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Association of genetic polymorphisms with interferon-induced haematologic adverse effects in chronic hepatitis C patients

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SUMMARY. Interferon (IFN)-based combination therapy with ribavirin has become the gold standard for the treatment of chronic hepatitis C virus infection. Haematologic toxicities, such as neutropenia, thrombocytopenia, and anaemia, however, frequently cause poor treatment tolerance, resulting in poor therapeutic efficacy. The aim of this study was to identify host genetic polymorphisms associated with the efficacy or haematologic toxicity of IFN-based combination therapy in chronic hepatitis C patients. We performed comprehensive single nucleotide polymorphism detection in all exonic regions of the 12 genes involved in the IFN signalling pathway in 32 healthy Japanese volunteers. Of 167 identified polymorphisms, 35 were genotyped and tested for an association with the efficacy or toxicity of IFN plus ribavirin therapy in 240 chronic hepatitis C patients. Multiple logistic regression analysis revealed that low viral load, viral genotypes 2 and 3, and a lower degree of liver fibrosis, but none of the genetic polymorphisms, were significantly associated with a sustained virologic response. In contrast to efficacy, multiple linear regression analyses demonstrated that two polymorphisms (*IFNAR1* 10848-A/G and *STAT2* 4757-G/T) were significantly associated with IFN-induced neutropenia ($P = 0.013$ and $P = 0.011$, respectively). Thrombocytopenia was associated with the *IRF7* 789-G/A ($P = 0.031$). In conclusion, genetic polymorphisms in IFN signalling pathway-related genes were associated with IFN-induced neutropenia and thrombocytopenia in chronic hepatitis C patients. In contrast to toxicity, the efficacy of IFN-based therapy was largely dependent on viral factors and degree of liver fibrosis.

Keywords: haematologic adverse effect, hepatitis C, interferon, single nucleotide polymorphism, sustained virologic response.

INTRODUCTION

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] and is a leading cause of chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma [2]. Currently, combination therapy with ribavirin (RBV) and either conventional interferon (IFN)-α or pegylated-IFN-α (peg-IFN-α) is the gold standard of treatment for chronic HCV infection [3,4], but the overall rate of a sustained virologic response (SVR) with these therapies ranges from only 54% to 63% [5–7]. The limited therapeutic efficacy might be due to the poor virologic response in some patients or to adverse effects of the IFN-based therapy, leading to low treatment tolerance [5,6].

Predictive factors associated with a virologic response to IFN-based therapy include viral and host factors. Several studies have recently reported a possible association between the efficacy of IFN-based therapy and polymorphisms in genes encoding cytokines, chemokines, or their receptors [8–14]. The reported single nucleotide polymorphisms (SNPs) associated with a virologic response to IFN-based therapy include the *IFNAR1* [8], *IL-10* [9,10], *TNF-α* [11], *IFN-γ* [12], *CCR5* [13], *osteopontin* [14] and *TLR7* [15] genes. These data, however, are controversial and inconclusive, because most of the previous studies analysed a selected single target gene. Indeed, such limited evaluation of only one or two SNPs might not be sufficient in determining association of genetic polymorphisms with a virologic response to IFN-based therapy. Moreover, few studies have involved patients treated with combination therapy using peg-IFN-α and RBV [16,17].

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; OR, odds ratio; PCR, polymerase chain reaction; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained virologic response.

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Among the side effects of IFN plus RBV combination therapy, haematologic toxicities are frequently observed and sometimes treatment must be discontinued or the drug dose reduced, resulting in reduced efficacy of the combination therapy [5,6,18]. However, the mechanisms and predictive factors in the occurrence of these adverse effects, especially the critical decrease in blood cell count, are not clear at present.

Many studies have clarified the molecular pathway of action of IFN in detail [4,19,20]. Binding of IFN-α to its receptor induces IFNAR1 and IFNAR2 dimerization, followed by the activation of IFNAR-associated tyrosine kinases (JAK1 and TYK2). These tyrosine kinases phosphorylate STAT1 and STAT2 monomers, leading to the induction of multiple IFN-stimulated genes. Moreover, type I IFNs induce IRF7 and IRF3, which are responsible for type 1 IFN induction mediated by the virus or Toll like receptors [21]. On the other hand, the mechanisms of IFN induction in response to viral infection were recently determined [22,23]. In HCV-infected cells, the cytoplasmic RNA helicase, RIG-I, recognizes the viral dsRNA and interacts with IPS-1, leading to activation of the transcription factors, IRF3 and NF-kB, which in turn transcribe type I IFN genes. In contrast, IRF2 negatively regulates the IFN signalling pathway and recent studies suggest that IRF2 modulates the differentiation of haematopoietic cells [24–26]. Despite the unveiling of the molecular pathway of IFN signalling, it remains unclear why IFN-based therapy induces divergent efficacy or adverse haematologic toxicities in different patients.

In the present study, therefore, in order to determine the genetic factors associated with not only the efficacy but also haematologic toxicity of IFN-based therapy, we focused on the genes involved in the IFN signalling pathway, and performed a large-scale and comprehensive analysis of the genetic polymorphisms in 12 genes among chronic hepatitis C patients receiving IFN plus RBV therapy. To identify the predictors of efficacy or haematologic toxicity of IFN-based therapy, we carried out multivariate analyses using various clinicopathological factors and genetic polymorphisms.

MATERIALS AND METHODS

Patients

DNA for SNP screening was extracted from blood samples of 32 healthy Japanese volunteers under the auspices of the Pharma SNP Consortium (Tokyo, Japan). The participants comprised 240 Japanese adult chronic hepatitis C patients receiving conventional IFN-α 2b (n = 157) or peg-IFN-α 2b (n = 83) plus RBV combination therapy (Schering-Plough, Kenilworth, NJ, USA) at Kyoto University and affiliated hospitals from February 2002 to August 2007. In Japan, peg-IFN-α 2b plus RBV combination therapy was approved in October 2004. Thus, the patients who participated before and after October 2004 received conventional IFN-α 2b and peg-IFN-α 2b, respectively. Indications for IFN-based therapy included high serum values of alanine aminotransferase (ALT) and positivity for serum anti-HCV and HCV RNA. Histological examination of liver biopsy specimens was avail able for 165 (68.8%) of the 240 enrolled patients. Liver histology was assessed by an experienced hepatopathologist using the METAVIR score [27]; the fibrosis stage was defined as: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis). The ethics committee at Kyoto University approved the studies, and informed consent for participation in the study was obtained from all patients.

IFN-α 2b or peg-IFN-α 2b plus RBV combination therapy

Patients receiving conventional IFN-α plus RBV therapy were treated with 6 million units of recombinant IFN-α 2b daily for 2 weeks and with 6 million units three times a week for the following assigned treatment period, in combination with daily oral RBV. The RBV dose was 600 mg/day in patients weighing less than 60 kg, and 800 mg/day in those weighing 60 kg or more. Patients receiving peg-IFN-α 2b plus RBV therapy were treated with peg-IFN-α 2b once per week, combined with daily oral RBV for the assigned period. The peg-IFN-α 2b dose was 1.5 μg/kg per week. Patients with genotype 1 received 48 weeks of combination therapy and patients with genotypes 2 and 3 received 24 weeks of combination therapy.

The dosage of IFN-α 2b or peg-IFN-α 2b was reduced by half if platelet counts dropped to $<80\ 000/\mu$L, if leucocyte counts dropped to $<1500/\mu$L, or if neutrophil counts dropped to $<750/\mu$L during therapy. IFN-α 2b or peg-IFN-α 2b was discontinued if platelet counts dropped to $<50\ 000/\mu$L, if leucocyte counts dropped to $<1000/\mu$L, or if neutrophil counts dropped to $<500/\mu$L during therapy. The RBV dosage was reduced to 400 mg/day or 600 mg/day if haemoglobin levels were less than 10 g/dL. RBV was discontinued if haemoglobin levels were less than 8.5 g/dL.

Sustained virologic response was defined as no detectable HCV RNA by qualitative assay for at least 24 weeks after cessation of therapy. Non-SVR was defined as no response or relapse after the cessation of therapy.

SNP screening of the IFN signalling pathway-related genes

We selected the following IFN signalling pathway-related genes, including seven genes involved in the intracellular IFN-mediated signalling pathway from the binding of IFN to its receptor to initiation of the transcription of various target genes [20]; four genes involved in the RIG-I signalling pathway, which triggers the IFN-induction pathway after viral infection [22,23], and one gene that negatively regulates the IFN signalling pathway [24] [IFNAR1 (NT_011512.10, NM_000629.2), IFNAR2 (NT_011512.10, NM_207585.1), JAK1 (NT_032977.7, NM_002227.1), TYK2 (NT_011295.10, NM_003331.3), STAT1 (NT_005403.15, NM_007315.2), STAT2 (NT_029419.10, NM_005419.2), IRF9 (NT_026437.11, NM_006084.3), RIG-I (NT_

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008413.16, NM_014314.2), IPS-1 (NT_011387.8, NM_020746.1), IRF3 (NT_011109.15, NM_001571.2), IRF7 (NT_035113.6, NM_004031.1), and IRF2 (NT_0022792.17, NM_002199.3)]. Genomic DNA was extracted from blood samples of 32 healthy Japanese volunteers using a DNA extraction kit (Genomix Kit; TALENT, Trieste, Italy), and the 179 exons, including the 5′- and 3′-untranslated regions and adjacent intronic regions of the 12 candidate genes, were amplified. The resultant polymerase chain reaction (PCR) products were used as templates for direct sequencing on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Segregating sites were identified and genotypes were confirmed directly from electrophorograms using Genalys (http://www.software.cng.fr/docs/genalys.html) [28].

SNP genotyping

Among the SNPs identified by the screening, we selected tag SNP markers that covered all of the common (>5% frequency) haplotypes using the minimal haplotype tagging method, one of the best methods to identify the smallest tagging set for an arbitrary region of the genome [29]. These tag SNPs allowed us to genotype the smallest possible number of SNPs for each gene while resolving all common haplotypes. We also included SNPs that existed in coding sequences or 5′ flanking regions with frequencies higher than 5%. These SNPs were genotyped using the ABI Taqman allelic discrimination method and an ABI 7900HT sequence detection system (Applied Biosystems). Primers and probes were designed by the manufacturer with SNP browser Software (Applied Biosystems), as shown in Tables S1 and S2. Amplification reactions were performed in a 3 μL volume, with 5 ng DNA, 1.5 μL universal PCR master-mix, and 0.0375 μL assay mix with the specific primers and probes. Seven SNPs that could not be detected using the Taqman assay were determined by direct sequencing of PCR products amplified with primers specific for each SNP (Table S3).

Statistical analysis

Genotype distributions were tested for Hardy–Weinberg equilibrium using exact tests. To identify predictors of SVR, we used univariate analysis of pre-treatment factors to compare all SVR and non-SVR patients who had completed the treatment. The following pre-treatment factors were considered: SNPs, sex (male *vs* female), age (in years), weight (in kilograms), serum ALT, IFN history (naive *vs* relapse *vs* nonresponse), HCV genotype (1 *vs* 2 and 3), HCV viral load (<100 *vs* 100 to <500 *vs* 500 to <850 *vs* ≥850 kIU/mL), and fibrosis stage (F0 *vs* F1 *vs* F3 *vs* F4). Allele and genotype frequencies were evaluated for their association with SVR using Fisher's exact tests. Sex, IFN history, and HCV genotype were evaluated using the chi-square test. Age, weight, and serum ALT were evaluated using the Mann–Whitney *U*-test. Fibrosis stage and viral load were evaluated using a trend chi-square test. We considered two-tailed *P*-values <0.05 to be statistically significant and calculated odds ratios (ORs) and 95% confidence intervals. Multiple logistic regression analysis was performed using STATISTICA (StatSoft, Tulsa, OK, USA) to evaluate the association between SVR and significant factors from the univariate analyses.

To identify predictors of cytopenia, we examined the association between decreased leucocyte, neutrophil, and platelet counts and haemoglobin levels, and the following patient characteristics and clinical features using linear regression analysis with STATISTICA: sex, age, weight, fibrosis stage and SNPs. Multiple linear regression analysis was performed to evaluate the association between the decreased peripheral blood cell numbers and significant factors from the univariate analyses.

RESULTS

Genetic variations and polymorphisms in IFN signalling pathway-related genes

By screening 32 healthy volunteers, we identified 167 genetic polymorphisms (153 SNPs and 14 insertions/deletions) in the 12 IFN signalling pathway-related genes (Table 1, Table S4). All identified polymorphisms were in Hardy–Weinberg equilibrium. Of these 167 polymorphisms, 60 (49 SNPs and 11 insertions/deletions) were novel and were not registered in Build 125 of the SNP database (http://www.ncbi.nlm.nih.gov) (Table 2). Among the 167 SNPs identified, 30 (16 nonsynonymous and 14 synonymous) were located in exons and we confirmed that 14 of the 30 SNPs identified in the exons were novel. Furthermore, we identified 10 novel nonsynonymous variants in the seven genes. Sixty-two SNPs were relatively uncommon (minor allele frequency <0.05) and were thus excluded from further analysis. Finally, 27 selected tag SNPs and eight additional SNPs that existed in coding sequences or 5′ flanking regions were subjected to further genotyping analyses in chronic hepatitis C patients (Table 2).

Variables associated with virologic response to IFN-based therapy

The relationship between baseline characteristics and virologic response to the IFN plus RBV combination therapy in chronic hepatitis C patients is summarized in Table 3. Combination therapy was discontinued in 37 patients during the assigned treatment period. These 37 patients were excluded from analysis of the virologic response. SVR was achieved in 98 of 203 (48.3%) patients, and 105 patients (51.7%) had a relapse of HCV infection after the end of therapy or showed no response to IFN-based therapy.

To determine the predictive factors for IFN-based therapy efficacy, we examined the correlation between virologic response, and clinical and viral factors. Of 56 patients with

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Table 1 Classification of polymorphisms identified in the IFN-signalling related genes

Gene	Number of base pairs screened	SNP	Ins/del	Total polymorphisms	Distribution by gene structure					
					5′FL	5′UTR	CDS (sSNP, nsSNP)	Intron	3′UTR	3′FL
IFNAR1	7522	8	2	10	2	1	0 (0, 0)	5	2	0
IFNAR2	4849	6	0	6	0	0	1 (0, 1)	4	1	0
JAK1	11312	18	2	20	0	0	2 (2, 0)	18	0	0
TYK2	8270	19	0	19	0	0	8 (3, 5)	10	1	0
STAT1	10647	20	3	23	0	0	3 (3, 0)	18	1	1
STAT2	8646	13	1	14	0	0	1 (0, 1)	11	2	0
IRF9	3171	3	0	3	0	0	0 (0, 0)	2	1	0
RIG-I	8819	26	1	27	0	1	4 (1, 3)	19	3	0
IPS-1	5105	11	1	12	0	0	3 (1, 2)	2	7	0
IRF3	3968	8	2	10	0	3	2 (1, 1)	4	0	1
IRF7	2589	8	1	9	1	0	4 (2, 2)	4	0	0
IRF2	5668	13	1	14	0	0	2 (1, 1)	10	1	1
Total	74898	153	14	167	3	5	30 (14, 16)	107	19	3

SNP, single nucleotide polymorphism; ins, insertion; del, deletion; UTR, untranslated region; FL, flanking region; CDS, coding region; sSNP, synonymous SNP; nsSNP, nonsynonymous SNP.

Table 2 One hundred and sixty-seven polymorphisms in the IFN-signalling related genes

IFNAR1 (10 polymorphisms)	**−347**	−6*	51	10595	**10848**	10927	24135	24469*
	33483*	**33741**						
IFNAR2 (6 polymorphisms)	14693	14983	22299*	**22687**	33267*	34057*		
JAK1 (20 polymorphisms)	91	365	12755	13212	13242*	21305	30599	30856*
	34934*	34999	35312	38993	39038	**40725**	40870	40871
	41498	42571*	46465	51217*				
TYK2 (19 polymorphisms)	2243*	12345*	12529	14003*	**14006**	14145*	15192*	15452
	15560	18074	18164*	18279	21293*	26247*	26378*	**26525**
	26561*	26854	29721*					
STAT1 (23 polymorphisms)	283	821*	**4270**	5384	6630*	6751	16036	16151
	16539	23416	24514	**27161***	27452	28838	30625	34532*
	35386*	35574*	37058	37178	39478	44152*	**45397**	
STAT2 (14 polymorphisms)	88	3706	3765*	**4757***	**4901***	9465	9488	9634
	9819	10543	11441*	16088*	18063	18306*		
IRF9 (3 polymorphisms)	621*	**1129***	4265*					
RIG-I (27 polymorphisms)	**90**	177	354	391*	408	**33794***	33971	**35083**
	35263	37764	38008*	38086	41043	46072	49075*	53235
	58363*	58590*	58615*	59861*	60046*	60133*	60139	**66873**
	69596	69667	70306					
IPS1 (12 polymorphisms)	10717	10748	**10952***	**15495**	**15538**	18908	**19354**	19653
	19836*	**20479***	20921*	20927				
IRF3 (10 polymorphisms)	**95**	175	188	244	418*	1389	2320	**2652**
	6206	6304*						
IRF7 (9 polymorphisms)	−198	390*	**457**	**789**	1335	1598*	2488	2686
	2829*							
IRF2 (14 polymorphisms)	45305	45371*	45420	55441*	56210	**66675**	75602*	**83546**
	83649*	83700*	83749*	**85509**	86327	87066		

Gene number is expressed as the nucleotide position from the first nucleotide of the transcriptional start codon. Polymorphisms in boldface are selected as tag SNP markers.
*Newly discovered polymorphisms.

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Table 3 Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy

Variable	SVR (n = 98)*	Non-SVR (n = 105)*	P-value
Sex			
Male	64	50	**0.011**
Female	34	55	
Age (years)†	56.0 (24–72)	58.5 (27–74)	**0.023**
Weight (kg)†	61.8 (43–91)	61.9 (41–94)	0.821
Pre-treatment ALT (IU/L)†	69 (17–285)	57 (16–304)	0.170
Interferon history‡			
Naive	58 (51.8%)	54 (48.2%)	0.311
Relapse	30 (47.6%)	33 (52.4%)	
Nonresponse	10 (35.7%)	18 (64.3%)	
HCV genotype			
1	50 (34.0%)	97 (66.0%)	**0.00000002**
2, 3	48 (85.7%)	8 (14.3%)	
HCV RNA titre (kIU/mL)			
<100	15 (93.7%)	1 (6.3%)	**0.004**
100–500	35 (49.3%)	36 (50.7%)	
500–850	22 (44.9%)	27 (55.1%)	
850≤	26 (38.8%)	41 (61.2%)	
Fibrosis score			
0	5 (62.5%)	3 (37.5%)	**0.0005**
1	38 (59.4%)	26 (40.6%)	
2	19 (45.2%)	23 (54.8%)	
3	5 (20.0%)	20 (80.0%)	
4	2 (28.6%)	5 (71.6%)	

SVR, sustained virologic response. P-values in boldface are significant.
*SVR and non-SVR were evaluated in patients who had completed therapy for 24 or 48 weeks.
†Values are median (range).
‡One hundred six patients had received previous treatment with IFN-α monotherapy for 24 weeks, but failed to respond or relapsed.

HCV genotypes 2 and 3, 48 (85.7%) had SVR, whereas 50 of 147 (34.0%) patients with HCV genotype 1 had SVR, indicating that HCV genotype 1 was significantly associated with non-SVR (P = 0.00000002). In addition, a lower viral load before treatment (P = 0.004), male sex (P = 0.011), young age (P = 0.023), and lower degree of liver fibrosis (P = 0.0005) were significantly associated with SVR.

SNP genotyping analyses revealed that the frequencies of all 35 polymorphisms detected in the 240 hepatitis C patients were not significantly different from those in healthy volunteers. The success scores of the Taqman assay were 96.4–100% and those of direct sequencing were 95.8–100%. Univariate analyses of 35 polymorphisms revealed that a *TYK2* exon8 15560-G/T polymorphism (rs2304256) was significantly associated with virologic response to IFN-based therapy [P = 0.050, OR = 0.66 (0.44–0.99)] (Table S5).

In contrast to the univariate analysis, however, multiple logistic regression analyses demonstrated that the rs2304256 was not significant (P = 0.675) (Table 4). As a host factor, only a lower degree of liver fibrosis before therapy (P = 0.007) was significantly associated with SVR in the multiple logistic regression model. On the other hand, HCV genotypes 2 and 3 (P = 0.00005) and a lower viral load before therapy (P = 0.027) were both significantly associated with SVR.

Genetic polymorphisms associated with the adverse effects of IFN-based therapy

A total of 132 of 240 (55.0%) patients required either a discontinuation or a dose reduction of IFN or RBV due to the following adverse events: anaemia (n = 50), neutropenia or leucocytopenia (n = 32), thrombocytopenia (n = 17), depression (n = 7), and other causes (malaise, alopecia, and abdominal discomfort). The relationship between baseline characteristics and occurrence of haematologic adverse effects of the IFN plus RBV combination therapy is summarized in Table S6.

To identify the host genetic polymorphisms associated with the haematologic adverse effects of IFN plus RBV therapy, we focused on decreases in blood cell counts during the therapy and analysed the association with the SNPs in IFN signalling pathway-related genes. Consistent with previous reports [30,31], leucocyte, neutrophil, and platelet counts and haemoglobin levels usually declined in the initial 2–4 weeks of treatment, then stabilized during treatment, and returned to baseline levels within 12 weeks from the end of treatment in patients receiving IFN plus RBV therapy (Fig. 1). Therefore, we evaluated the decreases in leucocyte, neutrophil, and platelet counts and haemoglobin level at 4 weeks of treatment. We first examined the predictive factors for neutropenia. In 240 patients, absolute neutrophil counts decreased by an average of 39.3% from baseline during the first 4 weeks of treatment. Univariate analyses of 32 polymorphisms and clinical features showed that two SNPs, an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), and a *STAT2* intron5 4757-G/T, were associated with neutropenia caused by IFN-based therapy [P = 0.038, P = 0.020] (Table 5, Table S7). Furthermore, multivariate linear regression analysis confirmed that both polymorphisms were significantly associated with the neutropenia (P = 0.013, P = 0.009). Next, we examined the predictive factors for leucocytopenia. Absolute leucocyte counts decreased by an average of 29.9% from baseline within the first 4 weeks of treatment. Univariate analyses indicated that an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), an *IRF2* intron6 66675-C/T polymorphism (rs2241500), and female sex were associated with leucocytopenia (P = 0.048, P = 0.026, P = 0.016,

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Table 4 Univariate and multiple logistic regression analyses of SNPs and clinical factors associated with the efficacy of IFN plus ribavirin combination therapy

Variable	Univariate analysis		Multiple logistic regression analysis	
	P-value	OR (95% CI)	*P*-value	OR (95% CI)
SNPs				
TYK2 15660-G/T	**0.050**	0.66 (0.44–0.99)	0.675	0.48 (0.14–1.67)
Clinical variables				
Sex	**0.011**	2.07 (1.17–3.66)	0.082	2.09 (0.90–4.84)
Age	**0.023**	0.16 (0.04–0.65)	0.347	0.69 (0.11–4.22)
HCV genotype	**0.00000002**	11.6 (5.09–26.6)	**0.00005**	7.35 (2.54–21.2)
Viral load	**0.004**	0.25 (0.10–0.62)	**0.027**	0.22 (0.06–0.88)
Fibrosis stage	**0.0005**	12.0 (2.63–54.8)	**0.007**	10.3 (1.72–62.3)

P-values in boldface are significant. SNP, single nucleotide polymorphism.

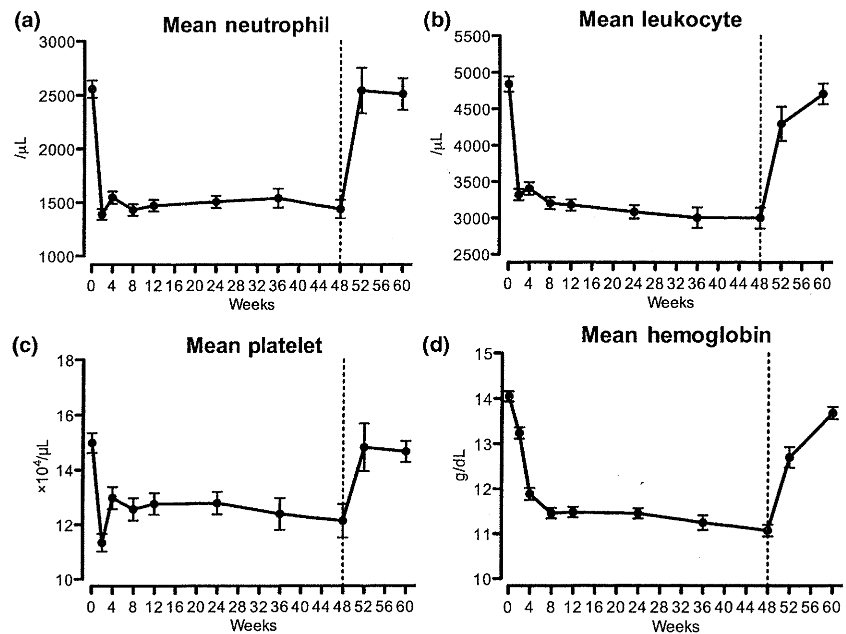

Fig. 1 Change in mean neutrophil (a), leucocyte (b), and platelet counts (c), and haemoglobin levels (d) during and after IFN plus RBV therapy. The results are shown as mean ± SEM.

respectively). Multivariate analysis, however, indicated that none of the factors, including *IFNAR1* rs2243594, *IRF2* rs2241500 and sex, were significant. Third, we examined the predictive factors for thrombocytopenia. Absolute platelet counts decreased by an average of 12.5% from baseline during the first 4 weeks of treatment. Univariate analyses showed that only an *IRF7* exon2 789-G/A (rs1061501) was associated with thrombocytopenia ($P = 0.031$). Finally, we examined the predictive factors for anaemia. Absolute haemoglobin concentration decreased by an average of 15.8% of baseline within the first 4 weeks of treatment. Univariate analyses revealed that anaemia was associated with older age ($P = 0.0004$), but not with any of the polymorphisms.

We examined the genotype results (variant allele carrier) of an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), a *STAT2* intron5 4757-G/T polymorphism, and an *IRF7* exon2 789-G/A polymorphism (rs1061501) for their association with various clinical and histologic features among 240 patients (Table S8). None of the factors, however, were associated with the SNPs identified.

DISCUSSION

In this study, we evaluated the influence of genetic polymorphisms on adverse effects and efficacy of IFN plus RBV combination therapy. Although several studies have evaluated the influence of host genetic polymorphisms on virologic response to IFN-based therapy, no studies have looked at possible association of adverse effects of the IFN-based therapy and host genetic polymorphisms. We report for the first time that certain SNPs in the IFN signalling pathway-related genes were associated with haematologic adverse effects in chronic hepatitis C patients undergoing IFN-based therapy.

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Table 5 Univariate and multiple linear regression analyses of SNPs and clinical factors associated with leucocytopenia, neutropenia and thrombocytopenia

Variable	Unit of B coefficient	Univariate analysis			Multiple analysis		
		P-value	*B* coefficient	SE *B*	*P*-value	*B* coefficient	SE *B*
Neutropenia							
IFNAR1 10848-A/G	%	**0.038**	6.94	3.31	**0.013**	6.43	2.57
STAT2 4757-G/T	%	**0.020**	−14.3	6.09	**0.011**	−13.8	5.41
Leucocytopenia							
IFNAR1 10848-A/G	%	**0.048**	4.14	2.08	0.109	1.62	1.61
IRF2 66675-C/T	%	**0.026**	3.44	1.53	0.054	3.00	1.54
Sex	%	**0.016**	7.79	3.20	0.134	3.30	2.20
Thrombocytopenia							
IRF7 789-G/A	%	**0.031**	4.15	1.92	ND	ND	ND
Anaemia							
Age	%/year	**0.0004**	0.28	0.08	ND	ND	ND

P-values in boldface are significant. SE, standard error. ND, not done because only one factor was significant in the univariate analysis.

The representative side effect of IFN-based combination therapy with RBV that causes poor therapeutic tolerance is haematologic toxicity, such as anaemia, neutropenia, and thrombocytopenia [4,32]. In fact, several studies reported that less than half the patients with hepatitis C were able to complete IFN plus RBV combination therapy at the assigned dose of both drugs, causing reduced therapeutic efficacy [5,6]. One thing to be noted is that the decrease in neutrophil and platelet counts induced by IFN-based therapy varies among patients, and thus it is difficult to predict the risk of haematologic toxicities in chronic hepatitis C patients receiving IFN-based therapy. The molecular mechanism of IFN-induced haematologic toxicities, however, is unknown. Several studies suggested the possibility that IFN treatment causes bone marrow suppression [33,34]. In agreement with this hypothesis, it was shown that a significant drop in platelet count after the initiation of IFN therapy is accompanied by a moderate increase in thrombopoietin levels in the failing liver, which may be insufficient to counteract the myelosuppressive action of IFN [35]. Another study suggested that IFN-mediated cytopenia may be due to rapid sequestration of platelets and leucocytes in the capillary beds of the liver and spleen [36]. Our current findings suggest that some of the IFN signalling pathway-related genes are involved in the decrease in neutrophil and platelet counts in response to IFN treatment. Interestingly, a recent study demonstrated that an intrinsic program for apoptosis controls platelet survival and dictates life span [37]. They revealed that platelets are genetically programmed to die by apoptosis and the antagonistic balance between antiapoptotic and proapoptotic molecules determines platelet life span. It is well known that IFN signalling induces the expression of multiple IFN-stimulated genes including molecules with proapoptotic or antiapoptotic function, such as tumour necrosis factor-related apoptosis-inducing ligand Fas, and X-linked inhibitor of apoptosis-associated factor 1 [38]. Thus, it is possible that IFNAR1, STAT2, and IRF7 contribute to the occurrence of neutropenia and thrombocytopenia by regulating the magnitude of IFN signalling involved in the apoptotic pathway in the haematopoietic cells in patients receiving IFN-based treatment.

In this study, three SNPs were associated with cytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. Among them, rs1061501 in the *IRF7* gene was located in the exon region but is a synonymous SNP. Recently, Kimchi-Sarfaty *et al.* demonstrated [39] that a synonymous SNP that did not affect amino acid sequence was capable of changing the function of the resultant protein. Indeed, the presence of a rare codon marked by a synonymous SNP in the *Multidrug Resistance 1* gene affects the timing of cotranslational folding and thereby alters the structure of substrate. Thus, it is possible that the synonymous rs1061501 contributes to a functional change in the IRF7 protein. On the other hand, rs2243594 in the *IFNAR1* gene and the SNP in the *STAT2* gene associated with neutropenia were located in an intronic region. In general, intronic SNPs provide little evidence for changes in protein structure or function, but an intronic mutation in the *p53* gene could have functional consequences by regulating gene expression, suggesting that the effect is mediated by a nonsynonymous and disruptive coding change in linkage disequilibrium with the associated intronic SNP or by a change in RNA splicing, editing, or expression [40]. Thus, it is possible that two intronic SNPs associated with neutropenia contribute to functional changes in the IFNAR1 and STAT2 proteins.

In contrast to the adverse effects of IFN plus RBV combination therapy, none of the host genetic polymorphisms in the IFN signalling pathway-related genes analysed were

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associated with therapeutic efficacy. The results indicated that viral factors, including viral genotype and pre-treatment viral load, and histological fibrosis grade were likely to have critical roles in treatment response. Consistent with many previous reports [41–43], we found that HCV genotypes 2 and 3, low viral load, and early fibrosis stage predict a favourable virologic response to IFN plus RBV combination therapy. On the other hand, it was reported that several SNPs in certain genes are associated with efficacy in IFN-based therapy [8–14,16,17]. Many of these previous studies, however, evaluated the association between the SNP and the treatment response using only univariate and not multivariate analyses that included viral factors. In fact, in our univariate analysis, one *TYK2* SNP (rs2304256) showed a possible association with therapeutic efficacy. Multivariate analysis, however, revealed that this SNP was not significant. Taken together, these findings suggest that the viral factors and host histological grade of liver fibrosis are important predictors of the treatment response in chronic hepatitis C infection. Although no significant association was observed between the efficacy and the IFN signalling pathway-related genes examined, it is possible that polymorphisms of other genes might play a role in the treatment response to IFN-based therapy.

In conclusion, we demonstrated that the SNPs in the *IFNAR1* and *STAT2* genes were associated with neutropenia and the SNP in the *IRF7* gene was associated with thrombocytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. In contrast, the virologic factors and histological grade of liver fibrosis are important predictors for virologic response to the IFN-based therapy, whereas no host genetic polymorphisms in IFN signalling pathway-related genes analysed affected the therapeutic efficacy. Further analyses are required to clarify the mechanisms of how those polymorphisms affect the biologic function of the IFN signalling and contribute to the occurrence of haematological adverse effects in IFN-treated patients.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Oligonucleotide sequences for primers and probes used for Taqman SNP genotyping assay.

Table S2 Assay ID of primers and probes used for Taqman SNP genotyping assay.

Table S3 Oligonucleotide sequences for primers used for PCR amplification and sequencing.

Table S4 List of discovered polymorphisms in 12 IFN-signalling related genes.

Table S5 Genotype frequency in the genotyped 35 polymorphisms of the IFN-signalling related genes.

Table S6 Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy.

Table S7 Linear regression analyses of 35 SNPs and clinical factors associated with haematologic adverse effects.

Table S8 Demographic, and clinical features according to three polymorphisms significantly associated with IFN-induced neutropenia and thrombocytopenia.

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BASIC—ALIMENTARY TRACT

Up-regulation of Activation-Induced Cytidine Deaminase Causes Genetic Aberrations at the *CDKN2b-CDKN2a* in Gastric Cancer

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BACKGROUND & AIMS: The DNA/RNA editing enzyme activation-induced cytidine deaminase (AID) is mutagenic and has been implicated in human tumorigenesis. *Helicobacter pylori* infection of gastric epithelial cells leads to aberrant expression of AID and somatic gene mutations. We investigated whether AID induces genetic aberrations at specific chromosomal loci that encode tumor-related proteins in gastric epithelial cells. **METHODS:** Human gastric epithelial cell lines that express activated AID and gastric cells from AID transgenic mice were examined for DNA copy number changes and nucleotide alterations. Copy number aberrations in stomach cells of *H pylori*-infected mice and gastric tissues (normal and tumor) from *H pylori*-positive patients were also analyzed. **RESULTS:** In human gastric cells, aberrant AID activity induced copy number changes at various chromosomal loci. In AID-expressing cells and gastric mucosa of AID transgenic mice, point mutations and reductions in copy number were observed frequently in the tumor suppressor genes *CDKN2A* and *CDKN2B*. Oral infection of wild-type mice with *H pylori* reduced the copy number of the *Cdkn2b-Cdkn2a* locus, whereas no such changes were observed in the gastric mucosa of *H pylori*-infected AID-deficient mice. In human samples, the relative copy numbers of *CDKN2A* and *CDKN2B* were reduced in a subset of gastric cancer tissues compared with the surrounding noncancerous region. **CONCLUSIONS: *H pylori* infection leads to aberrant expression of AID and might be a mechanism of the accumulation of submicroscopic deletions and somatic mutations in gastric epithelial cells. AID-mediated genotoxic effects appear to occur frequently at the *CDKN2b-CDKN2a* locus and contribute to malignant transformation of the gastric mucosa.**

Keywords: Gastric Cancer; Activation-Induced Cytidine Deaminase; Genetic Alterations; Tumor-Related Genes.

Increasing evidence indicates that cancer arises from a stepwise accumulation of genetic changes and that the incipient cancer cells acquire mutant alleles of tumor suppressor genes and/or proto-oncogenes.[1] Genetic alterations observed in cancers include point mutations, chromosomal number alterations, chromosomal translocations, and gene deletions or amplifications.[2] A number of human cancers have deletions and/or point mutations at specific gene loci, causing the putative inactivation of tumor suppressor genes. Thus, elucidation of the molecular mechanisms underlying the genetic alterations that occur at the loci encoding tumor suppressor proteins is important to gain a better understanding of tumorigenesis.

A novel mechanism of genetic alterations (ie, DNA/RNA editing by members of cytidine deaminases) was recently reported.[3–5] Among the 11 human cytidine deaminases identified, activation-induced cytidine deaminase (AID) is the only molecule that exerts genetic effects on human DNA sequences under physiologic conditions. AID is an essential enzyme for somatic hypermutation, class switch recombination (CSR), and gene conversion, all of which are crucial steps to achieve the diversification of the *immunoglobulin* (*Ig*) genes in activated B lymphocytes.[3] In sharp contrast to the physiologic role of AID in the editing of the *Ig* genes, we recently demonstrated a pathologic role of AID linking the accumulation of nucleotide alterations in tumor-related genes and human cancer development.[6–8] Indeed, aberrant expression of AID is induced in response to proinflammatory cytokine stimulation in gastric epithelial cells, colon epithelial cells, biliary ductal cells, and hepatocytes and leads to the accumulation of somatic mutations in various tumor-related genes in vitro.[9–13] Moreover, we showed that infection with *Helicobacter pylori*, a class 1 carcinogen for gastric cancer, induces aberrant AID expression in gastric epithelial cells, resulting in the accumulation of *TP53* tumor suppressor gene mutations.[13] Consistent with

Abbreviations used in this paper: **AID, activation-induced cytidine deaminase; BCL6, B-cell CLL/lymphoma 6; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; CGH, comparative genomic hybridization; CSR, class switch recombination; DSB, double-strand break; PCR, polymerase chain reaction; Tg, transgenic; WT, wild-type.**

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these in vitro findings, a mouse model with continuous and ubiquitous expression of AID develops cancers in several epithelial organs, including stomach, liver, and lung, via the accumulation of somatic mutations.[14–16] These findings elucidated a novel molecular mechanism linking inflammation, genetic mutations, and cancer development.

Because AID can trigger a CSR of the *Ig* gene, it is reasonable to assume that AID can also mediate chromosomal aberrations by triggering double-strand DNA breaks (DSBs) in lymphoid cells, in addition to somatic point mutations. Indeed, recent studies have shown that AID is required for the generation or accumulation of chromosomal translocations during lymphoma development.[17] For example, translocations between *c-myc* and the IgH locus (*Igh*) are induced in primary B cells within hours of AID expression, whereas *c-myc-Igh* translocations are absent in AID-deficient mice.[18–23] These findings prompted us to speculate that aberrant expression of AID in epithelial cells might cause not only somatic point mutations but also chromosomal alterations, both of which would play critical roles in the activation and/or inactivation of tumor-related genes. In this study, therefore, we investigated whether the genotoxic activity of AID could underlie the emergence of genetic aberrations at specific chromosomal loci encoding tumor-related proteins in human gastric epithelial cells and thus contribute to the development of gastric cancers.

Materials and Methods

Mice

The generation of transgenic (Tg) mice with constitutive and ubiquitous AID expression and AID-deficient mice was described previously.[15,24] Wild-type (WT) C57BL/6J mice were purchased from Japan SLC, Inc (Shizuoka, Japan). For infection, mice were challenged with 1.5×10^7 colony-forming units of *Cag*PAI-positive (TN2GF4) *H pylori* as described previously.[25–27] All experiments involving mice conformed to the relevant regulatory standards and were reviewed and approved by the Kyoto University School of Medicine Institutional Animal Care and Use Committee.

Cell Culture and Transfection

AGS human gastric epithelial cells and stable transfection of AID-expressing vector into AGS cells was described previously.[13]

Genomic Polymerase Chain Reaction, Reverse-Transcription Polymerase Chain Reaction, and Quantitative Real-Time Genomic and Reverse-Transcription Polymerase Chain Reaction

The oligonucleotide primers used are shown in Supplementary Table 1. Quantification of gene expression or gene copy numbers was performed by quantitative real-time reverse-transcription polymerase chain reaction (PCR) or genomic PCR using a Light Cycler 480 and Fast Start Universal Probe or SYBR Master (Roche, Mannheim, Germany).[9] To assess the quantity of isolated DNA, target DNAs were normalized to the DNA levels of the housekeeping reference gene human *ACTB* or mouse *Actb*. For simplicity, the ratios are represented as relative values of target gene/human *ACTB* or mouse *Actb*.

Comparative Genomic Hybridization Microarray Analysis

Genomic analyses were performed on Human 44K Agilent arrays by comparative genomic hybridization (CGH; Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. DNA extracted from AID-overexpressing AGS cells and reference DNA were both labeled by random priming with Cy3–deoxycytidine triphosphate and Cy5–deoxycytidine triphosphate for dye-swap experimental design. Arrays were scanned on an Agilent microarray scanner. Data were extracted and flagged with the Feature Extraction software. Agilent CGH Analytics software was used to identify regions of copy number alteration.

Fluorescence In Situ Hybridization Analysis

DNA probes specific to *CDKN2b-CDKN2a* locus and *ELAVL2* gene were amplified using the primers shown in Supplementary Table 1. These probes were labeled by nick-translation with either SpectrumGreen- or SpectrumOrange-labeled deoxyuridine triphosphate (Abbott Molecular Inc, Des Plaines, IL) and hybridized to chromosomes of AGS cells expressing AID for 21 days. Images were taken with the fluorescence microscope MD5000B (Leica, Wetzlar, Germany).

DNA Polymorphism Analysis

The DNA polymorphism analyses of restriction fragment length polymorphisms were performed to detect loss of heterozygosity. PCR was performed using the primer sets shown in Supplementary Table 1, and PCR products were digested with *Dde* I (for human *CDKN2A*) or *Psp* 1406 I (for human *CDKN2B*).

Southern Blot Analysis

Southern blot analysis of the PCR products was performed using AlkPhos Direct Labelling Reagents (GE Healthcare, Buckinghamshire, England), with DNA probes labeled using alkaline phosphatase, according to the manufacturer's protocol. The primer sets used are shown in Supplementary Table 1.

Subcloning and Sequencing of Tumor-Related Genes

The oligonucleotide primers used are shown in Supplementary Table 1. Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland), and the products were subcloned into a pcDNA3 vector (Invitrogen, Carlsbad, CA). The resulting plasmids were subjected to sequence analysis.[11]

Patients

The study group comprised 28 patients who had undergone potentially curative resection of primary gastric cancer at Kyoto University Hospital from 2006 to 2007. Written informed consent for the use of the resected tissues was obtained from all patients in accordance with the Declaration of Helsinki, and the Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved the study.

Statistics

Statistical significance ($P < .05$) was evaluated using the χ^2 test for sequence and fluorescence in situ hybridization analyses and the Mann–Whitney U test for quantitative real-time PCR analysis.

Results

AID Expression Induces Chromosomal Aberrations in Gastric Epithelial Cells

To view the overall landscape of the genetic alterations caused by AID activation in human gastric epithelial cells, we used a system that allows for conditional AID activation by constructing a stable transfectant of AID fused with the hormone-binding domain of the human estrogen receptor in the human gastric epithelial cell line AGS (Supplementary Figure 1). We then conditionally activated AID in the cells by introducing an estrogen analogue, 4-hydroxytamoxifen,[13,28] followed by CGH analyses performed on DNA samples extracted from the cells with or without AID activation. Copy number changes emerged in a number of submicroscopic areas in almost all chromosomes of the cells with AID activation compared with the control cells (Supplementary Figure 2). Most of the changes observed in the AID-expressing cells were submicroscopic deletions represented by copy number losses of various chromosomal loci, whereas large-scale deletions or changes in chromosomal number, such as monosomy, were not apparent in the cells with AID expression. In contrast, a submicroscopic copy number gain was observed in a few chromosomal loci on 3p, 10q, and 19p in AID-expressing cells (Supplementary Figure 2). Analysis of the time course changes in the copy numbers revealed that the number of submicroscopic chromosomal deletions increased depending on the duration of AID activation in gastric cells (Figure 1*A* and Supplementary Table 2). These findings suggested that AID expression caused the copy number changes, mainly by inducing submicroscopic chromosomal deletions, in gastric epithelial cells.

AID Induces Reductions of the CDKN2A *and* CDKN2B *Copy Numbers in Gastric Epithelial Cells*

Repeated CGH analyses on AGS cells showed that deletions at 2 specific loci, 9p21 and 3q27, commonly occurred after 1-week and 3-week AID activation, although there were many deleted regions observed. Notably, these chromosomal regions harbored the tumor suppressor genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) at 9p21 and B-cell CLL/lymphoma 6 (*BCL6*) at 3q27 (Figure 1*A*). It has been well recognized that *CDKN2A* and *CDKN2B* play crucial roles as tumor suppressor genes in the development of various human tumors.[29] Therefore, we further examined whether AID expression caused the deletion of the *CDKN2b-CDKN2a* locus at 9p21 using fluorescence in situ hybridization analyses with the probes specific for *CDKN2b-CDKN2a* locus and control *ELAVL2* gene. We found that significantly more deletions of *CDKA2b-CDKN2a* locus were present in human gastric cells with AID activation than those in the control cells (27.6% and 6.9%, respectively; $P < .001$; Figure 1*B* and Table 1). In contrast, there was no significant difference in the frequency of deleted signals for *ELAVL2* gene between AID-expressing cells and cells without AID activation (11.9% and 11.2%, respectively; Table 1). These results suggest that AID preferentially induces submicroscopic deletions of *CDKN2A* and *CDKN2B* genes in gastric epithelial cells.

Next, we analyzed the gastric mucosa of AID Tg mice, which develop various tumors, including gastric cancer, in association with the accumulation of somatic mutations.[16] We examined the relative copy number ratio of *Cdkn2a* and *Cdkn2b* at chromosome 4, and *Bcl6* at chromosome 16, in noncancerous gastric mucosa as well as in the gastric cancer tissue of AID Tg mice (Figure 2*A*). As a reference, we selected several genes, such as *Acot7* at chromosome 4 and *Actb* at chromosome 5, that were located at stable chromosomal sites in the AID-expressing cells in vitro. The relative copy number ratios of *Cdkn2a* and *Cdkn2b* were significantly lower in gastric epithelial cells of AID Tg mice compared with those of the WT mouse ($P < .05$; Figure 2*B* and *C*). Moreover, the gastric cancer tissues had substantially reduced amounts of *Cdkn2a* and *Cdkn2b* compared with the noncancerous gastric mucosa in the AID Tg mice (Figure 2*B* and *C*). All of the AID Tg mice also had significantly reduced copy number levels of *Bcl6* in the gastric mucosa compared with the WT mouse ($P < .01$; Figure 2*D*). In contrast, there was little difference in the copy numbers of the *Acot7* gene between the gastric mucosa of the WT and AID Tg mice (Figure 2*E*). Southern blotting analyses revealed that signals derived from the *Cdkn2a* gene in the gastric mucosa of the AID Tg mice were substantially reduced compared with that in the WT mouse (Figure 2*F*). These findings together suggested that constitutive expression of AID in normal gastric epithelial cells resulted in submicroscopic *Cdkn2a* and *Cdkn2b* gene defects at high frequency in vivo.

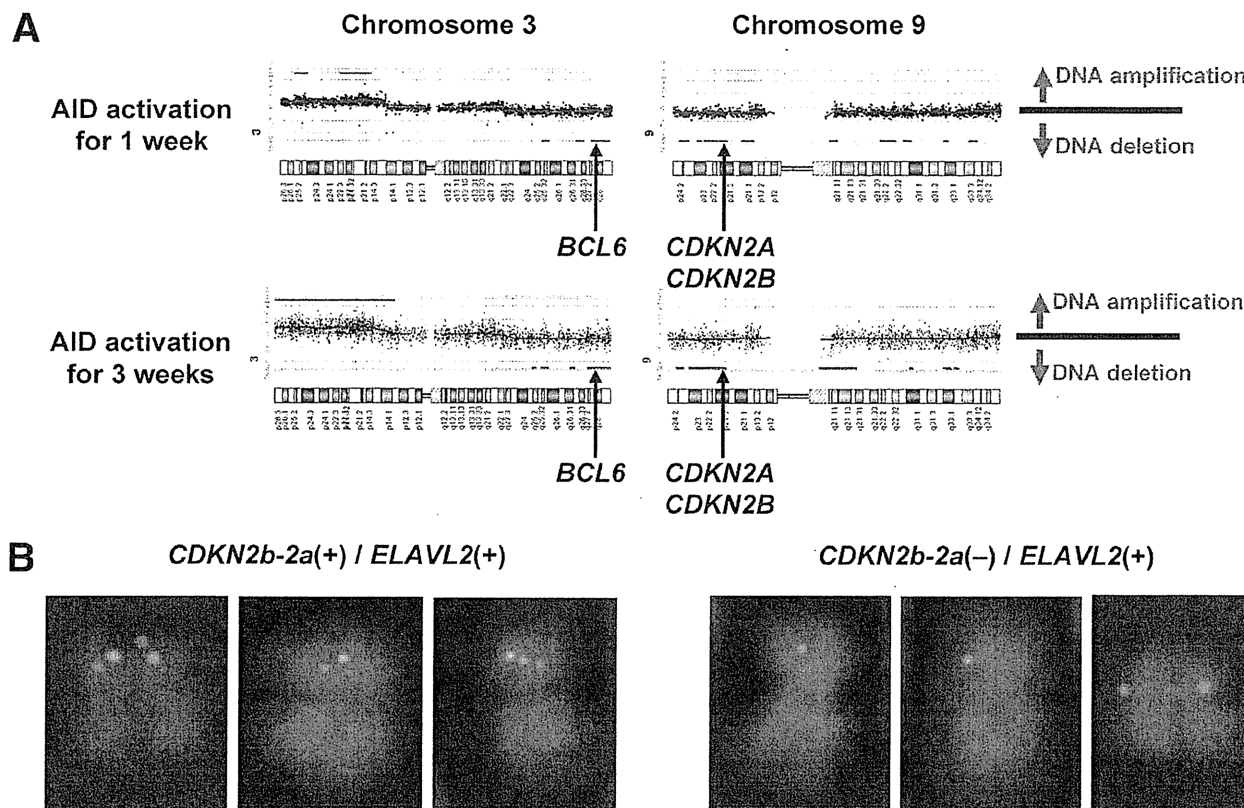

Figure 1. Copy number analyses of *CDKN2A* and *CDKN2B* genes in AGS cells with or without AID activation. (*A*) Copy number profiles on chromosomes 3 and 9 of AGS cells with AID activation for 1 or 3 weeks, using CGH analyses, are shown. The *BCL6* gene is located on chromosome 3, and the *CDKN2A* and *CDKN2B* genes are located on chromosome 9. *Red* and *green dots* represent copy number amplification and reduction, respectively. (*B*) Dual-color fluorescence in situ hybridization analyses for AID-expressing AGS cells. Representative images for the *CDKN2b-CDKN2a* locus (*green signals*) and *ELAVL2* gene (*red signals*) in cells with AID activation for 3 weeks. Chromosomes with both *CDKN2b-CDKN2a* locus and *ELAVL2* gene had green and red signals (*CDKN2b-2a*(+)/*ELAVL2*(+); *left panels*). Chromosomes without *CDKN2b-CDKN2a* signals had only red signals (*CDKN2b-2a*(−)/*ELAVL2*(+); *right panels*).

AID Expression in Gastric Epithelial Cells Caused Somatic Mutations in the CDKN2A *and* CDKN2B *Genes*

To further determine if the *CDKN2A* and *CDKN2B* genes are preferential targets of AID-mediated genotoxic effects, we examined whether somatic mutations are induced by AID activation in *CDKN2A* and *CDKN2B* genes of gastric cells in vitro and in vivo. In vitro, control AGS cells without AID activation contained only a single or 2 nucleotide alterations of the *CDKN2A* or *CDKN2B* gene sequences (Table 2). In contrast, gastric cells with AID activation had significantly higher frequencies of nucleotide alterations in the *CDKN2A* and *CDKN2B* genes than those in control cells ($P < .01$; Table 2). Of the 19 mutations in *CDKN2A* of AID-activated AGS cells, one was a nonsynonymous mutation and an-

Table 1. Frequency of Chromosomes With Deleted Signals Identified in AID-Expressing AGS Cells Using *CDKN2b-CDKN2a/ELAVL2* Dual-Color Fluorescence In Situ Hybridization Analyses

	AID(+)		Control	
Deleted genes	Frequency of the chromosomes without signals	Chromosomes without signals/total chromosomes	Frequency of the chromosomes without signals	Chromosomes without signals/total chromosomes)
CDKN2b-CDKN2a	27.6%	(97/352)[a]	6.9%	(24/347)
ELAVL2	11.9%	(42/352)	11.2%	(39/347)

NOTE. Data represent the frequency of chromosomes with the deletions of targeted genes, and values in parentheses indicate the number of chromosomes with deleted genes per number of total chromosomes examined.
[a]$P < .001$ vs control.

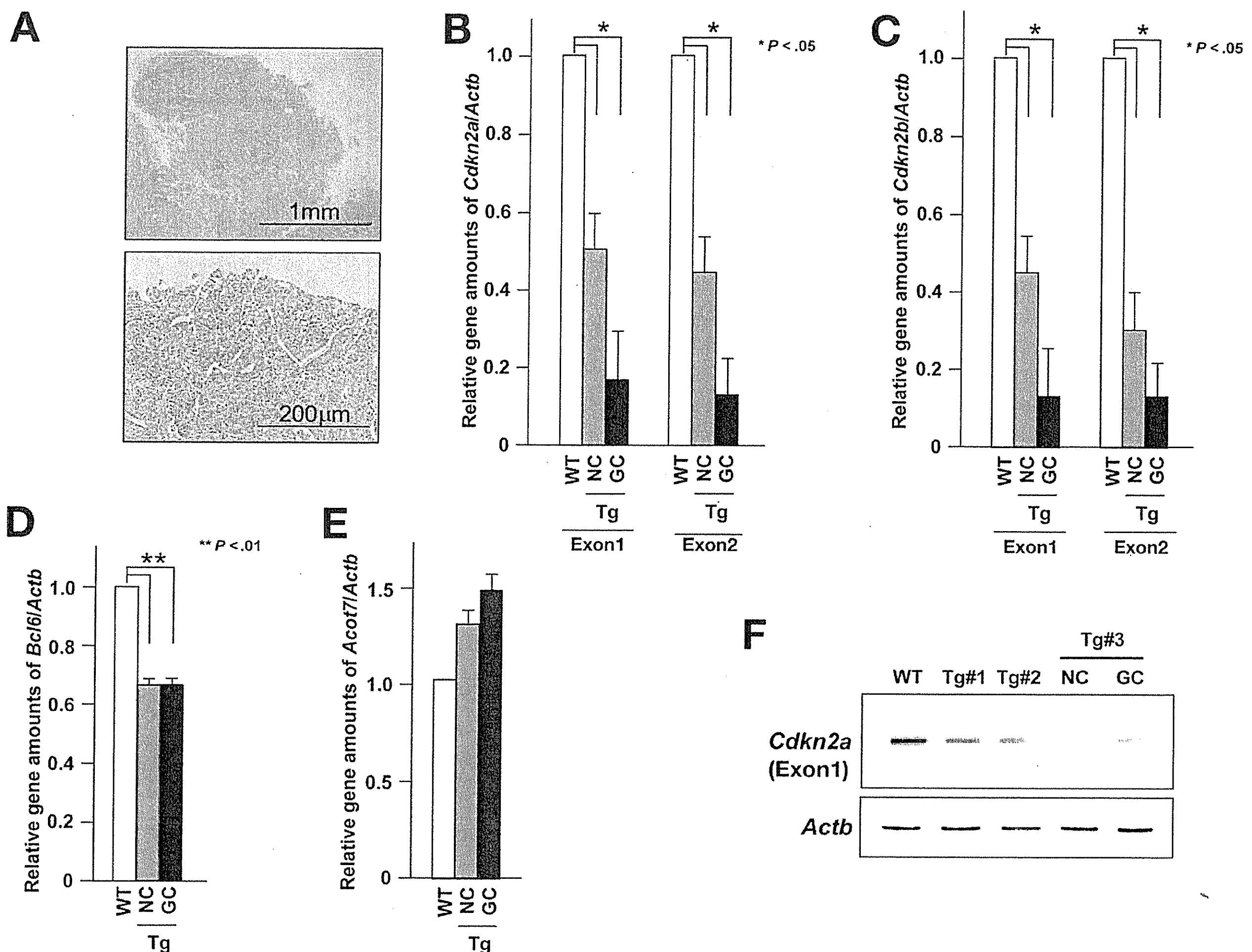

Figure 2. Copy number analyses of *Cdkn2a*, *Cdkn2b*, and *Bcl6* genes extracted from gastric epithelial cells of 1-year-old WT and AID Tg mice. (*A*) Microscopic images (H&E stain) of a representative gastric cancer developed in a 53-week-old AID Tg mouse (original magnification 40x [*upper panel*] and 200x [*lower panel*]). *Scale bars* are 1 mm (*upper panel*) and 200 μm (*lower panel*). (*B–E*) Relative copy number ratio of the (*B*) *Cdkn2a*, (*C*) *Cdkn2b*, (*D*) *Bcl6*, and (*E*) *Acot7* genes in the gastric epithelium of the WT and 3 AID Tg mice, one of which developed gastric cancer. Normal gastric mucosa of the WT mouse (WT), noncancerous mucosa of 3 AID Tg mice (NC), and gastric cancer of the AID Tg mouse (GC) were examined. *$P < .05$. **$P < .01$. (*F*) Southern blot analysis of the *Cdkn2a* gene in the gastric epithelium of the WT and 3 AID Tg (Tg#1-3) mice (*upper panel*) and that of the control *Actb* gene (*lower panel*).

other was a frameshift mutation resulting in loss of function. Three of 11 mutations in *CDKN2B* of AID-activated AGS cells were nonsynonymous mutations. Substantially higher mutation frequency was also observed in the *BCL6* gene in the cells with AID activation than that in control cells (Table 2).

Similar findings were obtained by analyses of the gastric mucosa from AID Tg mice. We detected only a single

Table 2. Mutation Frequency of *CDKN2A*, *CDKN2B*, and *BCL6* Genes in AGS Cells With or Without AID Activation

	AID(+)			Control		
	Mean mutation frequency	(Mutated bases/ total bases)	Mutated clones/ total clones	Mean mutation frequency	(Mutated bases/ total bases)	Mutated clones/ total clones
CDKN2A	$3.01/10^4$	(19/63,121)	16/199[a]	$0.15/10^4$	(1/67,726)	1/214
CDKN2B	$1.70/10^4$	(11/64,842)	11/202[a]	$0.29/10^4$	(2/68,052)	2/212
BCL6	$0.46/10^4$	(5/109,203)	5/178	$0.082/10^4$	(1/121,473)	1/198

NOTE. *CDKN2A*, *CDKN2B*, and *BCL6* sequences were amplified from AGS cells with or without AID activation for 21 days, followed by analysis of nucleotide sequences in randomly selected clones. Data represent mean mutation frequency (*left*), number of mutated bases per number of total base pairs sequenced (*middle*, in parentheses), and number of mutated clones per number of total clones examined (*right*).
[a]$P < .01$ vs control.

or 2 nucleotide alterations in either of the *Cdkn2a* or *Cdkn2b* genes in the gastric mucosa of WT mice (Table 3). In contrast, a number of somatic mutations were induced in the *Cdkn2a* and *Cdkn2b* genes in the gastric mucosa of AID Tg mice (Table 3). Significantly higher frequencies of nucleotide alterations in *Cdkn2a* and *Cdkn2b* genes were detected in noncancerous tissue as well as in gastric cancer in AID Tg mice than in control gastric tissue from WT mice ($P < .01$; Table 3). Interestingly, 6 of 11 (55%) nucleotide changes in noncancerous tissue and 10 of 14 (71%) in cancer tissue occurring in the coding sequences of the *Cdkn2a* gene were nonsynonymous mutations resulting in amino acid substitutions, and 1 of 11 (9%) nucleotide alterations in noncancerous tissue was a nonsense mutation resulting in a stop codon. Furthermore, 2 of 10 (20%) nucleotide changes in noncancerous tissue and 1 of 14 (7%) in cancer tissue in the coding sequences of the *Cdkn2b* gene were also nonsynonymous mutations. In contrast, there was no difference in the mutation frequencies of the *Bcl6* gene between AID Tg and WT mice.

Taken together, these findings suggested that AID-triggered genotoxic effects were preferentially aimed at the *CDKN2A* and *CDKN2B* genes and induced both submicroscopic deletions and nucleotide alterations of these tumor suppressor genes in gastric epithelial cells.

H pylori *Infection Triggered the Loss of the* Cdkn2a *and* Cdkn2b *Genes in Gastric Epithelial Cells*

We previously showed that *H pylori* infection induces aberrant expression of endogenous AID in gastric epithelial cells.[13] To determine whether persistent *H pylori* infection leads to changes in the *Cdkn2a/Cdkn2b* gene loci via AID expression, we orally infected WT C57BL/6J mice or AID-deficient mice with a *cag*PAI-positive pathogenic strain of *H pylori*, TN2GF4 (Supplementary Figure 3), and determined the relative copy number ratios of the *Cdkn2a* and *Cdkn2b* genes in the gastric mucosa. We first confirmed that oral infection of *H pylori* in WT mice up-regulated AID expression in the gastric mucosa (Figure 3*A*, *upper panel*), whereas endogenous AID expression was undetectable in uninfected mouse stomach (Figure 3*A*, *upper panel*) or in AID-deficient mice with *H pylori* infection (Figure 3*D*, *upper panel*). Interestingly, relative copy number ratios of *Cdkn2a* were significantly lower in most of the stomachs of WT mice infected with *H pylori* compared with uninfected mice ($P < .05$; Figure 3*B*). Similarly, there was a significant reduction in the copy number ratio of the *Cdkn2b* gene in the gastric mucosa in association with *H pylori* infection compared with the mice without *H pylori* infection ($P < .05$; Figure 3*C*). In contrast, there was no change in the relative copy number ratio of the *Cdkn2a* and *Cdkn2b* genes in the stomach of AID-deficient mice with *H pylori* infection (Figure 3*E* and *F*). These obser-

Table 3. Mutation Frequency of *Cdkn2a* and *Cdkn2b* Genes in Normal Gastric Mucosa of a 1-Year-Old WT Mouse (Control) and Gastric Cancer and Noncancerous Mucosa of a 1-Year-Old AID Tg Mouse

	AIDTg (gastric cancer)			AIDTg (noncancerous mucosa)			Control		
	Mean mutation frequency	(Mutated bases/ total bases)	Mutated clones/ total clones	Mean mutation frequency	(Mutated bases/ total bases)	Mutated clones/ total clones	Mean mutation frequency	(Mutated bases/ total bases)	Mutated clones/ total clones
Cdkn2a	$0.89/10^4$	(14/157,300)	14/270[a]	$0.72/10^4$	(11/152,347)	11/274[a]	$0.077/10^4$	(1/130,482)	1/319
Cdkn2b	$1.55/10^4$	(14/90,479)	14/106[a]	$1.05/10^4$	(10/95,186)	10/182[a]	$0.18/10^4$	(2/112,968)	2/216

NOTE. *Cdkn2a* and *Cdkn2b* sequences from mouse gastric mucosa were amplified, followed by analysis of nucleotide sequences in randomly selected clones. Data shown in this table are representative and are derived from one control and one AID Tg mouse that developed gastric cancer and represent mean mutation frequency (*left*), number of mutated bases per number of total base pairs sequenced (*middle*, in parentheses), and number of mutated clones per number of total clones examined (*right*).

[a]$P < .01$ vs control.

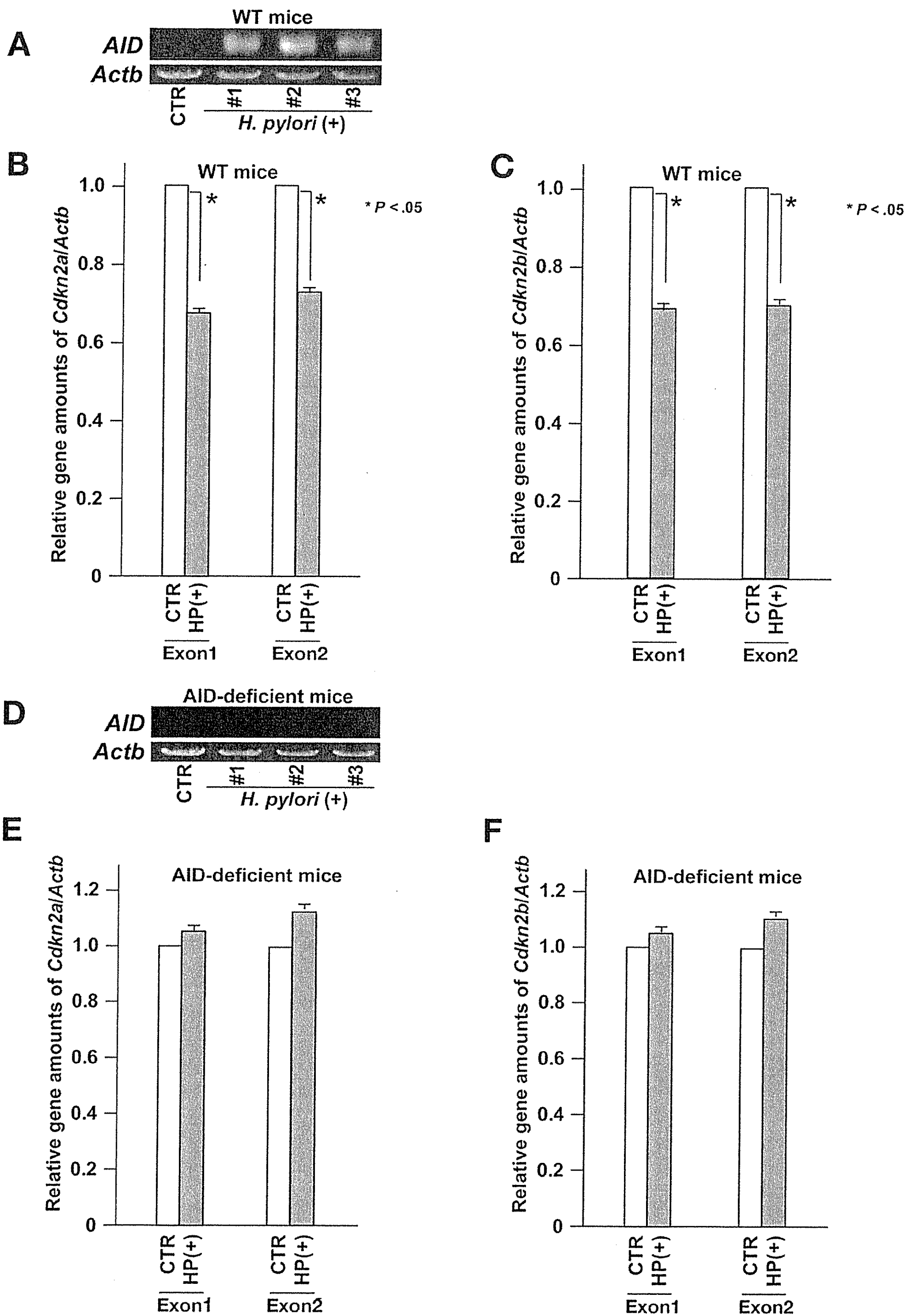

Figure 3. Copy number analyses of *Cdkn2a* and *Cdkn2b* genes extracted from gastric epithelial cells of WT mice with 2-year *H pylori* infection. (*A* and *D*) *AID* expression analyses using PCR were performed in gastric epithelial cells with *H pylori* infection of WT (*A*; *upper panel*) or AID-deficient mice (*D*; *upper panel*). Control *Actb* expression analyses are shown in *lower panels* of Figure 4*A* and *D*. (*B–F*) Relative copy number ratio of the *Cdkn2a* or *Cdkn2b* genes in gastric epithelium with or without *H pylori* infection in WT or AID-deficient mice. The data shown represent mean amounts of the *Cdkn2a* or *Cdkn2b* genes in (*B* and *C*) 3 WT mice or (*E* and *F*) 3 AID-deficient mice with *H pylori* infection (HP[+]) or the mice without infection (CTR). *$P < .05$.

vations suggested that *H pylori* infection triggered the submicroscopic deletion of *Cdkn2a* and *Cdkn2b* genes via endogenous AID expression in the gastric mucosa.

Although the CGH array analyses on the gastric mucosa of the *H pylori*-infected mouse detected a number of submicroscopic deletions in various chromosomal loci (Supplementary Figure 4), we did not detect a reduction in the copy number of the known tumor-related genes that are commonly reduced in the *H pylori*-infected mouse stomach and the cultured human gastric cells with AID expression.

Genetic Alterations of CDKN2A *and* CDKN2B *in* H pylori*–Positive Human Gastric Cancers*

Previous studies have shown that epigenetic changes in the promoter region contribute to the down-regulation of *CDKN2A* or *CDKN2B* gene expression, leading to inactivation of these tumor suppressor genes in various human cancers.[30–33] In contrast, little is known about the genetic alterations in *CDKN2A* and *CDKN2B* genes during development of gastric cancer in humans. Thus, we analyzed the relative copy number ratios of *CDKN2A* and *CDKN2B* in *H pylori*–positive human gastric cancer clinical specimens. The relative copy number ratio of the *CDKN2A* or *CDKN2B* genes was reduced to less than half in 10 of 28 tumors (36%) compared with those of the surrounding nontumorous gastric mucosa of patients with gastric cancers, and the copy number of the *CDKN2A* or *CDKN2B* genes was significantly lower in tumorous tissue than in surrounding nontumorous gastric mucosa among these cases ($P < .05$; Figure 4*A* and *B*). Real-time reverse-transcription PCR analyses revealed that the expression levels of both *CDKN2A* and *CDKN2B* transcripts were significantly lower in tumorous tissues than in nontumorous gastric mucosa in the clinical specimens with *CDKN2A* and *CDKN2B* gene copy number reductions ($P < .05$; Supplementary Figure 5*A* and *B*). Southern blot analyses revealed that genetic signals derived from the *CDKN2A* gene in cancer tissues were substantially smaller than those in the surrounding noncancerous gastric mucosa of the 10 patients with the reduced levels of *CDKN2A* and *CDKN2B* copy numbers (Figure 4*C*, *upper panel*). In addition, DNA polymorphism analyses showed that loss of heterozygosity in the *CDKN2A* or *CDKN2B* gene was detectable in 4 of 10 gastric cancer tissue specimens examined (Figure 4*D* and *E*). Taken together, these findings suggested that submicroscopic deletions of the *CDKN2b* and *CDKN2a* loci were present in a subset of human gastric cancer tissues.

Discussion

Various genetic alterations contribute to the inactivation of tumor suppressor genes during cancer devel-

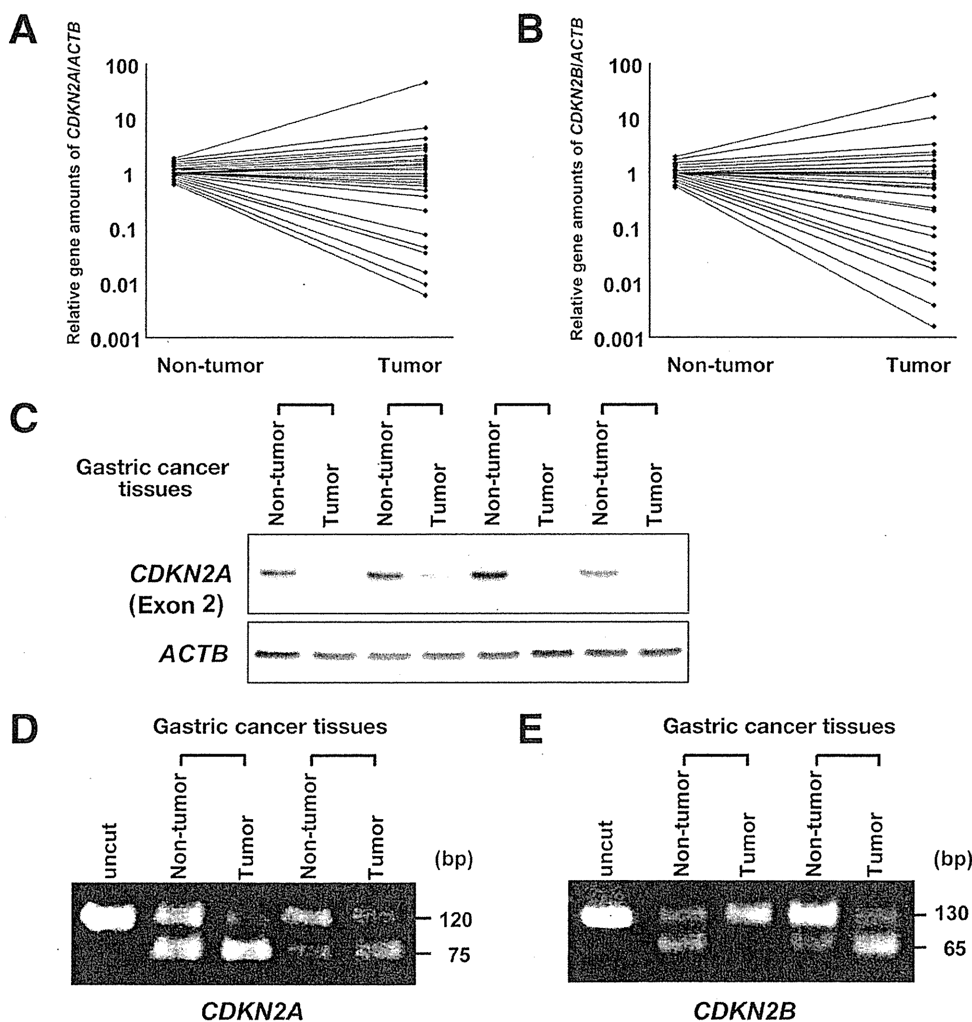

Figure 4. Copy number analyses of *CDKN2A* and *CDKN2B* genes extracted from the stomach of 28 patients with gastric cancer. (*A* and *B*) Relative copy number ratio of the (*A*) *CDKN2A* and (*B*) *CDKN2B* genes between nontumor and tumor tissues. (*C*) Southern blot analysis of the *CDKN2A* gene in nontumor and tumor tissues of 4 representative patients (*upper panel*) and that of the control *ACTB* gene (*lower panel*). (*D* and *E*) Loss of heterozygosity analyses of the (*D*) *CDKN2A* and (*E*) *CDKN2B* genes in 4 of the 6 informative patients for which DNA polymorphism analyses were performed.

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opment. We previously showed that *H pylori* infection and the resultant inflammatory response ectopically induce AID expression in human gastric epithelial cells, leading to the generation of somatic mutations in various tumor-related genes such as *TP53*.[13] On the other hand, deletion of specific chromosomal loci is another major genetic event that inactivates tumor suppressor genes.[34] The molecular processes underlying chromosomal deletions, including DSBs and subsequent joining of DNA ends during tumorigenesis, however, remain unclear. In the present study, we showed that aberrant AID expression caused chromosomal alterations, mainly submicroscopic deletions, at various genetic loci in gastric epithelial cells.

The molecular mechanism underlying AID-dependent chromosomal deletions in gastric epithelial cells remains unknown. In the case of the CSR of the *Ig* gene, which is achieved via the generation of DSBs, a nick or gap in the DNA sequence is generated during the repair process of AID-initiated C deamination at the preferred sites of the *Ig* gene. Consequently, AID could potentially induce a staggered DSB by generating closely positioned single-strand nicks on opposite DNA strands in certain hot spots of the IgH switch regions. DNA lesions intermediate to CSR are occasionally misrepaired, leading to chromosomal DSBs, translocations, and deletions in B lymphocytes.[17] A well-studied case involves the t(8;14)(q24; q32) chromosomal translocations that juxtapose the *Ig* heavy chain (IgH) to the proto-oncogene *c-Myc* in human Burkitt's lymphomas.[35-37] Indeed, several studies have shown the generation of *c-myc-Igh* translocations in the presence of AID in mouse models of developing lymphomas.[18,19,23] Thus, the catalytic activity of AID that is capable of initiating DSBs supports the idea that aberrant AID expression in gastric epithelial cells leads to chromosomal aberrations, resulting in submicroscopic deletions at various chromosomal loci.

The findings of the present study showed that aberrant AID expression preferentially caused chromosomal aberrations at the *CDKN2b-CDKN2a* locus in gastric epithelial cells. Chromosomal region 9p21 contains the *CDKN2b-CDKN2a* locus, which encodes 3 tumor suppressor proteins, $p16^{INK4a}$, $p15^{INK4b}$, and $p14^{ARF}$.[29] $p16^{INK4a}$ and $p14^{ARF}$ are potent tumor suppressors that regulate the activities of the retinoblastoma protein and the TP53 transcription factor.[38] Several mechanisms are involved in the inactivation of $p16^{INK4a}$ and $p14^{ARF}$, including chromosomal deletion, somatic mutation, and methylation of CpG islands extending from the promoter region to the first exon.[39] The deletion at chromosome 9p21 in humans, which removes the *CDKN2A* tumor suppressor gene, is a genetic alteration frequently observed in several human cancers,[40] prompting speculation that biochemical pathways regulated by these proteins must be disabled for normal cells to be transformed into tumor cells.[39] Consistent with these findings, we previously showed that AID expression in biliary cells induces somatic mutations in the promoter region of the *CDKN2A* gene.[10] Thus, the *CDKN2b-CDKN2a* locus might be a common target for AID-mediated genotoxic effects in both gastric and biliary epithelial cells.

In sharp contrast to the *CDKN2b-CDKN2a* locus, few of the other genes examined exhibited a common susceptibility to AID-mediated genotoxic effects in human and mouse gastric epithelial cells. It should be noted that the human *CDKN2b-CDKN2a* locus is located on chromosome band 9p21, whereas the cognate locus of the mouse genes is present on chromosome 4.[41] The reason for the selective AID attack of the *CDKN2b-CDKN2a* locus in both humans and mice irrespective of the chromosomal region is not clear. Consistent with the hypothesis that the *CDKN2A* deletion is triggered by AID activity, previous structural analyses of breakpoints for *CDKN2A* deletions in human cancers revealed that DSBs triggering deletions in leukemia cells are formed at a few defined sites by the illegitimate action of the RAG protein complex, whereas DSBs in solid tumors are formed at nonspecific sites in or near the *CDKN2a* locus by undefined factors.[40] It was recently shown that AID expression is required to introduce DNA single-strand breaks into both rearranged IgH variable region genes and the *CDKN2B* gene in leukemia cells,[42] and frequent deletion of *CDKN2A* and *CDKN2B* was also reported in the AID-positive lymphoid blast crisis leukemia cells.[43] In support of these findings, we confirmed that the upstream sequences of the *CDKN2b-CDKN2a* locus carry E box motifs (CAGGTG), which are tightly associated with AID hypermutation activity at both *Ig* and non-*Ig* genes,[44] at very high density (11 motifs within 10 kilo base pair sequences). Together these findings suggest that there are AID-preferential motifs in the sequences around the *CDKN2a* locus in both human and mouse, and thus the genotoxic activity of AID triggers preferential chromosomal deletions as well as somatic point mutations in the *CDKN2b-CDKN2a* locus.

A number of tumor suppressor genes exhibit epigenetic changes with resulting gene silencing in cancers, and it is well recognized that the *CDKN2A* gene is frequently inactivated by aberrant methylation.[32,45] In contrast, little is known about the genetic changes in the *CDKN2A* and *CDKN2B* genes in human gastric cancers. Here we showed that copy number losses of the *CDKN2b-CDKN2a* locus were present in a subset of human gastric cancer tissues. We also showed that *H pylori* infection triggered a reduction in the copy numbers of the *Cdkn2a* and *Cdkn2b* genes in the gastric mucosa of WT mice, and strikingly, the alterations of the *Cdkn2b-Cdkn2a* gene loci induced by *H pylori* infection were not observed in AID-deficient mice, indicating an indispensable role of AID in *H pylori*-induced genetic alterations of the *CDKN2b-CDKN2a* loci. Because a large population of patients with gastric cancer are commonly infected with *H pylori* and *H*

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pylori potently enhances AID expression in gastric epithelial cells, the present data strongly suggest that *H pylori* infection triggers inactivation of the *CDKN2A* and *CDKN2B* genes through AID-mediated genetic aberrations, contributing to the emergence of gastric cancers.

In conclusion, we showed that aberrant AID expression in gastric epithelial cells resulted in the accumulation of submicroscopic deletions in various chromosomal loci. The findings that AID preferentially targeted the tumor suppressor *CDKN2b-CDKN2a* locus in gastric epithelial cells suggest the significance of AID production in the development of gastric cancer. Further analyses are necessary to determine the precise multi-step process of genetic alterations in human gastric mucosa in association with *H pylori*-mediated AID expression.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.07.010.

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Conflicts of Interest

The authors disclose no conflicts.

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