2004 concluded that HP eradication reduced GC risk only in subjects who have not developed any premalignant lesions (29).

Avoiding the risk factors help to reduce the risk of developing GC but it cannot guarantee that GC will not occur. Early detection by screening as the secondary prevention is the best way to find cancer earlier and get cured especially in countries with high incidence rate. So far, mass screening was only available in Japan and Korea. Japan started population screening for GC using photofluorography (X-ray) in the 1960s, and remarkable improvement in survival rates from GC has been achieved as a result of early detection and consequently higher cure rates (113, 114). A nationwide GC screening program in Korea was initiated in 1999 as part of the National Cancer Screening Program (NCSP). The NCSP recommends that men and women over 40 years of age undergo GC screening every 2 years, with an upper

gastrointestinal series (UGIS) or by endoscopy (115). The preliminary data suggested the participation rate of the screening programme was low and more people underwent UGIS than endoscopy (78, 116). The effectiveness and efficacy of screening general population with high incidence rate of GC is still unclear.

In countries with low or intermediate incidence rate for GC, mass screening was not cost-effective as the benefit of early detection of a few GC could not offset the cost, and the risk of the screening programme caused by screening tests. Pepsinogen had attracted more and more attention in identifying people who were at high risk for GC as low pepsinogen level indicated the presence of atrophic gastritis which is the premalignant lesion for GC. Those identified high risk group would be referred for endoscopy screening. In Japan, a few prospective studies had proved that the combination of pepsinogen test and HP antibody test could predict GC risk and stratify people in low and high risk group for GC. More prospective studies are required to assess the effectiveness and efficacy of different screening programmes.

Screening is the key in early detection of GC to reduce mortality rate but not cost-effective in countries with intermediate incidence rate like Singapore, we conducted the following studies to determine the feasibility of applying targeted screening in the Singapore Chinese population by stratification into low or high risk for GC using known risk factors or blood markers:

Study 1 - Systematic endoscopic surveillance in a high-risk cohort is feasible for the detection of early gastric neoplasia

Study 2 - Clinical risk factors and premalignant lesions of gastric cancer in high risk cohort

Study 3 - Genetic risk factors for gastric cancer and premalignant lesion of gastric cancer

Study 4 - Using Pepsinogen and Hp antibodies to predict the risk of gastric cancer

Chapter II

STUDY POPULATIONS

The study populations of this thesis are from two data sources: Gastric cancer Epidemiology and Molecular Genetics Program (GCEP) and Singapore Chinese Health Study (SCHS).

2.1 Gastric Cancer Epidemiology and Molecular Genetics Program (GCEP)

The GCEP study is a prospective, multi-center cohort study of subjects who are at high risk of developing GC. GCEP was initiated in January 2004 with a planned enrolment of 3000 Chinese subjects age > 50 years, offering screening by endoscopy with systematic prospective follow-up over a minimum of 5 years. Study sites included all four major public hospitals in Singapore – National University Hospital, Tan Tock Seng Hospital, Singapore General Hospital and Changi General Hospital. In Singapore, 80% of hospitalization and specialist services are provided by public hospitals and 20% by private hospitals. Clinical information including demographics, medical history and family history were obtained. Informed consent was obtained from all subjects and the study was approved by the institutional review boards of all hospitals involved. The main outcome measurement is number of subjects who develop high grade dysplasia, intramucosal carcinoma or gastric adenocarcinoma. The primary objective of the GCEP study is to identify predictive risk factors from a high risk cohort and to develop an optimum approach and cost-effective algorithm for targeted screening for GC in the Singapore Chinese population.

The enrollment of 3000 subjects had been completed in Dec 2010 and the study is still in progress. 1300 subjects had completed the minimum 5 years of surveillance and the rest will be completed by 2015. Eighteen high grade dysplasia, intramucosal carcinoma or adenocarcinoma cases were detected so far with average follow up of 3 years.

Subject Selection

A total of 4085 Chinese, age above 50 years, which included patients seen in gastroenterology clinics during the period 2004-2010 and referred by the doctors, and subjects recruited from public forums. To be eligible, the subjects had to 1) be Chinese; 2) be greater than 50 years; 3) satisfies one or more of the following criteria: has (had) a history of dyspepsia of at least 4 weeks or more. Dyspeptic symptoms include bloating, epigastric discomfort and early satiety. Has a family history of GC. Has a medical condition for which an OGD is indicated. Participation in public forum and keen to undergo endoscopy screening.

The subjects who had bleeding disorders, such as haemophilia, in whom biopsies are contraindicated, were excluded from GCEP. The subjects with liver cirrhosis, previous total or partial gastrectomy, severe co-morbid illness, such as end-stage renal failure (ESRF), congestive cardiac failure (CCF), severe osteoarthritis (OA) and rheumatoid arthritis (RA) requiring long term non-steroidal anti-inflammatory drug (NSAID) therapy, other severe acute or chronic medical or psychiatric condition or laboratory abnormality that may interfere with the interpretation of study results. The subjects on aspirin, ticlopidine and clopidogrel must be able to undergo a one-week washout period before gastroscopy.

After interviewing patients and review of patients' case notes, 3094 Chinese who were over 50 years old signed the patient informed consent form and agreed to participate in the GCEP study. Reasons of not participating included 1) worrying about the discomfort and complications of endoscopy; 2) not willing to give blood; 3) not willing to join the research study; 4) medical condition which made one not suitable for taking biopsy; 5) having GC in the past. One hundred and nineteen subjects were later excluded from the study because they refused to have baseline endoscopy examination or no biopsy was taken during baseline endoscopy. 2975 subjects were included in the baseline analysis. (Figure 2.1)

Questionnaires

At enrolment, all subjects were interviewed by trained research nurses with baseline questionnaire, providing information on the demographics, personal medical history, indication of any previous OGD undergone, duration of symptoms, family history of GC or other cancers, previous history of peptic ulcer disease, any usage of antibiotics and acid suppressive therapy, smoking, alcohol consumption and any concomitant medical illness. The questionnaires were updated annually during yearly follow up.

OGD and Biopsy

Patients who had an endoscopy performed not more than 12 months prior to time of recruitment were enrolled in the study. The OGD reading was taken as baseline. Patients who had never had endoscopy or had an endoscopy performed more than 12 months prior to time of recruitment would undergo a prospective baseline endoscopy. A video-recording of the endoscopy was carried out during the

procedure for agreement, validation and comparison purposes. Subsequently, gastric mucosal biopsies were taken. The gastric mucosal biopsies were taken based on the Updated Sydney System as described in the next paragraph. In addition, cardia biopsies were taken (Figure 2.2). The locations are defined as follows:

- A1- lesser curvature of the antrum, within 2-3cm of the pylorus.
- A2- greater curvature of the antrum, within 2-3cm of the pylorus.
- IA- incisura angularis.
- B1- lesser curvature of the corpus, 4cm proximal to the angulus.
- B2- middle portion of the greater curvature of the corpus, 8cm from the cardia.
- Cardia (C) - within 1 cm below the OGJ (defined as the point where gastric folds disappear).

All biopsy samples were assessed for degree of chronic gastritis, HP infection, atrophic gastritis and intestinal metaplasia by two independent experienced pathologists. These were scored using the updated Sydney system classification. Intestinal metaplasia were classified into mild, moderate and marked using a special staining technique (Alcian blue, PAS.)

Blood collection

40mls of fasting blood were drawn from each subject at the baseline visit and when subjects reached study primary endpoint. 20mls were collected in plain tube (BD Vacutainer® Plus plastic serum tube) for serum and another 20mls were collected

with CPT tube (*BD* Vacutainer® *CPT*™ with Sodium Citrate) for plasma and mononuclear cell. During Feb 2006 and Apr 2009, EDTA tube (*BD* Vacutainer® *CPT*™ with Polymer gel and K_2EDTA) was used to obtain plasma before transfer to CPT tube for mononuclear cell. In the event that the primary endpoint was not attained, an additional 20mls of blood was drawn at the last visit of this surveillance programme. 10mls was in plain tube while another 10mls was in CPT tube. The serum/plasma and white blood cells were stored in -80 degree freezer or liquid nitrogen tank. The baseline serum was assayed for HP antibodies and serum pepsinogen routinely.

Follow up

Subjects were requested to undergo surveillance endoscopy at Year 1, 3 and 5. Biopsies were collected during these visits. If the subject, for clinical or medical reasons, required an endoscopy within 3 months prior to the scheduled surveillance endoscopy, then additional biopsies were carried out during the procedure. This was taken as the surveillance endoscopy reading.

All subjects were followed-up annually at the clinic or via telephone for symptom review when they were not due for endoscopy surveillance that year. Clinical data was collected and the database was updated.

Histology Analysis

All biopsy samples were assessed for degree of chronic gastritis, HP infection, atrophic gastritis and intestinal metaplasia by two independent experienced pathologists. These were scored using the updated Sydney system classification. Intestinal metaplasia was

classified into mild, moderate and marked using a special staining technique (Alcian blue, PAS.)

Endpoints

Subjects who developed high grade dysplasia, intramucosal carcinoma and adenocarcinoma were considered to reach endpoints.

Data Monitoring

The GCEP study used a web-based system for data collection. Data were entered directly from source document to the electronic case report form. All study coordinators and investigators were given unique identification and password to access the database. The source document was monitored by independent reviewers before entry into database.

Sample Size Calculation

Based on Singapore cancer registry data, the incidence rate for GC in Chinese males aged 50-70 is 25.9 per 100,000(117). The incidence rate of GC in high risk groups such as patients with premalignant lesions, was reported as 0.1-1.1% which included all age groups (118, 119). In this study, the outcome is number of subjects who develop GC, including adenocarcinoma, intramucosal carcinoma and high grade dysplasia. As GCEP is a 'high-risk' group comprising high-risk subjects of Chinese ethnicity, patients who were over 50 years, with HP infection, or known pre-malignant histology such as atrophic gastritis and intestinal metaplasia, we estimated an incidence rate of 0.5% based on the Singapore cancer registry data and literature search.

50 cases of GC or high grade dysplasia are required to detect putative risk factors with odds ratio (OR) >2.5 at power of 80% and p-value <0.05. Based on an estimated incidence rate of 0.5%, a cohort size of 3000 subjects under surveillance for at least 5-years was considered appropriate.

2.2 Singapore Chinese Health Study

The Singapore Chinese Health Study (SCHS) is a prospective cohort for long-term study of dietary, genetic and environmental determinants of cancer and other chronic diseases (120). Altogether, 63,257 Chinese men (n=27,959) and women (n=35,298), aged 45-74 years were recruited between April 1993 and December 1998 from permanent residents or citizens of Singapore residing in government-built housing estates (about 80% of Singaporeans reside in such estates). Recruitment was restricted to two major dialect groups of Chinese in Singapore: the Hokkiens and Cantonese. The details of the study design had been described. Written informed consent was obtained from all subjects enrolled in the SCHS. The study was approved by the Institutional Review Boards of the National University of Singapore.

Baseline interview

At recruitment, the subjects were interviewed in person at home by a trained interviewer using a structured questionnaire, which covered demographics, use of tobacco, current physical activity, menstrual/reproductive history (women only), occupational exposure, medical history and family history of cancer. Information on current diet, including alcohol consumption, was assessed using a 165-item food frequency questionnaire that has been validated against a series of 24-h dietary recall

interviews (120). The Singapore Food Composition Table was used to estimate average daily intake of 96 nutrient and non-nutrient compounds for each study subject.

Blood collection

Between April 1994 and December 1999, blood and single-void urine specimens were collected from a random 3% sample of study participants. Details of the biospecimen collection, processing and storage procedures have been described previously (121). Between January 2000 and April 2005, specimen collection was extended to all surviving cohort members and specimens from 32,543 subjects were collected, representing a consent rate of about 60% of surviving cohort participants at that time.

Incident GC cases occurring within the SCHS cohort were identified through the population-based cancer registry in Singapore. The nationwide cancer registry has been in place since 1968 and has been shown to be comprehensive in its recording of cancer cases. As of 12 Dec 2010, there were a total of 650 subjects diagnosed with GC. Amongst them, 222 GC subjects donated blood samples. 178 cases donated blood before the occurrence of GC.

Figure 2.1 Recruitment of subjects in GCEP study

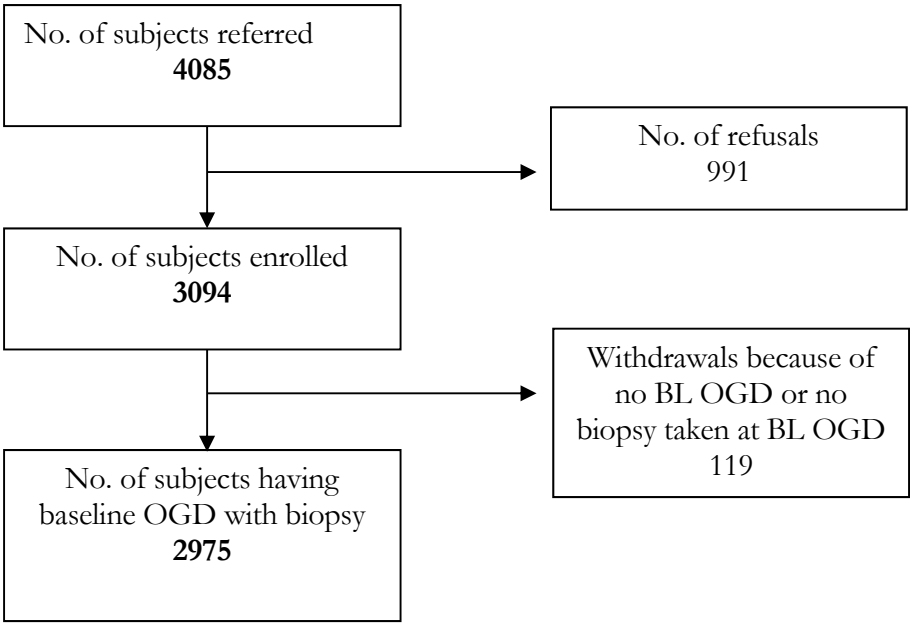
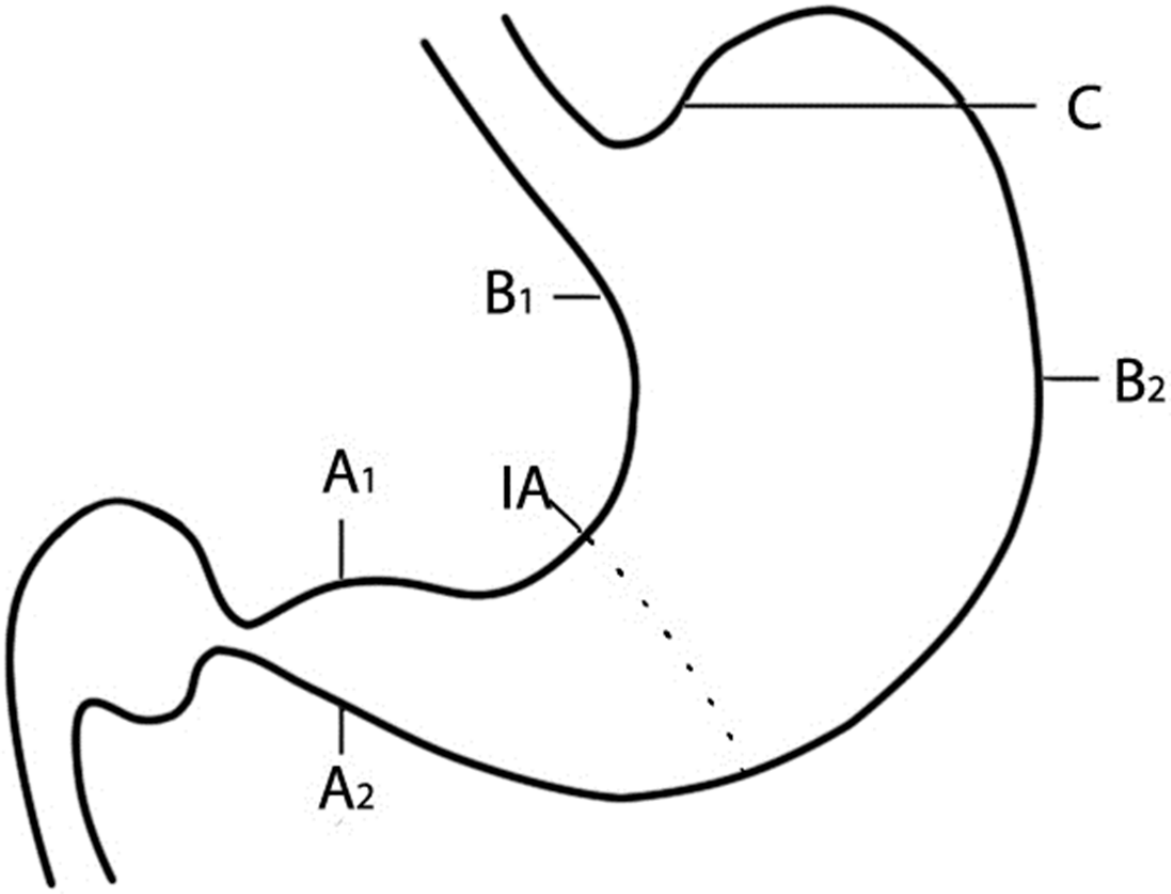


Figure 2.2 Biopsy protocol based on updated Sydney system in GCEP study



- A1- lesser curvature of the antrum, within 2-3cm of the pylorus.
- A2- greater curvature of the antrum, within 2-3cm of the pylorus.
- IA- incisura angularis.
- B1- lesser curvature of the corpus, 4cm proximal to the angulus.
- B2- middle portion of the greater curvature of the corpus, 8cm from the cardia.
- Cardia (C) - within 1 cm below the OGJ (defined as the point where gastric folds disappear).

Chapter III

FEASIBILITY OF ENDOSCOPY SURVEILLANCE AND RISK FACTORS OF EARLY GASTRIC NEOPLASIA IN A HIGH RISK COHORT

3.1 Introduction

Screening for GC is not commonly practiced in other countries because of the comparatively lower incidences of GC(122). More targeted screening in the high-risk subpopulation to detect early GC in countries where the incidence of GC is intermediate is a possible option (117, 123). In Singapore, the age-standardized rate of GC incidence rate was 10.9 per 100 000 in 2008 (4), which was in the intermediate level. The proven risk factors such as gender, age(2, 124), HP infection(13, 125), smoking, alcohol(34, 37, 126), dietary factors(127, 128), family history(43, 129, 130) and premalignant lesions such as intestinal metaplasia, atrophy gastritis could help select the subjects at high risk of GC. Various genetic factors and recently discovered molecular markers had also been reported to be associated with increased risk of GC. To define criteria of risk stratification in the intermediate population for targeted screening, and to develop the guidelines on the frequency of surveillance endoscopy, a large scale study in a multicentre setting is required.

The primary objective of the Gastric Cancer Epidemiology and Molecular Genetics Program (GCEP) is to identify predictive risk factors from a high risk cohort and to develop an optimum approach and cost-effective algorithm for targeted screening for GC in the Singapore Chinese population.

In this chapter, there are two studies based on the data from GCEP cohort. In the first study, we described the baseline demographics of the population, the prevalence of

gastric premalignant lesions in Singapore Chinese population who are at the high risk of GC. We also discussed the clinical characteristics and stage of 18 early GC or high grade dysplasia cases (early gastric neoplasia, EGN) diagnosed in the cohort. In the second study, we evaluated the association between clinical risk factors as well as the premalignant lesions and the early gastric neoplasia (EGN).

3.2 Method

A total of 2975 subjects who had had both baseline endoscopy and histology examination results were included in the analysis. Descriptive statistics were produced for demographic and baseline characteristics. In addition, the percentage of early gastric neoplasia detected in the GCEP programme was compared with the one in a hospital-based GC registry.

Identification of EGN cases

EGN cases include gastric adenocarcinoma, intramucosal carcinoma (IMC) or high grade dysplasia (HGD). 18 EGN cases were identified during the baseline OGD or surveillance OGD and confirmed by the histology examination.

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics (version 20), a 2-tailed P value of less than 0.05 was considered statistically significant. Descriptive statistics were produced for demographic and baseline characteristics. Means and SDs were generated for continuous variables. Frequencies and percentages were generated for categorical variables. The Pearson's chi-square test was used for categorical data to compare proportions of each candidate risk factor - age, gender, education level,

smoking, alcohol consumption, family history of GC, atrophic gastritis and intestinal metaplasia (IM). Multiple logistic regression models were used to analyze the risk factors for EGN.

Study 1 – Systematic Endoscopic Surveillance in a High-Risk Cohort is Feasible for the Detection of Early Gastric Neoplasia (EGN)

3.3 Result

A total of 2975 subjects with a mean age of 59±7 years (range 50-86 years), with baseline endoscopy and histology examination were included in the current analysis. 52% were males, 22% had current or previous history of smoking, 17% had alcohol consumption, 16% had family history of GC, and 39% had history of HP infection (Table 3.1).

Of the 2975 subjects, 14% had no risk factor, 23% had one risk factor, 30% had two risk factors and 33% had three or more risk factors (Table 3.2).

All subjects in GCEP must have one baseline endoscopy which could be either a clinical endoscopy performed no more than 12 months prior to recruitment or a prospective endoscopy performed under GCEP protocol. With the 2975 subjects, 88% had chronic gastritis, 21% presented with current HP infection, 18% had atrophic gastritis, 47% had intestinal metaplasia, 6% had fundic or neoplastic polyp and 6 subjects (0.2%) had low or moderate grade dysplasia during the baseline OGD (Table 3.3).

A total of 18 EGN were detected through surveillance endoscopy with an average follow up of 3 years; 12 of them were stage 0 with either high grade dysplasia (HGD) or intramucosal carcinoma (IMC). Six invasive cancers were detected in stages 1A and 1B (Table 3.4); 17 out of 18 had intestinal metaplasia, 15 out of 18 had atrophic gastritis and 16 out of 18 had had HP infection history. Eleven of them were male and the mean age was 65 years (Table 3.5). All subjects survived after treatment. Among 6

cases of invasive cancers (stage 1A and 1B), 4 were removed by subtotal or total gastrectomy and the other 2 were cured by endoscopic submucosa dissection (ESD). Out of 12 HGD or IMC, 2 underwent subtotal gastrectomy, 5 were cured by endoscopic mucosa resection (EMR) or ESD. The rest 5 cases of high grade dysplasia did not undergo any treatment because the lesion was not detected again in the repeat endoscopy.

Three gastric neoplasia cases were found in baseline endoscopy. The other 15 cases were detected during surveillance endoscopies with the average surveillance period of 28 months or median surveillance period of 23 months, ranging from 6 to 74 months (Figure 3.1). Out of 15 GC, 3 cases (no. 6, 10 and 18 in Table 3.6) had had the surveillance endoscopy 6 months or less prior to the detection of gastric neoplasia. This may indicate that the cancers were missed at the previous endoscopy or the lesion had developed within the 6 months interval period. Among the 12 other cases, the intervals between last endoscopy prior EGN and the endoscopy with EGN diagnosis were between 11 – 29 months. In our study, the interval between the most recent normal endoscopy and the diagnosis of cancer endoscopy did not affect the stages of cancer. The long term survival will be monitored and compared in the future. Out of 18 EGN, 9 cancers were detected at antrum, 5 from body and 4 from incisura. Four out of 18 neoplasia cases presented as polyps and another 3 as ulcers (Table 3.6).

The stage distribution in the 18 EGN detected in GCEP was compared with the hospital based GC registry (GASCADII study) (Table 3.7). GASCADII study recruited all newly diagnosed GC cases from National University Hospital and Tan Tock Seng Hospital. The study started in 2006. Based on histology examination, 310

Chinese were diagnosed with GC between 2006-2011; 135 patients(44%) were diagnosed in early stage (stage 0, 1A, 1B and 2) while 175 (56%) were in late stage (stage 3A, 3B and 4). In 175 late stage subjects, 111 were in stage 4. In GCEP study, all 18 cases (100%) were in early stage (stage 0, 1A or 1B), which was significantly more than the 44% diagnosed in early stage in GASCADII ($p<0.001$).

3.4 Discussion

With 18 EGN diagnosed in the programme, our study demonstrates that GC screening in selected subjects is feasible. Over 3000 Chinese subjects above 50 years old were recruited for our study. This population could be further refined to limit the screening programme to those with additional risk factors which could be determined with data from our cohort after more patient-years and more cancers are diagnosed with additional follow-up. Personal medical history and family history of GC were collected prospectively, and endoscopic surveillance with follow-up over a minimum of 5 years was offered. In addition to investigating the risk factors associated with GC and design of a cost-effective screening programme, many other questions could potentially be addressed, including the subtype of intestinal metaplasia, progression or regression of lesions, implication of HP eradication and cost-effectiveness of endoscopy screening in Singapore. In addition, interesting findings could be made through the natural follow up of this group of subjects. This includes the genomic characteristic of those early GC cases, predictive factors of recurrence and the steps of the progression of premalignant lesions to GC. The discovery or validation of genomics or proteomics biomarker is also feasible because tissue and blood have been collected prospectively.

The choice of the study population was based on scientific, practical and ethical considerations. In Singapore, the incidence of GC was only 13.9 per 100,000 people per year in males and 8.3 per 100,000 people per year in females in 2008 (4). As GC incidence was higher in Chinese than in Malays or Indians and cancer increases sharply in older people, the GCEP study targeted Chinese over 50 years old who were at high risk of GC. With the sample size of 3000, we could produce definitive relative risk for some risk factors. It was not feasible to recruit from the asymptomatic general population because endoscopy is invasive and the compliance of endoscopy screening may be poor in the general population who has no abdominal symptom. The overall participation rate in a national GC screening programme which offered either photofluorography or endoscopy in Korea was only 20.5% (116) and photofluorography was preferred among the participants. In our study, the compliance rate of endoscopy is about 81% which is the highest among all endoscopy screening or surveillance programmes (116, 118).

Although the highly selected study population was logistically advantageous and scientifically well founded, there are limits to the generalizability of the findings as we only investigated Chinese who were over 50 years and most of them were recruited at Gastroenterology clinics. Risk factors such as HP infection were removed when detected during the surveillance endoscopy. Dietary factors were not measured in detail because of the complexity of life-time dietary habits over time. As diet may interact with HP infection and genetic factors, we may not be able to report a justified effect of genetic susceptibility. Most of our predictive factors were associated with intestinal type GC. Little has been reported for diffuse type. The progression of diffuse type carcinoma will be difficult to assess. Due to the small number of cancer cases, we

may not be able to determine the risk factors associated with intestinal and diffuse type of GC by stratification analysis. The same applies for gastric cardia cancer which has a different etiology from the distal cancers.

The benefit of this surveillance programme to this group of high risk subjects is early detection of GC and the prevention of GC by the eradication of HP infection and the removal of premalignant lesion such as low/high grade dysplasia.

In patients diagnosed with EGN in our study, the interval between the last two endoscopies ranged from 2-29 months. In 3 cases, the interval was ≤ 6 months. One of them had history of moderate grade dysplasia and was under endoscopy surveillance every 2 months. Another two patients presented with symptoms and clinical OGDs were provided. A tubulovillous polyp was found in one of them and the histology of dissected tissue showed focal high grade dysplasia. Out of 12 HGD/IMC detected, lesions in 5 subjects (42%) were not detected in the subsequent repeat endoscopy. There are two possible reasons. First, the focal high grade dysplasia was so small that it had been removed during biopsy. Secondly, the lesion could be missed due to sampling error.

As endoscopic surveillance is costly, there were not many similar studies. Most of the past studies had small sample sizes, were retrospective or without standard protocol for biopsy taking. J L Whiting *et al.*(118) reported 14 cancers (8.4%) which were detected after 10 years follow up in a group of 166 subjects. In the study, 67% of cancer cases were detected in stage I or II. The interval between the most recent benign endoscopy and the endoscopy diagnostic of malignancy ranged from 1-50 months. The prolonged interval was the greatest factor in the diagnosis of later stage

disease. This was also due to the poor compliance rate of annual endoscopy of 50%. Because of the high compliance rate in our study, we were able to detect early GC in stage I or high grade dysplasia, intramucosal carcinoma.

Chung SJ *et al.* observed 58,849 patients who underwent endoscopy during health checkup at Seoul National University Hospital Healthcare System Gangnam Center in Korea (131). The patients were stratified into repeat OGD and infrequent OGD groups. Repeat OGD group was further split into annual endoscopic or biennial screening groups. The result showed annual surveillance improved the early detection of GC but there was no significant difference on 5-year survival rate between the groups. This is in agreement with what we have observed in our GCEP study. This Korea study had the biggest sample size among all endoscopic studies but the biopsy taking in the study was determined by individual endoscopist without following any standard protocol. In our study, we follow Updated Sydney recommendation and classification for the biopsy collection and reading. Our histology result is based on the consensus of two independent experienced pathologists.

Another study(132) conducted in Spain around 12 years ago included 478 subjects who had gastric biopsy with the diagnosis of normal mucosa, non-atrophic gastritis, atrophic gastritis, complete or incomplete intestinal metaplasia. A new biopsy was taken when the subject agreed to join the study. Twenty-three GC cases were detected (4.8%) with yearly incidence of 0.377%. Incomplete intestinal metaplasia and family history of GC were significantly associated with risk of GC with HR (95% CI) of 11.3 (3.8-33.9) and 6.1 (1.7-22.4) respectively. The study proved that the subtype of intestinal metaplasia was useful for the prediction of GC risk. However, the subjects in

this study were recruited retrospectively, so biopsies in the baseline endoscopy were not collected following a standard protocol. The number of biopsy was less in the baseline endoscopy. There was only one follow up endoscopy. This made the assessment of the progression of gastric premalignant lesions less reliable. Vries *et al.*(119) had conducted a retrospective review based on the Dutch nationwide histopathology registry (PALGA). The annual incidence of GC was 0.1% in atrophic gastritis, 0.25% in intestinal metaplasia, 0.6% in mild-moderate dysplasia and 6% in severe dysplasia within 5 years of follow-up. It showed that patients with premalignant gastric lesions are at higher risk of GC compared to those without any lesion.

In Japan, 5-year survival rate of all GC patients has remained at around 68% (5, 133). In Korea, the survival rate had increased dramatically from 42.8% during the period 1993-1995 to 63.1% during the period 2004-2008 (134). This could be explained by the early detection and the improvement of GC treatment. Based on a large scale population cohort study in Japan, the early stage GC was 59% of total number of GC detected in screened population, compared to 45% in unscreened population (113). The annual endoscopic screening observational study in Korea had reported better results – incidence of early GC was 98.6% in the group with annual endoscopy and 56.7% in the group with infrequent screening.(131) In our GCEP surveillance programme, the preliminary data showed that 100% of GC case detected was in early stage. We compared with the hospital GC registry data (GASCADII study) which indicated only 44% of GC detected in hospitals were in early stage ($p < 0.001$). Subjects in GASCADII and GCEP studies were all from public hospitals. Without GCEP study, most of the GC cases would be detected in late stage which has poorer

survival rate. This suggested that endoscopy surveillance is required in order to detect cancer in early stage.

As a prospective cohort study, the design of GCEP reduces the potential bias problems. By targeting on hospital subjects, we have had high compliance rate of endoscopy surveillance which helps detect GC in early stage. However, the study population does not reflect the general Chinese population and it represents a Chinese population who are at high risk of developing GC. The generalizability of the findings will limit to Chinese who were over 50 years with gastro symptoms.

The sample size may be underestimation as the incidence rate of GC continues falling. Besides, the subjects were mainly from the hospitals. It is possible that the subjects in our study had easy access to the hospital facility and were more willing to visit the hospital when they felt uncomfortable. This may suggest that this special group of people had better socioeconomic status and they may also have good awareness of cancer prevention through their contact with doctors. The incidence of GC in this high risk group may not be as high as we estimated.

In summary, GC surveillance is feasible in a select group of high risk subjects. The number of EGN detected in surveillance group is significantly higher than in the usual specialist clinic. With the multi-centre setting, cohort design and uniform prospective pathologic examination, further follow-up of our cohort will contribute data for the determination of risk factors associated with GC and aid in the understanding of the progression from premalignant lesions to GC. The findings will help provide recommendations for future screening guidelines of GC and the surveillance guidelines for gastric premalignant lesions.

Table 3.1 Baseline Characteristics of GCEP population

Total No. subjects		2975	100%
Mean Age ± SD		59±7	
Age	50-59	1620	55%
	60-69	1082	36%
	≥70	273	9%
Gender			
	Male	1540	52%
	Female	1435	48%
Education			
	Tertiary	659	22%
	Junior college	272	9%
	Secondary	1223	41%
	Primary and below	821	28%
Smoking (%)			
	Never	2313	78%
	Past	367	12%
	Current	295	10%
Alcohol consumption		520	17%
Family history of GC		468	16%
HP infection History (%) #		1160	39%
Peptic ulcer (current or previous)			
	Absent	2344	79%
	Gastric	383	13%
	Duodenal	204	7%
	Unknown	44	1%
Diabetes		420	14%
Hypertension		1193	40%
Personal history of cancer other than GC		154	5%
Family history of other cancers		1146	39%
Statin		714	24%

HP is considered as present if any one of the following is positive: HP infection history by reviewing the case notes, HP current infection based on histological examination.

Table 3.2 Risk factors in GCEP subjects

Risk factors include family history of GC, smoking, H.pylori infection (history, histology, serology), atrophy, IM

No. of Risk Factors	No. of subjects (%)
0	429 (14%)
1	696 (23%)
2	882 (30%)
≥3	968 (33%)
TOTAL	2975 (100%)

Table 3.3 Histological findings at the baseline endoscopy with biopsy

	Baseline endoscopy N=2975
Chronic Gastritis	2611 (88%)
Current *H.pylori* Infection	621 (21%)
Atrophy	538 (18%)
Intestinal Metaplasia	1409 (47%)
Fundic or neoplastic Polyp	188 (6%)
Low or moderate grade dysplasia	6

Table 3.4 Stage of early gastric neoplasia detected

Stage	N = 18	Treatment
0 (High grade dysplasia)	5	No treatment
0 (High grade dysplasia)	6	EMR, ESD, Gastrectomy
0 (Intramucosal carcinoma)	1	Gastrectomy
1A	5	Gastrectomy, ESD
1B	1	Gastrectomy

Table 3.5 Risk factors in early gastric neoplasia cases

Risk factors	N=18	
Intestinal metaplasia	17	94%
History of *H.pylori* infection#	16	89%
Atrophic gastritis	15	83%
Male	11	61%
Smoking	9	50%
Family history	3	17%

HP is considered as present if any one of the following is positive: HP infection history by reviewing the case notes, HP current infection based on histological examination, HP serology test

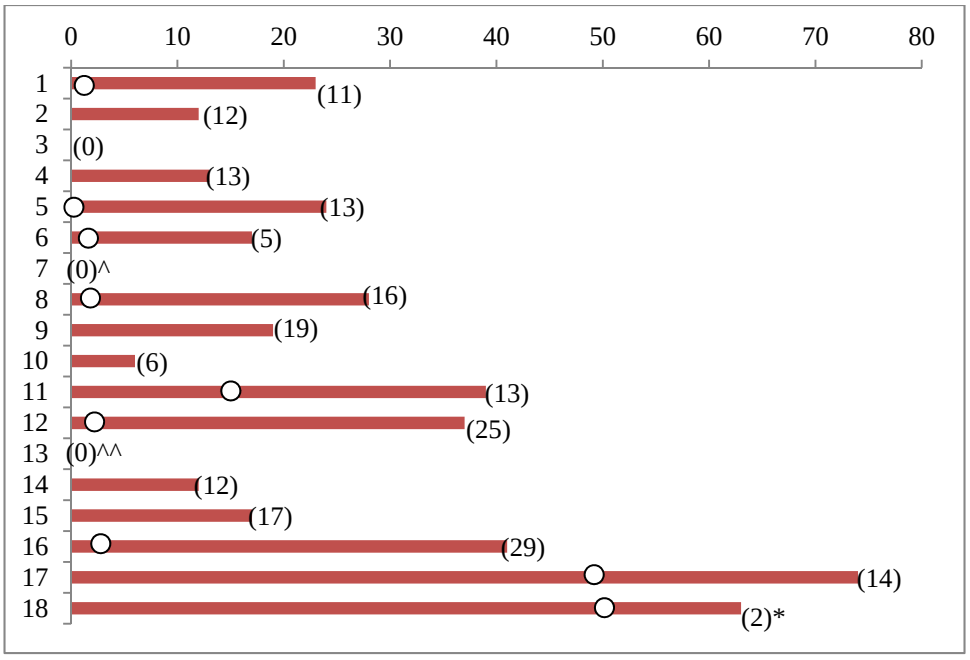
Table 3.6 The development of early gastric neoplasia

S. N	Gender	Age at enrol	Stage	Location	Initial endoscopy (D1)	Endoscopy before Cancer detected (D2)	Diagnostic endoscopy (D3)	Surveillance month (D3-D1) (month)	Interval between last two endoscopy (D3-D2) (month)	Treated
1	F	76	0	Antrum	Sep 2004	Sep 2005	Aug 2006	23	11	ESD
2	F	50	0	Incisura	Mar 2006	=D1	Mar 2007	12	12	No Rx
3	M	58	0	Incisura	Jan 2007	=D1	Jan 2007	0	0	Subtotal Gastrectomy
4	M	50	0	Antrum	June 2007	=D1	June 2008	13	13	ESD
5	F	55	1A	Antrum	Apr 2006	Apr 2007	Apr 2008	24	13	Subtotal Gastrectomy
6	F	77	0	Antrum	Jan 2007	Jan 2008	June 2008	17	5	ESD
7	M	75	0	Antrum	Aug 2004	Aug 2004	Aug 2004	0	0	EMR
8	M	54	1B	Body	Nov 2006	Nov 2007	Mar 2009	28	16	Total Gastrectomy
9	F	68	0	Incisura	Sep 2006	=D1	Apr 2008	19	19	No Rx
10	M	60	0	Antrum	Aug 2008	=D1	Feb 2009	6	6	No Rx
11	M	69	0	Body	Apr 2007	May 2009	June 2010	39	13	EMR
12	M	78	0	Antrum	Sep 2007	Sep 2008	Sep 2010	37	25	No Rx
13	M	73	0	Antrum	Dec 2010	Dec 2010	Dec 2010	0	0	Subtotal Gastrectomy
14	M	58	1A	Body	Jan 2010	=D1	Jan 2011	12	12	Total Gastrectomy
15	M	69	1A	Body	Sep 2009	=D1	Jan 2011	16	16	Total Gastrectomy
16	M	62	0	Incisura	Jan 2008	Jan 2009	June 2011	41	29	No Rx
17	F	67	1A	Body	June 2006	May 2011	July 2012	74	14	ESD
18	F	72	1A	Antrum	Aug 2007	Aug 2012	Oct 2012	63	2	ESD

Table 3.7 Difference of stages of GC between hospital cancer registry (GASCADII study in 2006-2012) and surveillance cohort (GCEP study)

Stage	GASCADII GC registry		GCEP endoscopy surveillance		
	No. of subject (N = 310)		No. of subject (N = 18)		
Early GC	**135**	**(44%)**	18	**(100%)**	$p < 0.001$
0	5	(1%)	12	(73%)	
1a	47	(15%)	5	(20%)	
1b	44	(14%)	1	(7%)	
2	39	(13%)	0		
Late GC	**175**	**(56%)**	0		
3a	40	(13%)	0		
3b	24	(8%)	0		
4	111	(36%)	0		

Figure 3.1 The interval between the diagnosis of EGN and the most recent endoscopy prior to diagnosis



Horizontal bars denote surveillance duration in months between baseline endoscopy to the development of EGN. The dot on the bar indicates the most recent endoscopy prior to detection of EGN and the number in brackets indicates the time in months from the latter to diagnosis.

^ case number 7 had one clinical endoscopy 4 months before enrolled in the study

^^ case number 13 had one clinical endoscopy 13 months before enrolled in the study

*case number 18 was diagnosed with moderate dysplasia two months before EGN was diagnosed

Study 2 – Clinical Risk Factors and Premalignant Lesions of Gastric Cancer in High Risk Cohort

3.5 Result

Univariate and multivariate analysis were performed for risk factors such as age, gender, education level, smoking, alcohol consumption, family history of GC, HP infection history as well as premalignant lesions – atrophic gastritis and IM (Table 3.8 and 3.9). Age above 70, primary education level or below, smoking, atrophic gastritis and IM were statistically significantly associated with early gastric neoplasia in both univariate and multivariate analysis with adjusted ORs (95% CI) of 3.24 (1.17-8.98), 2.72 (1.01-7.30), 2.89 (1.13-7.44), 3.71 (1.42-9.72) and 8.01 (1.05-60.86) respectively. HP was statistically significantly associated with early gastric neoplasia in univariate analysis with OR of 4.92 (1.13-21.44) but the effect was not significant after adjusted for other risk factors. Gender, alcohol consumption and family history of GC did not demonstrate difference between early gastric neoplasia group and control group.

The relationship between two premalignant lesions (atrophic gastritis and IM) and age, gender, education level, smoking, alcohol consumption, family history of GC and HP infection history were assessed. Subjects over 70 years old were more likely to have atrophic gastritis or IM. The proportion of past history of HP infection increased in the groups of normal or chronic gastritis, atrophic gastritis without IM and IM. The prevalence of HP infection in IM group was as high as 74%. Greater proportions of primary education or below and alcohol consumption were seen in IM group compared with the other two groups with less severe lesions. The overall prevalence of male gender, smoking and family history of GC were 52%, 22% and 16% respectively,

and there were no significant difference were observed among various groups. (Table 3.10)

3.6 Discussion

We performed interim analysis for the GCEP study, a large GC cohort with over 3000 subjects enrolled. Endoscopy surveillance was provided at 1-3 years interval. We found that age over 70 years old, primary education or below, smoking, atrophic gastritis and IM were strongly associated with early gastric neoplasia.

Eighteen cases of early gastric neoplasia were identified after an average of 3 years of follow up and the incidence rate was estimated to be about 0.2% per year. This is similar to a prospective study in China with incidence rate of 0.22% (135) and slightly lower than the result from a large prospective study in Japan in which 36 GC was detected in 1245 HP positive subjects and the incidence rate was estimated to be 0.37% (13), a Caucasian study in Spain (132). Those studies all targeted on high risk subjects but had long follow up (4.5 - 12 years).

The result showed IM was a strong and significant risk factor in the GC risk prediction in our high risk cohort. 17 out of 18 (94%) of early gastric neoplasia cases had IM. The only one case which was IM negative was diffuse type of GC. It showed IM is a necessary step in the development of high grade dysplasia or intestinal type GC. The proportion of IM cases in our cohort is 61% and the result was based on the all surveillance endoscopy examinations following with histology confirmation and the no. of endoscopy examinations varied from 1 - 5. If any one of the histology reports indicated subjects had IM, we will classify this subject as IM although sometime IM

may be absent in subsequent endoscopy or prior baseline endoscopy due to sampling error. There has been some debate on whether IM was reversible (112, 136-138) and it seemed IM was less likely to regress. Studies on IM progression or regression had small sample size or short follow up period and sampling error was the big problem in assessing the IM regression. The indirect evidence was from a randomized trial on the effect of HP eradication on GC prevention (29). HP eradication reduced GC incidence rate only in the subgroup without any premalignant lesions but there was no difference between placebo and eradication groups after 7.5 years follow up. It suggested the lesions were unlikely to return to the less severe lesions or normal status. Nevertheless, our estimation of the power of IM in predicting GC risk was similar to other studies (118, 119) and IM was proved to be an important risk factor in selecting high risk people. Some studies had also showed incomplete type of IM characterized by brush border cells and goblet cells was at elevated risk of developing GC compared with complete IM represented by hybrid intermediate non-goblet mucous columnar cells with cytoplasmic drops of different size (132).

The prevalence of atrophic gastritis was 27% in this cohort but it always co-existed with IM. Only 98 subjects had atrophic gastritis without IM. Atrophic gastritis was characterized by the loss of gastric glandular cells due to long term chronic inflammation. It is believed atrophic gastritis will eventually lead to IM, dysplasia and GC (64). Subjects with atrophic gastritis were reported to have 3.5 – 25-fold increased risk of GC and the risk was higher for intestinal type of GC than in diffuse type GC (139-141). In our cohort, two out of 18 GC were diffuse type and the adjusted OR for the risk of atrophic gastritis was 3.71. Blood pepsinogen level was used as marker for atrophic gastritis as it was believed to reflect the functional status of gastric mucosa

(142). Loss of gastric glandular cells resulted in low pepsinogen level (143, 144). Pepsinogen (PG) had been widely used as screening tool in Japan because it was less invasive and cheaper (145-147). In Chapter 5, we will discuss the usefulness of the HP-PG panel in GC risk prediction in Singapore Chinese population.

Sufficient evidence from cohort studies as well as case-control studies proved the causal relationship between HP infection and GC (12, 13, 148). The risk of HP reported from different studies varied greatly and the range of ORs were 2 – 8 (6-9, 65). The difference in the risk of HP infection could possibly be due to the various methods used to detect HP infection. In our study, we collected results from HP serology test, histology examination and also from reviewing case notes. If any one of the tests were positive for HP, we considered the subject had had HP infection currently or in the past. In the study with stratification analysis, HP infection was found to have much higher risk in noncardia GC with OR of 20 (149). Our study showed HP infection was associated with increased risk of GC in univariate analysis but failed to show statistically significant effect in multivariate analysis. This may be a common scenario in the high risk population and the premalignant lesion study in Spain which had the high prevalence of HP could not show significant effect of HP too (132).

Other than premalignant lesions, we found three more clinical risk factors – old age, low education level and smoking were statistically significantly associated with the development of GC. They were all well-known risk factors for GC. Education level represented socioeconomic status. A nested case-control study in a prospective cohort, the European Prospective Investigation into Cancer and Nutrition (EPIC), low

education level was found to be associated with GC with HR 1.56 (95% CI, 1.02-2.33) (150). An extreme high HR was observed when limit to intestinal type GC with HR of 7.69 (95% CI, 2.27-25). In the same EPIC cohort, ever smoker was found to have 1.45 (95% CI, 1.08–1.94) (151).

In this study we examined the possible risk factors associated with risk of IM. The results showed subjects who were age over 70, primary education or below and had history of HP infection were more likely to develop IM. It seemed the development of IM and GC had some common risk factors. In countries where GC incidence is too low, the risk factors of IM could be used to identify those at high risk of GC.

Our study here has confirmed that the premalignant lesions atrophic gastritis and IM were associated with increased risk of GC with adjusted OR of 3.71 and 8.01 respectively in a high risk Chinese cohort. The other clinical risk factors such as age over 70 years old, smoking, low education level were also validated in our cohort with significant ORs. A few strengths of using our cohort to estimate the predictive power of those known risk factors: prospective study reduces the chance of selection bias and recall bias. The variables were documented before the subjects reached endpoint. The majority of risk factors collected by interview were able to be verified by reviewing case notes because our subjects were recruited from the hospitals. The premalignant lesions were confirmed based on multiple surveillance endoscopies and this had reduced the chance of underestimation due to sampling error. There were some limitations here. Our study is not ready for stratification analysis to evaluate the difference of the effect of risk factor between intestinal type of GC and diffuse type of GC. The cardia GC and non-cardia GC were also considered to have different

pathway in GC development. We hope this limitation can be overcome after we complete five year surveillance for all 3000 subjects and accumulate more early gastric neoplasia cases. As our cohort is a high risk cohort, the prevalence of HP infection was high and the effect of this risk factor was clearly demonstrated.

In summary, atrophic gastritis, IM, age over 70 years old, smoking, low education level will help us stratify the subjects into low, moderate and high risk groups. In addition, More attention should be paid to IM subjects due to its significantly higher OR compared with other risk factors. More studies are required to understand the progression from IM to GC and identify those IM cases which should be under frequent surveillance in order to detect the GC at early stage.

Table 3.8 Characteristics of GCEP cohort

	GC Case (n=18)	GC free in the cohort (n=2957)	P value
Age at enrolment	64.2 (9.2)	59.5 (6.9)	0.004
Male (%)	11 (61)	1529 (52)	0.48
Education (%)			
Primary and below	11 (61)	803 (27)	0.003
Secondary and above	7 (39)	2154 (73)	
Smoking			
Never	9 (50)	2304 (78)	0.009
Former/Current	9 (50)	653 (22)	
Alcohol drinking			
No	13 (72)	2442 (83)	0.225
Previous/Current	5 (28)	515 (17)	
Family history of GC	3 (17)	465 (16)	0.99
History of HP infection#	16 (89)	1826 (62)	0.025
Chronic Gastritis	17 (94)	2594 (88)	0.72
Atrophic Gastritis *	15 (83)	807 (27)	0.003
Intestinal Metaplasia *	17 (94)	1817 (61)	0.003

HP is considered as present if any one of the following is positive: HP infection history by reviewing the case notes, HP current infection based on histological examination, HP serology test

*Based on all surveillance endoscopy examination including baseline endoscopy

Table 3.9 Relative risk and adjusted relative risk for age, education, smoking, atrophic gastritis and IM

Risk factors	No. of GC cases (n=18)	Odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age (y)			
50-69	6	1.00	1.00
≥70	12	5.04 (1.88-13.53)	3.24 (1.17-8.98)
Education			
Secondary and above	7	1.00	1.00
Primary and below	11	4.20 (1.62-10.87)	2.72 (1.01-7.30)
Smoking			
No	9	1.00	1.00
Previous or current	9	3.53 (1.39-8.92)	2.89 (1.13-7.44)
History of HP infection			
Absent	2	1.00	1.00
Present	16	4.92 (1.13-21.44)	4.05 (0.92-17.77)
Atrophic gastritis			
Absent	7	1.00	1.00
Present	11	4.17 (1.61-10.78)	3.71 (1.42-9.72)
Intestinal Metaplasia			
Absent	1	1.00	1.00
Present	17	10.61 (1.41-79.82)	8.01 (1.05-60.86)

Table 3.10 Characteristics of atrophic gastritis and IM

	Normal or chronic gastritis without IM and Atrophic gastritis (n=1043)	Atrophic Gastritis without IM * (n=98)	P value	IM* (n=1834)	P value	Total (n=2975)
Age at enrolment	57.8 (6.1)	59.4 (7.1)	0.01	60.5 (7.1)	<0.001	59.5 (6.9)
Male	52%	49%	0.57	52%	1.00	52%
Primary education or below	21%	21%	1.00	32%	<0.001	27%
Ever Smoker †	21%	15%	0.16	23%	0.22	22%
Alcohol drinking	16%	11%	0.19	19%	0.04	17%
Family history of GC	16%	17%	0.80	16%	1.00	16%
History of HP infection #	41%	62%	0.001	74%	0.009	62%

† Ever Smoker refers to current smoker and ex-smoker

HP is considered as present if any one of the following is positive: HP infection history by reviewing the case notes, HP current infection based on histological examination, HP serology test

*Based on all surveillance endoscopy examination including baseline endoscopy

Chapter IV

STUDY 3 - GENETIC RISK FACTORS FOR GASTRIC CANCER AND PREMALIGNANT LESION OF GASTRIC CANCER

4.1 Introduction

GC is known to be a heterogeneous disease with multiple environmental factors and genetic factors implicated in the various gastric carcinogenesis pathways. There are a small number of GCs that are caused by hereditary diffuse GC syndrome mainly due to the mutation of E-cadherin (CDH1) gene on the chromosome 16q22 (152). The syndrome has been well studied and gene mutation carriers who are also on HP eradication are recommended to undergo endoscopic surveillance at six monthly intervals or prophylactic gastrectomy after the age of 20.

Two studies with new generation sequencing using GC samples had been published recently (153, 154). Wang *et al.* (153) from Pfizer had performed whole-exome sequencing on 22 GCs using the Illumina platform. The study identified the top 20 genes using driver-gene score including known driver gene of carcinogenesis such as TP53, PTEN and TTK. The study had also showed for the first time that ARID1A gene (a tumor suppressor gene) was the most frequently mutated gene in 83% of GCs with microsatellite instability (MSI), and 73% of those with Epstein-Barr virus (EBV) infection. Zang *et al.* had reported consistent result on the ARID1A gene mutation and in addition, they found the mutation of a cadherin gene, FAT4 was also a key tumorigenic events (154).

The etiologies and incidence of GC varies geographically. GC is common in the East Asia but is rare in North America and Europe. This can be partially explained by the difference in prevalence of HP (155). In Singapore, the Chinese has the

highest GC incidence rate while Indians and Malays have much lower incidence rate. HP infection may play an important role in race-associated GC risk. Ang TL from Changi General Hospital had evaluated the race difference in HP and GC in the Singapore population and found that HP prevalence was similar in Chinese and Indian (50%) but significantly lower in Malay (27%) (156). It seems that some races are more susceptible to GC than the others, suggesting host genetic susceptibility plays an important role in GC development. Identification of those gene variants will help us better understand GC development and is also important in risk stratification and early detection. A few genome-wide association studies have been successful in exploring genetic susceptibility factors in Japanese, and Chinese (157, 158). The Japanese study published in 2008 found that the prostate stem cell antigen (PSCA) gene and Mucin 1 (MUC1) gene were significantly associated with diffuse type GC. This result has been validated in both Asian and Caucasian populations (159-162). In genome-wide association study (GWAS) in Chinese population (158), gene PRKAA1 (encoding protein kinase, AMP-activated, alpha 1 catalytic subunit) and ZBTB20 (encoding zinc finger and BTB domain containing protein 20) were identified as susceptibility genes in non-cardia GC in addition to the two genes found in the Japanese study. Another Chinese study found a shared susceptibility locus in *PLCE1* (encoding phospholipase Cε1) for GC and esophageal squamous cell carcinoma (163). The findings have been validated in other Chinese population and it had been shown that the PLCE1 gene variation was also associated with GC survival (164).

HP has been classified as class I carcinogen due to its causative role in the GC development. Differing inflammatory responses between hosts may help to explain different outcomes for persons with HP infection. Therefore, the

association between GC susceptibility and inflammation-related gene polymorphisms had been evaluated in many studies. A meta-analysis showed IL1RN2 carriers had increased risk for both intestinal type and diffuse type GC in Caucasian population (165). In Asian populations, IL1B-31C carrier was found to be associated with reduced risk of GC. These polymorphisms could be used in conjunction with HP status and together with dietary and environmental factors to target screening programs towards individuals deemed to be at high risk.

GC is thought to arise via a multi-step pathway that involves intestinal metaplasia (IM) as a precursor lesion (64). It has been estimated that 0.25-1.1% of IM lesions will progress to GC annually, representing an 18-78-fold increased lifetime risk of developing this disease in comparison to the general population (118, 119). In the present study, we have investigated a panel of 18 polymorphisms in 14 candidate genes for their association with IM precursor lesions in a Singapore-Chinese population considered to be at increased risk of GC because of age greater than 50 years. These polymorphisms were chosen for study because previous research has shown them to be risk factors for GC. They included SNPs in genes involved in the immune response (*IL-1*β, *IL-10*, *PTPN11*) (166-168), folate metabolism (*FR*-α, *MTHFR*) (169, 170), cell growth (*EGF*, *HER2*) (171-173), cell survival (*STCH*) (174), cell invasion (*MMP2*) (175) and DNA damage or repair (*NQO1*, *SULT1A1*, *TP53*, *ADPRT*) (176-180).

4.2 Methods

Subjects

Subjects were recruited from the Gastric Cancer Epidemiology and Molecular Genetics Program (GCEP) which was described in Chapter 2. Blood samples

from 374 individual subjects collected between April 2004 and December 2006 were used for genotyping in the present study.

IM was diagnosed from mucosal biopsies in three locations (antrum, body and cardia) for each subject and by consensus amongst three pathologists according to the updated Sydney System for the classification and grading of gastritis (181). In cases where HP was identified in biopsies, eradication therapy was administered according to standard clinical guidelines. For 339/374 (91%) individuals, the HP status was determined using the Helicoblot2.1 serology test (Genelabs Diagnostics, Singapore). In individuals where this test was not performed, the HP status was determined from histological examination of biopsies from the antrum, body and cardia, as well as from past medical history. Blood samples (8 mls) were collected into Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ) and the mononuclear cells isolated and stored at -80°C prior to DNA extraction using Tri-Reagent (MRC Inc, Cincinnati, OH).

Helicoblot 2.1 serology test

This serological assay uses a Western Blot nitrocellulose strip containing electrophoretically separated proteins from a bacterial lysate of an ulcer-causing type strain of HP and a recombinant antigen of HP (MP Biomedicals, Singapore). When incubated with diluted serum/plasma, specific antibodies against the various antigens, if present, will bind to the HP antigens on the strip. These bound antibodies appear as dark bands upon reaction with goat anti-human IgG conjugated with alkaline phosphatase and a 5-bromo-4-chloro-2-indolyl-phosphate/nitroblue tetrazolium substrate solution. In order to identify the various bands present, the strip is compared with reference strips of non-reactive (negative) and reactive (positive) controls run concurrently. Determination of HP

seropositivity was based on criteria recommended by the kit manufacturer. They consist of (1), 116 kD (CagA) positive band present with one or more of the following bands: 89 kD (VacA), 37 kD, 35 kD, 30 kD (UreA) and 19.5 kD together, or with the current infection marker, (2) the presence of any one band at 89 kD (VacA), 37 kD or 35 kD, with or without current infection marker, or (3) the presence of both 30 kD and 19.5 kD with or without current infection marker.

Selection of gene polymorphism panel

A systematic literature search in PubMed was carried out using the terms "gastric cancer" and "polymorphism". From a total of 78 candidate polymorphisms identified, 18 were found to be significantly associated with the risk of GC and were therefore included in the current investigation of IM.

Genotyping

Table 4.1 shows the PCR primers, annealing temperatures and product sizes for 17 SNPs investigated in this study by pyroseqeuncing. The 86-bp variable number of tandem repeats (VNTR) polymorphism in *ILRN* was genotyped using PCR followed by size analysis using gel electrophoresis. The primers and PCR conditions were the same as previously reported (182). Polymorphisms were recorded in their most commonly used notation for easy cross-referencing. For PCR, 50 ng DNA was amplified in a 25 μl reaction containing 1 × FastStart Reaction Buffer, 2 mM magnesium chloride, 10 μM deoxynucleotide mix, 500 nM each of the forward and reverse primers and 1 unit FastStart Taq Polymerase (Roche Diagnostics, Mannheim, Germany). PCR cycling comprised of 4 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at the

appropriate annealing temperature and 30 seconds at 72°C, before conclusion with 7 minutes at 72°C.

Pyrosequencing was performed by incubating the PCR products with 3 µl of streptavidin magnetic beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and 1× binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixing for 10 minutes at 37°C. The product mix was then denatured by 5 seconds incubation in 0.2 M NaOH solution and washed in annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate) for 10 seconds. The single-stranded products were transferred to an annealing buffer containing 15 pmol of the sequencing primer (Table 4.1) and incubated for 2 minutes at 80°C in a Hybaid Maxi 14 hybridization oven (Thermo Electron, USA). Pyrosequencing was then performed on a PSQ96MA pyrosequencer instrument (Biotage AB, Uppsala, Sweden). Samples that failed to give a genotype result after the first analysis were repeated up to two times. The genotyping success rate varied from 85-99% for the 18 polymorphisms.

Statistics

Univariate analyses were carried out by Pearson's chi-square or the Fisher's exact test to examine for associations between genotype distributions, IM status and clinical factors. As there were more than one polymorphism investigated in *IL10* and *STCH*, the haplotypes were also considered in the analyses. Variables found significantly associated with IM in the univariate analyses for all cases, and HP+ and HP- subgroups were entered in respective multivariate logistic regression models. The analyses were based on the assumption of a dominant genetic model. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL) software at the 5% significance level. The Woolf test was used to

test for homogeneity of OR between two strata. As each polymorphism was tested for association with IM independently, it was not necessary to control for the family-wise error rate. Thus, no adjustment was made for multiple testing.

4.3 Results

The characteristics of 374 subjects evaluated in this study are shown in Table 4.2. A total of 128 were diagnosed with IM and 246 without IM. No significant differences between IM+ and IM- groups were apparent for sex, family history of GC (including 1st degree and 2nd degree relatives), alcohol consumption (at least one unit of wine, beer or liquor per week) or smoking status (at least one cigarette per day for a minimum of one year). IM+ subjects showed a significantly higher incidence of HP infection and were also older ($P < 0.05$). Genotype frequencies for the 18 polymorphisms investigated for association with IM are presented in Table 4.3. All polymorphisms were in Hardy-Weinberg equilibrium ($P > 0.05$), with the exception of *IL10* -819T/C, *NQO1* 609C/T and *TP53* Arg72Pro. By univariate analysis, the *NQO1* 609 T allele was the only variant in the overall cohort that was significantly associated with IM (OR = 1.82, 95%CI: 1.05-3.15, $P = 0.032$). In HP- individuals, only the *PTPN11* rs2301756 A allele was significantly associated with IM (OR = 2.51, 95%CI: 1.16-5.40, $P = 0.019$). Three polymorphisms in HP+ individuals were associated with IM in univariate analysis: the *IL-10* 819 C allele (OR = 2.32, 95%CI: 1.21-4.43, $P = 0.011$), *NQO1* 609 T allele (OR = 2.61, 95%CI: 1.18-5.80, $P = 0.018$) and *PTPN11* A allele (OR = 0.46, 95%CI: 0.21-0.99, $P = 0.048$). The haplotypes in *IL10* and *STCH* were not significantly associated with IM in overall cohort, HP-, as well as HP+ groups.

In multivariate analysis that included all cases, HP status and age were significantly associated with IM, while the *NQO1* T allele showing borderline association (Table 4.4). In HP- individuals, the *PTPN11* A allele was the only factor associated with IM. However, in HP+ individuals the factors of older age and the *NQO1* 609 T allele, *IL-10* 819 C allele and *PTPN11* A allele were all significantly associated with IM. These results suggest that HP status is an effect modifier of the association between IM and the *PTPN11* A allele ($P = 0.002$). As it is possible that IM+/HP cases in this study had prior unrecorded HP infection (183), subgroup analysis on cases with a "revised HP+" status (either HP+/IM-, HP+/IM+ or HP-/IM+) was also performed. Age (OR = 2.10, 95%CI: 1.24-3.56, $P = 0.006$) and *IL-10* -819 C allele (OR = 1.82, 95%CI: 1.07-3.08, $P = 0.027$) were the only significant variables in this subgroup.

4.4 Discussion

In this study, 18 polymorphisms that were previously linked to GC were investigated for possible associations with IM in a Singapore-Chinese population. The assumption was made that IM represents a precursor lesion for the development of GC and hence should have similar genetic risk factors. The cohort evaluated here was considered to be at elevated risk for GC because of the selection of individuals aged >50 years (123). As expected, older individuals and those demonstrating seropositivity for HP showed a doubling in the frequency of IM (Table 4.4).

Following univariate analysis, 3 genotypes were found to be associated with IM. The *NQO1* 609 T allele was associated with IM, particularly in HP+ individuals. The *IL-10* -819 C allele was also significantly associated with IM in HP+ cases. Interestingly, the *PTPN11* An allele in intron 3 (rs2301756) was associated with

increased incidence of IM in HP- individuals but a decreased incidence in HP+ cases. In multivariate analysis, all 3 polymorphisms remained significantly associated with IM, with the exception of the *NQO1* 609 T allele which was associated with borderline significance in the overall cohort ($P = 0.056$).

Previous data lends support to our observations. *NQO1* (NAD(P)H: quinine oxidoreductase 1) codes for a cytosolic enzyme that protects cells from oxidative damage by preventing the generation of semiquinone free radicals and reactive oxygen species (184). The C to T substitution at nucleotide 609 in exon 6 results in a change of amino acid from Pro to Ser at codon 187 (185). Whereas the CC homozygous wildtype genotype (Pro/Pro) has full enzymatic activity, the TT genotype (Ser/Ser) completely lacks activity. The *NQO1* 609 TT genotype has been associated with an increased risk for various tumour types including gastrointestinal and urological cancers (186-189). An increased risk of GC in patients with a family history of upper gastrointestinal cancers was also reported for the *NQO1* 609 TT genotype in a study on Chinese subjects (176). Our observation of increased prevalence of IM in carriers of the *NQO1* 609 T allele concurs with earlier reports on its association with various cancers and can be explained by a decreased activity for the detoxification of environmental and dietary carcinogens.

The *NQO1* C609T polymorphism was previously associated with seropositivity to HP in a Japanese study (190), thus raising the possibility that it is an indirect risk factor for IM via association with HP infection. However, we found no association between the *NQO1* C609T polymorphism and HP infection in the present cohort (results not shown). Carriers of the *IL-10* -819 C allele express higher mucosal levels of *IL-10* (interleukin 10) mRNA and experience

colonization with more virulent HP strains (191). Similar to *NQO1* C609T, no association was observed here between the *IL-10* T-819C polymorphism and HP infection. The current result showing the *IL-10* -819 C allele is associated with IM is at odds with an Italian study that reported the TT genotype was associated with increased risk of IM (182). However, two studies in Chinese and German populations found no associations between *IL-10* T-819C and IM (191, 192).

Other common polymorphisms in the *IL-1*β and *TNF*-αcytokine genes have been proposed to influence the host response to HP and therefore the risk of developing GC (167, 182, 191-195). The *IL-1*β C-511T and *IL-10* A-1082G polymorphisms were investigated in this cohort, but no significant associations were found with seropositivity to HP or with the presence of IM (Table 4.3). Previous studies reported the *IL-1*β -511 T allele increased the risk of IM in some (191, 192), but not all populations (166). One study found an association between the *IL-10* A-1082G polymorphism and IM (166, 196), but 3 other studies did not[12,29,39]. *PTPN11* (protein tyrosine phosphatase, non-receptor type 11) encodes for SHP-2, a protein tyrosine phosphatase thought to play a key role in intracellular signaling elicited by growth factors and cytokines (197). Interactions between the HP cagA protein and SHP-2 in gastric epithelial cells are believed to contribute to the development of GC (198). The *PTPN11* AA genotype was associated with reduced risk of gastric atrophy in a Japanese population of HP seropositive individuals (168, 183). In those studies, the assessment of gastric atrophy was done with serology test (pepsinogen levels). The present results on IM in HP seropositive Singapore-Chinese support these earlier observations, although the number of AA genotype individuals (n = 6) did not allow separate evaluation of this group. The diagnosis of IM was based on histology

examination. The *PTPN11* intron 3 G/A SNP may be in linkage disequilibrium with a coding marker that influences the interaction of SHP-2 with cagA and subsequent downstream signaling. However, its association with increased frequency of IM in HP negative individuals suggests it may play a role independently of this factor.

In summary, we found 3 polymorphisms associated with IM in a Singapore-Chinese population that was at high risk for GC because of older age and seropositivity for HP. The value of these SNPs in facilitating more cost-effective surveillance programs awaits further validation in large, independent cohorts.

Table 4.1 PCR primers and dispensation sequences for pyrosequencing of 17 SNPs evaluated for association with IM.

Locus	PCR				Pyrosequencing	
	Forward Primer	Reverse Primer	°C	bp	Sequencing Primer	Dispensation
IL10 – 1082 A/G	CTCAATCAAAGGATCCCCAGAGAC	AGGCTGGATAGGAGGTCCCTTACT	60	253	ACACTACTAAGGCTTCTTTG	cgagcagta
IL10 – 819 T/C	GGCCAATTTAATCCAAGGTTT	TCTGCACTTGCTGAAAGCTTCTTA	60	207	CCTTGTACAGGTCATGTAA	gtcgatctc
IL-1B – 511 T/C	CATGAGATTGGCTAGGGTAACAG	GCCCTCCCTGTCTGTATTGA	60	230	CAATTGACAGAGAGCTCC	atctgagca
MMP2 – 1306 T/C	TTTCATCTCTGGGCCATTGT	TGAAGTTCTCCCTGTGACAACC	60	265	TCCCCACCCAGCACT	gctgactct
EGF +61 A/G	GTCATCCCTGCTTTCCTGTGTG	CAGAGCAAGGCAAAGGCTTAGA	60	266	CCCAATCCAAGGGTTGT	cagactgac
PTPN11 (int1) A/G	TGGACGAATGGCAAATTG	GATCAATCCCACCTGAGACAGA	60	182	TTGTCTCTAAAGGACTGTG	tgagctcat
NQ01 C609T	AACTGCATGGAATTGGTTGACTTA	TGGTGTCTCATCCCAAATATTCT	60	191	GTGGCTTCCAAGTCTTA	cgatcgtca
STCH rs2242661	AACTCGAATCCTGGACCTGATTAG	CTGGCGTTTATAATCAAACCTGTG	65	203	GCGGAAAGAGAAAGG	gctagtact
STCH rs1882881	CTATGGAAGGCTGCGAGAAC	ACTTCCAGCTACAGGCAACATT	65	213	GAGGCTTTTTCCATCA	gcagtcgtg
STCH rs12479	CTTGAAGGACCGTGTTGATGT`	GCAAAGGTCTCGGATAACAAAAA	60	312	ATGTTTCAGCACCAT	gatagctag
STCH rs9982492	TCGTGCTTACCTTGTTCACATT	AGTATGAGCCCTGCCATGA	60	193	CCACTTGTCCTTTAAGTCC	actcgactc
SULT1A1 G638A	GCCAGATCGCCTCTGAGGT	TGGGGGACGGTGGTGTAGT	65	233	CCTGGAGTTTGTGGG	tgcgagctc
ADPRT T2285C	GATACCTAAGTCGGGGGCTTTC	ACAAGCTTTCCAGGAGATCCTAAC	65	262	TGCTCCTCCAGGCCA	cagtctgat
HER2 +17ex17 A/G	GTCCCTCCCACCCCAAACTA	CTGCCGTCGCTTGATGAG	65	145	CCCTCTGACGTCCAT	gtcagatct
TP53 C215G	TCCCAAGCAATGGATGATTTGA	AAGCCCAGACGGAAACCGTAG	60	230	CAGAGGCTGCTCCCC	tgcagtgct
FR-a A1314G	AAGTGGAGACTGAGGCCCAGA	TGACCCCTCCCCACCAAC	60	183	GTGTGGCCTGCTCAA	cgagtacga
MTHFR C677T	ACTGTCATCCCTATTGGCAGGTTA	TCGGTGCATGCCTTCACAA	60	168	GAAGGTGTCTGCGGG	cgagtacga

Table 4.2 Characteristics of study subjects in relation to the presence of IM

	tal (%)	IM+ (%)	IM- (%)
Subjects	374	128	246
Mean age ± SD (range)	60.5 ± 7.8	62.9 ± 7.8	59.2 ± 7.5
Age 50-59 yrs	190 (51)	48 (38)*	142 (58)*
Age 60-69 yrs	133 (36)	55 (43)	78 (32)
Age ≥70 yrs	51 (13)	25 (19)	26 (10)
Male	207 (55)	72 (56)	135 (55)
Family history of GC	66 (18)	23 (18)	43 (17)
HP infection	191 (51)	84 (66)*	107 (43)*
Drinker	66 (18)	22 (17)	46 (19)
Smoker	90 (24)	30 (23)	60 (24)
Chronic gastritis	290 (78)	115 (90)	175 (71)
Atrophy gastritis	194 (52)	97 (76)	97 (39)
Dysplasia	1 (0.3)	1 (0.8)	0

* $P<0.05$

Table 4.3 Distribution of genotype frequencies according to IM and HP infection status

Gene polymorphism (rs number)	Genotype	IM-	IM+	HP-		HP+	
				IM-	IM+	IM-	IM+
ADPRT Val762Ala	TT	71	31	33	9	38	22
(rs1136410)	TC	117	60	64	24	53	36
	CC	33	16	13	6	20	10
EGF +61A/G	AA	22	5	13	1	9	4
(rs4444903)	AG	103	55	54	20	49	35
	GG	110	58	50	18	60	40
FR-a 1314A/G	GG	164	95	74	30	90	65
(none)	GA	74	31	43	12	31	19
	AA	6	1	5	0	1	1
HER2 Ile/Val	AA	174	92	87	32	87	60
(rs1801200)	AG	60	30	29	8	31	22
	GG	1	1	1	1	0	0
IL1RN 86-bp VNTR	44	212	101	112	33	100	68
(none)	24	28	18	9	4	19	14
	34	1	2	0	2	1	0
	54	0	1	0	0	0	1
	22	2	2	0	1	2	1
IL-1β -511C/T	CC	64	35	33	10	31	25
(rs16944)	CT	119	62	63	21	56	41
	TT	48	23	20	10	28	13
IL-10 -819T/C	TT	131	55	57	21	**74***	**34**
(rs1800871)	TC	78	46	39	15	39	31
	CC	22	16	17	3	5	13
IL-10 -1082A/G	AA	207	100	98	37	109	63
(rs1800896)	AG	21	14	13	3	8	11
	GG	2	0	2	0	0	0
MMP2 -1306C/T	CC	178	79	85	28	93	51
(rs243865)	CT	46	22	26	8	20	14
	TT	3	2	2	0	1	2

Gene polymorphism (rs number)	Genotype	IM-	IM+	HP-		HP+	
				IM-	IM+	IM-	IM+
MTHFR 667C/T	CC	132	77	64	23	68	54
(rs1801133)	CT	98	42	50	16	48	26
	TT	14	7	8	2	6	5
NQO1 609C/T	CC	**64***	**21**	27	10	**37***	**11**
(rs1800566)	CT	143	80	78	25	65	55
	TT	28	22	13	4	5	18
TP53 Arg72Pro	CC	45	16	22	7	23	9
(rs1042522)	CG	126	78	66	23	60	55
	GG	51	26	24	10	27	16
PTPN11 rs2301756	GG	175	85	**92***	**24**	**83***	**61**
(rs2301756)	GA	58	28	26	16	32	12
	AA	4	2	0	1	4	1
STCH rs12479	GG	102	58	51	20	51	38
(rs12479)	GA	106	39	52	12	54	27
	AA	22	15	10	8	12	7
STCH rs1882881	AA	58	34	24	13	34	21
(rs1882881)	AC	123	58	67	15	56	43
	CC	57	31	26	13	31	18
STCH rs2242661	AA	69	31	34	12	35	19
(rs2242661)	AG	106	46	52	13	54	33
	GG	44	26	22	13	22	13
STCH rs9982492	CC	85	45	41	15	44	30
(rs9982492)	CT	105	39	53	12	52	27
	TT	28	18	14	9	14	9
SULT1A1 638G/A	GG	221	108	112	33	109	75
(rs9282861)	GA	16	10	6	6	10	4
	AA	1	0	1	0	0	0

* Bold type denotes significant difference in genotype frequencies

Table 4.4 Multivariate logistic regression analysis for associations with IM

	OR for IM (95% CI)	*P*
All cases		
HP (positive *vs* negative)	2.16 (1.35 - 3.45)	0.001
Age (>60 *vs* <60 yrs)	2.21 (1.40 - 3.49)	0.001
NO1 (CT/TT *vs* CC)	1.74 (0.99 - 3.06)	0.056
HP- cases		
Age (>60 *vs* <60 yrs)	1.92 (0.92 - 4.00)	0.082
PTPN11 (GA/AA *vs* GG)	2.51 (1.16 - 5.40)	0.019
HP+ cases		
Age (≥60 *vs* <60 yrs)	2.19 (1.15 - 4.17)	0.017
NQO1 (CT/TT *vs* CC)	2.61 (1.18 - 5.80)	0.018
IL-10 -819 (TC/CC *vs* TT)	2.32 (1.21 - 4.43)	0.011
PTPN11 (GA/AA vs GG)	0.46 (0.21 - 0.99)	0.048

Chapter V

STUDY 4 - USING PEPSINOGEN AND HP ANTIBODIES TO PREDICT THE RISK OF GASTRIC CANCER

5.1 Introduction

In Singapore, GC is the 5th most common cancer in males and 7th most common in females. The age standardized rate of GC for the period 2006 to 2010 is 14.3 per 100,000 per year in Singapore Chinese males and 7.9 per 100,000 per year in Singapore Chinese females (199). The risk of GC incidence in Singapore is at intermediate level, in contrast with high incidence countries such as Japan and Korea and low incidence countries such as India and the United States (4). Population screening in Singapore is not considered to be cost-effective (117). It is therefore important to have an efficient and cost-effective screening method which could select people who are at high risk of developing GC and provide screening accordingly.

It has been well established that gastric carcinogenesis begins with and progresses from superficial gastritis to subsequent development of atrophic gastritis, metaplasia, dysplasia and finally culminating in adenocarcinoma (64). *Helicobacter pylori* (HP) infection is a strong risk factor for the development of both atrophic gastritis and GC (9, 13, 200-202). There are broadly two divergent responses of long-term HP infection, namely antral-dominant gastritis with little atrophy and corpus-dominant gastritis with multi-focal atrophy which suggests high risk of GC (203-205). Atrophic

gastritis is a condition characterized by loss of glands and specialized cells in the stomach, and is hence a precursor lesion of GC (64). It can be diagnosed by histological examination of gastric biopsies, measuring gastric maximum acid output, or measuring serum or plasma concentration of proteins released from the gastric cells, such as pepsinogens (142, 206). Pepsinogens are proteinases that are mainly secreted by gastric cells and are classified into two major types: pepsinogen I (PG I) and pepsinogen II (PG II). PG I is secreted from the gastric fundic mucosa and PG II is secreted from the cardiac, fundic, and antral mucosa of the stomach, as well as the duodenal mucosa (143, 144). A low concentration of PG I or a low PG I to PG II ratio (PG I : II ratio) in the serum or plasma is an indicator of atrophic gastritis and is associated with an elevated GC risk (207, 208).

The combined use of plasma pepsinogen (PG) measurement and plasma HP antibodies has been widely used as a screening tool for GC in Japan (145, 208-210). Several prospective and case-control studies have shown that the HP-PG panel was effective at predicting the risk of GC and it could reduce the number of subsequent endoscopy screening and surveillance for the early detection of GC by targeting on high risk group of people(11, 146, 211-213). Recently, K Miki had reviewed the latest results of studies and reported the current status of using HP-PG ('ABC method') for screening GC risk (214). The 'ABC method', a screening system proposed in 2007, classified subjects into four risk groups and offered endoscopy screening at different frequencies according to the risk level of the subjects. Although the effectiveness of this screening method remains unknown, evidence based on

Japanese studies has supported the use of HP-PG as a stratification tool for GC. We aim to validate the 'ABC method' in our cohort of selected subjects in the Singapore Chinese Health Study. We also aim to examine the different antibodies of HP and their associations with GC.

5.2 Methods

Study population

The subjects in this study were selected from the Singapore Chinese Health Study (SCHS), a prospective cohort for long-term study of dietary, genetic and environmental determinants of cancer and other chronic diseases. The details of the study design had been described in Chapter 2.

Identification of gastric cancer cases

Incident GC cases occurring within the Singapore Chinese Health Study cohort were identified through the population-based cancer registry in Singapore (215). The nationwide cancer registry has been in place since 1968 and has been shown to be comprehensive in its recording of cancer cases (215). As of 12 Dec 2010, there were total 650 subjects who were diagnosed with GC. 222 GC subjects donated blood samples. Of these, 178 cases donated blood before the occurrence of GC. After 7 cases were excluded as they were either lymphoma or stromal tumor (GIST), 171 cases were included in the study.

Control selection

For each of the 171 cases, 3 control subjects were selected among the participants who had donated blood samples, who were still alive and free of any cancer. The controls were matched to the index case on age at study enrollment (±3 years), gender, dialect and the year blood was collected. There were 9 cases where only 2 eligible controls were found. The study consists of 171 cases and 504 controls.

Blood tests (HP and PG)

HP antibodies were measured by Western Blot Assay (Helico Blot 2.1, MP Biomedicals, Singapore), a qualitative assay for the detection of IgG antibodies to HP in human serum or plasma. After individual strips were incubated with diluted serum or plasma specimens, specific antibodies to the various antigens, if present, bound to the HP antigens on the strips. This allowed differentiation of reactivity to each of the various HP antigens including CagA, VacA, UreA etc. HP seropositivity was determined by the recommended criteria designed by the manufacturer, which is any one of the following conditions: 1) 116kD (CagA) positive, where CagA has to be present with one or more of the following bands: 89kD (VacA), 37kD, 35kD, 30kD (UreA), 19.5kD, or with current infection marker. 2) Presence of any one band at 89kD, 37kD or 35kd, with or without current infection marker. 3) Presence of both 30kD and 19.5kD with or without current infection marker. Through validation against histology, culture, rapid urease test and/or urea breath test (UBT), the sensitivity and specificity reported in the protocol of the kit by the manufacture is 96% and 95% respectively.

PG I and II were measured by using latex agglination turbidimetric immunoassay kit (LZ Test "Eiken" Pepsinogen I and II, Tokyo, Japan) and automated analyzer (Siemens Advia 2400). Results were defined as "atrophic gastritis" if PG I level was < 70 ng/ml and PG ratio was < 3, as recommended by manufacture. This criteria has been used widely in the mass screening for GC in Japan.

All measurement was done by lab personnel blinded to the case/control status and the samples were analysed in a set of 4 (one case and three controls) or 3 (one case and two controls).

Statistical analysis

The chi-square test and the Student's t-test were used to compare the distributions of selected demographic, lifestyle and dietary factors between cases and controls. The distributions of blood markers measured were markedly skewed with a long tail toward high values, which were corrected, to a large extent, by transforming the original values to logarithmic values. Therefore, geometric (as opposed to arithmetic) means are presented to minimize the effect of high values. The conditional logistic regression method was used to examine the associations between plasma markers measured and risk of GC. Statistical computing was carried out using SAS version 9.2. The statistical significance level was set at two-sided P value of 0.05.

5.3 Results

Compared to control cases, a higher percentage in the GC group had family history of GC, or had primary school education and below. Except for these statistically

significant differences, baseline characteristics were similar across both groups (Table 5.1). The median time interval between the baseline interview and GC diagnosis was 11 years (range: 1 -17 years). The mean time interval from blood collection to the diagnosis of cancer was 4.3 (SD 2.9) years.

The mean values of PG I and PG ratio were significantly lower in cases than in controls ($P < 0.0001$ for both). PG II was slightly higher in cases ($P = 0.045$) (Table 5.3). Of 171 cases, 61 were positive for atrophic gastritis according to manufacturer's criteria (PG I < 70 ng/ml and PG ratio < 3); 68 out of 504 controls were positive for atrophic gastritis.

Six HP antibodies (CagA, VacA, UreA, 35kD, 37kD and 19.5kD) were individually associated with GC, with statistically significantly increased ORs after adjustment for multiple potential confounding factors. The ORs (95% CI) of CagA, VacA, UreA, 35kD, 37kD and 19.5kD for the risk of GC were 5.82 (2.22-15.27), 4.17 (2.14-8.12), 2.81 (1.64-4.81), 2.06 (1.40-3.01), 2.46 (1.62-3.73) and 1.87 (1.27-2.77) (Table 5.3). The overall HP seropositivity based on the manufacturer's recommendation was also associated with increased risk of GC with OR (95% CI) 5.11 (2.14-12.22). Subjects with atrophic gastritis as determined by PG index had 4.02 times the GC risk of those without atrophic gastritis (95% CI 2.56-6.30).

Subjects were classified into three groups based on positivity of HP CagA antibody and PG index (Table 5.4). Group A (HP-/PG-) was considered as low risk group with negative for CagA and negative PG index. Group B (HP+/PG-) continued with negative PG index but positive CagA antibody. Group C (HP+/PG+) had positive

PG index with positive CagA antibody. There was no subject who had positive PG index but negative for HP CagA antibody. The adjusted ORs were calculated in each group. Risk of GC increased from A to C with group C having the highest ORs. Using the group A as reference group, the ORs (95% CI) for group B, C were 4.02 (1.51-10.73), 13.95 (5.01-38.87) respectively.

5.4 Discussion

Our study is the first outside Japan to investigate the association between the combination of plasma PG and HP antibodies and the risk of GC in a Chinese population recruited from the community. A few prospective studies had been done in Japan (146, 211). A study based on the Shanghai Women Cohort (10) demonstrated that low PG ratio was associated with higher risk of GC but HP was not found to be significantly associated with the risk of GC due to the high prevalence of HP in both case group and control group. Our study is more representative of the general population because it was not limited by gender.

The results from our study are consistent with one case-control study in Japan (11) and showed that serum PG index and HP antibodies were able to stratify the study population into groups with low risk (group A), intermediate risk (group B) and high risk (group C) of developing GC. There were no subjects who had PG index positive without HP infection. In prospective studies from Japan, subjects who had atrophic gastritis determined by PG index without HP infection were considered to be at very high risk with about 15-30 times higher risk than the low risk group (146, 211, 213). The high risk group C comprising subjects with atrophic gastritis and positive for

CagA had 13.95 times higher risk of getting GC, compared to low risk group A who were negative for HP antibodies and without atrophic gastritis. The ORs increased steadily from group A to group C. The atrophic gastritis indicated by the PG level was strongly associated with increased risk of GC. The persistent chronic inflammation was found to play an important role in initiation and promotion of cancer development (216). Atrophic gastritis was the result of chronic (long term) inflammation in stomach. Two mechanisms of how chronic gastritis initiates gene mutation were suggested. In a mouse model of HP-induced GC, bone-marrow stem cells were found in the stomach epithelium in chronic inflammation environment and underwent malignant progression which gave rise to gastric tumor (217). Another way is the high concentration of free radicals due to the excessive demand of oxygen in the chronic inflammation, leading to subsequent DNA damage. As the result, tissue stem cells were mutated and cancer development was initialized (218).

Amongst 675 subjects, 12% was in group A, 69% was in intermediate group (group B), 19% was in group C (high risk). A possible reason for the high percentage in intermediate group and low percentage in group A is that our study population contains elderly Chinese who are more likely to be HP positive compared to the general population. A second possible reason is a relatively high ratio of GC to controls in this nested case-control study. We propose both low risk group and intermediate group (81% of the total population) could be exempted from frequent endoscopic surveillance in countries with intermediate level of GC risk. Similar recommendation has been made in a prospective study in Japan where the annual incidence of GC in males over 60 years old in group with HP+/PG- (0.4%) was

similar to that in group with HP-/PG- (0.3%) (146). In comparison, the GC incidence in group with HP+/PG+ (1.0%) was much higher than group with HP+-/PG-. It seems HP status does not significantly affect the risk of GC in people who are negative for PG index. Risk stratification based on non-invasive blood test enables targeted endoscopic screening in people who are at high risk of GC.

In addition, we found that HP antibodies CagA, VacA, UreA, 35kD, 37kD and 19.5kD were all significantly associated with increased risk of GC with ORs which lies within the range 1.87 – 5.82. Among them, CagA had the highest OR and was the most sensitive antibody with the prevalence of 97% in cancer group. Many studies had evaluated CagA which was believed to be related to higher degree of gastric inflammation and higher risk of GC (27, 219). We found that by using CagA alone we were able to indicate the HP infection status and best predict the GC risk.

There are a few strengths in our study. Subjects were recruited from the community and the subjects were asymptomatic at the enrolment and had been observed for long term. The two prospective studies from Japan (146, 211) recruited subjects who were undergoing health screening or from a special workplace and the potential selection bias had to be taken into consideration when we interpreted the result. The 1:3 ratio of case/control maximized statistical power. Blood samples in our study were collected before cancer was diagnosed to minimize the potential influence from disease development and subsequent treatment.

Cardia GC, which have been suggested to have little association with HP, were not subjected to stratification analysis here because of lack of information, similarly for intestinal type and diffuse type GC.

In summary, the HP-PG panel was able to predict the risk of GC in our population. The OR of the high risk group (atrophic gastritis with HP CagA seropositive) was 13.95 compared with the low risk group where both atrophy gastritis determined by PG index and HP were negative. The study indicated the HP-PG panel was useful to stratify the population into low and high risk group. In order to justify risk-stratified screening at national level, future prevention study on GC using HP-PG panel in Singapore's general population is required. In addition, we found that CagA alone was able to predict the GC risk.

Table 5.1 Baseline characteristics of case and control subject

	Case (n=171)	Control (n=504)	P value
Age at blood draw	66.6 (7.6)	66.4 (7.4)	0.749
Male (%)	113 (66.1)	334 (66.3)	0.964
Education (%)			
None	45 (26.3)	115 (22.8)	0.025
Primary	96 (56.1)	248 (49.2)	
Secondary and above	30 (17.5)	141 (28.0)	
Smoking status			
Never	92 (53.8)	285 (56.6)	0.712
Former	28 (16.4)	85 (16.8)	
Current	51 (29.8)	134 (26.6)	
Alcohol drinking			
None-monthly	140 (81.9)	438 (86.9)	0.264
Weekly	24 (14.0)	50 (9.9)	
Daily	7 (4.1)	16 (3.2)	
Family history of gastric cancer	7 (4.1)	5 (1.0)	0.008
Body mass index	23.2 (3.2)	23.2 (3.2)	0.918
Total vegetables intake (g/day)	113.5 (64.9)	110.2 (60.9)	0.551
Total fruit intake (g/day)	201.0 (188.7)	208.4 (161.8)	0.649
Sodium intake (mg/day)	1180.9 (619.1)	1100.0 (555.0)	0.110

Table 5.2 Geometric means between cases and controls

	Cases (n=171)	Controls (n=504)	2-sided *P* *
PGI #	38.67 (2.3)	52.78 (1.8)	<0.0001
PGII #	13.06 (1.9)	11.81 (1.8)	0.045
PG ratio	2.97 (1.9)	4.47 (1.7)	<0.0001

The unit of PGI and PGII is ng/ml

*Adjusted for age at blood taking

Table 5.3 Adjusted ORs of GC risk with HP antibodies or PG index alone (conditional logistic regression)

	Case (n=171)	Control (n=504)	Adjusted ORs * (95% CI)
Cag A			
Negative	6 (3.5)	73 (14.5)	1.00
Positive	165 (96.5)	431 (85.5)	5.82 (2.22-15.27)
VacA			
Negative	13 (7.6)	117 (23.2)	1.00
Positive	158 (92.4)	387 (76.8)	4.17 (2.14-8.12)
UreA			
Negative	20 (11.7)	128 (25.4)	1.00
Positive	151 (88.3)	376 (74.6)	2.81 (1.64-4.81)
19.5 kD			
Negative	58 (33.9)	239 (47.4)	1.00
Positive	113 (66.1)	265 (52.6)	1.87 (1.27-2.77)
35 kD			
Negative	70 (40.9)	284 (56.3)	1.00
Positive	101 (59.1)	220 (43.7)	2.06 (1.40-3.01)
37 kD			
Negative	56 (32.7)	253 (50.2)	1.00
Positive	115 (67.3)	251 (49.8)	2.46 (1.62-3.73)
PG index			
Negative	110 (64.3)	436 (86.5)	1.00
Positive	61 (35.7)	68 (13.5)	4.02 (2.56-6.30)
HP positivity **			
Negative	8 (4.7)	84 (16.7)	1.00
Positive	163 (95.3)	420 (83.3)	5.11 (2.14-12.22)

* Adjust for education, smoking, alcohol, BMI, sodium, vegetables, fruits

** HP status determined by manufacture's recommendation

Table 5.4 ORs associated with PG index and CagA (conditional logistic regression)

SCHS	**Group A**	**Group B**	**Group C**
n =675	**(low risk)**		
PG index	Negative	Negative	Positive
CagA	Negative	Positive	Positive
Controls	73 (14.5)	363 (72.0)	68 (13.5)
Cases	6 (3.5)	104 (60.8)	61 (35.7)
OR (95% CI)	1.00	4.02 (1.51-10.73)	13.95 (5.01-38.87)

Adjust for education (D5_2_2), smoking (D14_1), alcohol (D37_1_2), BMI (D2_2), sodium (N3_14), vegetables (F3_6), fruits (F3_7)

Note: There was no subject who was negative for anti-CagA but positive for PG index.

Chapter VI

CONCLUSION AND FUTURE STUDIES

The development of GC is a multifactorial and multistep process. So far, there is no effective prevention programme to reduce the incidence of GC. Screening and early detection is still the key strategy to improve GC-related mortality. The successful screening experience in Japan has suggested that screening will help reduce GC mortality rate if we can target the high risk group in a country like Singapore where the incidence rate is at intermediate level.

In this thesis, we made use of two Singapore Chinese cohort studies – GCEP and SCHS – to investigate gene polymorphisms and the use of the blood marker HP-PG panel in GC risk stratification. This allows us to use immediately available data and biological samples to achieve sufficient statistical power to detect real association. It will also make possible the validation of other biomarker for GC risk stratification or early detection; similarly, for future gene-environmental interaction studies. Through genotyping of blood samples from GCEP subjects, we were able to investigate the genetic factors associated with increased risk of IM, a precursor lesion of GC.

In study one, we described the baseline characteristics of the GCEP cohort study and investigated the feasibility of endoscopic surveillance in the high risk group. The results look positive. All 18 early gastric neoplasia detected in the programme were in stage 0 or stage I. It seemed that endoscopic surveillance with frequency of two years was able to detect the GC at early stage. As the study is still ongoing, we expect more conclusive

suggestions on surveillance frequency, and recommendations on how often we should follow up with those people with precursor lesions could be drawn after year 2015 when all subjects complete at least 5 years surveillance. One limitation we have to highlight here is that the study recruited from amongst patients attending the gastroenterology clinic, a targeted population which is known to be at higher risk of developing GC. This should be taken into consideration during interpretation of the result, especially from the perspective of application in the general population.

In study two, we examined the clinical risk factors as well as the premalignant lesions of GC in the risk of developing GC. With 18 gastric neoplasia cases detected in GCEP cohort, we found that age over 70 years old, primary education or below, smoking, atrophic gastritis and IM were statistically significantly associated with early gastric neoplasia. These risk factors may not be conclusive at the moment as we have not completed the 5 years surveillance for all subjects. The HRs of five risk factors mentioned in the study were between 2.72 - 8.01 and it suggested the results were unlikely to be due to chance. Subjects with IM lesions alone had 8 times high risk of GC compared with those who are negative for IM. Surveillance endoscopy should be considered for these IM subjects.

In study three, we aimed to identify the genetic factors which could help in risk stratification. Three polymorphisms *NQO1*, *IL-10* and *PTPN11,* in combination with HP status, were found to be associated with the increased risk of IM and could be used to identify individuals who are more likely to develop IM and therefore GC. Based on literature search, it is difficult to use gene polymorphism in risk stratification

because of inconsistent result in case-control studies thus far. However, some promising results were seen from next genome sequencing (NGS) studies and genome wide association studies (GWAS). ARID1A gene (a tumor suppress gene) mutation was found in two NGS studies. Prostate stem cell antigen (PSCA) gene polymorphism and *PLCE1* (encoding phospholipase Cε1) were also found to be associated with susceptibility of GC. Based on results from NGS and GWAS, the candidate gene approach could be carried out to identify the specific SNP in predicting GC risk by taking into consideration the subject's race and HP status. Validation using a large cohort study to identify the SNPs associated with intestinal type, diffuse type or cardia GC or non-cardia GC should also be done before the results can be used in clinic.

In study four, the first study of its kind outside Japan, we validated the HP-PG panel in a Singapore Chinese elder population. The results showed that risk of GC increased from group A (HP-/PG-) to group B (HP+/PG-) and group C (HP+/PG+) with group C having the highest OR. Using the group A as reference group, the ORs (95% CI) for group B, C were 4.02 (1.51-10.73), 13.95 (5.01-38.87) respectively. This proved that the HP-PG panel was able to stratify subjects into low, moderate and high risk groups. There are some limitations with the use of the HP-PG panel. First of all, HP-PG stratification was based on assumption that GC carcinogenesis was initialized by chronic gastritis and progressed to atrophy gastritis, IM, dysplasia and eventually became GC. So the result was only able to pick up intestinal type GC. The HP-PG panel may not be able to stratify risk of other types of GC such as diffuse type or GC in cardia location. More risk factors for each type of GC will be identified in

prospective cohort. Genetic factors are believed to be significant in development of diffuse type GC. It is important to combine the HP-PG panel together with gene mutation candidate or gene polymorphism which is associated with GC risk in order to identify all subjects who are at high risk of GC. Secondly, the HP-PG panel should be validated in a prospective study such as the GCEP study.

Collectively, we have found that endoscopic surveillance was feasible for early detection of gastric neoplasia and five risk factors were confirmed in Singapore Chinese high risk cohort. Using three gene polymorphisms can help identify people associated with increased risk of IM and therefore GC. HP-PG panel was effective in risk stratification for GC which was consistent with the results from Japan. In future, we expect more studies to be performed in the following four areas:

First, a large scale prospective study recruiting subjects from the general population should be carried out in order to validate findings related to biomarkers for either GC risk prediction or host susceptibility. The study should include dietary factors which are important in GC development. These dietary factors include salt intake, fruit and vegetable intake, nitrate or nitrosamine in processed meat *et al*. The collection of dietary factors is complicated and requires large amount of resources because of wide range of food items, various cooking method and different sauces used in dialect groups. Standardization of the estimation of food intake is required when quantifying the amount of specific food ate. Data collected on dietary factors can only reflect the most recent status of food intake. Long term recall of food intake is not feasible and some justifications are required when the subject is immigrant or there is change of

cooking style *et al*. This large scale study should also have enough power to detect the risk factor with small OR and be able to investigate the possible gene-environmental interactions.

Secondly, we need more biological functional studies on genes found to be associated with GC risk based on NGS or GWAS studies. This will help us understand the effect of gene mutation or gene polymorphism and contribute to our understanding of GC carcinogenesis. Those studies could be *in vitro* or in animal model. Validation or meta-analysis of the panel of gene polymorphisms which can predict host susceptibility is needed, before they can be applied in the clinic. The validation study should also take into consideration of intestinal and diffuse type of GC. The findings on risk stratification in this thesis focused on intestinal type GC. Using genetic markers to identify people who are at high risk for diffuse type GC may help improve the screening strategy. One study had reported CDH1 mutation screening in 43 families with hereditary diffuse GC and 30% of the families were found to have CDH1 mutation (67). The results should also be reproducible in different populations with different study designs. As there are only 300 new GC cases in Singapore per year, this kind of validation study should combine GC cases from similar studies in other countries in order to have conclusive results.

Thirdly, it is well known that people with IM are at high risk of GC and the prevalence of IM is about 50% in GCEP cohort. Although we observed that IM positive subjects had 8 times higher risk of GC compared with IM negative subjects, only 0.9% of IM positive subjects had GC. There were a large number of subjects who had IM but did

not progress into GC. The studies had shown incomplete IM represented by brush border cells and goblet cells was associated with increased risk of GC compared with complete IM represented by hybrid intermediate nongoblet mucous columnar cells with cytoplasmic drops of different size. It will be interesting to find out the other clinical characteristics or genetic effect modifiers in the progression from IM to GC.

Fourthly, early GC or high grade dysplasia is difficult to diagnose and new endoscopic image techniques are required to improve the sensitivity of conventional endoscopy. In GCEP cohort, out of 18 early gastric neoplasia including high grade dysplasia and early stage GC, three subjects with high grade dysplasia had had endoscopy done 6 months or less prior to diagnosis of gastric neoplasia . This indicated that high grade dysplasia may have been missed at the previous endoscopy. Projects on biomarker discovery for GC early detection will also be useful. GC specific miRNA panel or DNA methylation markers could help identify suspected GC cases and a comprehensive endoscopy checkup will be offered to confirm the GC.

Lastly, a targeted screening study based on known risk factors at community level should be started. The aim is to determine if targeted screening reduces the mortality rate and the difference in incidence rate among groups with various risk level of developing early gastric neoplasia. Subjects will be divided according to the number of risk factors they have. In the low risk group with no risk factor or with some risk factors but the low ORs, no intervention is required. In the intermediate risk group, endoscopy surveillance every 5 years may be indicated. Frequent endoscopy surveillance with 2-3 years interval should be considered in the high risk group. With

the histology result from surveillance endoscopy, the subjects will be further stratified. The cost-effectiveness of screening based on risk stratification could also be evaluated.

Risk stratification and targeted screening will be the trend for GC prevention due to the decline of GC incidence worldwide. The results from this thesis have identified five risk factors which are associated with early gastric neoplasia. Using these five risk factors together with HP-PG panel and gene polymorphism markers, we are able to stratify Singapore Chinese into low, intermediate and high risk for GC. If validated, this will give a possible solution to reduce the mortality rate in Chinese community where incidence rate of GC is at intermediate level. Risk stratification before population screening could also be similarly applied in the prevention of other common and life-threatening diseases.

Chapter VII

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Annex A List of publication

This thesis is based on the following three papers:

1. **Feng Zhu**, Marie Loh, Jeffrey Hill, Sumarlin Lee, King Xin Koh, Kin Wai Lai, Manuel Salto-Tellez, Barry Iacopetta, Khay Guan Yeoh, Richie Soong and the Singapore Gastric Cancer Consortium

   Genetic factors associated with intestinal metaplasia in a high risk Singapore-Chinese population: a cohort study

   BMC Gastroenterology 2009, **9**:76

2. **Feng Zhu**, Lee Guan Lim, Khek Yu Ho, Chung King Chia, Christopher Khor, Choon-Jin Ooi, Kwong Ming Fock, Jimmy So, Wee Chian Lim, Khoon Lin Ling, Ang Tiing Leong, Andrew Wong, Jaideepraj Rao, Andrea Rajnakova, Ming Teh, Manuel Salto-Tellez, Supriya Srivastava, Richie Soong, Kee Seng Chia, Khay Guan Yeoh

   Systematic Endoscopic Surveillance in a High-risk Cohort is able to Successfully Detect Early Gastric Neoplasia

   In manuscript.

3. **Feng Zhu**, Woon-Puay Koh, Benjamin Yip, Lee Guan Lim, Ai-Zhen Jin, Ren-Wei Wang, Jian-Min Yuan, Khay Guan Yeoh

   Plasma Pepsinogen and H.pylori Antibody Levels Predicts Gastric Cancer Risk in the Singapore Chinese Health Study (SCHS)

   In manuscript.