RESEARCH COMMUNICATION

Lack of an Association between Serum Level of Transforming Growth Factor β-1 and Stomach Cancer Risk in the JACC Study

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Abstract

Alterations in the serum concentration of transforming growth factor β-1 (TGFβ1) have been observed in gastric cancer patients. No study, however, has ever examined the association between the serum TGFβ1 level and stomach cancer prospectively. We conducted a prospective, nested case-control analysis among apparently healthy men and women who were followed for up to 8 years in the JACC Study to assess whether serum level of total TGFβ1 is associated with a subsequent risk of stomach cancer. The concentration of serum TGFβ1 in previously collected blood samples was analyzed by ELISA for 209 individuals in whom a diagnosis of stomach cancer was documented, and for 409 controls matched with them for gender, age and study area. Baseline blood levels of TGFβ1 were not related to the risk of stomach cancer in either men or women, a finding unchanged even after adjustment for potential confounders. The multivariate-adjusted odds ratio of stomach cancer in men and women was 1.10 (95% CI, 0.82 to 1.48) and 1.09 (95% CI, 0.80 to 1.48), respectively, for each increase of 1 SD in the TGFβ1 value. In conclusion, serum TGFβ1 levels were not associated with increased risks of subsequent stomach cancer.

Key Words: transforming growth factor β - stomach cancer - nested case-control study - *Helicobacter pylori* - JACC study

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Introduction

Transforming growth factor β-1 (TGFβ1) is a member of a family of dimeric polypeptide growth factors that virtually every cell in the body produces and has receptors for (Gold, 1999). TGFβ1 exerts profound regulatory effects on many developmental and physiological processes. Mutations in the TGFβ1, its receptors, or intracellular signaling molecules associated with TGFβ1 are considered important in the pathogenesis of cancer (de Caestecker et al, 2000; Miyazono et al, 2003).

Elevated serum TGFβ1 levels have been observed in gastric cancer patients with poor prognoses (Saito et al, 2000), or in those of advanced stage with poorly differentiated or invasive type adenocarcinoma (Niki et al, 1996). The severity of the disease stage was also related to the degree of loss of Runx3, a transcriptional factor in TGFβ1 signaling (Li et al, 2002; Moss, 2003).

Approximately half of human gastric cancer cells lack expression of Runx3 due to hemizygous deletion and DNA methylation of the promoter of Runx3. The gastric epithelial cells of $Runx3^{-/-}$ mice were found resistant to the growth-

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inhibiting and apoptosis-inducing effects of TGFβ1 (Li et al, 2002). They demonstrated that restoring the Runx3 function by gene transfection in cells lacking Runx 3 caused a marked inhibition of tumour growth, showing that loss of the Runx3 function is a cause rather than an effect of gastric malignant transformation (Li et al, 2002).

It is possible that there may be latent disturbances in the TGFβ1/Runx3 signaling pathway in the stomach of apparently healthy individuals who will eventually develop gastric cancer, and this may lead to alterations in their TGFβ1 level. Therefore, in a case-control study nested within a large-scale cohort of Japanese men and women, we examined the serum level of TGFβ1 to assess whether inter-individual variability in the level of serum TGFβ1 is associated with a subsequent incidence of stomach cancer.

Subjects and Methods

JACC Study

This study was part of the Japan Collaborative Cohort Study For Evaluation of Cancer Risk Sponsored by the Ministry of Education, Culture, Sports, Science and Technology of Japan (JACC Study), a nationwide multicenter collaborative study to prospectively evaluate the various risks or protective factors involved in cancer mortality and incidence. The JACC study was started between 1988 and 1990, enrolling apparently healthy subjects living in 45 areas of Japan, and collecting baseline data using a self-administered questionnaire, which covered the medical history and included lifestyle-related items such as drinking and smoking, level of education, and family history of several medical conditions including cancer. Sampling methods and detailed protocols of the JACC study are described elsewhere (Ohno and Tamakoshi, 2001; Yatsuya et al, 2002; Yatsuya et al, 2004). We followed 110,792 subjects (46,465 men and 64,327 women), aged 40 to 79 years at baseline. About one-third of the cohort members (n=39,293) also donated a residual serum sample (about 2 ml) used for the general health checkup. Each sample was partitioned into 0.3 to 0.5 ml aliquots and stored at -80 C until laboratory analysis. Informed consent procedures were approved by the Ethics Committee of Medical Care and Research, University of Occupational and Environmental Health, Kitakyushu with which the chief investigator of the stomach cancer group is affiliated, and the Ethical Board of the Nagoya University School of Medicine, Japan with which the present chairman of the JACC study is affiliated.

Follow-up and Identification of Stomach Cancer Cases, and Selection of Control Subjects

Vital statuses of the participants were checked annually at each regional research center with permission to review their population-register sheets from the Ministry of Public Management, Home Affairs, Posts and Telecommunications. The incidence of cancer was ascertained in 24 study areas (n=65,184) and coded according to the tenth revision of International Classification of Diseases and the second edition of International Classification of Diseases for Oncology. These data were collected at the central office of the Research Committee.

We first restricted the subjects to those who lived in the study areas where the cancer incidence was ascertained. We then excluded 857 participants with a self-reported history of cancer at any site. Among the remaining 64,327 subjects, a diagnosis of stomach cancer 12 or more months after cohort recruitment was documented in 804 cases up to the end of 1997. Serum had been obtained from 218 out of the initial 804 cases. However, 8 cases lacking enough serum for laboratory analysis, and one case without an eligible control subject were excluded. Thus, the study reported here included 209 cases (109 men and 100 women) in total. Lag times between blood sampling and stomach cancer diagnosis varied between 12 and 113 months (median 50 months). Each of these subjects was matched with two control subjects for gender, age at recruitment (as near as possible) and study area, who had also provided an adequate baseline blood sample and who were alive and remained free of confirmed cancer as of the end of 1997. Owing to a lack of eligible subjects, a few sets (n=10) contained only 1 control; thus, a total of 409 controls were available for the present analysis. Because information on the location of cancer within the stomach or its histological type was not available in all cases, we did not use it to classify cases.

Laboratory Assays

Serum samples from each case and matched controls were retrieved from storage and shipped on dry ice to a single laboratory (SRL, Inc., Hachioji, Japan) for the assay by trained staff who were blinded to the case/control status of the samples. None of the samples had been previously defrosted. The total TGFb1 was measured by sandwich enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN). Results were expressed in nanograms per milliliter (ng/ml). The assay range was 20-2,180 ng/ml. Intra- and inter-assay coefficients of variation were 2.67-6.79% and 4.17-6.16%, respectively.

H. pylori infection was investigated serologically using HM-CAPTM (Enteric Products, Westbury, NY, USA) with antigen from Japanese subjects (J-HM-CAP); and a serum titer of immunoglobulin G antibodies 2.3 or greater was defined as positive infection.

Definition of Confounding Variables

Risk factors that could potentially confound the relation between serum TGFβ1 and the stomach cancer risk other than *H. pylori* infection (Hamajima et al, 2004) were collected at baseline, using self-administered questionnaires (Yatsuya et al, 2002; Hoshiyama et al, 2002; Fujino et al, 2002; Mizoue et al, 2003; Khan et al, 2004). A family history of stomach cancer was defined as any subject having at least one first-degree relative with a history of stomach cancer. A drinking habit was first categorized into three statuses (none, past, present). If present, it was further categorized into two

levels by weekly consumption (light, heavy), i.e., daily alcohol consumption times days of drinking per week. Smoking status was also classified into three categories (never, past, current). Consumption frequency of vegetables, citrus fruits and green tea was initially assessed at five levels (everyday, 3-4 times a week, 1-2 times a week, 1-2 times a month, and seldom); for the present analysis, the former 2 and the latter 3 categories were combined. Salty-food preference was categorized into three categories (dislike, neutral, like). Information on educational levels was measured as the age at which formal schooling was completed, and was further classified into two categories: <=15-years-old (corresponds to <=9 years of schooling) and >=16-years-old (corresponds to >=10 years of schooling). Missing values in each variable were treated as an additional category within the variable, and were included in the analyses.

Statistical Analysis

We compared the baseline characteristics of case subjects and control subjects by the one-way analysis of variance for continuous variables and the *chi*-squared test for categorical variables. We then performed logistic regression analysis, conditioned on the matching variables of gender, age and study area. Because serum TGFβ1 was normally distributed (Kolmogorov-Smirnov test), we treated it as a continuous variable, and presented the odds ratios (ORs) that represented a change in risk per increment of 1 SD (8.0 in men and 8.2 in women), which was calculated from the distribution among the control subjects. Adjusted estimates of risk were obtained using multivariate models that also controlled for the covariates listed above. TGFβ1 was temporarily treated as a categorical variable (quartile) in another logistic model with no assumption about the relationship between TGFβ1 and the incidence of stomach cancer. This model yielded identical results (data not shown), and we only presented the results using TGFβ1 as a linear contributor to the log-odds. The 95% confidence intervals (95% CIs) are presented for all ORs. All reported P values are two-sided. All analyses were performed separately for men and women with the SPSS statistical package for Windows version 12.0.

Results

Serum TGFβ1 levels in control subjects ranged from 15.0 to 65.3 ng/ml (mean±SD: 36.5±8.0) in men, and 17.6 to 58.8 ng/ml (35.8±8.2) in women. Pearson's correlation coefficients between serum TGFβ1 level and age were -0.21 and -0.17 in male and female controls, respectively (both P<0.05). Table 1 shows the baseline characteristics of the 209 cases and the 409 matched controls. The proportion of individuals infected with *H. Pylori* was high even in control subjects (80.1% and 79.3% for men and women, respectively). However, it was higher in cases with stomach cancer among men and significantly higher among women (87.2% and 91.0% for men and women, respectively: P values for the *chi*-squared test were 0.12 in men, and 0.013 in women; case vs. control). In this sample, women diagnosed with stomach cancer were more likely to have a family history of stomach cancer, whereas men were not. The proportion of case subjects who reported a history of stomach cancer in a first-degree relative was 14.7% and 24.0% in men and women, respectively vs. 15.6% in men and 14.6% in women among control subjects (P values for the *chi*-squared test were 0.97 in men, and 0.14 in women; case vs. control). Cases and controls for both sexes did not differ significantly in terms of smoking status, alcohol intake, or other diet-related items. The proportion of women with a higher educational level seemed to be higher in controls compared to that in cases. The levels of TGFβ1 were higher in subjects with *H. pylori* infection than those without such an infection (36.9 ng/ml vs. 35.7 ng/ml in men, respectively, and 36.1 ng/ml vs. 35.0 ng/ml in women, respectively), but the differences were statistically significant in neither sexes (P=0.30 and 0.37 for men and women, respectively). The serum level of TGFβ1 was not related to smoking status in this sample (data not shown).

Table 2 shows the relation of baseline blood levels of TGFβ1 to the risk of stomach cancer. The levels of TGFβ1 were unrelated to the risk of stomach cancer incidence in both men and women. The multivariate-adjusted odds ratios of stomach cancer in men and women were 1.10 (95 % confidence interval, 0.82 to 1.48) and 1.09 (95 % confidence interval, 0.80 to 1.48), respectively, for each increase of 1 SD in the TGFβ1 value.

Discussion

This is the first study to examine the association between serum level of TGFβ1 and stomach cancer risk in a prospective, nested case-control study. We found no association between TGFβ1 levels and a subsequent risk of stomach cancer. In previous clinical studies, elevated serum TGFβ1 levels have been observed in gastric cancer patients with poor prognoses (Saito et al, 2000), those in an advanced stage, or those with poorly differentiated or invasive type adenocarcinoma (Niki et al, 1996). Others, however, did not necessarily find such an association of blood TGFβ1 levels with tumour stages (Maehara et al, 1999). The blood TGFβ1 level has also been proposed as a tumour marker for colorectal cancer (Shim et al, 1999; Narai et al, 2002), and nasopharyngeal cancer (Xu et al, 1999), but the results are inconsistent with the spectrum of cancers (Ghellal et al, 2000).

TGFβ1 is a multifunctional polypeptide, and its negative regulation of cellular proliferation has been shown to constitute a tumour-suppressor pathway. A reduction in TGFβ1 signaling in tumour cells is often accompanied by an increased secretion of TGFβ1 itself, which would promote tumourigenesis through its positive effects on angiogenesis, the accumulation of extracellular matrix glycoproteins and cell adhesion proteins, and immune suppression (Gold 1999; de Caestecker et al, 2000; Miyazono et al, 2003). We

Table 1. Baseline Characteristics of The Study Participants

Characteristic	Men (n=320)			Women (n=298)		
	Cases (*n*=109)	Controls (*n*=211)	p^a-value	cases (*n*=100)	Controls (*n*=198)	p^a-value
Age category: no. (%)						
40-49	6 (5.5)	12 (5.7)	Matching	9 (9.0)	18 (9.1)	Matching
50-59	23 (21.1)	44 (20.9)	factor	33 (33.0)	66 (33.3)	factor
60-69	53 (48.6)	107 (50.7)		40 (40.0)	79 (39.9)	
70-79	27 (24.8)	48 (22.7)		18 (18.0)	35 (17.0)	
Age (years): mean±SD	63.7±7.9	63.4±7.9		61.6±8.2	61.5±8.3	
H. pylori Infection: no. (%)						
Present	95 (87.2)	169 (80.1)	0.12	91 (91.0)	157 (79.3)	0.013
Absent	14 (12.8)	42 (19.9)		9 (9.0)	41 (20.7)	
Family history of stomach cancer: no. (%)						
Present	16 (14.7)	33 (15.6)	0.97	24 (24.0)	29 (14.6)	0.14
Absent	88 (80.7)	168 (79.6)		73 (73.0)	163 (82.3)	
Missing	5 (4.6)	10 (4.7)		3 (3.0)	6 (3.0)	
Smoking status: no. (%)						
Never	16 (14.7)	37 (17.5)	0.24	89 (89.0)	174 (87.9)	0.52
Past	31 (28.4)	62 (29.4)		1 (1.0)	1 (0.5)	
Current	60 (55.0)	99 (46.9)		4 (4.0)	4 (2.0)	
Missing	2 (1.8)	13 (6.2)		6 (6.0)	19 (9.6)	
Alcohol intake: no. (%)						
None	21 (14.1)	44 (20.9)	0.37	71 (71.0)	150 (75.8)	0.82
Past	8 (7.3)	7 (3.3)		3 (3.0)	4 (2.0)	
Light drinker	37 (33.9)	82 (38.9)		13 (13.0)	21 (10.6)	
Heavy drinker	23 (21.1)	34 (16.1)		0 (0.0)	1 (0.5)	
Missing	20 (18.3)	44 (20.9)		13 (13.0)	22 (11.1)	
Educational level: no. (%)						
<= 9 years of schooling	28 (25.7)	70 (32.2)	0.39	35 (35.0)	51 (25.8)	0.21
>= 10 years of schooling	60 (55.0)	105 (49.8)		46 (46.0)	110 (55.6)	
Missing	21 (19.3)	36 (17.1)		19 (19.0)	37 (18.7)	
Salty-food preference: no. (%)						
Dislike	13 (11.9)	36 (17.1)	0.69	25 (25.0)	31 (15.7)	0.13
Neutral	39 (35.8)	72 (34.1)		38 (38.0)	100 (50.5)	
Like	41 (37.6)	73 (34.6)		19 (19.0)	37 (18.7)	
Missing	16 (14.7)	30 (14.2)		18 (18.0)	30 (15.2)	
Tomatoes: no. (%)						
<= 1-2 times/week	53(48.6)	113 (53.6)	0.68	49 (49.0)	98 (49.5)	0.61
>= 3-4 times/week	45 (41.3)	77 (36.5)		45 (45.0)	82 (41.4)	
Missing	11 (10.1)	21 (10.0)		6 (6.0)	18 (9.1)	
Citrus fruits: no. (%)						
<= 1-2 times/week	48 (44.0)	80 (37.9)	0.51	29 (29.0)	68 (34.3)	0.64
>= 3-4 times/week	48 (44.0)	99 (46.9)		59 (59.0)	109 (55.1)	
Missing	13 (11.9)	32 (15.2)		12 (12.0)	21 (10.6)	
Spinach and green vegetables: no. (%)						
<= 1-2 times/week	28 (25.7)	63 (29.9)	0.54	30 (30.0)	60 (30.3)	0.96
>= 3-4 times/week	65 (59.6)	112 (53.1)		58 (58.0)	112 (56.6)	
Missing	16 (14.7)	36 (17.1)		12 (12.0)	26 (13.1)	
Carrots and pumpkins: no. (%)						
<= 1-2 times/week	47 (43.1)	91 (43.1)	0.93	36 (36.0)	81 (40.9)	0.67
>= 3-4 times/week	53 (48.6)	100 (47.4)		54 (54.0)	101 (51.0)	
Missing	9 (8.3)	20 (9.5)		10 (10.0)	16 (8.1)	
Green tea: no. (%)						
<= 1-2 times/week	11 (10.1)	13 (6.2)	0.41	11 (11.0)	21 (10.6)	1.00
>= 3-4 times/week	92 (84.4)	183 (86.7)		83 (83.0)	165 (83.3)	
Missing	6 (5.5)	15 (7.1)		6 (6.0)	12 (6.1)	

[a] P-value by m x n *chi-squared* test.

Table 2. Multivariate Conditional Logistic Regression Models Examining the Relation Between the Serum Transforming Growth Factor β-1(TGFβ1) and The Risk of Stomach Cancer[a]

Variables adjusted for	Men (n=320)			Women (n=298)		
	No. of Cases/ No. of Subjects	OR (95%CI)	*p*-value	No. of Cases/ No. of Subjects	OR (95%CI)	*p*-value
Univariate	109/320	1.03 (0.79-1.35)	0.83	100/298	1.03 (0.79-1.35)	0.83
H. pylori infection		1.04 (0.81-1.35)	0.75		1.04 (0.79-1.37)	0.77
H. pylori infection and family history of stomach cancer		1.04 (0.81-1.35)	0.75		1.06 (0.80-1.40)	0.69
Multivariate-adjusted		1.10 (0.82-1.48)	0.51		1.09 (0.80-1.48)	0.60

[a] The level of TGFβ1 was analyzed as a continuous variable. The odds ratios (ORs) are per increment of 1 SD (8.0 in men and 8.2 in women) in TGFβ1 value. CI denotes confidence interval, and *H. pylori* is *Helicobacter pylori*.
[b] Adjusted for H. pylori infection, family history of stomach cancer, smoking status (never, past, current), drinking habits self-rated preference for salty foods (dislike, neutral, like), consumption of green-yellow vegetables, citrus fruits and green tea (<= 3-4 times a week, >= 1-2 times a week), and educational level (<= 9 years of schooling, >= 10 years of schooling). Missing values in each variable were treated as an additonal category.

conducted the present analysis to examine serum levels of TGFβ1 in apparently healthy individuals who would develop stomach cancer during the follow-up as indicated by elevated levels of TGFβ1 mRNA in tumour tissues and elevated levels of TGFβ1 in the serum or plasma of patients with various malignant tumors that have been described (Saito et al, 2000; Niki et al, 1996; Shim et al, 1999; Narai et al, 2002; Xu et al, 1999; Ghellal et al, 2000).

Our finding of a null association may be related to study designs and limitations. First, the source of serum TGFβ1 has not been identified; increased tissue TGFβ1 expression and levels have been observed not only in gastric cancer patients but in subjects with a family history of gastric cancer (Ebert et al, 2000). It is possible that there might have been differences in TGFβ1 expression or tissue TGFβ1 levels between cases and controls even in the present sample (Maehara et al, 1999). Further study is needed that includes measurements of tissue TGFβ1 levels or their expression.

The mean TGFβ1 levels observed in the present study were comparable to those in previous studies that measured serum TGFβ1 levels in normal controls (Shim et al, 1999; Xu et al, 1999; Wu et al, 2002). In the present study, we measured total TGFβ1 levels, the use of which may possibly constitute another limitation, since it combines two different pools of TGFβ1, i.e., the active and the latent pools (Xu et al, 1999). TGFβ1 is secreted in a latent form, which appears to be activated by a poorly understood mechanism before interacting with ubiquitously occurring TGFβ receptors on cells. Under normal physiological conditions, the concentration of the active form is usually less than one-third of total TGFβ1, whereas the proportion of the active form of TGFβ1 had increased to more than half of the total value in the serum of nasopharyngeal cancer patients (Xu et al, 1999). Thus, the lack of an association in our study may be due to the fact that we did not obtain the proportion of the active pool.

Since our analyses are based on a single baseline measurement, and may not accurately reflect TGFβ1 levels over long periods. This source of variability, i.e., random misclassification, could have biased our results toward the null hypothesis.

We did not classify cases by the location of cancer within the stomach or by the histological type because the relevant information was not available in all cases. It was reported that serum TGFβ1 level differed by histopathological features of the tumour (Niki et al, 1996). This may be another reason for the null association.

One of the strengths of the present study is the control for *H. pylori* infection, since it may be associated with both TGFβ1 levels and the incidence of stomach cancer (Lindholm et al, 1998). Cases in the present study were more likely to be infected with *H. pylori* among men (P=0.12) and significantly among women (P=0.013). We found that the serum TGFβ1 levels of both men and women in those with *H. pylori* infection were higher than those without such an infection, though not to a significant degree. Increased expression of TGFβ1 in *H. pylori*-infected individuals has been observed in some studies but not in others (Lindholm et al, 1998; Ohana et al, 2003; Crabtree et al, 2004). Since age may interact with *H. pylori* infection against the TGFβ1 level, we included an age-infection interaction term in the model, but still found identical results (data not shown).Further study is needed to elucidate the effect of *H. pylori* infection on TGFβ1 levels.

In summary, this prospective data did not support the hypothesis that serum TGFβ1 levels were related to a risk of stomach cancer. Elevated TGFβ1 serum levels observed in certain gastric cancer patients may be due to an existing tumour. Further study is necessary to obtain both active and latent forms of serum TGFβ1 together with measurements of gastric tissue TGFβ1 levels.

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