Table 3. Regression analysis for association with IL-6 levels.

	Univariate		Multivariate (adjusted for age and gender)		Multivariate (fully adjusted[c])	
Factor[a]	Per cent increment per unit[b]	*p*	Per cent increment per unit[b]	*p*	Per cent increment per unit[b]	*p*
Age	21.2 (15.9–26.5)	0.0001			20.0 (14.4–25.7)	0.0001
Gender	11.0 (−0.1–20.9)	0.05			2.4 (−10.0–13.5)	0.69
Smoking	115.8 (−14.8–247.4)	0.08	174.8 (40.2–310.6)	0.01	163.8 (31.0–297.6)	0.02
BMI	0.6 (−1.2–2.4)	0.52	1.3 (−0.4–3.0)	0.12	1.0 (−0.8–2.7)	0.27
TC	−3.5 (−5.1–−1.9)	0.0001	−2.5 (−4.1–−0.9)	0.002	−2.5 (−4.2–−0.8)	0.005
HDL	−0.3 (−0.7–−0.1)	0.13	−0.3 (−0.7–0.1)	0.16	−1.7 (−2.7–0.9)	0.57
TG	−0.1 (−0.7–0.5)	0.78	0.2 (−0.4–0.8)	0.59	0.2 (−0.5–1.8)	0.84
SBP	7.0 (4.5–9.6)	0.0001	4.3 (1.7–6.8)	0.001	3.7 (1.1–6.3)	0.005
MI	62.5 (11.2–137.3)	0.01	43.5 (0.5–104.7)	0.04	47.2 (4.4–107.6)	0.03
Radiation dose	9.3 (3.1–15.9)	0.003	32.9 (16.2–51.9)	0.0001	9.8 (4.1–15.9)	0.0007

See notes to table 2.

T-cell proportion for the change in the levels of CRP and IL-6, statistical evaluation for any such threshold was difficult. Thus, regression analysis was performed. As shown in table 4, both CRP and IL-6 levels were negatively associated with $CD4^+$ T-cell proportion. This negative association was still highly significant after adjustment for the various factors that appeared to be significantly associated with CRP or IL-6 levels (tables 2 and 3).

4. Discussion

Our present study shows that plasma CRP levels, which are often used as a sensitive marker of inflammatory activity, appear to increase with radiation dose in a cohort of A-bomb survivors. Since the correlation between CRP levels and radiation dose remained significant even after adjustments for other factors that are either known or widely assumed to cause plasma CRP levels to change, it could reasonably be inferred that A-bomb radiation exposure is likely to have been an important factor in the increases in CRP levels detected in A-bomb survivors up to half a century after the bombing. In accordance with the established fact that IL-6 acts as a primary inducer of CRP production in the liver (Kishimoto 1989, Heinrich *et al.* 1990), we found that CRP levels correlated very well with the increase in IL-6 levels in our present study of A-bomb survivors. Also, given that IL-6 is rapidly produced in response to various inflammatory stimuli (Heinrich *et al.* 1990), our present results with elevated IL-6 levels strongly support our conclusion that inflammatory processes are active (albeit at quite low levels) in radiation-exposed subjects.

It is not known whether plasma CRP and IL-6 levels reached their present levels soon after the bombing (i.e. in 1945 or shortly thereafter) or were steadily rising during the years before 1995–97, which is when we obtained the samples for the tests reported here. However, the results of biennial medical examinations of the A-bomb survivors in earlier clinical examination cycles (in the 1960s) indicate that there were already significant dose-related changes in white blood cell counts and erythrocyte sedimentation rates (Sawada *et al.* 1986). Since the levels of both markers are known to rise in response to a variety of inflammatory stimuli (Heinrich *et al.* 1990), it seemed reasonable to assume that low levels of inflammation may well have been discernible in the survivors for very long periods, up to and including the sampling period in 1995–97.

Since the opinion of several recent investigators is that even quite low levels of inflammation may well constitute an independent risk factor for CVD (Ridker *et al.* 1997, 2001, Koenig *et al.* 1999, Danesh *et al.* 2000, Mendall *et al.* 2000), it would be of great interest to find out whether the dose-dependent

increases in CRP levels that we observed in A-bomb survivors are also associated with an increased risk of CVD. Moreover, although the sources of the low levels of inflammation that seem to be associated with CVD risk in apparently healthy individuals have not yet been determined, a recent report suggests that increased levels of IL-6 may also be predictive of an increased risk of MI (Ridker *et al.* 2000). Thus our finding of correlated increases in CRP and IL-6 levels in radiation-exposed A-bomb survivors could well be indicative of low levels of persistent inflammation that could presage an increased risk of CVD for the survivors concerned. Clearly, a prospective study will be required to test this hypothesis directly.

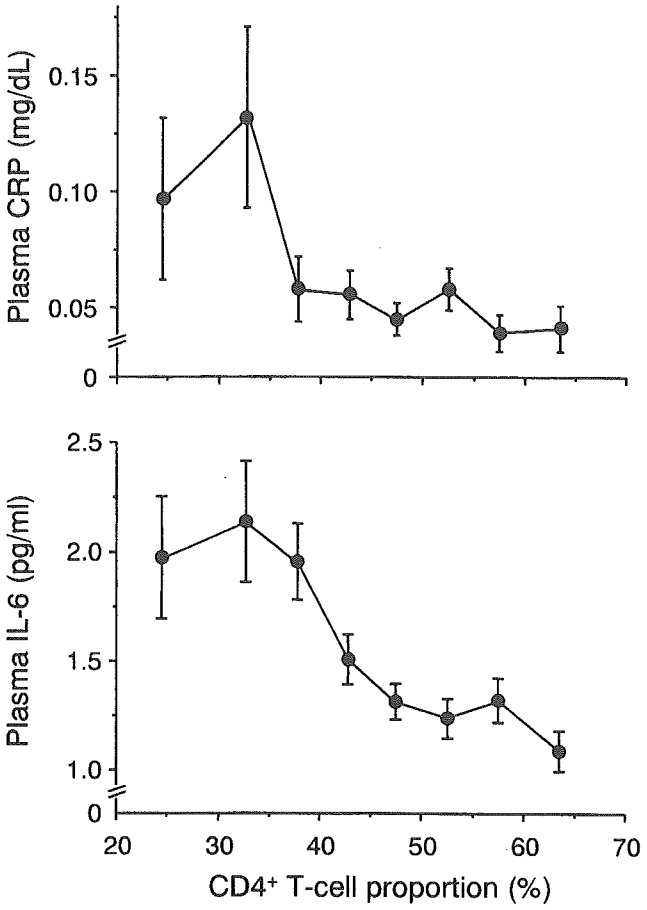

Figure 1. Negative association of the $CD4^+$ T-cell proportions with the plasma CRP and IL-6 levels. Each plot shows the mean and standard error of plasma levels of CRP (upper) and IL-6 (lower) in each 5% category of $CD4^+$ T-cell proportion.

The mechanisms underlying the persistently increased inflammatory responses that we detected in A-bomb survivors have not yet been determined. Increases in blood levels of IL-6 after total-body irradiation have been observed in patients who had recently undergone whole-body irradiation for bone marrow transplantation (Girinsky *et al.* 1994). These elevated IL-6 levels were found to have returned to baseline within 24 h, however, indicating that the more immediate effects of whole-body irradiation on this particular manifestation of an inflammatory response do not persist for terribly long periods in these patients. Comparable results were obtained in animal (rat) experiments in which the increased levels of IL-6 that occurred in response to whole-body irradiation had also dropped back to baseline levels within 24 h (Haveman *et al.* 1998). However, long-term experiments have not been performed to test whether IL-6 levels could increase again after their initial return to baseline levels in both humans and animals.

It is of course also possible that certain of the less direct effects of whole-body irradiation on humans cause—or contribute to—the persistently increased (but still relatively mild) symptoms of inflammatory activity that we see in A-bomb survivors. It may therefore be worth noting that we have previously reported and presented here a dose-dependently decreased representation of $CD4^+$ T-cells in A-bomb survivors (Kusunoki *et al.* 1998). Our observation that the CRP and IL-6 levels measured in this study were negatively correlated with the proportions of $CD4^+$ T-cells in A-bomb survivors may therefore be of some considerable interest. Since $CD4^+$ T-cells

Table 4. Regression analysis for association of CRP and IL-6 levels with $CD4^+$ T-cell proportion.

	Effect of 5% increase in $CD4^+$ T-cell proportion			
Adjustment	Per cent change in CRP[a]	*p*	Per cent change in IL-6[a]	*p*
Univariate	−11.4 (−17.9 to −4.8)	0.0008	−8.7 (−11.5 to −5.9)	0.0001
Multivariate (adjusted for age and gender)	−9.0 (−15.9 to −1.9)	0.01	−5.3 (−8.1 to −2.5)	0.0002
Multivariate (fully adjusted[b])	−7.0 (−13.7 to −0.2)	0.04	−5.0 (−7.7 to −2.2)	0.0007

[a]Log-transformed values of CRP and IL-6 as dependent variables were used in the linear regression analyses. Numbers in parentheses denote the 95% confidence interval.

[b]Adjusted for age, gender, smoking, body mass index, total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure and radiation dose.

have an important role in defending the body against infectious agents, it is not unrealistic to suggest that A-bomb survivors with decreased $CD4^+$ T-cell numbers may be somewhat more susceptible to infections of various sorts than their non-exposed counterparts. Consistent with this expectation is the fact that A-bomb survivors appear to display a radiation dose-related increase in mortality due to pneumonia (Shimizu *et al.* 1999). Viral infections may also occur at elevated frequencies in A-bomb survivors, as suggested by a finding that the prevalence of hepatitis B surface antigen appears to increase with increasing A-bomb irradiation (Neriishi *et al.* 1995). One may also envisage asymptomatic infectious events being of some relevance to the increased inflammatory responses observed in our present study.

Clearly, we are still obtaining new and unexpected information about the long-term effects of ionizing radiation on humans. Our present study establishes that one of the more sensitive markers of inflammatory status, an individual's CRP levels, still seem to be dose-dependently elevated in the A-bomb survivors more than half a century after the bombing of Hiroshima. We also found that there was a correlation between increased CRP levels and contemporaneous plasma levels of IL-6. Although we can not say much about the significance of increased levels of CRP and IL-6 in diseases of A-bomb survivors as yet, we believe there is a good chance that such inflammatory reactions we have detected will prove to be associated with an increase in CVD risk, in much the same way as they were seen to be in numerous epidemiological studies of unirradiated and otherwise seemingly normal individuals (Ridker *et al.* 1997, 2001, Koenig *et al.* 1999, Danesh *et al.* 2000, Mendall *et al.* 2000). Thus our present results appear to imply that further studies of radiation-associated inflammatory responses are likely to provide us with important new information about one or more of the less well-documented, but nonetheless far from trivial, long-term effects of ionizing radiations in humans.

Acknowledgements

This paper was based on research performed at the Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan. RERF is a private, non-profit foundation funded equally by the Japanese Ministry of Health, Labour and Welfare and the US Department of Energy through the National Academy of Sciences.

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ELSEVIER

HLA Haplotype is Associated With Diabetes Among Atomic Bomb Survivors

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ABSTRACT: We examined radiation effects on the relationship between diabetes development and genetic background in atomic-bomb (A-bomb) survivors. Our main aim in this study was to shed light on the role of genetic background in diabetes onset among A-bomb survivors by studying possible relationships between human leukocyte antigen (HLA) genotypes and the diabetes in patients and controls. We examined the effects of different HLA haplotypes on type 2 diabetes development by determining the DQA1 and DRB1 alleles of Hiroshima A-bomb survivors (111 diabetic patients and 774 controls) using the polymerase chain reaction-sequence specific oligonucleotide probes (PCR-SSOP) method. We noted an increased risk of diabetes in the higher dose group among these patients (trend $p = 0.001$). The risk of the most heavily exposed group was significantly higher than that of the unexposed group or the low-dose group especially in survivors with the DQA1*03-DRB1*09 or DQA1*0401-DRB1*08 haplotypes (trend $p = 0.002$ or $p = 0.05$, respectively). By contrast, in people with other haplotypes, the risk did not increase significantly with increasing dose. These results suggest that individuals with specific HLA haplotypes may have an increased risk of diabetes with increased-dose categories. *Human Immunology 64, 910–916 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: diabetes; HLA; haplotypes; atomic bomb; radiation

ABBREVIATIONS

A-bomb	atomic bomb
AHS	adult health study
ATB	age at the time of the bombing
ATE	age at the time of examination
BMI	body mass index
GTT	glucose tolerance test
HLA	human leukocyte antigens
MHC	major histocompatibility complex
OR	odds ratio
SSOP	sequence-specific oligonucleotide probes

INTRODUCTION

Genetic background and environmental factors are both known to be important in the development of diabetes. One especially important genetic factor appears to be the major histocompatibility complex (MHC) locus, which in humans is referred to as the human leukocyte antigen (HLA) locus. The literature contains many reports of a close association between the development of type 1 diabetes and certain HLA class II alleles, with a small number of DQ and DR alleles being particularly prominent [1–3]. Similar suggestions of a relationship between type 2 diabetes and certain HLA haplotypes have also begun to appear, but details of the association are far from clear at present [4–6].

A careful analysis of the data obtained in a 1992–1994 study of atomic bomb (A-bomb) exposed patients indicated a significant positive correlation between type 2 diabetes prevalence and radiation dose in individuals who were exposed to the A-bomb in Hiroshima. A different study we conducted previously [7] has already determined HLA class II types for a subset of the survivor population, and an analysis of genotype effect on type 2 diabetes prevalence seemed feasible for those survivors whose HLA class II types were known. In the present

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Received May 2, 2003; revised June 17, 2003; accepted June 17, 2003.

Human Immunology 64, 910–916 (2003)

0198-8859/03/$–see front matter
doi:10.1016/S0198-8859(03)00157-5

study, we used these HLA class II typing data to determine whether evidence exists of a relationship between the development of diabetes and HLA class II haplotypes that might differ with A-bomb radiation doses to which the patients were exposed.

MATERIALS AND METHODS

Patients

The study patients were originally selected for the purpose of HLA analysis of the A-bomb survivors in a previous study [7], and analyzed for the prevalence of diabetes mellitus. Briefly, a total of 3269 participants underwent clinical examination for diabetes mellitus from 1992 to 1994 in an epidemiologic follow-up study of A-bomb survivors (adult health study [AHS]) that has been conducted since 1958. Among them, HLA genotypes that had been determined in the previous study [7] were available for 892 individuals. Persons who developed diabetes mellitus (DM) as a consequence of chronic liver disease were excluded, and the remaining 855 survivors were analyzed for effects of their HLA genotypes on the relationship between radiation dose and DM prevalence. Thus, patients in this study included the following: those nonexposed (< 0.005 Gy, n = 328) examined for diabetes mellitus; those exposed to a low-dose level (0.005–1.15 Gy, n = 278); and those exposed to a high-dose level (>1.15 Gy, n = 279). The prevalence of diabetes may be biased by the accumulation of first- and second-degree family members among AHS patients. Although we have investigated, based on information of family relationships, whether the accumulation of first- and second-degree family members occurred in the patients and controls, such an overlap of family members was not found in these patients. Estimated bone marrow doses were based on the 1986 Dosimetry System known as DS86; basically this involves calculating a free-in-air radiation dose estimate for the patient's reported location and then adjusting the value obtained to reflect shielding information [8]. A diagnosis of diabetes was made according to the guidelines of the Japan Diabetes Society [9], which referred to the new American Diabetes Association criteria [10]. Definite patients were defined as a fasting serum glucose level of 126 mg and over, or a casual plasma glucose level or a 2-hour glucose tolerance test (GTT) level of 200 mg and over. In addition, people undergoing treatment for diabetes were categorized as definite patients. All diabetes patients in this study were classified as having type 2 diabetes according to the Japan Diabetes Society criteria [9]. Institutional approval has been obtained from the Human Investigation Committee.

Laboratory Methods

The HLA-DQA1 and DRB1 typing was conducted with amplified DNA as described by Hayashi *et al.* [7]. HLA-DQA1 alleles are separable by assay into the following eight groups: *DQA1*0101, *0102, *0103, *0201, *03* (**0301* and **0302*), **0401, *0501,* and **0601*. In the present study, DNA allele typing of each DRB1 gene was not attempted because the large number of distinct alleles (there are more than 30 alleles for the DRB1 gene alone, for example) would require excessive effort to classify. In addition, the purpose of the present study was to identify the functional allele types (*i.e.*, antigen groups) and not the exact genotypes; therefore, it was deemed sufficient to classify our study patients into the following ten HLA-DRB1 allele groups: *DRB1*01, *02* (**15* and **16*), **03, *04, *05* (**11* and **12*), **06* (**13* and **14*), **07, *08, *09*, and **10*.

Statistical Analysis

The expected frequencies of alleles and haplotypes were estimated by logistic regression, the standard procedure for data of this nature [11]. Calculations were accomplished using the binary logistic GAMBO program in the Epicure package (Hirosoft International, Seattle, WA, USA [12]). Statistical significance was evaluated by likelihood ratio tests assuming that the deviance was chi-squared in distribution [11]. Each trend p value, which represented the dose effect in each allele or haplotype frequency, was used for the test of dose response in logistic regression using continuous dose for each allele and haplotype, but not for that of each odds ratio in each dose group.

RESULTS

Table 1 presents the characteristics of the study subjects. There were no significant differences in mean age at the time of the bombing (ATB), mean age at the time of examination (ATE), or mean body mass index (BMI) by dose levels. The prevalence of patients who had developed diabetes was found to be significantly higher in the highest dose group (trend p = 0.001). This indicated that radiation effects on the prevalence of diabetes may be significant in the A-bomb survivor population, and also suggested that dose and/or unrelated factors associated with dose categories may affect the development of diabetes among survivors approaching the age at which a diagnosis of type 2 diabetes is increasingly likely.

Next, we examined the effects of different HLA alleles, namely certain DQA1 and DRB1 alleles, on diabetes development among the A-bomb survivor population. The odds ratio (OR) of diabetes was obtained for each dose group with each of the specific DQA1 alleles (Table 2). We detected no statistically significant differ-

TABLE 1 Characteristics of the study patients

	Radiation dose groups			*p* Value for trend[c]
	Nonexposed (n = 328)	Low[a] (n = 278)	High[b] (n = 279)	
Radiation dose (Gy)*	0	0.55 ± 0.36	2.32 ± 0.82	
Age ATB (years)*	19.7 ± 10.7	20.8 ± 10.4	17.8 ± 10.1	
ATE (years)*	67.4 ± 10.7	68.5 ± 10.3	65.5 ± 10.2	
Gender (F/M)†	183/145	149/129	175/104	
BMI				
All patients	22.5 ± 3.4	22.6 ± 3.6	22.5 ± 3.3	
Controls	22.5 ± 3.4	22.5 ± 3.5	22.4 ± 3.2	
Diabetes cases	23.0 ± 3.1	22.9 ± 4.5	23.1 ± 3.8	
Diabetes†, n (%)	30 (9.1)	32 (11.5)	49 (17.6)	0.001

Data are: * values are mean ± SD; † number of participants.

ATB denotes at the time of bombings and ATE age at the time of examination; BMI indicates body mass index.

Comparison of characteristics across three radiation dose groups: [a] estimated dose in dose categories 0.005–1.15 Gy; [b] estimated dose in dose categories > 1.15 Gy; [c] trends were tested by simple regression analysis for continuous values and logistic analysis for age and gender.

ences in the distribution of specific HLA-DQA1 alleles among the various exposure groups (*i.e.*, the nonexposed, the 0.005–1.15 Gy, and the > 1.15 Gy groups). The OR was significantly or suggestively increased with increasing radiation dose in groups comprising individuals with the DQA1*0103, DQA1*03, or DQA1*0401 allele (trend p = 0.05, 0.01, and 0.06, respectively). No such trend, however, was evident for the ORs in individuals with other DQA1 alleles, except for DQA1*0501. The trend p value of DQA1*0501 was significant but the allele's OR was slightly decreased in the higher dose category.

Table 3 presents the ORs of diabetes that were obtained for each dose group with each of the specific DRB1 alleles. The distribution of HLA-DRB1 alleles did not appear to significantly differ among the different dose groups. However, the OR of diabetes did appear to rise with increasing radiation dose categories comprising individuals with either the DRB1*08 or DRB1*09 allele (trend p = 0.09 and 0.004, respectively). Such radiation effects were not observed for the ORs in any dose groups of individuals who had the other DRB1 alleles.

Given that the DQ genes and the DR genes are tightly linked on chromosome 6 and that the linkage disequilibrium between DQA1*03 and DRB1*09 or DQA1*0401 and DRB1*08 is established [13], we decided to further examine the data to look for possible radiation effects on the development of diabetes among people with these haplotypes. In survivors with the DQA1*03-DRB1*09 the ORs for the most heavily exposed group (> 1.15 Gy) were significantly higher (1.62) than those observed for either the nonexposed group (0.57) or the low-dose group (0.49; trend p = 0.002; Table 4). Similarly, a significant increasing trend (p = 0.05) with increasing radiation dose categories was also found in the survivors with the DQA1*0401-DRB1*08 haplotype: ORs for the radiation dose categories were 1.96, 0.65, and 1.11 in the heavily exposed, nonexposed, and the low-dose group, respectively. By contrast, the ORs were not significantly increased with increasing radiation dose categories in individuals with neither the DQA1*03-DRB1*09 or DQA1*0401-DRB1*08 haplotype (trend p = 0.14). Although the DQA1*0103 and DRB1*02, DQA1*0103 and DRB1*08, and DQA1*03 and DRB1*04 alleles are also linked, the ORs did not appear to increase with increasing dose categories in survivors with these haplotypes. Figure 1 illustrates cumulative prevalence rates of dia-

TABLE 2 Distribution of DQA1 alleles in diabetic patients and healthy controls among A-bomb survivors

	Nonexposed (n = 328)			Low* (n = 278)			High† (n = 279)			
DQA1 alleles	Patients (n = 30) n	Controls (n = 298) n	OR (CI)[a]	Patients (n = 32) n	Controls (n = 246) n	OR (CI)[a]	Patients (n = 49) n	Controls (n = 230) n	OR (CI)[a]	Trend p value[b]
0101	4	87	0.37 (0.13–1.10)	6	77	0.51 (0.20–1.28)	7	70	0.38 (0.16–0.89)	0.25
0102	7	60	1.21 (0.50–2.95)	10	55	1.58 (0.71–3.53)	12	48	1.23 (0.60–2.54)	0.24
0103	6	96	0.53 (0.21–1.33)	10	83	0.89 (0.40–1.97)	12	68	0.77 (0.38–1.57)	**0.05**
03	20	196	1.04 (0.47–2.31)	18	154	0.77 (0.37–1.62)	33	148	1.14 (0.59–2.20)	**0.01**
0401	1	21	0.46 (0.06–3.51)	2	16	0.96 (0.21–4.38)	4	19	0.99 (0.32–3.04)	**0.06**
0501	8	49	1.85 (0.78–4.39)	7	31	1.94 (0.78–4.87)	14	41	1.84 (0.91–3.74)	0.03
0601	3	9	3.57 (0.91–13.97)	1	11	0.69 (0.09–5.52)	2	7	1.36 (0.27–6.73)	0.73

The DQA1*0201 allele was not observed in either diabetic or control populations.

* Estimated dose in dose categories 0.005–1.15 Gy; † estimated dose in dose categories > 1.15 Gy.

[a] The ORs were calculated for each dose group comparing the numbers of diabetic patients and controls; [b] adjusted for age ATB, and sex. Bold numbers indicate significant trends.

Abbreviations: ATB = at the time of bombing; CI = 95% confidence interval; n = total number of alleles; OR = odds ratio.

TABLE 3 Distribution of DRB1 alleles in diabetic patients and healthy controls among A-bomb survivors

DRB1* alleles	Nonexposed (n = 338)			Low* (n = 278)			High† (n = 279)			Trend p value[b]
	Patients (n = 30)	Controls (n = 298)		Patients (n = 32)	Controls (n = 246)		Patients (n = 49)	Controls (n = 230)		
	n	n	OR (CI)[a]	n	n	OR (CI)[a]	n	n	OR (CI)[a]	
01	4	57	0.65 (0.22–1.94)	4	49	0.57 (0.19–1.71)	4	43	0.39 (0.10–1.13)	0.61
02	5	95	0.43 (0.16–1.15)	11	80	1.09 (0.50–2.36)	11	72	0.64 (0.26–1.26)	0.17
04	18	118	2.29 (1.06–4.92)	14	92	1.30 (0.62–2.74)	19	90	0.99 (0.42–1.57)	0.29
05	7	46	1.67 (0.68–4.11)	7	39	1.49 (0.60–3.68)	11	38	1.46 (0.48–2.58)	0.05
06	7	72	0.96 (0.39–2.32)	7	65	0.78 (0.32–1.89)	11	62	0.78 (0.39–1.78)	0.18
08	6	62	0.95 (0.37–2.43)	9	59	1.24 (0.54–2.83)	12	59	0.94 (0.48–2.11)	0.09
09	5	87	0.49 (0.18–1.31)	6	62	0.69 (0.27–1.74)	16	54	1.58 (0.87–3.52)	**0.004**

The DRB1*03 and DRB1*10 alleles were not observed in either diabetic or control populations. The DRB1*02, DRB1*05, and DRB1*06 groups contain the DRB1*15, 16, DRB1*11, 12, and DRB1*13, 14 alleles, respectively.

* Estimated dose in dose categories 0.005–1.15 Gy; †estimated dose in dose categories > 1.15 Gy.

[a] The ORs were calculated for each dose group comparing the numbers of diabetic patients and controls; [b] adjusted for age, ATB, and sex. Bold numbers indicate significant trends.

Abbreviations: ATB = at the time of bombing; CI = 95% confidence interval; n = total number of alleles; OR = odds ratio.

betes among individuals who have either the DQA1*03-DRB1*09 or DQA1*0401-DRB1*08 haplotype, but have neither the DQA1*03-DRB1*09 alleles nor the DQA1*0401-DRB1*08 haplotype after adjusting for male sex and ATB of 20 years. The prevalence of diabetes increased with higher radiation dose categories among individuals with either the DQA1*03-DRB1*09 or DQA1*0401-DRB1*08 haplotype (trend p = 0.0003). No significant difference in prevalence of diabetes was found among individuals with neither the DQA1*03-DRB1*09 alleles nor the DQA1*0401-DRB1*08 haplotype (trend p = 0.14). The incidence of diabetes among individuals with these haplotypes was significantly higher than that among individuals without these haplotypes (p = 0.03).

DISCUSSION

In the present study we observed that increased dose category is associated with increased risk of diabetes among A-bomb survivors and found that there appears to be significant differences in diabetes prevalence between

TABLE 4 Distribution of selected DQA1-DRB1 haplotypes in diabetic and healthy A-bomb survivors

DQA1-DRB1 haplotypes	Nonexposed (n = 328)			Low* (n = 278)			High† (n = 279)			Trend p value[b]
	Patients (n = 30)	Controls (n = 298)		Patients (n = 32)	Controls (n = 246)		Patients (n = 49)	Controls (n = 230)		
	n	n	OR (CI)[a]	n	n	OR (CI)[a]	n	n	OR (CI)[a]	
0103-02	2	63	0.27 (0.06–1.15)	5	53	0.67 (0.25–1.84)	4	38	0.45 (0.15–1.32)	0.14
0103-08	4	44	0.88 (0.30–2.67)	6	40	1.19 (0.46–3.07)	6	35	0.78 (0.31–1.96)	0.18
0103-02 or 08	6	92	0.56 (0.22–1.42)	10	81	0.93 (0.42–2.05)	10	65	0.65 (0.31–1.38)	0.07
03-04	18	118	2.29 (1.06–4.92)	14	92	1.30 (0.62–2.74)	18	89	0.92 (0.49–1.74)	0.33
03-09	5	87	0.49 (0.18–1.31)	5	60	0.57 (0.21–1.56)	16	53	1.62 (0.83–3.17)	**0.002**
0401-08	1	15	0.65 (0.08–5.11)	2	14	1.11 (0.24–5.10)	4	10	1.96 (0.59–6.51)	**0.05**
03-09 or 0401-08	6	100	0.50 (0.20–1.25)	6	72	0.56 (0.22–1.41)	20	62	1.87 (0.97–3.46)	**0.0003**
Not (03-09 or 0401-08)	24	198	2.02 (0.80–5.10)	26	174	1.79 (0.71–4.54)	29	168	0.54 (0.28–1.02)	0.14

* Estimated dose in dose categories 0.005–1.15 Gy; † estimated dose in dose categories > 1.15 Gy.

[a] The ORs were calculated for each dose group comparing the numbers of diabetic patients and controls; [b] adjusted for age ATB, and sex. Bold numbers indicate significant trends.

Abbreviations: ATB = at the time of bombing; CI = 95% confidence interval; n = total number of haplotypes; OR = odds ratio.

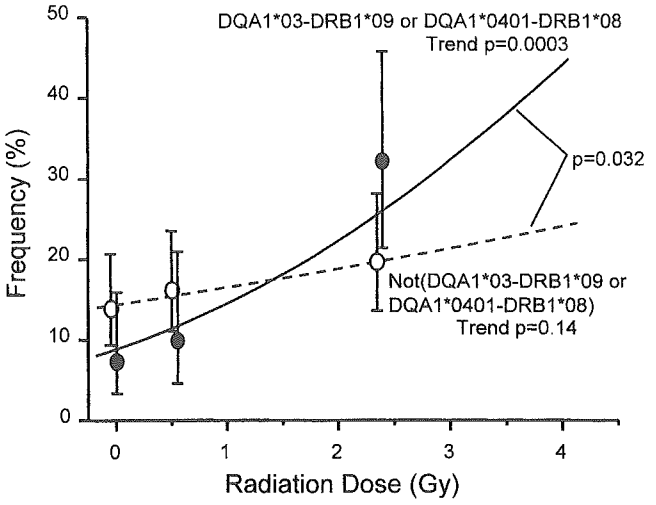

FIGURE 1 Cumulative prevalence rates of diabetes by human leukocyte antigen (HLA) haplotyping in radiation dose categories. Prevalence of diabetes increased with higher radiation dose categories among individuals who have either the DQA1*03-DRB1*09 or DQA1*0401-DRB1*08 haplotype (trend $p = 0.0003$). No significant association was found between radiation and diabetes among individuals who have neither the DQA1*03-DRB1*09 alleles nor the DQA1*0401-DRB1*08 haplotype (trend $p = 0.14$). The prevalence of diabetes among individuals with these haplotypes was significantly higher than that among individuals without these haplotypes ($p = 0.032$).

exposed and low-dose or nonexposed survivors with different HLA class II haplotypes. These results did not stem from population bias among different dose categories in terms of the distribution of HLA class II haplotypes. Our previous results indicated that no significant differences in certain allele frequencies among A-bomb survivors were found among the control, low-dose, and high-dose groups in Hiroshima [7]. We believe ours is the first report describing that development of a particular disease can be affected by radiation dose categories in individuals with different genetic backgrounds.

Most studies to identify the putative susceptibility loci have focused on candidate genes implicated in the pathways that are impaired in individuals with type 2 diabetes, although a few investigators have examined the roles of loci encoding HLA [4–6, 14]. In a previous study, Ghabanbasani *et al.* [5] reported a significant association between homozygosity for the allele encoding HLA-DQα1^{Arg52} and susceptibility to type 2 diabetes. Pandy *et al.* [6] also reported that particular homozygous genotypes of TNF-α and immunoglobulin allotypes epistatic interact with HLA-DQα1^{Arg52} and contribute to an increased relative risk of type 2 diabetes. This HLA-DQα1^{Arg52} genotype includes both the DQA1*03 and the DQA1*0401 alleles. Because type 2 diabetes has a heterogeneous etiology [15–17], several factors might collaborate partially or particular factors might cause a certain subtype of the disease. As suggested by Hitman and Metcalf [18], there might be a type 2 diabetes subtype as a milder form of type 1 diabetes, it is hypothesized that a particular subgroup among the younger survivors may develop the disease because of an autoimmune process, as in type 1 diabetes. It is known that the frequency of DRB1*09 allele is significantly higher in patients with type 1 diabetes than controls [19–22]. Our present study also suggests an increased OR in radiation-exposed individuals who have DRB1*09 allele (Table 3), indicating that the DRB1*09 allele may be involved in the development of a part of type 2 diabetes. An extensive analysis of HLA genotype together with detailed clinical characterization of diabetes patients will be necessary to disclose HLA and disease association.

Our previous studies on A-bomb survivors enabled us to identify significant dose-dependent impairment of immune responses in a cohort of the A-bomb survivor population [23, 24]. It may be that this impairment led to many of these individuals being less immunologically responsive to a range of infectious microorganisms [25]. It has been also reported that the prevalence of autoimmune hypothyroidism to which thyroid autoantibodies are accompanied is higher among A-bomb survivors [26], suggesting that the survivors are somewhat susceptible to a type of autoimmune disease. The reason why the radiation effect on diabetes development can be observed among A-bomb survivors is not clear. One possible mechanism is molecular mimicry, a phenomenon that arises when an infectious organism with an antigen that closely resembles a host antigen infects the host and provokes an immune response, which then crossreacts with an autoantigen in individuals with particular HLA types [27–29]. Thus, it is hypothesized that some additional autoimmune diabetes patients may have a complex etiology in which individuals whose immune responsiveness has been impaired due to radiation exposure may be at increased risk of microbial infection, and as a result may mount an autoimmune response that induces diabetes because the immune system crossreacts with a host antigen that mimics a microbial antigen [27–29]. To explore whether some autoimmune mechanisms are possibly involved in the development of a part of diabetes we can observe in the survivor population, we are planning further studies detecting diabetes-related autoantibodies, such as anti-GAD and anti-insulin antibodies, and analyzing their associations with radiation-induced immunologic changes, such as impaired cellular immunity to microbes.

It is of course also possible that certain other polymorphic genes closely linked to the HLA class II gene loci are involved in the radiation-related development of

diabetes. For example, it has been reported that polymorphism of the closely linked TNF-α gene may also influence the onset of type 2 diabetes [30]. To confirm our findings a further investigation will be required in future studies on possible confounding factors including genetic clustering among patients with diabetes, specifically in the high-dose category.

ACKNOWLEDGMENTS

We thank Dr. D. G. MacPhee for his careful reading of the manuscript. This publication is based on research performed at the Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan. RERF is a private nonprofit foundation funded equally by the Japanese Ministry of Health, Labour and Welfare, and the US Department of Energy through the National Academy of Sciences. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan.

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Oncology

Laboratory/Clinical Translational Research

Oncology 2003;64:423–429
DOI: 10.1159/000070302

Reduced Expression of the TSP1 Gene and Its Association with Promoter Hypermethylation in Gastric Carcinoma

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Key Words
DNA methylation · Gastric carcinoma · TSP1 · p53

Abstract
Thrombospondin-1 (TSP1) is a potent peptide shown in some tumor systems to be linked with angiogenesis. Epigenetic alteration of *TSP1* has been reported in various primary tumors. However, the expression pattern of *TSP1* has not been characterized in gastric carcinoma. We measured levels of *TSP1* mRNA expression using quantitative RT-PCR in 30 gastric carcinomas and 10 non-neoplastic mucosae. In addition, we examined the correlation of the levels of *TSP1* mRNA expression levels with promoter methylation status of *TSP1* monitored by methylation-specific PCR as well as *p53* mutation status detected by PCR-single-strand conformation polymorphism. Promoter hypermethylation of the *TSP1* gene was found in 10 (33%) of 30 gastric carcinomas, and *TSP1* mRNA expression levels were associated with promoter hypermethylation of *TSP1* ($p = 0.017$; Mann-Whitney U test). *p53* mutation was found in 5 (17%) of 30 gastric carcinomas, however, *TSP1* mRNA expression was not associated with *p53* mutation status ($p = 0.858$; Mann-Whitney U test). There was no correlation between *TSP1* mRNA expression levels and T grade, N grade, tumor stage, or histological type. Our results suggest that transcriptional inactivation of *TSP1* by aberrant DNA methylation of the promoter region may participate partly in stomach carcinogenesis through *TSP1* down-regulation.

Introduction

Intratumoral microvessel density is believed to reflect the overall degree of angiogenesis in cancers [1]. Tumor cells secrete various growth factors [2] resulting in neovascularization, which supports tumor expansion and metastasis [3]. In gastric carcinoma, the microvessel densities increase with histological stage and the densities are significantly higher in tumors with lymph node metastases than in those without metastases [4]. The expression of vascular endothelial growth factor (VEGF) directly correlates with vessel involvement, lymph node metastasis, and liver metastasis in gastric carcinoma [5]. The level of IL-8 mRNA in gastric neoplasms strongly correlates with vascularization [6].

Thrombospondin-1 (TSP1) is a multifunctional glycoprotein that contributes to angiogenesis [7]. TSP1 is secreted from platelets [8, 9] and modulates platelet aggre-

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gation, wound healing, and protease activity [10–13]. TSP1 is also synthesized and secreted by various types of cells including fibroblasts [11], smooth muscle cells [13], monocytes, macrophages [12], osteoblasts, [10] and a variety of neoplastic cells. Several studies have indicated that TSP1 is an inhibitor of angiogenesis. Transition from a resting to a sprouting phenotype and mitogenic activity of cultured endothelial cells was inhibited by TSP1 [14, 15]. The down-regulation of endogenous TSP1 appears to facilitate endothelial cell chemotaxis and capillary morphogenesis [16]. In addition, experiments on cultured fibroblasts from Li-Fraumeni patients showed that tumor cells switch to an angiogenic phenotype coincident with simultaneous loss of the wild-type *p53* allele and decrease in TSP1 expression [17]. In human cancers, an inverse association has been found between TSP1 expression and *p53* mutations [18–21]. Although, no correlation exists between *p53* alterations and TSP1 mRNA expression, a significant correlation exists between *p53* alterations and high VEGF protein expression in lung carcinoma neovascularization [22]. Production of TSP1 by breast carcinoma cells can exert an inhibitory effect on tumor progression [23]. Expression of TSP1 inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines [24].

Recently, promoter hypermethylation of the *TSP1* gene has been found in some primary human carcinomas including gastric carcinomas [25, 26]. Methylation status of the CpG island in promoter regions is an important determinant of gene expression [27, 28]. Aberrant hypermethylation of the CpG island is associated with silencing of tumor suppressor genes in various tumors. However, the correlations between the *TSP1* expression pattern and clinicopathological features, promoter hypermethylation, and *p53* mutation have not been characterized in gastric carcinoma.

In the present study, we measured the expression level of *TSP1* mRNA in 30 primary gastric carcinoma tissues and 10 non-neoplastic mucosae using a quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) method. We compared the expression level of *TSP1* with respect to clinicopathological features. Moreover, to determine whether transcriptional silencing of the *TSP1* gene is caused by promoter methylation, we compared DNA methylation of CpG islands of the *TSP1* gene with expression of *TSP1* mRNA in 30 primary gastric carcinoma tissues. We also compared the *p53* mutation status of each specimen with *TSP1* mRNA expression.

Materials and Methods

Tissue Samples

A total of 30 gastric carcinoma tissue samples from 30 patients were studied. Tumors and corresponding non-neoplastic mucosae were surgically removed, frozen immediately in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor tissue specimens consisted mainly (>80%) of carcinoma tissue and that non-neoplastic mucosa did not exhibit any tumor cell invasion or show significant inflammatory involvement. Histological classification and tumor staging were done according to the Lauren classification system [29] and the TNM Stage Grouping [30].

Quantitative RT-PCR Analysis

Quantitation of mRNA levels of *TSP1* was carried out by a real-time fluorescence detection method as described previously [31, 32]. In brief, after RNA isolation with an RNeasy Mini Kit (QIAGEN, Valencia, USA), 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The *TSP1* cDNA and internal control cDNA (*beta-actin*: *ACTB*) were PCR-amplified separately with an oligonucleotide probe having a 5′ fluorescent reporter dye (6FAM) and a 3′ quencher dye (TAMRA). Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined by the threshold cycles for the *TSP1* gene and the *ACTB* gene. Reference samples (gastric carcinoma cell line, HSC-39) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. The PCR amplification was performed using a 96-well optical tray and caps with a 25-μl final reaction mixture consisting of 600 n*M* of each primer; 200 n*M* probe; 5 units of AmpliTaq Gold (Applied Biosystems); 200 m*M* each of dATP, dCTP, and dGTP; 400 m*M* dUTP; 5.5 m*M* $MgCl_2$; 1 unit of AmpErase uracil N-glycosylase, and 1× TaqMan buffer A containing a reference dye at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 55°C for 1 min. *TSP1* primer and probe sequences were: 5′-AAC TAC CTG GGC CAC TAT AGC G-3′, 5′-6FAM-CCC CAT GTA CCG CTG CGA GTG C-TAMRA-3′, and 5′-GCC AGC GTA GCC AGG CT-3′. *ACTB* primer and probe sequences were: 5′-TCA CCG AGC GCG GCT-3′, 5′-FAM-CAG CTT CAC CAC CAC GGC CGA-TAMRA-3′, and 5′-TAA TGT CAC GCA CGA TTT CCC-3′. In all cases, the first sequence is the forward PCR primer, the second sequence is the TaqMan probe, and the third sequence is the reverse PCR primer.

Genomic DNA Extraction and Methylation-Specific PCR (MSP)

To examine the DNA methylation patterns in the promoter regions of the *TSP1* gene, we performed methylation-specific PCR [33]. In brief, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by 3 *M* sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA purification resin (Promega, Madison, Wisc., USA), treated with NaOH, precipitated with ethanol, and resuspended in 25 μl water. PCR was performed with 2 μl aliquots of the above DNA preparations as templates. Sequences of methylation-specific PCR primers of *TSP1* were: sense, 5′-TGC GAG CGT TTT TTT AAA TGC-3′; and antisense, 5′-TAA ACT CGC AAA CCA ACT CG-3′ (74 bp) for methylated primer, sense, 5′-GTT TGG TTG TTG TTT ATT GGT TG-3′; and antisense, 5′- CCT AAA CTC ACA AAC CAA CTC A-3′ (115 bp) for unmethylated primer. Each

target sequence was amplified in a 25-μl reaction volume containing 0.2 μ*M* dNTP, 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.3 μ*M* of each primer, and 0.75 units of Ampli Taq Gold (Perkin-Elmer, Norwalk, CT). PCR amplification consisted of 35 cycles (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s) after the initial Taq Gold activation step (95 °C for 10 min). All PCRs were performed with positive controls consisting of methylated alleles and negative controls consisting of no template DNA. CpG sites of genomic DNA isolated from cell line MKN-28 were methylated by treatment with *Sss I* methylase (New England Biolabs, Inc., Beverly, Mass., USA) and served as the positive control for *TSP1* methylation assay. Each PCR product (15 μl) was loaded onto 8% nondenaturing polyacrylamide gels, stained with ethidium bromide and visualized under UV light. This methylation assay detects methylation of the regions of primer sequences in the promoter.

p53 Mutation Analysis

The presence or absence of exons 5–8 of the *p53* gene was determined as described previously by PCR single-strand conformation polymorphism (SSCP) analysis with 10 sets of primers [34].

Statistical Methods

Statistical analysis were performed with Fisher's exact and Mann-Whitney U test. p values less than 0.05 were regarded as statistically significant.

Table 1. mRNA expression levels and *TSP1* methylation status in gastric carcinomas

Methylation status	Case	*TSP1* mRNA expression level in tumor[a]	*p53* mutation status	Stage[b]	T[c] grade	N[d] grade	Histology[e]
Unmethylated	8	2.82	Wild-type	IB	2	0	intestinal
	26	24.96	Wild-type	IB	2	0	diffuse
	14	1.17	Wild-type	II	3	0	intestinal
	22	4.16	Wild-type	II	3	0	intestinal
	5	1.47	Wild-type	II	2	1	diffuse
	27	1.42	Wild-type	II	2	1	intestinal
	16	0.10	Wild-type	II	2	1	intestinal
	12	1.14	Wild-type	II	2	1	diffuse
	15	2.39	Wild-type	II	2	1	intestinal
	30	1.98	Wild-type	II	2	1	diffuse
	17	0.50	Wild-type	IIIA	4	0	intestinal
	18	22.45	Wild-type	IIIA	3	1	diffuse
	7	0.33	Wild-type	IV	3	3	intestinal
	24	92.53	Wild-type	IV	3	3	diffuse
	20	8.23	Wild-type	IV	4	3	diffuse
	10	2.59	Mutant-type	II	2	1	intestinal
	19	0.69	Mutant-type	II	3	0	intestinal
	21	0.20	Mutant-type	IIIA	3	1	diffuse
	2	91.89	Mutant-type	IIIB	3	2	intestinal
	11	2.83	Mutant-type	IV	3	3	diffuse
Methylated	3	0.99	Wild-type	IB	2	0	intestinal
	28	0.12	Wild-type	IB	2	0	intestinal
	4	0.54	Wild-type	IIIA	3	1	diffuse
	29	0.88	Wild-type	IB	2	0	intestinal
	23	0.08	Wild-type	IIIA	2	2	diffuse
	6	6.99	Wild-type	IIIB	3	2	intestinal
	9	0.94	Wild-type	IV	3	3	intestinal
	13	0.26	Wild-type	IV	3	3	intestinal
	1	0.37	Mutant-type	IB	2	0	diffuse
	25	1.13	Mutant-type	IIIA	2	2	diffuse

[a] The units are arbitrary, and we calculated the *TSP1* mRNA expression in tumor tissues by standerdization with 1 μg total RNA of the HSC-39 gastric carcinoma cells, taken as 1.0.
[b] Stage was classified according to the criteria of the UICC TNM Stage Grouping 5 th edition, 1997, Stomach.
[c] T garde was classified according to the criteria of the UICC TNM Stage Grouping 5 th edition, 1997, Stomach.
[d] N grade was classified according to the criteria of the UICC TNM Stage Grouping 5 th edition, 1997, Stomach.
[e] Histology was classified according to the criteria of Lauren.

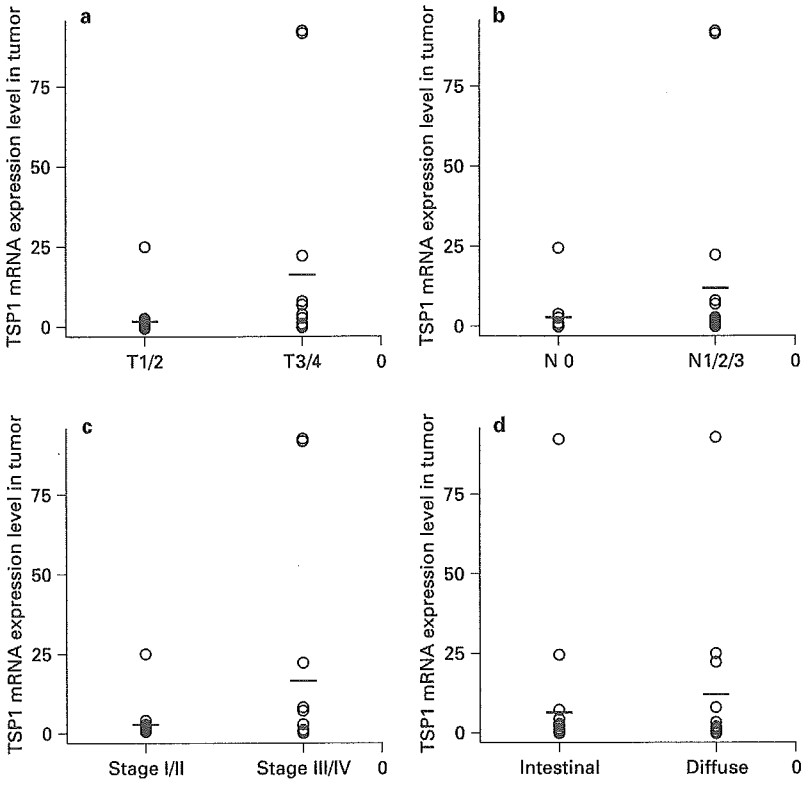

Fig. 1. *TSP1* mRNA expression level in gastric carcinoma. Each point represents the *TSP1* mRNA expression level of an individual tumor. Horizontal bar represents the mean of *TSP1* mRNA expression levels. The *TSP1* mRNA expression levels were not significantly associated with the T grade (p = 0.384; Mann-Whitney U test, **a**), the N grade (p = 0.524; Mann-Whitney U test, **b**), the tumor stage (p = 0.918; Mann-Whitney U test, **c**), and histological type (p = 0.427; Mann-Whitney U test, **d**).

Results

mRNA Expression Levels of TSP1 in Gastric Carcinoma Tissues

We measured *TSP1* mRNA expression levels by quantitative RT-PCR in 30 gastric carcinoma tissues and 10 non-neoplastic mucosae. The overall results of quantitative RT-PCR analysis are summarized in table 1. As shown in figure 1, the *TSP1* mRNA expression levels were not significantly associated with the T grade (depth of tumor invasion) (p = 0.384; Mann-Whitney U test), the N grade (degree of lymph node metastasis) (p = 0.524; Mann-Whitney U test), the tumor stage (p = 0.918; Mann-Whitney U test), or histological type (p = 0.427; Mann-Whitney U test).

TSP1 Promoter Methylation Status and mRNA Expression Levels in Gastric Carcinoma Tissues

To determine whether promoter hypermethylation of the *TSP1* gene is associated with transcriptional silencing, the 30 gastric carcinoma samples were assayed by MSP. Representative results are shown in figure 2. A methylated allele was detected in 2 specimens (specimens 9 and 13). The overall results of MSP are summarized in table 1. In total, promoter hypermethylation of the *TSP1* gene was found in 10 (33%) of the 30 gastric carcinomas. As shown in table 1 and figure 3a, the *TSP1* mRNA expression levels in tumor tissues with promoter hypermethylation of *TSP1* (1.23 ± 2.06; mean ± standard deviation) were significantly lower than in those without promoter hypermethylation (13.20 ± 27.89) (p = 0.017; Mann-Whitney U test) and in the non-neoplastic mucosae (2.19 ± 1.25) (p = 0.029; Mann-Whitney U test). Hypermethylation of the *TSP1* gene did not correlate with the T grade (p = 0.700; Fisher's exact test), the N grade (p = 0.690; Fisher's exact test), the tumor stage (p = 1.000; Fisher's exact test), or the histological type (p = 1.000; Fisher's exact test) (table 2).

p53 Mutation Status and mRNA Expression Levels of the TSP1 Gene in Gastric Carcinoma Tissues

We examined whether a correlation exists between *TSP1* mRNA expression level and *p53* mutation status.

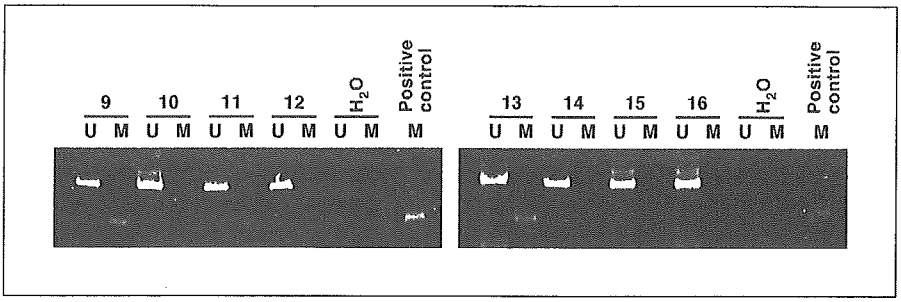

Fig. 2. MSP of *TSP1*. Primer sets were used, unmethylated (U), methylated (M). The methylated allele was detected in specimens 9 and 13.

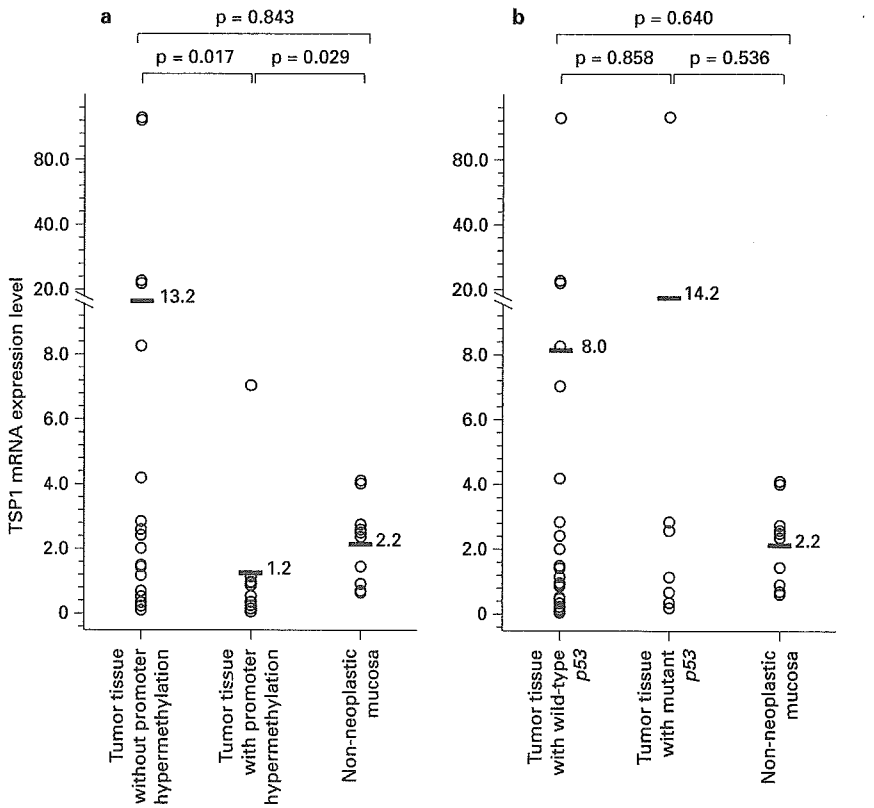

Fig. 3. *TSP1* mRNA expression level in gastric carcinoma. Each point represents the *TSP1* mRNA expression level of an individual specimen. Horizontal bar represents the mean of *TSP1* mRNA expression levels. The *TSP1* mRNA expression level was significantly associated with the promoter methylation status (p = 0.017; Mann-Whitney U test, **a**), however, was not significantly associated with *p53* mutation status (p = 0.858; Mann-Whitney U test, **b**).

Our previous results of *p53* PCR-SSCP analysis are summarized in table 1 [34]. As shown in table 1 and figure 3b, *TSP1* mRNA expression level was not significantly associated with *p53* mutation status (p = 0.858; Mann-Whitney U test), and *p53* mutation status did not correlate with *TSP1* promoter hypermethylation (p = 1.000; Fisher's exact test) (table 2). We compared the mRNA expression level of *TSP1* between wild-type *p53* and mutant *p53* in gastric carcinoma cases without promoter hypermethylation of *TSP1*, however, we did not find association between *TSP1* mRNA expression level and *p53* mutation status (*TSP1* mRNA expression level: wild-type p53, 11.05 ± 23.86 (mean ± SD); mutant p53, 19.65 ± 40.40; p = 1.000; Mann-Whitney U test).

Discussion

We found promoter hypermethylation of the *TSP1* gene in gastric carcinomas with low levels of *TSP1* mRNA expression, suggesting that hypermethylation of the *TSP1* promoter region plays an important role in the inactiva-

Table 2. Association between *TSP1* methylation status and clinicopathological features and *p53* mutation status in gastric carcinomas

		TSP1 methylation status		p value[a]
		methylated	unmethylated	
T grade	T0, T1, T2	6	9	0.700
	T3, T4	4	11	
N grade	N0	3	6	0.690
	N1, N2, N3	7	14	
Stage	Stage I/II	5	11	1.000
	Stage III/IV	5	10	
Histological classification	intestinal	6	11	1.000
	diffuse	4	10	
p53 mutation status	mutant *p53*	2	5	1.000
	wild-type *p53*	8	16	

[a] Fisher's exact test.

tion of *TSP1* in gastric carcinomas. However, there were some exceptions that did not show reduced expression of *TSP1* mRNA even with promoter hypermethylation. Because detection of promoter methylation by MSP shows only the presence of some methylated DNA molecules in the sample analyzed, only one allele can be methylated , or only a small fraction of cancer cells might have methylated allele. Our study showed that the mutation status of *p53* was not associated with *TSP1* mRNA expression. In addition, though p53 is known to induce *TSP1* expression when the promoter of *TSP1* is functioning, there was no association between *TSP1* mRNA expression level and *p53* mutation status in gastric carcinoma cases without promoter hypermethylation of *TSP1* gene. However, earlier studies by other investigators showed an inverse correlation between TSP1 gene expression and p53 accumulation by immunohistochemical methods [18–21]. In fact, it has been observed that colon cancer cells expressed TSP1 protein and p53 accumulation reciprocally in the same nests using double-immunohistostaining analysis [20]. Thus, our negative results may be due to differences in the methodology for detecting *TSP1* gene expression and *p53* mutation status. To resolve these differences, it may be necessary to measure TSP1 gene expression and p53 accumulation by immunohistochemical staining.

A number of different methyl-CpG binding proteins (MBD1 to 4 and MeCP2) compete with transcription factors and prevent them from binding to promoter sequences [35]. Promoter hypermethylation of the *TSP1* gene prevents transcription factors such as p53 from binding to promoter sequences of the *TSP1* gene. Another gene that is regulated by promoter hypermethylation or p53 is *MGMT* [36, 37]. In our previous observation [38], we found that *MGMT* gene expression is associated with promoter hypermethylation and not *p53* mutation status.

We found no correlation between *TSP1* mRNA expression levels and clinicopathological features. The present study did not include measurement of microvessel density. Therefore, future studies should investigate the relation between TSP1 gene expression and microvessel density.

In conclusion, our results suggest that transcriptional inactivation of *TSP1* by aberrant DNA methylation of the promoter region may participate partly in stomach carcinogenesis through *TSP1* down-regulation.

Acknowledgements

We thank Mr. K. Tominaga, Mr. K. Ogawa, Ms. M. Oda, Ms. Y. Ouchi, and Ms. K. Nakamura for excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. This work was supported, in part, by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports, and Technology of Japan, and from the Ministry of Health, Labor, and Welfare of Japan.

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Cytological Grading For Ductal Carcinoma Of Breast Is Effective On Estimating Hormone receptors and Her2 Gene Amplification.

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Aim; To clarify the efficacy of cytological grading on the fine needle aspiration cytology (FNAC) for ductal carcinoma of breast.

Background; In the FNAC of breast tumors, descriptive reporting of cellular change is generally considered to be better than reporting only of malignancy or not. However, description on cellular change is subjective and uncertain whether it is representing the clinicopathological status of tumors.

Materials and Methods; Sixty-seven tumors of ductal carcinoma of breast were enrolled in the present study. They were all graded according to the classification by Robinson et al. (Lancet, 1994) on preoperative FNAC and were then resected surgically. Immunoreactivities for estrogen receptor (ER), progesterone receptor (PgR) and Her2 gene were graded from none to 3+ on formalin-fixed, paraffin-embedded specimens. Her2 gene amplification was calculated by FISH with fresh samples obtained at surgery. Moreover, cytological findings were compared among ten CTs unfamiliar with grading.

Results; Out of 67 tumors, 35 (52.2%), 27 (40.3%) and five (7.5%) were graded as 1, 2 and 3, respectively. Their mean ages were 61.5, 58.6 and 57.6 years old. As cytological grade increased, scirrhous carcinoma tended to decrease while papillo-tubular and solid-tubular carcinomas tended to increase. ER and PgR expressions were reversely correlated with cytological grading while Her2 gene amplification was correlated with cytological grading. In the cytological findings, estimation of nuclear size and edge was mostly variable among ten CTs unfamiliar with the grading and their findings has improved after a brief teaching.

Conclusion; Cytological grading of FNAC by Robinson et al. for ductal carcinoma of breast is effective on estimating the hormone receptors and Her2 gene amplificaion when it is performed by trained CTs for the grading.

発行者　新規がん予防・早期発見システムを用いた
包括的ながん予防の開発研究班
主任研究者　田原　榮一
平成 18 年 3 月 31 日発行

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