as 0·05, taking account of three cases (two or more, three or more and four risk factors) in a Bonferroni-type multiple comparison. All analyses were conducted using SAS software running on a UNIX System (SAS/STAT Software, Release 9·0. Cary, NC, SAS Inst. Inc.).

Results

The basic characteristics of controls and subjects with subclinical hypothyroidism are shown in Table 1. Men with subclinical hypothyroidism (mean age: 72·1 years) were older than controls (mean age: 68·3 years, $P < 0·001$). The sex ratios of controls (2134/3243, 65·8% of women) and subjects with subclinical hypothyroidism (194/306, 63·4% of women) did not differ ($P = 0·40$). Less smokers were in the group of subjects with subclinical hypothyroidism ($P < 0·001$). In thyroid function tests, as anticipated, TSH levels were significantly elevated, and free T4 levels were significantly lower in subjects with subclinical hypothyroidism ($P < 0·001$). The positive rates for TPOAb and TgAb were significantly higher in subjects with subclinical hypothyroidism. No difference between the subjects with subclinical hypothyroidism and controls was observed in terms of either the exposed atomic-bomb thyroid radiation dose or the city of residence (Hiroshima: Nagasaki; 124:182 and 1289:1954, respectively, $P = 0·79$, not shown in Table 1).

The clinical and laboratory metabolic parameters are shown in Table 2. There were no differences in BMI, body temperature, systolic blood pressure, diastolic blood pressure, glucose, HbA1c, total cholesterol, LDL cholesterol, HDL cholesterol or triglycerides after adjusting for age in both men and women. In the analyses in men, the total cholesterol level was suggestively higher ($P = 0·06$) and the HDL cholesterol level was significantly higher in subjects with subclinical hypothyroidism ($P = 0·03$). In women, the uric acid level was significantly higher in subjects with subclinical hypothyroidism ($P = 0·02$).

The four major metabolic CVD risk factors of hypertension, diabetes mellitus, dyslipidaemia and hyperuricaemia were further defined by diagnostic criteria, which incorporated metabolic parameter levels and medications. We investigated possible associations between subclinical hypothyroidism and those metabolic CVD risk factors by adjusting for age, BMI and smoking status (Table 3). The ORs of hypertension, diabetes mellitus and hyperuricaemia were positive in men with subclinical hypothyroidism (OR: 1·13–1·38), but did not reach a significant level ($P > 0·13$). On the contrary, subclinical hypothyroidism was significantly associated with the prevalence of dyslipidaemia in men [OR: 1·64, 95% CI: 1·09, 2·46, $P = 0·02$]. In contrast, the analyses among women did not exhibit any differences in any of the risk factors. The possible association between uric acid levels and subclinical hypothyroidism observed in the laboratory data among women was no longer observed in the analysis of hyperuricaemia ($P = 0·09$). Because the ORs for hypertension, diabetes mellitus and hyperuricaemia tended to be high and the OR for dyslipidaemia was significantly high in men, we evaluated the relationship between subclinical hypothyroidism and a cluster of metabolic risk factors. Subclinical hypothyroidism exhibited an increasing trend with a number of metabolic risk factors (hypertension, diabetes mellitus, dyslipidaemia and hyperuricaemia) after adjusting for age, BMI, and smoking status in men ($P = 0·004$, Fig. 1), while no significant trend was observed in women ($P = 0·86$, Fig. 1). Furthermore, as shown in Table 4, subclinical hypothyroidism was significantly associated with a cluster of metabolic risk factors (a number of metabolic risk factors of three or more) after adjustment for age, BMI, and smoking status in men (OR: 1·83, 95% CI: 1·13–2·94, $P = 0·01$), which was a significant result taking multiple comparisons into account (a Bonferroni-type correction). In the subset analysis of mild subclinical hypothyroidism (4·5 < TSH levels <10 mU/l), a significantly increased OR for a cluster of metabolic risk factors was also observed in men (Table 4). The analyses among women showed no association between subclinical hypothyroidism and the cluster of three or more metabolic risk factors (Fig. 1 and Table 4). Because the cohort comprises atomic-bomb survivors, we further conducted an analysis by an additional adjustment for atomic-bomb radiation dose. The significant results of a cluster of metabolic risk factors in subclinical hypothyroidism remained in men (OR:

Table 1. Basic characteristics and thyroid status of participants

	Men			Women		
	Controls	Subjects with subclinical hypothyroidism	*P* value	Controls	Subjects with subclinical hypothyroidism	*P* value
Number	1109	112		2134	194	
Age (years, mean ± SD)	68·3 ± 8·6	72·1 ± 9·3	<0·001	71·4 ± 8·4	72·2 ± 7·9	0·25
Smoker (%)	82·6	71·4	<0·001	15·8	12·4	<0·001
TSH (mean ± SD, mU/l)	2·06 ± 0·93	6·59 ± 1·98	<0·001	2·17 ± 0·91	5·98 ± 1·67	<0·001
Free T4 (mean ± SD, pmol/l)	13·13 ± 2·06	12·23 ± 2·19	<0·001	13·00 ± 2·06	11·97 ± 1·93	<0·001
Positive for TPOAb (%)	8·2	18·8	<0·001	10·5	23·7	<0·001
Positive for TgAb (%)	14·3	23·2	0·018	23·85	30·4	0·04
Thyroid-atomic bomb radiation dose (Gy)	0·52 ± 0·77 (838)	0·48 ± 0·80 (81)	0·74	0·42 ± 0·65 (1670)	0·36 ± 0·59 (154)	0·38

All *P* values except that for 'age' are adjusted for age. Parentheses indicate the number of analysed subjects for thyroid radiation dose excluding those exposed *in utero*, those not in the city at the time of atomic bombings, or those with an unknown radiation dose according to the Dosimetry system 2002.

Table 2. Clinical and laboratory data

	Men			Women		
	Controls	Subjects with subclinical hypothyroidism	*P* value	Controls	Subjects with subclinical hypothyroidism	*P* value
Number	1109	112		2134	194	
BMI	22·7 ± 3·1	22·6 ± 3·0	0·74	23·0 ± 3·9	22·9 ± 3·7	0·89
Body temperature (degree centigrade)	36·5 ± 0·3	36·5 ± 0·3	0·88	36·6 ± 0·3	36·6 ± 0·3	0·86
Systolic blood pressure (mmHg)	133 ± 19	137 ± 21	0·10	130 ± 19	133 ± 19	0·19
Diastolic blood pressure (mmHg)	80 ± 11	78 ± 11	0·71	78 ± 11	77 ± 11	0·80
Glucose (mmol/l)	6·11 ± 2·44	6·00 ± 2·16	0·44	5·83 ± 2·00	5·77 ± 1·72	0·67
HbA1c (%)	5·6 ± 0·9 (1061)	5·6 ± 0·9 (106)	0·61	5·5 ± 0·9 (2037)	5·5 ± 0·9 (188)	0·91
Total cholesterol (mmol/l)	5·12 ± 0·86	5·23 ± 0·89	0·06	5·53 ± 0·87	5·53 ± 0·88	0·90
LDL cholesterol (mmol/l)	3·01 ± 0·76 (546)	3·10 ± 0·79 (40)	0·41	3·20 ± 0·76 (1072)	3·19 ± 0·70 (91)	0·99
HDL cholesterol (mmol/l)	1·44 ± 0·39	1·53 ± 0·45	0·03	1·66 ± 0·42	1·67 ± 0·42	0·73
Triglyceride (mmol/l)	1·53 ± 1·15	1·52 ± 1·22	0·68	1·35 ± 0·75	1·43 ± 0·78	0·18
Uric acid (mol/l[‡])	339·0 ± 83·3	345·0 ± 77·3	0·21	279·6 ± 71·4	297·4 ± 83·3	0·02

Data were expressed as mean ± SD. All *P* values are adjusted for age. Parentheses indicate the number of analysed subjects for HbA1c and LDL cholesterol (see Section Clinical examination and laboratory methods for details).

Table 3. Risk for metabolic abnormalities in subclinical hypothyroidism

	Controls		Subjects with subclinical hypothyroidism					
	Total no.	No. of cases (%)	Total no.	No. of cases (%)	OR (95%CI)*	*P* value*	OR (95% CI)[†]	*P* value[†]
Hypertension								
Men	1109	607 (54·7)	112	73 (65·2)	1·40 (0·93–2·12)	0·11	1·38 (0·91–2·10)	0·13
Women	2134	1253 (58·7)	194	110 (56·7)	1·03 (0·75–1·40)	0·38	0·87(0·64–1·19)	0·39
Diabetes mellitus								
Men	1109	284 (26·5)	112	33 (29·5)	1·10 (0·72–1·70)	0·66	1·13 (0·73–1·74)	0·59
Women	2134	428 (20·1)	194	36 (18·6)	0·89 (0·61–1·31)	0·56	0·91 (0·62–1·34)	0·64
Dyslipidaemia								
Men	1109	534 (48·2)	112	64 (57·1)	1·56 (1·05–2·33)	0·03	1·64 (1·09–2·46)	0·02
Women	2134	64 (66·7)	194	130 (67·1)	1·03 (0·75–1·40)	0·87	1·03 (0·75–1·41)	0·86
Hyperuricaemia								
Men	1109	203 (18·3)	112	25 (22·3)	1·30 (0·81–2·09)	0·28	1·36 (0·84–2·20)	0·21
Women	2134	107 (5·0)	194	15 (7·7)	1·54 (0·88–2·72)	0·13	1·65 (0·93–2·92)	0·09

*Adjusted for age; [†]Adjusted for age, BMI and smoking status.

2·24, 95% CI: 1·32–3·77, $P = 0·003$), suggesting that subclinical hypothyroidism was associated with a cluster of metabolic risk factors independent of atomic-bomb radiation.

We further evaluated combinations of risk factors in 174 control men and 27 men with subclinical hypothyroidism having three or more risk factors. The most frequent combination of risk factors was hypertension, dyslipidaemia and diabetes in both controls ($n = 73$, 42·0%) and subjects with subclinical hypothyroidism ($n = 12$, 44·4%), and we observed no significant difference in the frequency of this combination between the two groups in the analysis adjusted by age, BMI and smoking status ($P = 0·95$).

We also observed that the association between subclinical hypothyroidism and a cluster of all four metabolic risk factors was not significant in men (OR: 1·91, 95% CI: 0·64–5·71, $P = 0·25$), but it was significant in women (6/194, 3·1% in subjects with subclinical hypothyroidism and 13/2, 134, 0·6% in controls, OR: 6·14, 95% CI: 2·24–16·95, $P < 0·001$) after adjustment for age, BMI and smoking status. On the contrary, we did not observe a significant association between subclinical hypothyroidism and a cluster of two or more risk factors in both men and women ($P > 0·1$). TSH levels in three or more risk-clustering cases in men (mean ± SD: 6·73 ± 2·11 mU/l) were not different from those in men with

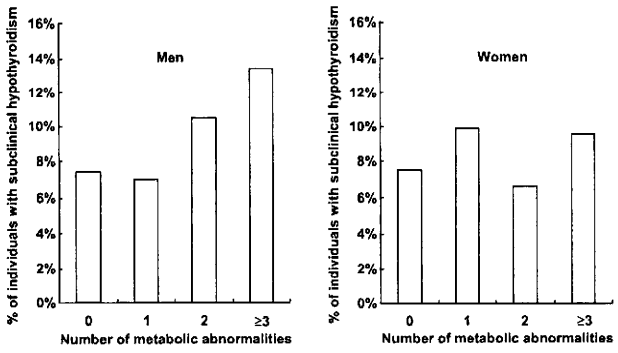

Fig. 1 Subclinical hypothyroidism by number of accompanying metabolic CVD risk factors. The rate of subjects with subclinical hypothyroidism in a total of 3549 study subjects according to number of accompanying metabolic CVD risk factors (hypertension, diabetes mellitus, dyslipidaemia and hyperuricaemia) is shown. Logistic regression analyses after adjusting for age, BMI and smoking status showed a significant positive trend in men (P = 0·004) but no significant trend in women (P = 0·86).

less than three risk factors (6·55 ± 1·95 mIU/l) in subclinical hypothyroidism (P = 0·70). TSH levels in all four risk-clustering cases in women (mean ±SD: 5·38 ± 0·50 mIU/l) were not different from those in women with less than four risk factors (mean ± SD: 5·99 ± 1·69 mIU/l) in subclinical hypothyroidism (P = 0·93).

Discussion

Our results suggest an apparent association between subclinical hypothyroidism and a cluster of metabolic CVD risk factors consisting of hypertension, diabetes mellitus, dyslipidaemia and hyperuricaemia, although we did not observe a significant relationship between subclinical hypothyroidism and each of these factors alone, except for dyslipidaemia in men. A major strength of our study is the use of a large population-based cohort of older men and women and clear diagnostic criteria for metabolic risk factors. Furthermore, we excluded individuals taking thyroid medication or having a history of thyroid surgery or radiation, so that we could investigate metabolic risk factors in individuals with spontaneous subclinical hypothyroidism.

In terms of the lipid profile, we did not observe an apparent abnormality in each of the laboratory levels of total cholesterol, LDL cholesterol or triglycerides. This result is consistent with our previous study showing no association with the total cholesterol level,[11] and is in broad agreement with a recent large US study suggesting no adverse effects on the levels of total cholesterol and LDL cholesterol.[3] On the contrary, various studies including this study reported inconsistent results of HDL cholesterol levels in people with subclinical hypothyroidism.[16,17] However, in this study, dyslipidaemia, as defined by a combination of lipid parameters consisting of total cholesterol, HDL cholesterol, triglycerides and lipid-lowering medication use, was significantly associated with subclinical hypothyroidism in men. Recent RCT studies investigating the effects of L-thyroxine therapy in approximately 50 subclinical hypothyroidism subjects showed an improvement of the lipid profile.[18–21] The results support the possibility that subclinical hypothyroidism may have adverse effects on lipid metabolism.

The putative associations between subclinical hypothyroidism and hypertension, diabetes mellitus and hyperuricaemia are not well established, although certain studies have reported an increased risk of hypertension.[22,23] In the present study, the association of metabolic risk factors such as hypertension, diabetes mellitus or hyperuricaemia with subclinical hypothyroidism did not achieve statistical significance, although the ORs in these metabolic risk factors were positive in men. However, we demonstrated that subclinical hypothyroidism is significantly associated with a clustering of metabolic risk factors in individuals independent of age, obesity, and smoking status in men. Recently, metabolic syndrome has become well recognized as a cluster of metabolic abnormalities such as abdominal obesity, hypertriglyceridaemia, a low HDL cholesterol level, high blood pressure and a high fasting glucose level related to the risk for future CVD. Roos demonstrated the relationship between low normal free T4 and certain components of the metabolic syndrome.[24] Although we did not ascertain the metabolic syndrome based on the criteria of the United States,[25] WHO[26] and Japan[27] in the present study, the result suggests that subclinical hypothyroidism clusters with several abnormal metabolic conditions and is potentially related to the increased risk for CVD in subclinical hypothyroidism. The fact that >10% of subjects with a cluster of more than three metabolic risk factors had

Table 4. Risk of a cluster of metabolic CVD risk factors (number of risk factors ≧ 3) in subclinical hypothyroidism

	Controls		Subjects with subclinical hypothyroidism					
	Total no.	No. of cases (%)	Total no.	No. of cases (%)	OR (95% CI)*	*P* value*	OR (95%CI)†	*P*-value†
Controls *vs.* subjects with subclinical hypothyroidism (4·5 mU/l < TSH)								
Men	1109	174 (15·7)	112	27 (24·1)	1·71 (1·07–2·73)	0·02	1·83 (1·13–2·94)	0·01
Women	2134	266 (12·5)	194	28 (14·4)	1·16 (0·76–1·77)	0·50	1·21 (0·79–1·85)	0·39
Controls *vs.* subjects with mild subclinical hypothyroidism (4·5 < TSH < 10 mU/l)								
Men	1109	174 (15·7)	105	26 (24·8)	1·77 (1·10–2·85)	0·02	1·87 (1·15–3·03)	0·01
Women	2134	266 (12·5)	189	28 (14·8)	1·20 (0·79–1·84)	0·40	1·26 (0·82–1·93)	0·29

*Adjusted for age; †Adjusted for age, BMI and smoking status.

subclinical hypothyroidism (Fig. 1) indicates the importance of examining thyroid function in subjects with metabolic syndrome. Further studies in other populations are necessary to confirm a significant association between subclinical hypothyroidism and a cluster of CVD metabolic risk factors. Recent studies indicate a possible higher risk of insulin resistance in individuals with subclinical hypothyroidism.[28,29] Insulin-stimulated rates of glucose transport in isolated monocytes were decreased due to impaired translocation of GLUT4 glucose transporters on the plasma membrane in subclinical hypothyroidism patients.[30] Insulin resistance may be associated with a cluster of abnormal metabolic conditions in subclinical hypothyroidism. Furthermore, in hypothyroid patients, the hyperuricaemia is attributed to decreased renal plasma flow and impaired glomerular filtration[31] and T4 replacement therapy results in normalization of serum uric acid.[32] A similar mechanism may affect uric acid metabolism in subclinically hypothyroid patients.

Few studies have demonstrated significant associations between mild subclinical hypothyroidism, with TSH levels of < 10 mU/l, and metabolic risk factors. Clinical consensus for hormone replacement therapy in mild subclinical hypothyroidism with TSH levels of <10 mU/l has not been reached, while most clinicians do agree on the necessity of hormone replacement therapy in cases of subclinical hypothyroidism with TSH levels equal to or > 10 mU/l. In the present study, we also demonstrated that even mild subclinical hypothyroidism with TSH levels of <10 mU/l was associated with a cluster of metabolic risk factors independent of age, obesity and smoking status in men. Our result suggests the necessity of careful follow-up in individuals with mild subclinical hypothyroidism.

Although the present results were obtained in atomic-bomb survivors, the conclusion can be generalized to the Japanese population because: (1) the atomic-bomb radiation dose was not associated with subclinical hypothyroidism; and (2) the significant association between subclinical hypothyroidism and a cluster of metabolic risk factors was observed after an adjustment for the radiation dose, suggesting that subclinical hypothyroidism was independently associated with a cluster of metabolic risk factors.

The prevalence of subclinical hypothyroidism in men is similar to that in women in this cohort, while most studies have reported higher frequency in women because of the higher frequency of autoimmune thyroid diseases. In Japan, one of the major causes of subclinical hypothyroidism is excess iodine intake.[33] High iodine intake in Japanese may be related to the absence of a gender difference in the prevalence of subclinical hypothyroidism. Interestingly, the association between subclinical hypothyroidism and lipid abnormality and a cluster of metabolic risk factors was apparent in men in the present study. That result is consistent with our previous study suggesting that an association between subclinical hypothyroidism and CVD was apparent in men.[11] It has been suggested that influences of subclinical hypothyroidism on cardiovascular risk factors are reflected by differences in age, gender and ethnicity of the subjects tested. [1] The gender difference in the association between subclinical hypothyroidism and CVD risk factors and the CVD observed in this Japanese population may be related to genetic factors or life-style factors. However, we cannot deny a possible association between subclinical hypothyroidism and a cluster of metabolic CVD risk factors in women because a cluster of all four metabolic risk factors was significantly associated with subclinical hypothyroidism in women in this study. A further study with other Japanese populations will be required to resolve this issue.

A limitation of this study is that it is cross-sectional and we cannot clarify the longitudinal effects of subclinical hypothyroidism on metabolic risk factors. This large-scale study, however, may nevertheless provide certain clues for a better understanding of the relationship between a cluster of metabolic risk factors and subclinical hypothyroidism. Furthermore, diagnosis of metabolic CVD risk factors using information on medications prescribed by practitioners might have some biases. Another limitation is that the prevalence or incidence of CVD was not evaluated in this population, although our previous study among residents in Nagasaki showed a high prevalence of ischaemic heart disease in individuals with subclinical hypothyroidism. A follow-up study evaluating an association between subclinical hypothyroidism and CVD in this cohort is needed in the future.

Our study suggests that subclinical hypothyroidism might be associated with a cluster of abnormal metabolic CVD risk factors such as hypertension, dyslipidaemia, diabetes mellitus and hyperuricaemia. Further study is necessary to evaluate whether a clustering of those risk factors is related to a high risk of CVD in individuals with subclinical hypothyroidism.

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Competing interests/financial disclosure

Nothing to declare.

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ORIGINAL ARTICLE

Fatty liver incidence and predictive variables

Akira Tsuneto[1,2], Ayumi Hida[2], Nobuko Sera[2], Misa Imaizumi[2], Shinichiro Ichimaru[2], Eiji Nakashima[3], Shinji Seto[1], Koji Maemura[1] and Masazumi Akahoshi[2]

Although fatty liver predicts ischemic heart disease, the incidence and predictors of fatty liver need examination. The objective of this study was to determine fatty liver incidence and predictive variables. Using abdominal ultrasonography, we followed biennially through 2007 (mean follow-up, 11.6 ± 4.6 years) 1635 Nagasaki atomic bomb survivors (606 men) without fatty liver at baseline (November 1990 through October 1992). We examined potential predictive variables with the Cox proportional hazard model and longitudinal trends with the Wilcoxon rank-sum test. In all, 323 (124 men) new fatty liver cases were diagnosed. The incidence was 19.9/1000 person-years (22.3 for men, 18.6 for women) and peaked in the sixth decade of life. After controlling for age, sex, and smoking and drinking habits, obesity (relative risk (RR), 2.93; 95% confidence interval (CI), 2.33–3.69, $P<0.001$), low high-density lipoprotein-cholesterol (RR, 1.87; 95% CI, 1.42–2.47; $P<0.001$), hypertriglyceridemia (RR, 2.49; 95% CI, 1.96–3.15; $P<0.001$), glucose intolerance (RR, 1.51; 95% CI, 1.09–2.10; $P=0.013$) and hypertension (RR, 1.63; 95% CI, 1.30–2.04; $P<0.001$) were predictive of fatty liver. In multivariate analysis including all variables, obesity (RR, 2.55; 95% CI, 1.93–3.38; $P<0.001$), hypertriglyceridemia (RR, 1.92; 95% CI, 1.41–2.62; $P<0.001$) and hypertension (RR, 1.31; 95% CI, 1.01–1.71; $P=0.046$) remained predictive. In fatty liver cases, body mass index and serum triglycerides, but not systolic or diastolic blood pressure, increased significantly and steadily up to the time of the diagnosis. Obesity, hypertriglyceridemia and, to a lesser extent, hypertension might serve as predictive variables for fatty liver.
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INTRODUCTION

The recent increase in obesity caused by excess food intake has led to an increased incidence in metabolic syndrome and visceral fat accumulation,[1–3] both of which are associated with non-alcoholic fatty liver disease.[4–11] As non-alcoholic fatty liver disease is also associated with the classical coronary risk factors of obesity, hypertension, dyslipidemia and glucose intolerance,[4,7,8,10–15] non-alcoholic fatty liver disease serves as a surrogate marker for visceral fat accumulation or metabolic syndrome.

Non-alcoholic fatty liver disease correlates with the remodeling of coronary artery lesions or lipid core plaques when evaluated by multislice computed tomography and with coronary artery stenosis evaluated by coronary angiography.[16,17] Moreover, non- alcoholic fatty liver disease might be a stronger predictor than metabolic syndrome of cardiovascular disease.[18] In obese children, non-alcoholic fatty liver disease is associated with dyslipidemia, hypertension and glucose intolerance, and predicts development of these conditions.[5,7] Few reports, however, describe the incidence of fatty liver and its predictive variables.[19,20] In this study, we selected atomic bomb survivors in Nagasaki who were confirmed by abdominal ultrasonography during 1990 to 1992 (baseline) as not having fatty liver and followed them through 2007 to examine the incidence of, and predictive variables for, fatty liver.

METHODS

Subjects

As part of the follow-up program of the Radiation Effects Research Foundation (RERF, formerly the Atomic Bomb Casualty Commission), 7564 atomic bomb survivors (3374 men) have undergone biennial examinations in Nagasaki since 1958. A detailed description of this program has been published elsewhere (Atomic Bomb Casualty Commission, Technical Report and RERF, Research Plan for RERF Adult Health Study, Hiroshima and Nagasaki, RERF Research Protocol 2-75, 1975). RERFs Research Protocol Review Committee and the Human Investigation Committee approved the original program in 1975 and this study in 2008 (RP-A 08-08).

At the baseline examination (November 1990 through October 1992), 2015 subjects underwent clinical examination, biochemical measurements and abdominal ultrasonographic examination. The 123 fatty liver cases that were detected then were excluded from this study, as were subjects who were positive ($n=167$) or indeterminate ($n=90$) for hepatitis B virus surface antigen and/or

[1]Department of Cardiovascular Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto, Nagasaki, Japan; [2]Department of Clinical Studies, Radiation Effects Research Foundation, Nakagawa, Nagasaki, Japan and [3]Department of Statistics, Radiation Effects Research Foundation, Minami-ku, Hiroshima, Japan
Correspondence: Dr A Tsuneto, Department of Cardiovascular Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8120, Japan.
E-mail: atsuneto@rerf.or.jp
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anti-hepatitis C virus antibody. We included the remaining 1635 subjects in this study.

Baseline data collection

At each examination, a trained nurse collected clinical and life-style (past and current smoking and drinking habits) information. The nurse also measured sitting blood pressure (mm Hg) on the left arm with a sphygmomanometer after a sufficient sedentary period using the first Korotkoff phase for systolic blood pressure (SBP) and the fifth for diastolic blood pressure (DBP). We classified subjects as having hypertension if their SBP was $\geqslant$130 mm Hg and/or their DBP was $\geqslant$85 mm Hg from a preventive point of view based on Guidelines for the Management of Hypertension (JSH2009) and metabolic syndrome.[21,22]

Standing height (in m) and body weight (in kg) were measured without socks and outer clothing. Body mass index (BMI) was calculated as body weight divided by the square of the standing height ($kg\,m^{-2}$). Obesity was defined as a BMI of $\geqslant 25\,kg\,m^{-2}$, in accordance with the definition of the Japan Society for the Study of Obesity.

Fasting blood samples were drawn for biochemical measurements. Serum total cholesterol (mg per 100 ml), high-density lipoprotein cholesterol (HDL-cholesterol, mg per 100 ml), serum triglyceride (mg per 100 ml), and fasting blood glucose (mg per 100 ml) were measured by an automated procedure (Hitachi 7050 and 7170S; Hitachi, Tokyo, Japan) with quality control monitored in accordance with the College of American Pathologists (Northfield, IL, USA). Hypercholesterolemia was defined as total serum cholesterol $\geqslant$220 mg per 100 ml, low HDL-cholesterol as serum HDL-cholesterol $<$40 mg per 100 ml and hypertriglyceridemia as serum triglyceride $\geqslant$150 mg per 100 ml. Subjects with a fasting blood glucose $\geqslant$110 mg per 100 ml and those undergoing medical treatment for diabetes mellitus or impaired glucose tolerance were defined as having glucose intolerance.

Radiologists, without making reference to the subjects' history or data, conducted abdominal ultrasonographic examinations using an Aloka SSD-650, SSD-2000 (Aloka, Tokyo, Japan) and GE LOGIQ-500 (GE Healthcare Japan, Tokyo, Japan) and diagnosed fatty liver when there was accentuation of liver–kidney contrast, blurring of the hepatic vessel wall or deep attenuation of echogenicity.[23] Ultrasonography is a sensitive and reasonably accurate diagnostic tool for assessing fat infiltration of the liver. Others have found 83% sensitivity and 100% specificity in comparison with histology, and 100% sensitivity and 56% specificity in comparison with CT.[23,24] In this study, a second radiologist blindly reviewed 40 films originally classified as fatty liver and 80 films from age- and sex-matched persons that were not so classified. The positive agreement rate by the second rater was 80% and the negative agreement rate was 95%, yielding a kappa coefficient of agreement of 0.77.[25]

Follow-up

All subjects visited RERF biennially and underwent the same clinical, biochemical and ultrasonographic examinations as at the baseline examination. Follow-up began on the date of the baseline examination and ended on the date of the diagnosis of fatty liver, the date of death or 31 December 2007, whichever came first. The criteria for fatty liver were the same as at baseline examination.

Radiation dose

We based total radiation dose on Dosimetry System 2002 (DS02) with a weighting factor of 10 for neutrons relative to γ rays.[26] To reduce radiation effect estimation bias,[27] we adjusted the gamma and neutron doses for 35% dose error after truncation at 4 Gy for doses over 4 Gy. The mean radiation dose was 0.517 Gy (range, 0–3.455).

Statistical analysis

We calculated the incidence of fatty liver using the person-year method. We used the Wilcoxon rank-sum test to compare continuous variables (age, BMI, total cholesterol, HDL-cholesterol, serum triglycerides, SBP, DBP and radiation dose) and the χ^2 test to compare prevalences (sex, smoking and drinking habits, and glucose intolerance) between incident fatty liver cases and non-incident fatty liver cases at baseline. We used the Cox proportional hazard model to examine the predictive variables (obesity, hypercholesterolemia, low HDL-cholesterolemia, hypertriglyceridemia, glucose intolerance and hypertension) for incident fatty liver after controlling for age, sex, smoking and drinking habits, and atomic bomb radiation dose.

When fatty liver was diagnosed in someone for the first time at follow-up, we randomly selected two sex- and age-matched controls from non-incident fatty liver cases. For example, if fatty liver was diagnosed for the first time in 1995 in a 63-year-old male, we randomly selected two male controls who were aged 60–64 years in 1995. As the predictive variables for fatty liver were obesity, hypertriglyceridemia and hypertension (see multivariate analysis in results section), we plotted BMI, serum triglycerides, SBP and DBP at –6, –4, –2 (before the diagnosis), 0 (at the diagnosis), 2, 4 and 6 years after the diagnosis in cases and at the corresponding times in controls, and we used the Wilcoxon rank-sum test to compare the values. We set a *P*-value of <0.05 for over-all significance and of <0.0084 ($\fallingdotseq 0.05/6$) for a Bonferroni-type multiple comparison in the analysis of BMI, triglycerides, SBP and DBP. We conducted all analyses using SAS software running on a UNIX System (SAS/STAT Software, Release 9.0., SAS Institute, Cary, NC, USA).

RESULTS

Table 1 shows the baseline characteristics of the 1635 subjects by sex. As indicated from the mean age of our study subjects, our study cohort consisted of middle aged/elderly subjects (–49-year old, 113 subjects; 50–59 years old, 284 subjects; 60–69 years old, 863 subjects; 70–79 years old, 289 subjects; 80– years old, 86 subjects). Among them, 323 (124 men) were newly diagnosed with fatty liver during the follow-up period. The mean follow-up period was 11.6 years (s.d., 4.6; median, 14.0; range, 1.3–17.1).

Table 1 Baseline characteristics of the study population by sex

Risk factors	*Total (n=1635)*	*Men (n=606)*	*Women (n=1029)*	P
Age	63.1±8.9	62.4±9.6	63.4±8.4	0.002
Smoking (%)	34.6	76.9	9.7	<0.001
Drinking (%)	41.0	78.7	18.8	<0.001
BMI ($kg\,m^{-2}$)	22.5±3.0	22.2±2.7	22.7±3.1	0.003
T-chol (mg per 100 ml)	206.5±34.7	193.1±30.6	214.4±34.5	<0.001
HDL-C (mg per 100 ml)	54.9±14.5	52.0±14.7	56.7±14.0	<0.001
TG (mg per 100 ml)	115.3±61.0	122.6±72.3	110.9±52.9	0.004
Glucose intolerance (%)	10.5	14.2	8.4	<0.001
SBP (mm Hg)	127.3±19.4	129.4±18.2	126.1±20.0	<0.001
DBP (mm Hg)	79.3±11.0	81.2±10.4	78.1±11.2	<0.001
Atomic radiation dose (mGy)[a]	522.6±741.7 (*n*=1077)	527.0±719.8 (*n*=403)	520.0±754.8 (*n*=674)	0.42

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T-chol, total cholesterol; TG, triglyceride.
[a]As radiation dose was not determined in all subjects, the numbers of subjects was reduced to 1077 (403 men, 674 women).
Atomic radiation dose, atomic bomb radiation dose estimated by RERF 2002 criterion. Unless otherwise indicated, values are expressed as the mean±s.d.

Table 2 Baseline characteristics of incident fatty liver cases and non-incident fatty liver cases

	Incident fatty liver	*Non-incident fatty liver*	P
Number	323	1312	
Age	59.0 ± 7.3	64.1 ± 9.0	<0.001
Sex (men %)	38.4	36.7	0.58
Smoking (%)	34.4	34.7	0.92
Drinking (%)	45.2	39.9	0.09
BMI ($kg\,m^{-2}$)	24.1 ± 2.8	22.1 ± 2.9	<0.001
T-chol (mg per 100 ml)	210.2 ± 35.1	205.6 ± 34.5	0.029
HDL-C (mg per 100 ml)	50.9 ± 13.1	56.0 ± 14.6	<0.001
TG (mg per 100 ml)	143.1 ± 85.0	108.4 ± 51.3	<0.001
Glucose intolerance (%)	13.0	9.9	0.10
Hypertension (%)	56.3	47.4	0.004
Atomic radiation dose (mGy)	510.1 ± 726.6 (*n*=214)	525.6 ± 745.6 (*n*=863)	0.92

Abbreviations are the same as in Table 1. Unless otherwise indicated, values are expressed as the mean ± s.d.

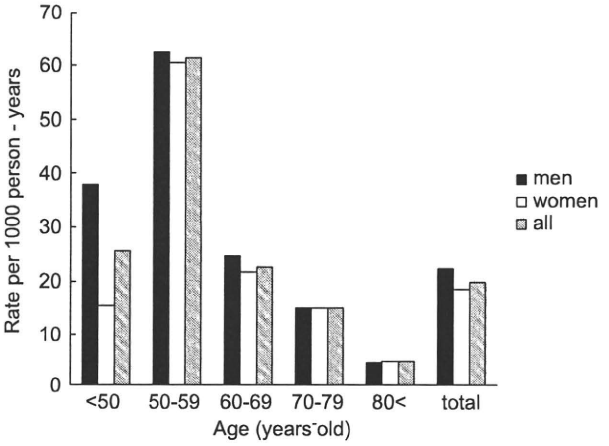

Figure 1 Incidence of fatty liver. Black bars represent men, white bars women and gray bars all subjects.

Table 2 shows the baseline characteristics of subjects who did and did not develop fatty liver. The two groups did not differ in sex ratio, smoking or drinking habits, prevalence of glucose intolerance, or radiation dose. The group that developed fatty liver, however, had higher mean values for BMI, total cholesterol level and triglyceride level, a higher prevalence of hypertension, and lower mean values for age and HDL-cholesterol level. The incidence of fatty liver per 1000 person-years was 19.9 (22.3 for men, 18.6 for women). It peaked in the sixth decade of life and decreased thereafter in both sexes (Figure 1).

In Cox's proportional hazard model adjusted for age, sex, and smoking and drinking habits, obesity, low HDL-cholesterol, hypertriglyceridemia, glucose intolerance, and hypertension were statistically significant predictors of fatty liver (Table 3). In multivariate analysis including all variables, obesity, hypertriglyceridemia and hypertension remained positive predictors (Table 3). Radiation dose was not a predictive variable.

Subjects who developed fatty liver had significantly higher BMIs and serum triglyceride levels than control subjects throughout the 12-year study period (Figures 2a and b). Moreover, their BMI levels increased steadily and significantly during the 6 years before the diagnosis to the diagnosis ($23.9 \pm 2.8\,kg\,m^{-2}$ at –6 years and $24.1 \pm 3.0\,kg\,m^{-2}$ at –4 years to $25.0 \pm 3.0\,kg\,m^{-2}$ at 0 years ($P<0.001$ for two intervals)) and remained elevated, while the level remained constant in controls (Figure 2a). Similarly, serum triglycerides levels in those who developed fatty liver increased steadily and significantly up to the time of diagnosis (130.0 ± 54.6 mg per 100 ml at –6 years to 159.2 ± 71.9 mg per 100 ml at 0 years, $P<0.001$) and remained elevated, while the level remained constant in controls (Figure 2b). Although hypertension was a predictive variable, SBP and DBP were not consistently higher in cases than in controls and did not increase significantly during the observation period (Figures 2c and d).

DISCUSSION

In this study of 1635 Nagasaki atomic bomb survivors who were followed for 12 years, our finding of an incidence of fatty liver of 19.9/1000 person-years was similar to the incidence of 18.5/1000 person-years reported by Bedogni *et al.*[19] for a general Italian population followed for a median time of 8.5 years. In a study of 3147 Japanese adults who consumed <20 g of ethanol per day, Hamaguchi *et al.*[20] reported that 10% of subject developed non-alcoholic fatty liver disease at follow-up examination (1.13 ± 0.35 years later). And while we did not find a sex difference in the development of fatty liver, Hamaguchi *et al.* reported a higher incidence among men, which might be explained by the fact that the mean age of participants at baseline examination in their cohort study (48.1 years for men, 46.6 for women) was younger than it was in our study (62.4 years for men, 63.4 for women). Indeed, as shown in Figure 1, the incidence of fatty liver in participants <50 years old was higher in men than in women.

Our finding that the incidence of fatty liver in both sexes peaked in the sixth decade of life and decreased thereafter may follow from that fact that BMI generally follows that age pattern.[28,29] Our calculation of fatty liver incidence in participants <50 years old, however, was based on only four male and two female incident cases. That may have biased our results, leading to the possibility that peak fatty liver incidence might occur in the fifth decade instead of the sixth. Cohort studies including younger subjects are needed to confirm the age of peak fatty liver incidence.

That obesity, low HDL-cholesterol, hypertriglyceridemia, glucose intolerance and hypertension—classic risk factors for cardiovascular disease—were predictive of fatty liver in Cox regression analysis (after controlling for age, sex, and smoking and drinking habits) and that obesity, hypertriglyceridemia and hypertension remained predictive in multivariate Cox regression analyses fit in with our understanding of the pathophysiological pathway of fatty liver.[12,30] Obesity (from excess nutrition intake) leads to visceral fat accumulation. Visceral fat has high metabolic activity and releases free fatty acids and adipokines such as leptin, tumor necrosis factor-α, and adiponectin.[31–34] Insulin resistance caused by increasing tumor necrosis factor-α secretion or decreasing adiponectin secretion induces hypertension and glucose intolerance.[31] Leptin and angiotensinogen secreted from visceral fat also induces hypertension.[33] In this way, visceral fat accumulation associated with obesity serves as a risk factor for hypertension and glucose intolerance. In contrast, free fatty acids released from visceral fat enter the liver through the portal vein, and increased free fatty acid influx from the portal vein stimulates triglyceride synthesis in liver. Fatty liver is the condition of triglyceride deposited in liver, and fatty

Table 3 Relative risk of predictive variables for fatty liver by means of multiple Cox's regression analysis

	RR (95% CI)[a]	P	*RR (95% CI)*[b]	P
Age (older decade)			0.54 (0.45–0.64)	<0.001
Sex (women)			0.93 (0.61–1.42)	0.73
Smoking			0.92 (0.64–1.34)	0.67
Drinking			0.95 (0.68–1.32)	0.75
Obesity (BMI ⩾ 25 kg m^{-2})	2.93 (2.33–3.69)	<0.001	2.55 (1.93–3.38)	<0.001
Hypercholesterolemia (T-chol ⩾ 220 mg per 100 ml)	1.12 (0.88–1.42)	0.35	0.98 (0.73–1.30)	0.88
Low HDL-cholesterolemia (HDL-C < 40 mg per 100 ml)	1.87 (1.42–2.47)	<0.001	1.19 (0.83–1.70)	0.35
Hypertriglyceridemia (TG ⩾ 150 mg per 100 ml)	2.49 (1.96–3.15)	<0.001	1.92 (1.41–2.62)	<0.001
Glucose intolerance	1.51 (1.09–2.10)	0.013	1.31 (0.90–1.90)	0.16
Hypertension	1.63 (1.30–2.04)	<0.001	1.31 (1.01–1.71)	0.046
Atomic radiation dose (mGy) (n=1077)	0.99 (0.83–1.18)	0.89	0.92 (0.78–1.10)	0.38

Abbreviations: CI, confidence interval; RR, relative risk.
Other abbreviations are the same as in Table 1.
[a]Adjusted for age, sex, smoking and drinking habits.
[b]All variables are included.

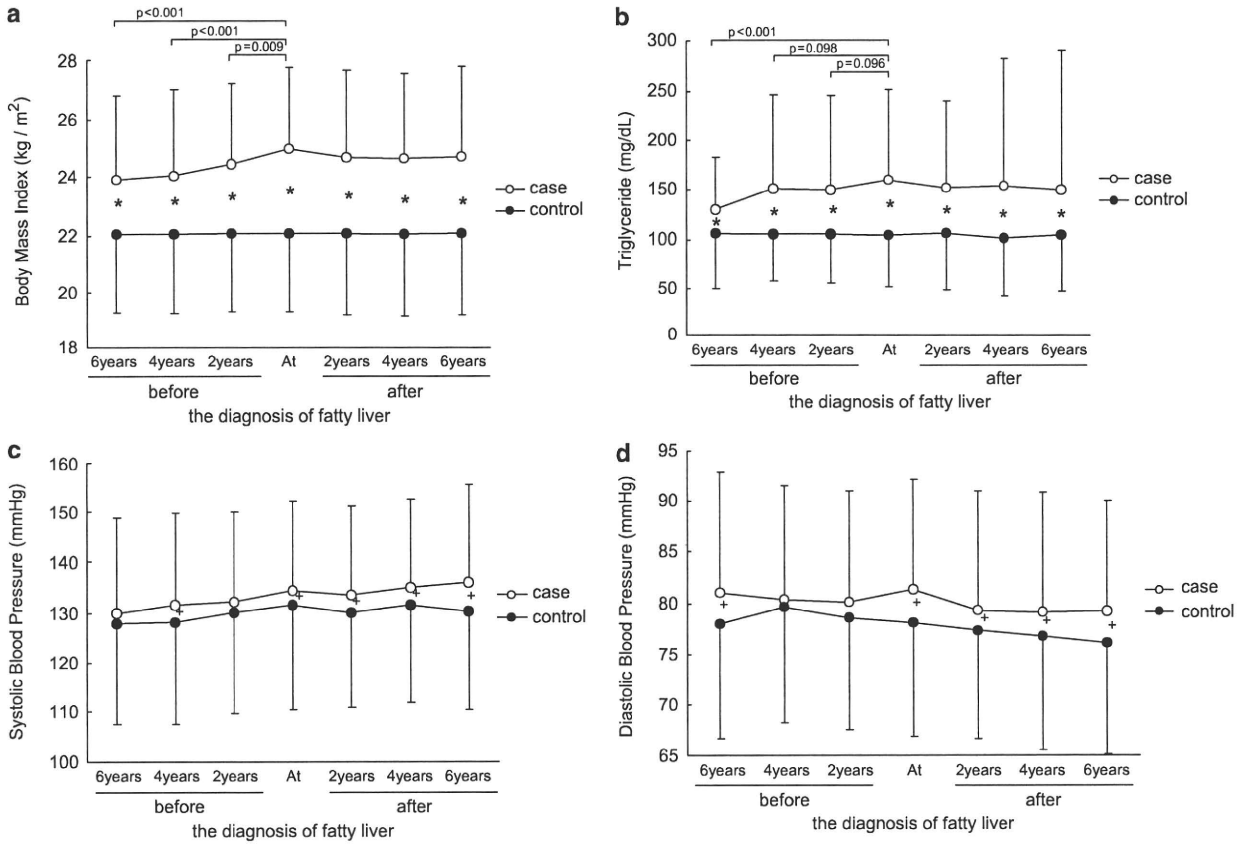

Figure 2 (**a**) BMI trends. Values are expressed as means ± s.d. Comparisons were made by means of analysis of covariance. *P<0.001 between fatty liver cases and controls. (**b**) Triglyceride trends. Values are expressed as means ± s.d. Comparisons were made by means of analysis of covariance. *P<0.001 between fatty liver cases and controls. (**c**) SBP trend. Values are expressed as means ± s.d. Comparisons were made by means of analysis of covariance. +P<0.05 between fatty liver cases and controls. (**d**) DBP trend. Values are expressed as means ± s.d. Comparisons were made by means of analysis of covariance. +P<0.05 between fatty liver cases and controls.

liver is thus a surrogate marker of visceral fat accumulation and clusters the classic risk factors for cardiovascular disease—obesity, hypertension, dyslipidemia and glucose intolerance.

Hamaguchi *et al.*[20] reported that metabolic syndrome and weight gain were predictive of non-alcoholic fatty liver disease development. Although we could not evaluate whether metabolic syndrome predicted the development of fatty liver because we did not measure waist circumference at baseline examination, our results are consistent with their results in the following aspects; (1) coronary risk factors associated with metabolic syndrome, that is, obesity, dyslipidemia

and hypertension, predict the development of fatty liver, and; (2) increases in BMI and serum triglycerides are related to the development of fatty liver as suggested by observed trends (Figure 2).

LIMITATIONS

(1) The mean age of study participants was 63.1 ± 8.9 at baseline (November 1990 through October 1992), which means that many of our study participants had taken excess nutrition since their middle age. Thus, our calculated age of peak incidence may shift to older age and our overall incidence estimate (19.9/1000 person-years) may be calculated to be low. Further studies including younger subjects are necessary to evaluate fatty liver incidence in the contemporary era of excess nutrition intake.

(2) Our subjects were atomic bomb survivors in Nagasaki, Japan, which means that our results might not be generalizable. Radiation dose was not a predictive variable for incident fatty liver, however, so we believe that the present results can be generalized, although further studies in non-atomic bomb survivors are necessary.

(3) Although we incorporated alcohol intake into the analysis and found it not to be a predictive variable for incident fatty liver, we could not negate the possibility that fatty liver cases related to alcohol intake were included in the study because we did not take amount of alcohol intake into account. (For the diagnosis of non-alcoholic fatty liver, alcohol intake should not exceed 20 g per day[35]).

CONCLUSION

In this middle aged/elderly subjects cohort, fatty liver incidence (19.9/1000 person-years) peaked in the sixth decade of life and decreased thereafter. Although obesity, hypertriglyceridemia and hypertension were predictive of fatty liver, BMI and serum triglyceride level, but not SBP or DBP, increased steadily up to the time of diagnosis. These data suggest that obesity and hypertriglyceridemia, rather than hypertension, are closely associated with development of fatty liver.

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LTA 252GG and *GA* Genotypes are Associated with Diffuse-Type Noncardia Gastric Cancer Risk in the Japanese Population

Gen Suzuki,* Harry Cullings,† Saeko Fujiwara,‡ Shinsuke Matsuura,‡ Takeshi Kishi,‡ Waka Ohishi,‡ Masazumi Akahoshi,§ Tomonori Hayashi¶ and Eiichi Tahara**

*International University of Health and Welfare Clinic, Ohtawara City, Tochigi, Japan, †Departments of Statistics and ‡Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan, §Department of Clinical Studies, Radiation Effects Research Foundation, Nagasaki, Japan, ¶Department of Radiation Biology/Molecular Epidemiology, Radiation Effects Research Foundation, Hiroshima, Japan, **Hiroshima Cancer Seminar Foundation, Hiroshima, Japan

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Epidemiology, gastric cancer, *LTA*, smoking, radiation.

Reprint requests to: Gen Suzuki, International University of Health and Welfare Clinic, 2600-6 Kitakanemaru, Ohtawara City, Tochigi 324-8501, Japan.
E-mail: gensuzki@iuhw.ac.jp

Abstract

Background: There are limited numbers of reports on the association of *lymphotoxin-alpha* (*LTA*) genotypes with gastric cancer.

Methods: A nested case–control study was carried out in the longitudinal cohort of atomic bomb survivors using stored sera before diagnosis (mean, 2.3 years) and blood cells. Enrolled were 287 cases with noncardia gastric cancer of diffuse and intestinal types and three controls per case selected from cohort members matched on age, gender, city, and time and type of serum storage and counter-matched on radiation dose.

Results: *LTA 252GG* and *GA* genotypes were associated with the prevalence of *Helicobacter pylori* IgG seropositivity and higher antibody titer against *H. pylori* cytotoxin-associated gene A (CagA) protein in controls and they were an independent risk factor for noncardia gastric cancer of diffuse type (RR = 2.8 (95% CI: 1.3–6.3), $p = .01$, and RR = 2.7 (95% CI: 1.5–4.8), $p < .001$), but not for intestinal type, after adjusting for *H. pylori* IgG seropositivity, CagA antibody titers, chronic atrophic gastritis, smoking, and radiation dose. Cessation of smoking (RR = 0.4 (95% CI: 0.2–0.7), $p < .001$) and never smoking (RR = 0.4 (95% CI: 0.3–0.6), $p < .001$) were both protective for future noncardia gastric cancer. Radiation dose was associated with noncardia gastric cancer in subjects with both the *LTA 252G*-allele and never smoking/quit smoking histories (RR = 3.8 (95% CI: 1.7–5.9), $p = .009$).

Conclusion: The *LTA 252* genotype is associated with noncardia gastric cancer of diffuse type in Japan and interacted with radiation dose.

Introduction

Gastric cancer (GC) ranks as the fourth most common cancer and the second most common cause of cancer death worldwide [1]. In Japan, GC is the most common cancer and the second most common cause of cancer death. As in other cancers, the carcinogenesis of GC is a multistep event and many risk factors contribute to the process [2,3]. *Helicobacter pylori* plays a decisive role in the development of noncardia GC [4], but only a small fraction of *H. pylori*-infected subjects develop GC. Other factors are also important in the development of GC than *H. pylori* infection, i.e., different virulence among *H. pylori* strains, smoking, a diet high in salt and low in fresh fruit or vegetables, and finally host genetic variation [2,5]. As the levels of pro- or anti-inflammatory cytokines produced in response to *H. pylori* may be under genetic control, polymorphisms in genes coding for pro- or anti-inflammatory cytokines have been investigated as a candidate-genetic risk factor for GC. El-Omar et al. first reported the association of genotypes of interleukin-1β (IL-1β) coding gene (*IL1B*) and interleukin-1 receptor antagonist coding gene (*IL1RN*) with GC [6,7]. Since then, many groups have investigated the association of *IL1B* and other gene polymorphisms coding for pro- or anti-inflammatory cytokines with GC in different ethnic populations in Europe, the USA, Japan, China, and Korea [8–14]. However, the

results of such studies were mixed. One reason for such discrepancy may be the lack of other risk factors in the analyses than gene polymorphisms, especially life-style factors that differ among subjects as well as among ethnic groups [15]. Such life-style factors may attenuate or strengthen the biological effect of gene polymorphism. Another reason may be that a particular genetic polymorphism may be just a surrogate for other functional genetic polymorphisms in an adjacent gene, of which frequency differs from population to population. Thus, it is important to analyze gene polymorphism as a haplotype of several genes' cluster. In this study, we wished to investigate the association of the haplotype composed of the single-nucleotide polymorphisms (SNPs) of *HLA-associated transcript 1* (*BAT1*), *NFκB inhibitor-like 1* (*NFKBIL1*), and *LTA* in the class III major histocompatibility complex (MHC) region of chromosome 6 [16,17] with GC in a nested case–control study, where *H. pylori* CagA status, chronic atrophic gastritis (CAG), radiation dose, and smoking were included.

Interleukin-1β and tumor necrosis factor-alpha (TNF-α) are proinflammatory cytokines that augment immune-inflammatory response and modulate gastric acid secretion [18,19]. LT-α (or TNF-β) is also a proinflammatory cytokine belonging to the TNF family. TNF-α and LT-α mediate their biological activities through binding to common receptors, TNF receptor-I (TNFR-I) and TNFR-II. *LTA* and TNF-α-coding gene (*TNFA*) are located in the class III MHC region of chromosome 6. While *TNFA* polymorphisms are rare in the Japanese population, *LTA* polymorphisms are sizable. *LTA 252A/G* SNPs are almost completely linked to *LTA −449G/A*, *+265C/A*, and *804C/A*, *NFKBIL1 −63T/A*, and *BAT1 −22G/C* polymorphisms, and these SNPs make up a haplotype block, i.e., "*LTA −449G, 252A, 265C, 804C, NFKBIL1 −63T, BAT1 −22G*" and "*LTA −449A, 252G, 265A, 804A, NFKBIL1 −63A, BAT1 −22C,*" in the class III MHC region in Japanese and German populations [16,17]. *LTA 252G* and *LTA 804A* SNPs are associated with higher transcription of *LTA* and higher biological activity of lymphotoxin-α, respectively [16]. *BAT1* is a putative anti-inflammatory gene and the *BAT1 −22C* SNP was associated with a high level of cytokine production [20,21]. *LTA 252G*-carriage was associated with high serum concentration of C-reactive protein (CRP), a biomarker of inflammation, in Japanese and European populations [22,23], indicating that this haplotype is functioning in Japanese as well as in Caucasians.

In this study, we demonstrated that *LTA 252G*-carriage was associated with a higher prevalence of *H. pylori* infection in controls and a future risk for noncardia GC of diffuse type after adjusting for other risk factors in a Japanese cohort.

Materials and Methods

Cases and Controls

Study design was a nested case–control study using stored sera and blood cells in the Adult Health Study (AHS) longitudinal cohort of atomic bomb survivors in Hiroshima and Nagasaki [24,25]. Cancer incidence cases were detected from the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registries, where the histological classification was based on the Japanese Research Society for Gastric Cancer (JRSGC) classification until 1986, and then on the WHO coding system (ICD-O, ICD-O-2, and ICD-O-3), which was converted into Lauren's classification as reported [26,27]. In the previous study [28], we had misclassified 11 mucinous adenocarcinoma cases as diffuse type, and in the present study they were reclassified as intestinal type. In the present study, 297 noncardia GC cases of diffuse and intestinal types with full serologic data were subjected to genotype analysis. Three controls per case were selected from the cohort members matched on age, gender, city, and time and type of serum storage, and counter-matched on radiation dose. Out of these cases and controls, 1 case and 12 controls did not allow us to conduct genotype analyses and nine cases and seven controls did not store blood cells suitable for genotype analyses. As a result, 287 cases and 1023 controls were analyzed. Demographic features of the GC cases and controls are shown in Table 1.

Radiation Dose

Stomach radiation dose was estimated by the DS02 dosimetry system [29]. A weighted sum of the gamma dose in gray plus 10 times the neutron dose in gray was used.

Serologic Test and Other Risk Factors

Serologic tests for *H. pylori* and CagA were carried out using an enzyme immunoassay kit (AutoAce *H. pylori*G; Alfresa Pharma, Osaka, Japan) and an ELISA kit (CagA IgG EIA WELL; Radim, Roma, Italy). According to the manufacturers' recommendations, negative antibody titer was determined as <16.5 units/mL in an AutoAce *H. pylori*G kit and <15 relative units/mL in a CagA IgG EIA WELL kit. Anti-CagA antibody titer of ≥15 and <23 units/mL was defined as "low" titer and that of ≥23 units/mL was defined as "high" titer. Grade II CAG was diagnosed by the criteria of Miki et al. using pepsinogen measurements (i.e., pepsinogen (PG) I of <50 mg/L and a PG I/PG II ratio of <3.0) [30].

Table 1 Demographic features of cases and controls

	Noncardia GC case		
	Intestinal	Diffuse	Control
n	159	128	1023
Male; female	97; 62	74; 54	601; 422
Age at time of bombing[a]	29 (10)	26 (12)	26 (11)
Age at diagnosis[a]	72 (9.3)	67 (11)	(–)
Age at serum storage[a]	69 (9.4)	65 (11)	67 (11)
City (Hiroshima; Nagasaki)	105; 54	71; 57	627; 396
Stomach dose, gray[b] [no. with unknown dose]	0.38 (0, 3.0) [24]	0.49 (0, 32.9) [15]	0.41 (0, 3.2) [92]
Anti-*H. pylori* IgG (+)	140/159 (88%)	117/128 (91%)	806/1023 (79%)
Anti-CagA IgG (+)	118/159 (74%)	107/128 (84%)	735/1023 (72%)
CAG	118/159 (74%)	81/128 (63%)	494/1023 (48%)
Current/former/never [unknown]	84/13/57 [5] (53%/8%/36% [3%])	75/8/38 [7] (59%/6%/30% [5%])	431/147/411 [34] (42%/14%/40% [3%])

GC, gastric cancer; CAG, cytotoxin-associated gene.
[a]Numbers represent mean (SD in parentheses).
[b]Numbers represent mean (upper and lower boundaries of 95% confidence interval in parentheses).

Smoking Information

Smoking status was obtained from three sources: AHS interview in 1963–1965, Mail survey in 1968, and Mail survey in 1978–1980. The most recent data on smoking before GC diagnosis or equivalent time for matched controls were used. Smoking habit was divided into "never," "former," and "current smoking."

SNP Analyses

Genomic DNA was extracted using Qiagen QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan) either from stored blood clots on paper sheet or blood smear on glass plate covered with a cover slip. In the case of blood smear, genomic DNA was amplified by a whole genome amplification method, improved-primer-extension-preamplification –polymerase chain reaction (I-PEP-PCR), using Roche Expand High Fidelity PLUS PCR System (Roche, Mannheim, Germany) as reported [31]. After the first PCR amplification using a specific primer set as shown below by a thermal cycler (Whatman Biometra, Goettingen, Germany), the amplicon was subjected to the second PCR for genotyping by the fluorescence resonance energy transfer (FRET)–PCR technique with subsequent melting curve analysis using a real-time PCR instrument (Light Cycler; Roche Diagnostics) [32]. PCR primers and fluorescence probes used were: forward primer for *LTA* (5′-CAAGGTGAGCAGAGGGAGACA-3′), reverse primer for *LTA* (5′-GGGTTTGGTTTTGGTTTCCTTC-3′), sensor probe for *LTA* (5′-CAGAGAGGAACCATGGCAGA-3′-FITC), and anchor prove for *LTA* (Red640-5′-CAGAGAATGTGTGACAGAGACAAT-3′). The first thermocycling procedure consisted of 2 minutes of denaturation at 95 °C following 40 cycles of denaturation at 95 °C for 1 minutes, annealing at 60 °C for 30 seconds, and extension at 72 °C at 1 minute. After 40 cycles, additional extension was performed at 72 °C for 10 minutes. The second thermocycling procedure for genotyping consisted of 5 minutes denaturation at 95 °C following 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 65 °C for 8 seconds, and extension at 72 °C at 6 seconds. After 40 cycles, a melting curve analysis was performed by changing the temperature from 95 °C for 1 seconds to 40 °C for 15 seconds and then by gradually raising the temperature to 70 °C over a period of 7 minutes as reported [22]. Accuracy of genotyping using FRET–PCR method was validated in 20 each of blood clot and smear samples by comparing the results with sequence data obtained by a PCR-based direct sequencing method. Genotyping results by FRET–PCR using blood smears were completely concordant with those by FRET–PCR using blood clots and those by direct sequencing.

Ethical Consideration

Because of the nature of the present study, i.e., a nested case–control study from 1970 to 2001, informed and written consent has been obtained from living participants in 2003. This study (Radiation Research Foundation Research Protocol 20-04) was performed according to the Declaration of Helsinki, and reviewed and approved by the Ethics Committee for Genome Research on 26 February 2004.

Statistical Analyses

The relative risk was estimated as a product of a linear term in radiation dose and a log-linear combination of other risk factors: $RR = (1 + \beta_0 D)e^{\beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \cdots}$, where D is the radiation dose, the x_is are other risk factor variables, and the β_is are coefficients to be estimated. Categorical variables with more than two categories were coded with dummy variables to compare the adjusted RR of each category to a reference category. When interactions of radiation with other risk factors were modeled, the product of the dummy variables was added to the linear term, allowing a different linear slope of the radiation dose response for each category of the other risk factor. The nested case–control design is based on risk sets formed by associating each case with a set of controls chosen at random from all persons in the AHS who were at risk (i.e., had not developed a cancer) at the time when the case was diagnosed [33]. The probability to be maximized in the regression is the combined probability that in each risk set the person who actually developed GC was the case, i.e., the product over risk sets i of the conditional probabilities p_i = RR(case$_i$)/[RR(case$_i$) + RR(control$_{i1}$) + RR(control$_{i2}$) + RR(control$_{i3}$)]. Because the controls are chosen within strata defined by the matching and counter-matching criteria, the probabilities are weighted to account for the counter-matching by using a weight for each control that is proportional to the number of persons in the total pool at risk for the associated case who met the matching criteria for that case (*Helicobacter* serology, smoking status, etc.) and the counter-matching criteria for that control (dose stratum). The conditional logistic regression models were fitted with the GMBO procedure of Epicure software (Hirosoft International Corp., Seattle, Washington, USA).

The distribution of *LTA 252GG, GA,* and *AA* genotypes in the control group was assessed after eliminating redundant controls and was well within statistical bounds for Hardy–Weinberg equilibrium (p = .97 by the Pearson χ^2 statistic).

Results

The *LTA 252G*-Allele was Associated with Antibody Titers Against *H. pylori* CagA in Controls

The number of *LTA 252G* allele was significantly associated with the prevalence of *H. pylori* seropositivity (p = .049; Table 2A) as well as with the levels of anti-CagA titer (p = .024) in controls (Table 2B). We also calculated 1, the odds of a positive titer using a logistic regression model, and 2, a multiple linear regression with log-transformed anti-*H. pylori* IgG antibody titer as a dependent variable; both types of regression included the age at serum collection and *LTA 252G*-carriage as explanatory variables. After adjusting for age at serum collection, *LTA 252G*-carriage was significantly associated with anti-*H. pylori* antibody titer in controls (OR = 1.5 (95% CI: 1.1–2.1), p = .02), especially in controls without CAG (OR = 1.6 (95% CI: 1.1–2.5), p = .02). Thus, gene polymorphism associated with higher inflammatory phenotype is associated with lower eradication rate and higher IgG antibody response to *H. pylori*.

Table 2 The number of controls with *LTA 252* polymorphisms and serological status for *H. pylori* and CagA

	Anti-*H. pylori* antibody status		
	P(−)	P(+)	Total
(A)			
LTA 252AA	81 (45.8)	227 (36.1)	308
LTA 252GA	71 (40.1)	307 (48.8)	378
LTA 252GG	25 (14.1)	95 (15.1)	120
Total	177	629	806[a]

	Anti-*H. pylori* and CagA antibody status[b]				
	P(−)	P(+)&C(−)	P(+)&C(low)	P(+)&C(high)	Total
(B)					
LTA 252AA	57 (45.6)	36 (40.0)	20 (40.0)	171 (35.0)	284
LTA 252GA	49 (39.2)	42 (46.7)	22 (44.0)	243 (49.7)	356
LTA 252GG	19 (15.2)	12 (13.3)	8 (16.0)	75 (15.3)	114
Total	125	90	50	489	754

Values in parenthesis represent percentages.
[a]In a nested case–control design, some individuals may serve as controls for multiple cases at different times. In this table, we eliminated redundant controls, and 806 nonredundant controls were analyzed.
[b]P(−) and P(+) represent seronegative and seropositive for *H. pylori*, respectively, while C(−), C(low), and C(high) represent antibody status for CagA: CagA antibody negative, low titer, and high titer, respectively. Part (A) has a p = .049 by a nonparametric test for trend based on rank sums, p = .058 by Pearson's χ^2 test, and p = .059 by Fisher's exact test. Part (B) has a p = .069 for the nonparametric test for trend within *LTA* genotypes across serology categories, but the same test gives p = .024 within *LTA 252G*-carrier status across serology categories. This table omits 52 individuals who were negative for *H. pylori* IgG but positive for CagA. If those are added as the second of five columns in (B), the p-values are .033 for *LTA* genotype and .012 for *LTA 252G*-allele carriage.

Protective Effect of Abstinence from Smoking on Future Noncardia GC

As already reported and discussed previously [28], CAG, serological status for *H. pylori* and CagA, current

smoking, and radiation were risk factors for future noncardia GC. In order to evaluate the protective effect of smoking cessation, the "current smoking" category was coded as a reference category and "former smoking" and "never smoking" were analyzed as separate categorical variables in the present model. The average of the minimal time since smoking cessation, i.e., the date of last questionnaire reporting quitting smoking, was longer for cases (25th, 50th, and 75th percentiles were 9.5, 18.1, and 27.2 years, respectively) than controls (25th, 50th, and 75th percentiles were 8, 11.5, and 18 years, respectively). As shown in Table 3, former smoking and never smoking were both protective from noncardia GC (RR = 0.4 (95% CI: 0.2–0.7), $p < .001$; RR = 0.4 (95% CI: 0.3–0.6), $p < .001$, respectively), especially from diffuse type (RR = 0.3 (95% CI: 0.1–0.9), $p < .03$; RR = 0.2 (95% CI: 0.1–0.5), $p < .001$, respectively).

Table 3 Relative risk of risk factors for future nocardia gastric cancer of intestinal and diffuse types

	RR	95% CI	*p*
(A) Intestinal and diffuse types combined			
CAG	2.7	1.9–3.8	<.001
H. pylori IgG (+), cagA (–)	1.8	1.0–3.4	.06
H. pylori IgG (+), cagA (low)	3.1	1.6–5.9	<.001
H. pylori IgG(+), cagA (high)	1.7	1.0–2.8	.03
Former smoker	0.4	0.2–0.7	<.001
Never smoker	0.4	0.3–0.6	<.001
LTA 252GA	1.5	1.1–2.2	.01
LTA 252GG	1.3	0.8–2.2	.2
Radiation dose (1 Gy)	1.5	1.0–1.9	.03
(B) Intestinal type			
CAG	3.2	2.0–5.1	<.001
H. pylori IgG (+), CagA (–)	2.3	1.0–5.3	.04
H. pylori IgG (+), CagA (low)	4.0	1.7–9.6	.002
H. pylori IgG(+), CagA (high)	1.4	0.7–2.7	.3
Former smoker	0.4	0.2–0.8	.008
Never smoker	0.6	0.4–1.1	.1
LTA252GA	1.0	0.6–1.5	>.5
LTA252GG	0.8	0.4–1.6	>.5
Radiation dose (1 Gy)	1.1	0.7–1.6	>.5
(C) Diffuse type			
CAG	2.4	1.4–4.0	<.001
H. pylori IgG (+), CagA (–)	1.2	0.4–3.6	>.5
H. pylori IgG (+), CagA (low)	3.0	1.0–8.6	.04
H. pylori IgG(+), CagA (high)	2.4	1.0–5.5	.03
Former smoker	0.3	0.1–0.9	<.03
Never smoker	0.2	0.1–0.5	<.001
LTA252GA	2.7	1.5–4.8	<.001
LTA252GG	2.8	1.3–6.3	.01
Radiation dose (1 Gy)	2.2	1.1–3.3	.03

CAG, cytotoxin-associated gene; 95% CI, 95% confidence interval.

LTA252GG and *GA* Genotypes were Associated with Noncardia GC of Diffuse Type

As shown in Table 3A, *LTA 252GA* genotype was an independent risk factor for future noncardia GC (RR = 1.5 (95% CI: 1.1–2.2), $p = .01$) while *LTA 252GG* genotype showed an increased RR but at nonsignificant level. When the endpoint was confined to noncardia GC of diffuse type, *LTA 252GA* and *GG* genotypes were both significant risk factors (RR = 2.7 (95% CI: 1.5–4.8), $p < .001$ and RR = 2.8 (95% CI: 1.3–6.3), $p = .01$, respectively; Table 3C). *LTA 252* polymorphism did not associate with the risk of noncardia GC of intestinal type (Table 3B).

Interaction Between *LTA 252G*-Carriage, Smoking, and Radiation

Next, we investigated the interaction among risk factors. Terms for multiplicative interaction in the log-linear part of the model between *LTA 252GA* and *LTA 252GG* genotypes versus CAG, the levels of anti-CagA antibody titer, and smoking were not significant at the 0.05 level, while radiation dose positively interacted separately with smoking and *LTA 252GA* genotype (data not shown). In order to investigate the interaction among these three factors, we wished to increase the statistic power in the analysis and re-categorized smoking status into two categories (current smoker vs. noncurrent smoker, i.e., never or quit-smoking subject) and *LTA 252* genotypes into two categories (*LTA 252AA* vs. *LTA 252G*-carriage). As shown in Table 4, radiation dose was a risk factor for noncardia GC only among subjects with *LTA 252G*-carriage and noncurrent smokers (RR = 3.8 (95% CI: 1.7–5.9), $p = .009$).

Discussion

In this study, we have demonstrated first of all that the number of *LTA 252G* alleles, which was a surrogate maker of a haplotype composed of SNPs in *LTA*, *NFKBIL1*, and *BAT1* genes in the class III MHC region of chromosome 6 [16,17], was associated with a higher prevalence of *H. pylori* infection and a higher antibody response to *H. pylori* CagA among controls. Second, the number of *LTA 252G* alleles in the genome was significantly associated with future risk of noncardia GC of diffuse type, but not of intestinal type, after adjusting for CAG, *H. pylori* IgG seropositivity, anti-CagA IgG seropositivity, smoking status, and radiation dose. Third, quitting smoking and never smoking were both protective from noncardia GC, especially of diffuse type.

Table 4 Interaction among three risk factors: radiation risk for noncardia gastric cancer is restricted to subjects with *LTA* 252G-carriate and smoking status

Risk categories	RR	95% CI	*p*
Radiation dose (1 Gy)	0.8	0.5–1.2	.3
Radiation dose (1 Gy) for current smoker with *LTA 252G-carriage*	1.3	0.6–1.9	.4
Radiation dose (1 Gy), for noncurrent smoker with *LTA 252AA*	2.0	0.6–3.4	.2
Radiation dose (1 Gy) for noncurrent smoker with *LTA 252G-carriage*	3.8	1.7–5.9	.009

In order to see the interaction between radiation dose, *LTA*, and smoking status, first four new categories composed of *LTA* and smoking status were created, i.e., current smoker with *LTA 252G*-carriage, current smoker with *LTA 252AA*, noncurrent smoker with *LTA 252G*-carriage, and noncurrent smoker with *LTA 252AA*. Then the interaction terms between radiation dose and these categories were put into a model, where current smoker with *LTA 252AA* was used as a reference, in the presence of other risk factors as in Table 3. Only radiation risk data are shown for noncardia gastric cancer of intestinal and diffuse types for the sake of simplicity.

Forth, radiation risk differed according to the host-genetic polymorphism and smoking status.

The AHS cohort is not fully representative of the general population. Because many full-aged healthy men became soldiers and were absent from Hiroshima and Nagasaki at the time of atomic bombings, the proportion of male and female genders in the atomic bomb survivors was distorted and about 65% of the population was female in the AHS population [24]. In Japan, there was a gender effect on both the histological types of GC and incidence of GC. According to Kaneko's report where 161,067 GC cases in the JRSGC registry were analyzed, the frequencies of diffuse-type GC from 1975 to 1989 were 34–40% and 49–58% in males and females, respectively [27]. According to the population-based Hisayama study, men were about three times more likely to suffer from GC than women in Japan [34]. Thus, in our study population, it was expected that the proportion of diffuse-type GC and female cases would be higher than in other populations.

Proinflammatory cytokines such as IL-1β, TNF-α, and LT-α may modify the immune/inflammatory responses upon *H. pylori* infection in two different ways. One is the augmentation of immune-inflammatory response, which may result in either the eradication of *H. pylori* from the stomach or stronger chronic inflammation if *H. pylori* continue to colonize. Another is the suppression of gastric acid secretion [18,19], which may favor the colonization of *H. pylori* in the stomach and reduce eradication rate. Thus, the net effect of gene polymorphisms associated with high inflammatory cytokine secretion is unpredictable and must be evaluated by different endpoints, i.e., the prevalence of *H. pylori* infection, antibody titer, and cancer risk. Thus, we first evaluated whether *LTA 252G*-carriage was associated with the prevalence of *H. pylori* infection or antibody titers in controls. We excluded cases from such an analysis because CAG might confound antibody titer by reducing the burden of *H. pylori* in the stomach. The present results indicated that *LTA 252G*-carriage was not associated with an increased eradication rate but higher immune response to *H. pylori* that might contribute to the cancer genesis.

In the previous study, we reported low antibody titer to CagA was a risk factor for future noncardia GC, and hypothesized that either gene polymorphisms controlling immune responsiveness to CagA or gene polymorphisms suppressing CagA expression in the stomach might be responsible for the phenomenon [28]. It is known that CagA expression is under the control of *pH* in the stomach [35] and that proinflammatory cytokines suppress gastric acid secretion [18,19]. Therefore, we wished to investigate whether *LTA* gene polymorphism would confound the risk of low antibody titer against CagA. However, the present result has demonstrated that low antibody titer to CagA serves as a risk factor for GC independently from *LTA 252G/A* polymorphisms, and mechanisms associated with low antibody titer to CagA remain to be elucidated in future work.

There are two major carcinogenesis pathways to noncardia GC after *H. pylori* infection [3,36,37]. One is a pathway to noncardia GC of intestinal type, where CAG and intestinal metaplasia are intermediate stages proceeding to cancer. Another is a pathway to noncardia GC of diffuse type, where *H. pylori* infection is an indispensable risk factor while neither CAG nor intestinal metaplasia is essential [4]. The spectrum of molecular alterations differs between intestinal and diffuse types in tumor suppressor genes, oncogenes, and gene expression coding for adhesion molecules [3,36]. It was speculated that additional carcinogenic elements besides *H. pylori* such as *N*-nitrosamines [36,38] might be operating more profoundly in noncardia GC of diffuse type than in those of intestinal type. Although biological mechanisms remain elusive, we demonstrated here that the *LTA 252G*-containing haplotype in the *LTA*, *NFKBIL1*, and *BAT1* gene regions was associated with carcinogenesis only in noncardia GC of diffuse type. As the biological function of the *LTA 252G*-containing haplotype is not fully explored except for higher production of proinflammatory cytokines *in vitro* and higher plasma CRP levels in the general population, further studies are needed to understand mechanisms by which this

haplotype facilitates carcinogenesis for noncardia GC of diffuse type.

There is only limited knowledge about the association of *LTA* genotype and noncardia GC [11,12,39,40]. Seno did not find a positive association between haplotypes composed of three SNPs in *TNFA* and *LTA* genes, but did not investigate *LTA −449G/A, 252G/A, 265C/A,* and *804C/A* SNPs [12]. Shimura et al. reported a positive association between the *LTA 252AA* genotype and better prognosis in GC patients in Japan [40]. Although Shimura did not investigate the histological type of GC, it is well known that diffuse-type GC shows generally poor prognosis. Thus, Shimura's result would not conflict with ours. On the contrary, Lee and Garcia-Gonzalez did not observe a positive association between *LTA 252G*-carriage and GC in Korean [39] and Spanish [11] populations, respectively. The allele frequency of *LTA 252G* was higher in Korea (0.44) and lower in Spain (0.26) than in Japan (0.38 in the present study and 0.36 in ref. [16]). Moreover, in Lee's study, a very high proportion of 69% in GC cases was diffuse type and *H. pylori* seropositivity was not included in the analyses. Thus, it is possible that his cases might possess a unique risk factor for diffuse-type GC, which is not playing a dominant role in our cases. In Garcia-Gonzalez's study, none of the gene polymorphisms in pro- or anti-inflammatory cytokine genes showed significant association with noncardia GC regardless of any histological types. As discussed by McColl et al., each ethnic group has different dietary factors that protect against oxidative stress and cancer progression and the high intake of fresh fruits and vegetables by Spanish people might obscure the biological effect of polymorphisms in proinflammatory cytokine genes [15]. Further studies are needed to investigate an association between *LTA 252* genotype and GC in different populations.

Smoking is a known risk factor for GC [41]. However, there are a limited number of reports where smoking and other risk factors have been analyzed in the same model [42–44]. If our understanding is correct, this is the first study that has evaluated the risk of stopping smoking for noncardia GC in the presence of other risk factors. It was shown that both former smokers and never smokers were protected from noncardia GC. However, it is noteworthy that our study cannot specify the biological mechanisms of cancer protection governing in former smokers. It is speculated that stopping smoking might slow down a carcinogenesis process operating in active smokers.

Radiation risk for lung cancer was less prominent in smokers in the atomic-bomb survivors (present data and Pierce et al. [45]) as well as in miners [46]. Assuming that radiation carcinogenesis is the acceleration of the promotion step as discussed by Nakamura [47], our results and miners' results suggest that smoking and radiation have a common promotion mechanism where smoking plays a dominant role over radiation in terms of the promotion activity. Chronic oxidative stress in the descendants of irradiated progenitor cells might be one candidate mechanism of driving forces in the promotion step [48,49], and *LTA/NFKBIL1/BAT1* polymorphism might augment the inflammatory responses to oxidative stress and thus promote the carcinogenesis in irradiated subjects. These assumptions need to be tested in future work.

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