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ORIGINAL ARTICLE

A polymorphism of C-to-T substitution at *–31 IL1B* is associated with the risk of advanced gastric adenocarcinoma in a Japanese population

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Abstract Proinflammatory cytokine gene polymorphisms have been demonstrated to associate with gastric cancer risk, of which *IL1B–31T/C* and *–511C/T* changes have been well investigated due to the possibility that they may alter the *IL1B* transcription. The signal transduction target upon interleukin 1 beta (IL1β) stimulation, the nuclear factor of kappa B (NFκB) activation, supports cancer development, signal transduction in which is mediated by FS-7 cell-associated cell surface antigen (FAS) signaling. Based on recent papers describing the prognostic roles of the polymorphisms and the NFκB functions on cancer development, we sought to determine if Japanese gastric cancer patients were affected by the *IL1B –31/–511* and *FAS–670* polymorphisms. A case-control study was conducted on incident gastric adenocarcinoma patients (n=271) and age–gender frequency-matched control subjects (n=271). We observed strong linkage disequilibrium between the *T* allele at –511 and the *C* allele at –31 and between the *C* allele at –511 and the *T* allele at –31 in *IL1B* in both the cases and controls (R^2=0.94). Neither *IL1B–31, –511* nor *FAS–670* polymorphisms showed significantly different risks of gastric adenocarcinoma. Though *FAS–670* polymorphisms did not show any significant difference, the proportion of subjects with *IL1B–31TT* (or *IL1B–511CC*) increased according to stage (trend P=0.019). In particular, subjects with stage IV had a two times higher probability of having either *IL1B–31TT* (or *IL1B–511CC*) genotype compared with stage I subjects. These observations suggest that *IL1B–31TT* and *IL1B–511CC* are associated with disease progression.

Keywords Gastric cancer · Polymorphism · *IL1B* · *FAS* · NFκB

Abbreviations

NFκB	Nuclear factor of kappa B
HP	*Helicobacter pylori*
PCR-CTPP	Polymerase chain reaction with confronting two-pair primers
HERPACC	The Hospital-based Epidemiologic Research Program at Aichi Cancer Center

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Introduction

Chronic inflammation appears to support the formation of epithelial tumors by which a causal relationship between inflammation and cancer has long been suspected. As described in earlier studies, tumor promotion and progression appear to be maintained by the interaction between transformed cells and their close surrounding cells as innate immune cells, fibroblasts, and endothelial cells in chronic inflammation although the exact mechanism is almost unknown (Cordon-Cardo and Prives 1999; Balkwill and Mantovani 2001; Clevers 2004). Accumulated information on chronic inflammation of gastric mucosa has revealed that infection by the gram-negative bacteria *Helicobacter pylori* (HP) is one of the causative factors in gastric carcinogenesis (Group 2001; Uemura et al. 2001; Peek and Blaser 2002).

Another characteristic feature of gastric cancer risk appears to be mediated by polymorphisms of *IL1B*, one of the proinflammatory cytokines induced on inflammation. Indeed, since a first report demonstrated the positive association between higher gastric cancer risk and *IL1B–31C* allele in patients in the United States (El-Omar et al. 2000, 2001), epidemiological studies in Portugal (Machado et al. 2003), China (Zeng et al. 2003), the United States (El-Omar et al. 2003), and Mexico (Sicinschi et al. 2006) have demonstrated supportive evidence on either *IL1B–31C* or *IL1B–511T* that is genetically linked with *IL1B–31C*. Concerning biological functions of the polymorphism, a first report indicated that *IL1B–31C to T* change generates a *TATA* sequence providing a transcriptional factor binding element at *–31IL1B* gene (El-Omar et al. 2000) and that no increased binding of transcription factor was seen among *IL1B–31C*, *ILB–511C*, and *IL1B–511T* in human monocytes by lipopolysaccharide (LPS) stimulation (El-Omar et al. 2000). Through the original (El-Omar et al. 2000) and the erratum articles (El-Omar et al. 2001), *IL1B–31T* was finally suggested for the probable hypo interleukin 1 beta (IL1β) production allele. Indeed, this finding was supported by in vivo studies demonstrating that the *IL1B–31C* allele was a higher IL1β protein secretion allele than the *IL1B–31T* allele (Hwang et al. 2002; Hall et al. 2004). However, inconsistent epidemiological results on *ILB–511C* linked with *IL1B–31T* were also reported from Chinese (Yang et al. 2004), Italian (Palli et al. 2005), and Korean cancer patients (Chang et al. 2005), and a higher production of IL1β or transcription of the *IL1B* gene was also reported in *IL1B–31T* allele in in vivo (Chang et al. 2005; Xuan et al. 2005) and in vitro (Kimura et al. 2004) studies. Thus, the exact biological role of the nucleotide substitutions on gastric cancer risk remains unclear.

Signaling through IL1β and the receptor IL1R is generally believed the main response to the infection, and mucosal injury generates transcriptional activation through the nuclear factor kappa B (NFκB) that belongs to the reticulo-endotheliosis (Rel) family of proteins (Karin et al. 2002; Senftleben and Karin 2002; Lin and Karin 2003; Bonizzi and Karin 2004). NFκB is also a key player in anti-apoptotic function (Balkwill and Mantovani 2001; Karin et al. 2002; Lin and Karin 2003; Bonizzi and Karin 2004; Clevers 2004) in which activation correlates with gastric cancer (Sasaki et al. 2001) and colitis-associated cancer development (Greten and Karin 2004). Because the activation is needed to generate a microenvironment helping the progression of transformed cells, cancer progression appears to be promoted by the activation. The activation signaling pathway can be mediated by the dissociation between myeloid differentiation factor 88 (MyD88) and FS-7 cell-associated cell surface antigen (FAS) (CD95; Apo-1)-associated death domain protein (FADD) due to ligation and oligomerization by FAS ligand (Ma et al. 2004). FAS ligation on macrophages promotes chronic inflammation. As the *FAS–670* polymorphism was demonstrated to alter the amounts of expression (Huang et al. 1997; Kanemitsu et al. 2002; Sibley et al. 2003), we hypothesized that the polymorphism might associate with the NFκB activation magnitude.

In this study, we sought to determine if Japanese patients were affected by the polymorphism of *IL1B* at –31 and –511 positions in a case-control study, and further, whether the risk was also altered by the *FAS–670* polymorphism. We show that although these polymorphisms are not associated with gastric cancer risk, *IL1B* at –31 and –511 polymorphisms may be associated with disease progression while the *FAS–670* polymorphism was neutral for the risk per se.

Materials and methods

Subjects

We have reported in detail elsewhere our ongoing Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) featuring a self-administered questionnaire completed by first-visit outpatients at the Aichi Cancer Center Hospital (ACCH) (Hamajima et al. 2001a, b). In this framework, more than 95% of all first-visit outpatients have participated in the questionnaire study, and

approximately 60% of them agreed to provide blood samples. Before diagnosis, subjects completed a questionnaire with items including lifestyle factors. Subjects in the present study were enrolled between February 2001 and December 2003 in the framework of HERPACC. Among them, subjects diagnosed pathologically as having adenocarcinoma of the stomach were recruited as cases in this study. A total of 271 non-cancer controls, patients who had visited ACCH during the same period and had never been diagnosed as having cancer, were frequency-matched for age–gender strata (age <45, 45–54, 55–65, 65–74, and 75 or older according to gender) with cancer subject cases. After providing written informed consent, study subjects donated a 7-ml sample of peripheral venous blood. Genomic DNA was extracted from the blood samples, as described previously (Ikehara et al. 1998, 2001). This study was approved by the Institutional Review Board of Aichi Cancer Center.

Genotyping

Genotypes of *IL1B–31T/C* were determined by polymerase chain reaction with confronting two-pair primers (PCR-CTPP) (Hamajima et al. 2001a, b). PCR-CTPP was performed in 25 μl of PCR mixture containing 25 pmol each of four primers (IL1B–31 F1: 5′-ACTTCTGCTTTTGAAAGCC-3′; IL1B–31 R5: 5′-TCAGCTGTTAGATAAGCAGTATC-3′; IL1B–31 F4: 5′-AATGTGGACATCAACTGCA-3′; IL1B–31 R2: 5′-CTCCCTCGCTGTTTTTATA-3′), 200 μM dNTPs, 1.0 mM $MgCl_2$, 50 mM KCl_2, 10 mM Tris–HCl (pH 8.5), and 0.5 U AmpliTaq Gold polymerase (PE Applied Biosystems, NJ, USA) under 35 thermal cycling conditions consisting of 1 min at 94°C for denaturing, 1 min at 54°C for annealing, and 1 min at 72°C for extension. PCR product was resolved in 2.0% agarose gels containing ethidium bromide. The genotype was determined under ultraviolet (UV) illumination, with a C allele fragment length of 345 bp and T allele of 266 bp. Genotypes of *IL1B–511C/T* were determined by PCR-based restriction fragment length polymorphism (PCR-RFLP) using specific primer pairs (IL1B–511 F1: 5′-GCATTGATCTGGTTCATCCATG-3′; IL1B–511 R: 5′-GTTCATGGAAGGGCAAGGAG-3′). The reaction was performed in 200 μM dNTPs, 1.0 mM $MgCl_2$, 50 mM KCl_2, 10 mM Tris–HCl (pH 8.5), and 0.5 U AmpliTaq Gold polymerase (PE Applied Biosystems) under 35 thermal cycling conditions of 90 s at 95°C for denaturing, 90 s at 56°C for annealing, and 1 min at 72°C for extension. *Ava*I restriction enzyme-digested product was resolved in 2.0% agarose gels containing ethidium bromide. The genotype was determined under UV illumination, with a T allele fragment length of 530 bp and C allele fragments of 190 and 340 bp that were digested by *Ava*I. *FAS–670* was determined by PCR-RFLP using specific primer pairs (Fas F: 5′-CTACCTAAGAGCTATCTACCGTTC-3′; Fas R: 5′-GGCTGTCCATGTTGTGGCTGC-3′) (Kanemitsu et al. 2002). The reaction was performed in 200 μM dNTPs, 1.0 mM $MgCl_2$, 50 mM KCl_2, 10 mM Tris–HCl (pH 8.5), and 0.5 U AmpliTaq Gold polymerase (PE Applied Biosystems) under 35 thermal cycling conditions of 30 s at 95°C for denaturing, 1 min at 58°C for annealing, and 1 min at 72°C for extension. *Mva*I restriction enzyme-digested product was resolved in 2.0% agarose gels containing ethidium bromide. The genotype was determined under UV illumination, with a G allele fragment length of 188 bp and A allele of 232 bp.

Statistical analysis

A chi-squared test and exact test were applied to test distribution difference, as well as Hardy–Weinberg disequilibrium when appropriate. The continuous values were tested with Wilcoxon's signed rank test or *t* test according to variable distribution. An unconditional logistic regression model was applied to calculate age–gender-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for *IL1B–31*, *IL1B–511*, and *FAS–670* genotypes. An ordinal logistic regression was applied to evaluate the impact of genotypes on clinical stage (I, II, III and IV). We defined *P* value less than 0.05 as statistically significant. In each multivariate model, age was treated as a continuous variable and gender was treated as indicator variable. All analyses were conducted with STATA ver.8 (STATA Corp., College Station, TX, USA).

Results

Subject characteristics are summarized in Table 1. There was no statistically significant difference between patients and controls in terms of age and gender, indicating that the age–gender strata matching was adequate. Although not statistically significant, ever smokers were relatively dominant among cases rather than controls. Distributions of clinical stages on 271 gastric cancers were as follows: 154 for stage I (56.8%), 20 for II (7.4%), 29 for III (10.7%), and 68 for IV (25.1%).

Initial experiments were performed to determine polymorphisms of *IL1B–31*, *IL1B–511*, and *FAS–670* in subjects using the PCR technique, and genotype

Table 1 Characteristics of cases and controls

	Case	Control	*P* value
Gender (M, F)	198, 73	198, 73	1.00
Age (range, mean)	29–79, 58.3	23–79, 59.2	0.38
Smoking			
Never smoker (*n*)	90	107	
Ever smoker (*n*)	181	164	0.13
Stage			
Ia	129	–	–
Ib	25	–	–
II	20	–	–
IIIa	16	–	–
IIIb	13	–	–
IV	68	–	–

distributions are presented in Table 2. *IL1B–31C/C* was inversely associated with *IL1B–511T/T*. Allele frequencies for *FAS–670G* and *IL1B–31C* were 51.1% and 44.8% in cases and 49.8% and 45.4% in control subjects. No significant deviations in control subjects were seen in observed genotype frequencies of *FAS–670 G* to *A*, *IL1B–31C* to *T*, and *IL1B–511T* to *C* polymorphisms, inferring the expected results from the Hardy–Weinberg equilibrium (P=0.22, 0.54, and 0.26, respectively). As expected, a tight linkage disequilibrium was observed between *IL1B–31C* to *T* and *IL1B–511T* to *C* polymorphisms. Coefficients for linkage disequilibrium were D' 0.97 and R^2 0.94.

To detect a possible association between polymorphisms and gastric cancer risks, we compared allele frequencies among *IL1B–31C/T*, *IL1B–511T/C*, and *FAS–670 G/A* genotypes among case patients and control subjects (Table 2). We did not find any significant difference between cases and controls in terms of genotype distributions. To evaluate the risk for gastric cancer according to genotypes, an unconditional logistic regression model analysis adjusted for age and gender was performed. Results demonstrated that both *IL1B–31C/T* and *IL1B–511T/C* polymorphisms were neutral in terms of the risk of gastric carcinogenesis in our study. No increasing OR was seen in *FAS–670 G to A* polymorphisms on gastric cancer risk.

Since some studies pointed out a smoking habit mildly associated with increased gastric cancer risks, we evaluated the possibility of whether or not a higher individual smoking status may have caused an increasing risk by the polymorphism in the Japanese population. Regarding the *FAS–670* polymorphism, smokers harboring the *G* allele showed a statistically significant increased risk of gastric cancer (OR=1.70, 95% CI 1.04–2.79) whereas no such effect was observed in never smokers (OR=0.74, 95% CI 0.37–1.50) compared with patients with *FAS–670 A* allele homozygote in ever-smoker patients. However, we could not find any effect modification due to smoking habit for *IL1B–31* and *IL1B–511* polymorphisms. These results suggest that persons with the *FAS–670 G* allele exhibited a higher transcriptional activity on the *FAS* gene showing susceptibility to gastric cancer in terms of their smoking habit and that the *IL1B–31C* allele linking with *IL1B–511T* did not cause an increase in the risk of gastric adenocarcinoma in the Japanese population.

To determine why the positive association between *IL1B–31C* allele and gastric cancer risk was not seen in Japanese populations despite its presence in Caucasians, we further explored the association between the tumor, node, metastases (TNM) stage of gastric cancer and *IL1B* and *FAS* genotypes. Of 271 gastric cancer cases, 56.8% (154 cases) were in stage I while 43.2% (117 cases) were classified in stages II–IV. Frequencies for homozygous alleles, *IL1B–31T*, *IL1B–511C*, and *FAS–670G* were 24.7, 24.0, and 24.0% among stage I and 36.8, 34.5, and 24.7% among stages II–IV, respectively, demonstrating an accumulation of the *IL1B–31TT* and *IL1B–511CC* genotype in stages II–IV. As shown in Table 3, the proportion of subjects with both *IL1B–31TT* and *IL1B–511CC* increased according to stage (trend P=0.019, trend P=0.040). In particular, subjects with stage IV showed a two times higher probability of having the *IL1B–31TT* genotype compared with stage I subjects. However, this trend was not observed for the *FAS* polymorphism. This might suggest that the *IL1B–31C/T* polymorphism,

Table 2 Gastric cancer risk on *IL1B–31*, *IL1B–511*, and *FAS–670* polymorphism

Overall	*CC*	*CT*	*TT*	*CT* and *TT*
IL1B–31 polymorphism				
Cancer/control	53/61	137/124	81/86	218/210
Odds ratio	1	1.289	1.085	1.205
95% CI		0.828–2.006	0.673–1.749	0.796–1.824
Overall	*TT*	*TC*	*CC*	*TC* and *CC*
IL1B–511 polymorphism[a]				
Cancer/control	51/58	142/123	77/86	219/209
Odds ratio	1	1.320	1.024	1.198
95% CI		0.844–2.066	0.630–1.666	0.786–1.828
Overall	*AA*	*AG*	*GG*	*AG/GG*
FAS–670 polymorphism				
Cancer/control	62/71	141/130	68/70	209/200
Odds ratio	1	1.251	1.113	1.202
95% CI		0.825–1.897	0.690–1.793	0.812–1.780

Adjusted for age and gender in the unconditional logistic regression model

[a] Three controls and a case were excluded

Table 3 Stage of gastric cancer and *IL1B–31*, *IL1B–511*, and *FAS–670* polymorphism

TNM stage	No. of cases	*IL1B–31C/T*	
		CC/CT (%)	*TT* (%)
IL1B–31 polymorphism			
I	154	75.3	24.7
II	20	70.0	30.0
III	29	65.5	34.5
IV	68	60.3	39.7
TNM stage	No. of cases	*IL1B–511T/C*	
		TT/TC (%)	*CC* (%)
IL1B–511 polymorphism[a]			
I	154	76.0	24.0
II	20	70.0	30.0
III	29	69.0	31.0
IV	67	62.7	37.3
TNM stage	No. of cases	*FAS–670A/G*	
		AA/AG (%)	*GG* (%)
*FAS–670*polymorphism			
I	154	76.0	24.0
II	20	80.0	20.0
III	29	69.0	31.0
IV	68	73.5	26.5

TNM tumor, node, metastases

[a] One case was excluded

linking with *IL1B–511T/C*, has a specific impact on the progression of gastric cancer.

Discussion

Through this case-control study, we found evidence that the progression of gastric adenocarcinoma is enhanced by the presence of the *IL1B–31T* allele that is strongly linked with *IL1B–511C*. Although it remains to be elucidated whether cancer tissues of subjects with *IL1B–31T* express IL1β rather than those of subjects with *IL1B–31C*, the present results demonstrated a causal relationship between advanced gastric adenocarcinoma and the *IL1B–31T* allele.

The first implication of this study is that the presence of the *IL1B–31T* allele has a higher risk for advanced gastric cancer in the Japanese population. The overall results did not demonstrate accumulation of any alleles in Japanese gastric cancer patients while we found an increased frequency of *IL1B–31TT* but not for *IL1B–31CC* in patients with advanced gastric cancer defined as greater than stage II. This was corroborated by the fact that *IL1B–31TT* was strongly linked with *IL1B–511CC* that was inversely increased in advanced gastric cancer patients. Therefore, the *IL1B–31T* allele is associated with the risk of cancer progression and may be considered to be a higher IL1β productive allele. In support of this possibility, the in vivo (Chang et al. 2005; Xuan et al. 2005) and in vitro (Kimura et al. 2004) experiments of earlier studies were consistent with higher production of IL1β by *IL1B–31T* allele. On the other hand, *IL1B* polymorphisms were not significantly different between the control and stage IV gastric cancer. Moreover, *P* value of *CC* versus *CT* and *TT* between control and stage I was lowest (P=0.08). This is not consistent with the result that these polymorphisms associated with gastric cancer progression. It is reasonable to consider the result was due to the bias of patient. And further, the higher transcriptional activity on *IL1B–31C* than *IL1B–31T* reportedly corresponded to a higher risk of gastric cancer in Portugal (Machado et al. 2003), China (Zeng et al. 2003), the United States (El-Omar et al. 2003), and Mexico (Sicinschi et al. 2006), and no mention was made as to any associations between TMN stages and the genotypes. This controversial issue should be addressed in further biochemical and epidemiological studies on *IL1B* polymorphisms.

The second implication of this study is that *FAS–670A/G* polymorphism does not interact with the risk of either incidence or development of gastric adenocarcinoma. A-to-G substitution at *FAS–670* altered FAS transcriptional activity by the disruption of STAT1 binding sequence, the polymorphism of which was earlier pointed out to be associated with the risk of esophageal squamous cell carcinoma (Sun et al. 2004) and acute myeloid leukemia (Sibley et al. 2003). However, the results of our case-control study did not support this hypothesis, presumably because not all gastric tumors express FAS as reported (positive cases up to 30% of gastric cancer) (Vollmers et al. 1997; Osaki et al. 2001). Based on previous epidemiological studies of the polymorphism, the positive and negative correlation between the polymorphism and a risk of cervical squamous cell carcinoma has been disregarded (Lai et al. 2003; Engelmark et al. 2004). Immunohistochemical analysis using pathological specimens would help to provide a biological explanation to resolve this discrepancy.

Among the potential methodological limitations of the present study, one issue is admittedly the selection of the base population for controls. We recruited noncancer patients at the ACCH for this purpose because it is reasonable to assume our cases arose within this population base. A notable characteristic of our control population is its similarity to the general

population in terms of exposure of interest, in this case smoking and drinking (Inoue et al. 1997). Similarity in genotype distribution for the *IL1B–31C/T* polymorphism between our controls and the general population has also been reported (Yoshimura et al. 2003). No previous report in terms of the *FAS* polymorphism is another limitation. The medical background of controls is still another potential source of bias; however, our previous study focusing on women demonstrated a limited impact. More than 66% of noncancer outpatients at ACCH do not have any specific medical condition. The remaining 34% have specific diseases, such as benign tumors and/or nonneoplastic polyps (13.1%), mastitis (7.5%), digestive disease (4.1%), or benign gynecological disease (4.1%) (Hamajima et al. 1995). As for men, the circumstances are similar although not reported. This situation is very different from that in other developed countries where people visit local general clinics first and are then referred to hospitals that function as secondary and/or specific facilities for further medical treatment. We conclude, therefore, that it is feasible to use noncancer outpatients at ACCH as referents in HERPACC-type epidemiological studies. In addition, the present study was free of questionnaire response information bias because all data were collected prior to diagnoses. Our case-control study has a statistical power of more than 90% under the assumption that the proportion of subjects with *IL1B* or *FAS* genotype of interest is 25–30% and OR for those genotypes is 2.0. Therefore, there is no conclusive risk of an association between *IL1B/FAS* polymorphisms and gastric cancer. However, the association between *IL1B* and stage should be carefully interpreted because the analysis was made in an exploratory fashion.

Yet another potential limitation was not being able to examine HP infection status. HP infection is known to induce chronic inflammation and IL1β expression to activate NFκB that can promote cancer cell growth and survive. Given high cytokine production by HP infection, it is tempting to speculate that the infection enhances growth of gastric adenocarcinoma by NFκB activation through IL1R. An alternative hypothesis is that virulent factors from HP continue to transcriptionally activate NFκB in transformed cells. Further investigation of the functional consequences of HP infection will be needed to define its exact role in cancer development though some gastric cancers arise long after infection has disappeared because of unfavorable conditions for HP in the precancerous changes. Nevertheless, high IL1β production by HP infection remains of particular interest in the context of gastric cancer because it is the main cause of chronic gastritis.

In conclusion, this study showed that there was no association between *IL1B–31* and *FAS* polymorphisms and the risk of gastric cancer among the Japanese population. The observed trend in which *IL1B–31 TT* genotype was more prevalent with the advanced stage of stomach cancer, although of interest, needs further clarification.

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