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Upregulation of activation-induced cytidine deaminase causes genetic aberrations at the *CDKN2b-CDKN2a* in gastric cancer

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Short Title: Aberration of *CDKN2b-CDKN2a* locus by AID activity

Abbreviations used: 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; DSBs, double-strand breaks; LOH, loss of heterozygosity.

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Abstract

BACKGROUND & AIMS: The DNA/RNA editing enzyme activation-induced cytidine deaminase (AID) is mutagenic and has been implicated in human tumorigenesis. *Helicobacter pylori* (*H.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 non-cancerous 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 the *CDKN2b*-*CDKN2a* locus and contribute to malignant transformation of the gastric mucosa.

KEY WORDS: gastric cancer; activation-induced cytidine deaminase; genetic alterlations; tumor-related genes

Introduction

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, i.e., 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 pro-inflammatory 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* (*H.pylori*), a class one 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 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 revealed 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, while *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 x 10^7 cfu *Cag*PAI-positive (TN2GF4) *H.pylori* as described previously[25-27]. All experiments involving mice conform to the relevant regulatory standards and were reviewed and approved by 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 PCR, RT-PCR, and quantitative real-time genomic and RT-PCR

The oligonucleotide primers used are shown in Supplementary Table 1. Quantification of gene expression or gene copy numbers was performed by quantitative real-time RT-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, target gene/human *ACTB* or mouse *Actb*.

Comparative genomic hybridization (CGH) microarray analysis

Genomic analyses were performed on Human 44K Agilent arrays 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-dCTP and Cy5-dCTP 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 (FISH) 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 dUTP 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 (LOH). 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, UK), 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 ($P < 0.05$) was evaluated using the χ^2 test for sequence and FISH 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 two specific loci, 9p21 and 3q27, commonly occurred after 1-week and 3-week AID activation, though 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 (FISH) 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; $P < 0.001$; 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 non-cancerous gastric mucosa (NC) as well as in the gastric cancer tissue (GC) 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 < 0.05; Figure 2*B* and *C*). Moreover, the gastric cancer tissues had substantially reduced amounts of *Cdkn2a* and *Cdkn2b* compared with the non-cancerous 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 < 0.01; 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*.

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 two 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 < 0.01; Table 2). Of the 19 mutations in *CDKN2A* of AID-activated AGS cells, 1 was a nonsynonymous mutation and another 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 or two 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 non-cancerous tissue as well as in gastric cancer in AID Tg mice than in control gastric tissue from WT mice ($P < 0.01$; Table 3). Interestingly, 6 of 11 (55%) nucleotide changes in non-cancerous 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 alteration in non-cancerous tissue was a nonsense mutation resulting in a stop codon. Furthermore, 2 of 10 (20%) nucleotide changes in non-cancerous 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 demonstrated 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 upregulated 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 ($P < 0.05$; 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 ($P < 0.05$; 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 observations 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 revealed that epigenetic changes in the promoter region contribute to the downregulation 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 human gastric cancer development. 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 (36%) tumors compared with those of the surrounding non-tumorous 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 non-tumorous gastric mucosa among these cases ($P < 0.05$; Figure 4*A* and *B*). Real-time RT-PCR analyses revealed that the expression levels of both *CDKN2A* and *CDKN2B* transcripts were significantly lower in tumorous tissues than in non-tumorous gastric mucosa in the clinical specimens with *CDKN2A* and *CDKN2B* gene copy number reductions ($P < 0.05$; 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 non-cancerous 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 LOH in *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 development. We previously demonstrated 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 demonstrated 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 hotspots 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 demonstrated 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 demonstrated 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 three 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 demonstrated 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, while 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, while DSBs in solid tumors are formed at nonspecific sites in or near the *CDKN2a* locus by undefined factors [40]. It was recently demonstrated 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 carries 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 kbp 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 demonstrated 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 gastric cancer patients are commonly infected with *H.pylori* and *H.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 demonstrated 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 gastric cancer development. Further analyses are necessary to determine the precise multistep process of genetic alterations in human gastric mucosa in association with *H.pylori*-mediated AID expression.

Figure legends

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 FISH 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).

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 (hematoxylin and eosin stain) of a representative gastric cancer developed in a 53-week-old AID Tg mouse (original magnification ×40, upper panel, and original magnification ×200, lower panel). Scale bars are 1 mm (upper panel) and 200 μm (lower panel). (*B*, *C*, *D* and *E*) Relative copy number ratio of the *Cdkn2a* (*B*), *Cdkn2b* (*C*), *Bcl6* (*D*), and *Acot7* (*E*) genes in the gastric epithelium of the WT and three AID Tg mice, one of which developed gastric cancer. Normal gastric mucosa of the WT mouse (WT), non-cancerous mucosa of three AID Tg mice (NC), and gastric cancer of the AID Tg mouse (GC) were examined. *, $P<0.05$. **, $P<0.01$. (*F*) Southern blot analysis of the *Cdkn2a* gene in the gastric

epithelium of the WT and three AID Tg (Tg#1-3) mice (upper panel) and that of the control *Actb* gene (lower panel).

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 Figures 4*A* and *D*. (*B, C, E* and *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 three WT mice (*B* and *C*) or three AID-deficient mice (*E* and *F*) with *H. pylori* infection (HP[+]) or the mice without infection (CTR). *, $P<0.05$.

Figure 4

Copy number analyses of *CDKN2A* and *CDKN2B* genes extracted from stomach of 28 patients with gastric cancer.

(*A* and *B*) Relative copy number ratio of the *CDKN2A* (*A*) and *CDKN2B* (*B*) genes between non-tumor and tumor tissues. (*C*) Southern blot analysis of the *CDKN2A* gene in non-tumor and tumor tissues of 4 representative patients (upper panel) and that of the control *ACTB* gene (lower panel). (*D* and *E*) LOH analyses of the *CDKN2A* (*D*) and *CDKN2B* (*E*) genes in 4 of the 6 informative patients for which DNA polymorphism analyses were performed.

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References

1. Hahn WC, Weinberg RA. Rules for making human tumor cells. N Engl J Med 2002;347:1593-603.
2. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998;396:643-9.
3. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 2002;20:165-96.
4. Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature 2003;424:94-8.
5. Wedekind JE, Dance GS, Sowden MP, Smith HC. Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. Trends Genet 2003;19:207-16.
6. Kinoshita K, Nonaka T. The dark side of activation-induced cytidine deaminase: relationship with leukemia and beyond. Int J Hematol 2006;83:201-7.
7. Marusawa H. Aberrant AID expression and human cancer development. Int J Biochem Cell Biol 2008;40:1399-402.
8. Chiba T, Marusawa H. A novel mechanism for inflammation-associated carcinogenesis; an important role of activation-induced cytidine deaminase (AID) in mutation induction. J Mol Med 2009.
9. Endo YM, H. Kou, T. Nakase, H. , Fujii SF, T. Kinoshita, K. Honjo, T., and Chiba T. Activation-induced cytidine deaminase links between inflammation to colitis-associated colorectal cancers. Gastroenterology 2008;135:889-98, e881-3.
10. Komori J, Marusawa H, Machimoto T, Endo Y, Kinoshita K, Kou T, Haga H, Ikai I, Uemoto S, Chiba T. Activation-induced cytidine deaminase links bile duct inflammation to human cholangiocarcinoma. Hepatology 2008;47:888-96.
11. Endo Y, Marusawa H, Kinoshita K, Morisawa T, Sakurai T, Okazaki IM, Watashi K, Shimotohno K, Honjo T, Chiba T. Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. Oncogene 2007;26:5587-95.
12. Kou T, Marusawa H, Kinoshita K, Endo Y, Okazaki IM, Ueda Y, Kodama Y, Haga H, Ikai I, Chiba T. Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. Int J Cancer 2007;120:469-76.
13. Matsumoto Y, Marusawa H, Kinoshita K, Endo Y, Kou T, Morisawa T, Azuma T, Okazaki IM, Honjo T, Chiba T. Helicobacter pylori infection triggers aberrant expression

of activation-induced cytidine deaminase in gastric epithelium. Nat Med 2007;13:470-6.

14. Takai A, Toyoshima T, Uemura M, Kitawaki Y, Marusawa H, Hiai H, Yamada S, Okazaki IM, Honjo T, Chiba T, Kinoshita K. A novel mouse model of hepatocarcinogenesis triggered by AID causing deleterious p53 mutations. Oncogene 2009;28:469-78.
15. Okazaki IM, Hiai H, Kakazu N, Yamada S, Muramatsu M, Kinoshita K, Honjo T. Constitutive expression of AID leads to tumorigenesis. J Exp Med 2003;197:1173-81.
16. Morisawa T, Marusawa H, Ueda Y, Iwai A, Okazaki IM, Honjo T, Chiba T. Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression. Int J Cancer 2008;123:2735-40.
17. Casellas R, Yamane A, Kovalchuk AL, Potter M. Restricting activation-induced cytidine deaminase tumorigenic activity in B lymphocytes. Immunology 2009;126:316-28.
18. Dorsett Y, Robbiani DF, Jankovic M, Reina-San-Martin B, Eisenreich TR, Nussenzweig MC. A role for AID in chromosome translocations between c-myc and the IgH variable region. J Exp Med 2007;204:2225-32.
19. Ramiro AR, Jankovic M, Eisenreich T, Difilippantonio S, Chen-Kiang S, Muramatsu M, Honjo T, Nussenzweig A, Nussenzweig MC. AID is required for c-myc/IgH chromosome translocations in vivo. Cell 2004;118:431-8.
20. Kovalchuk AL, duBois W, Mushinski E, McNeil NE, Hirt C, Qi CF, Li Z, Janz S, Honjo T, Muramatsu M, Ried T, Behrens T, Potter M. AID-deficient Bcl-xL transgenic mice develop delayed atypical plasma cell tumors with unusual Ig/Myc chromosomal rearrangements. J Exp Med 2007;204:2989-3001.
21. Robbiani DF, Bothmer A, Callen E, Reina-San-Martin B, Dorsett Y, Difilippantonio S, Bolland DJ, Chen HT, Corcoran AE, Nussenzweig A, Nussenzweig MC. AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. Cell 2008;135:1028-38.
22. Ramiro AR, Jankovic M, Callen E, Difilippantonio S, Chen HT, McBride KM, Eisenreich TR, Chen J, Dickins RA, Lowe SW, Nussenzweig A, Nussenzweig MC. Role of genomic instability and p53 in AID-induced c-myc-Igh translocations. Nature 2006;440:105-9.
23. Takizawa M, Tolarova H, Li Z, Dubois W, Lim S, Callen E, Franco S, Mosaico M, Feigenbaum L, Alt FW, Nussenzweig A, Potter M, Casellas R. AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. J Exp Med 2008;205:1949-57.
24. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a

potential RNA editing enzyme. Cell 2000;102:553-63.

25. Matsumoto Y, Blanchard TG, Drakes ML, Basu M, Redline RW, Levine AD, Czinn SJ. Eradication of Helicobacter pylori and resolution of gastritis in the gastric mucosa of IL-10-deficient mice. Helicobacter 2005;10:407-15.
26. Matsumoto Y MH, Kinoshita K, Honjo T and Chiba T. Detection of Activation-induced Cytidine Deaminase in Gastric Epithelial Cells Infected with cag pathogenicity island-positive Helicobacter pylori. Nature Protocols 2007;10.1038/nprot.2007.237
27. Uchida K, Okazaki K, Debrecceni A, Nishi T, Iwano H, Inai M, Uose S, Nakase H, Ohana M, Oshima C, Matsushima Y, Kawanami C, Hiai H, Masuda T, Chiba T. Analysis of cytokines in the early development of gastric secondary lymphoid follicles in Helicobacter pylori-infected BALB/c mice with neonatal thymectomy. Infect Immun 2001;69:6749-54.
28. Doi T, Kinoshita K, Ikegawa M, Muramatsu M, Honjo T. De novo protein synthesis is required for the activation-induced cytidine deaminase function in class-switch recombination. Proc Natl Acad Sci U S A 2003;100:2634-8.
29. Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol 2006;7:667-77.
30. Leung WK, Yu J, Ng EK, To KF, Ma PK, Lee TL, Go MY, Chung SC, Sung JJ. Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. Cancer 2001;91:2294-301.
31. To KF, Leung WK, Lee TL, Yu J, Tong JH, Chan MW, Ng EK, Chung SC, Sung JJ. Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. Int J Cancer 2002;102:623-8.
32. Ushijima T, Nakajima T, Maekita T. DNA methylation as a marker for the past and future. J Gastroenterol 2006;41:401-7.
33. Tamura G. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. World J Gastroenterol 2006;12:192-8.
34. Canning S, Dryja TP. Short, direct repeats at the breakpoints of deletions of the retinoblastoma gene. Proc Natl Acad Sci U S A 1989;86:5044-8.
35. Boerma EG, Siebert R, Kluin PM, Baudis M. Translocations involving 8q24 in Burkitt lymphoma and other malignant lymphomas: a historical review of cytogenetics in the light of todays knowledge. Leukemia 2009;23:225-34.
36. Bornkamm GW. Epstein-Barr virus and the pathogenesis of Burkitt's lymphoma: more questions than answers. Int J Cancer 2009;124:1745-55.
37. Korsmeyer SJ. Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu Rev Immunol 1992;10:785-807.

38. Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. Cell 2006;127:265-75.
39. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev 2003;13:77-83.
40. Kohno T, Yokota J. Molecular processes of chromosome 9p21 deletions causing inactivation of the p16 tumor suppressor gene in human cancer: deduction from structural analysis of breakpoints for deletions. DNA Repair (Amst) 2006;5:1273-81.
41. Gallagher SJ, Kefford RF, Rizos H. The ARF tumour suppressor. Int J Biochem Cell Biol 2006;38:1637-41.
42. Feldhahn N, Henke N, Melchior K, Duy C, Soh BN, Klein F, von Levetzow G, Giebel B, Li A, Hofmann WK, Jumaa H, Muschen M. Activation-induced cytidine deaminase acts as a mutator in BCR-ABL1-transformed acute lymphoblastic leukemia cells. J Exp Med 2007;204:1157-66.
43. Klemm L, Duy C, Iacobucci I, Kuchen S, von Levetzow G, Feldhahn N, Henke N, Li Z, Hoffmann TK, Kim YM, Hofmann WK, Jumaa H, Groffen J, Heisterkamp N, Martinelli G, Lieber MR, Casellas R, Muschen M. The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. Cancer Cell 2009;16:232-45.
44. Michael N, Shen HM, Longerich S, Kim N, Longacre A, Storb U. The E box motif CAGGTG enhances somatic hypermutation without enhancing transcription. Immunity 2003;19:235-42.
45. Ushijima T. Epigenetic field for cancerization. J Biochem Mol Biol 2007;40:142-50.

Figure 1 Matsumoto et al.

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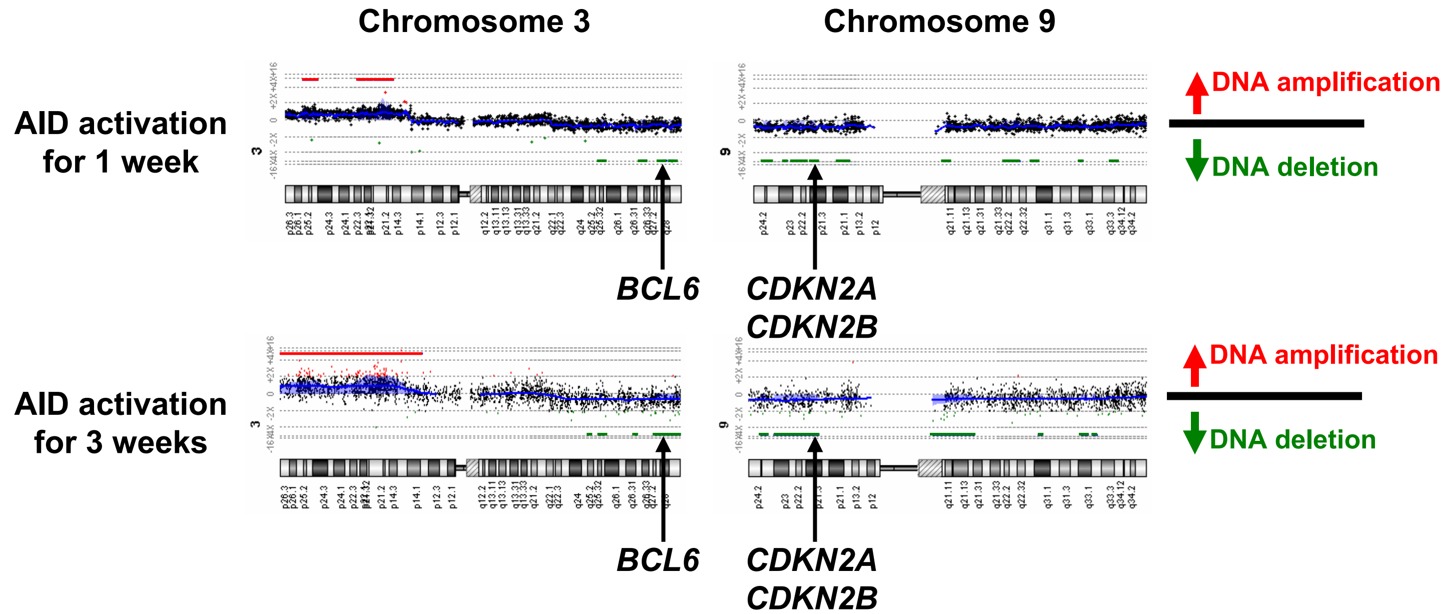

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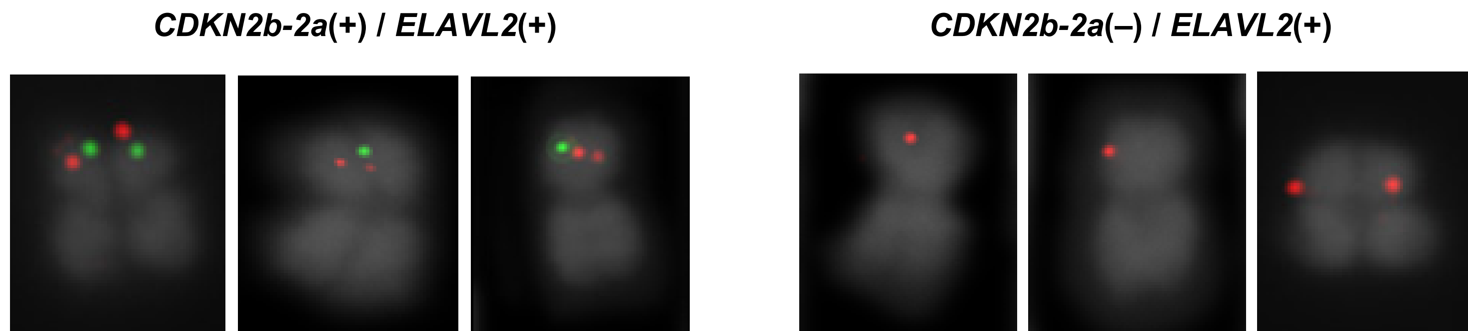

Figure 2 Matsumoto et al.

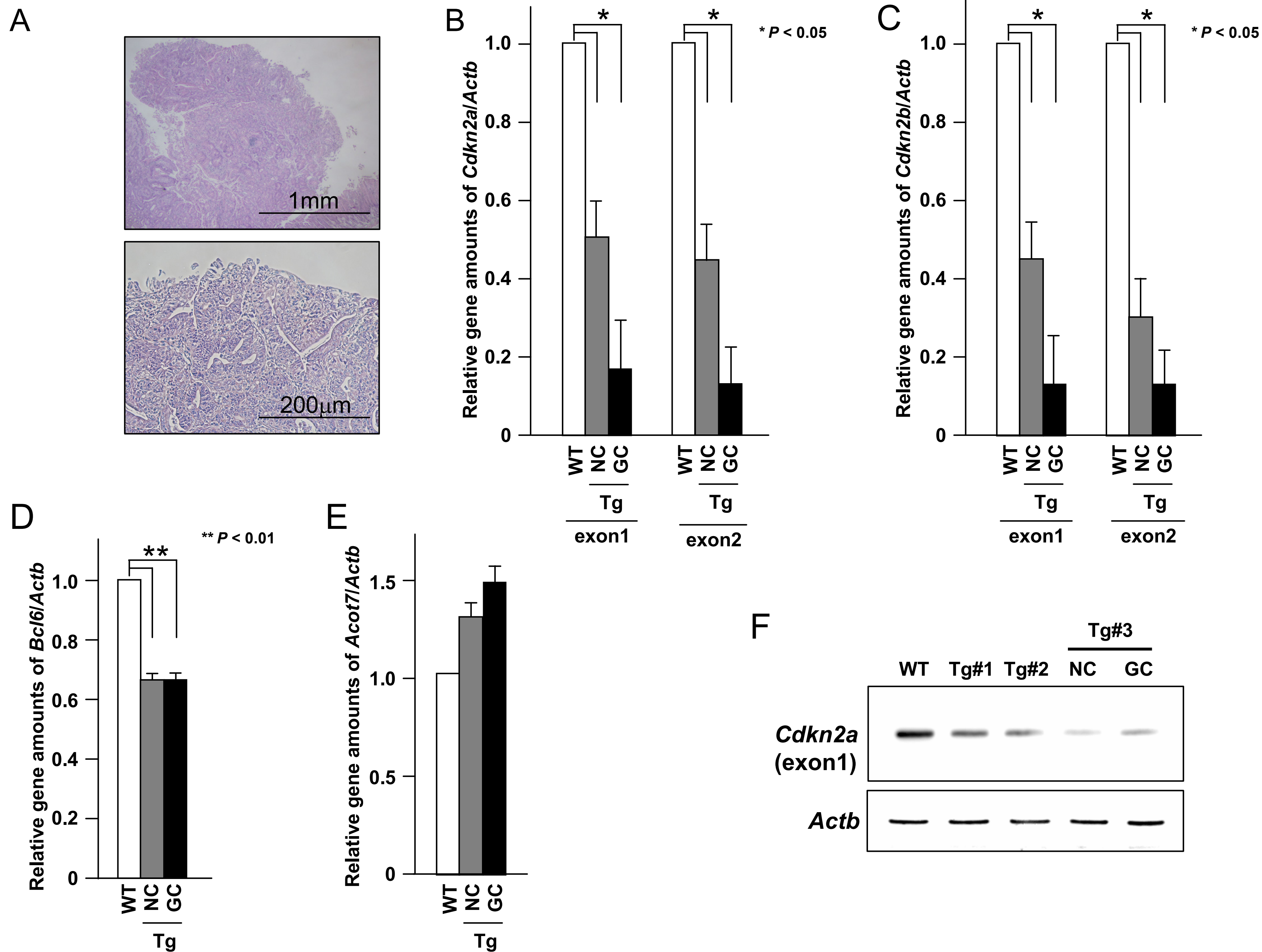

Figure 3 Matsumoto et al.

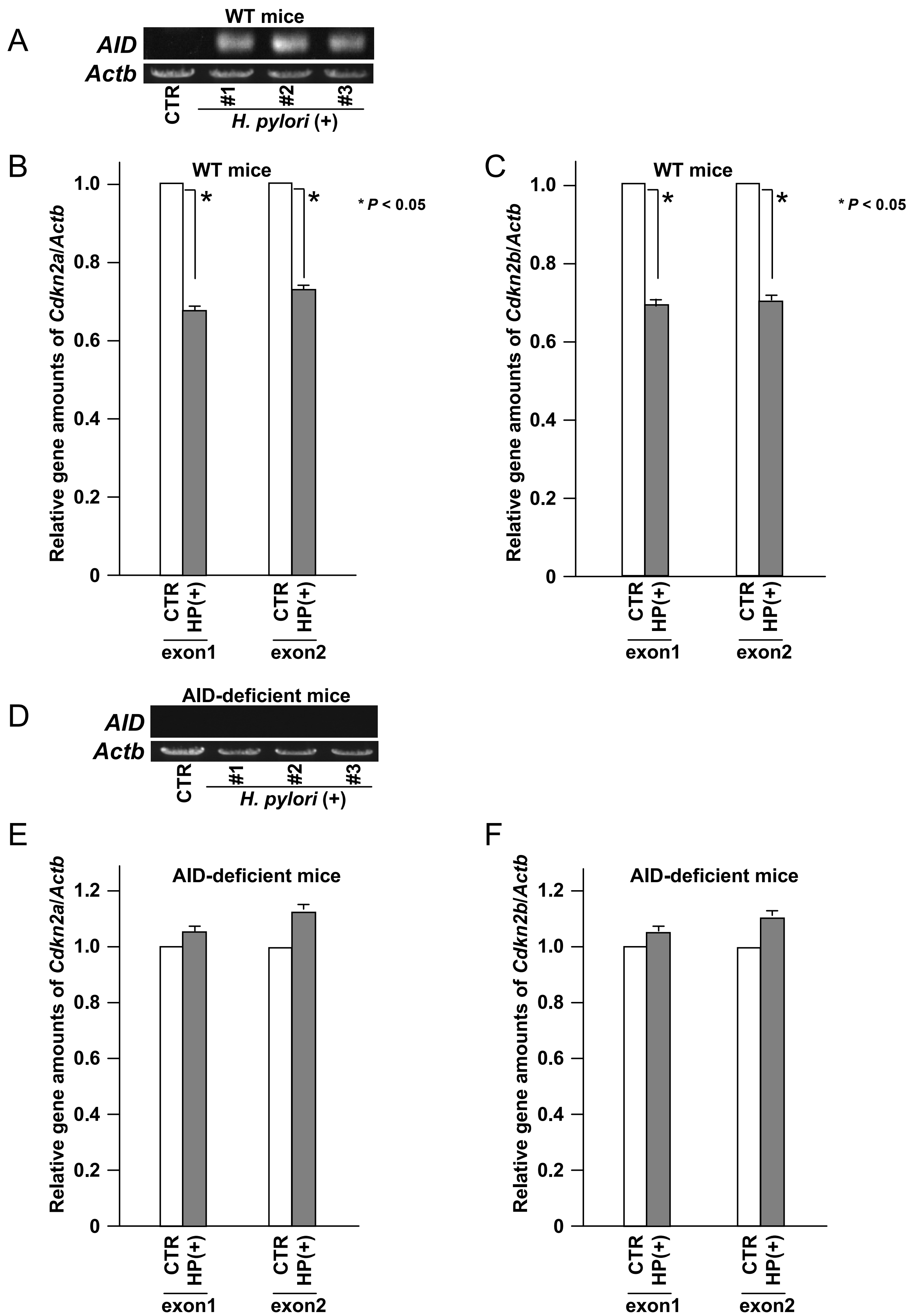

Figure 4 Matsumoto et al.

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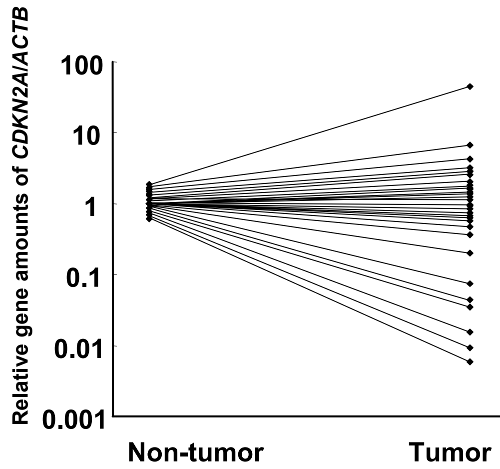

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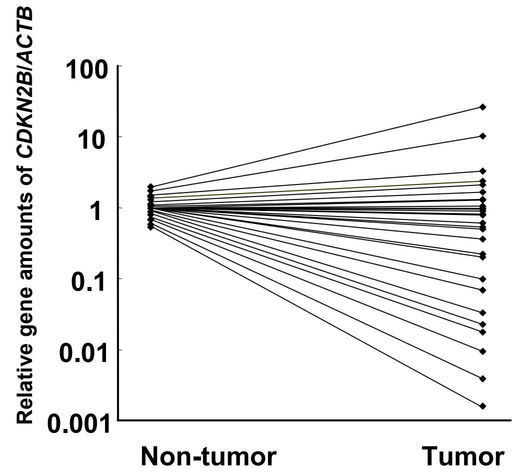

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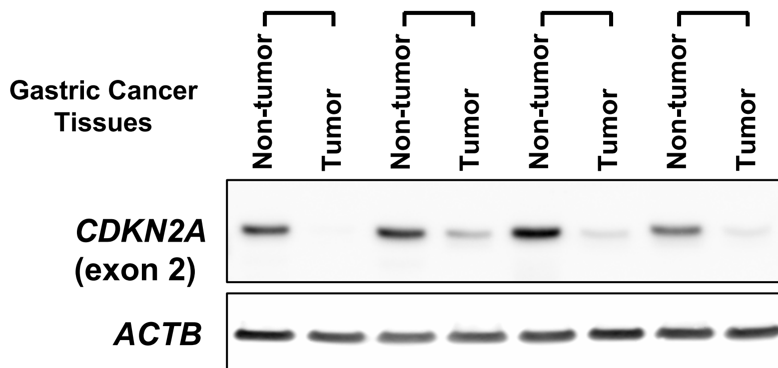

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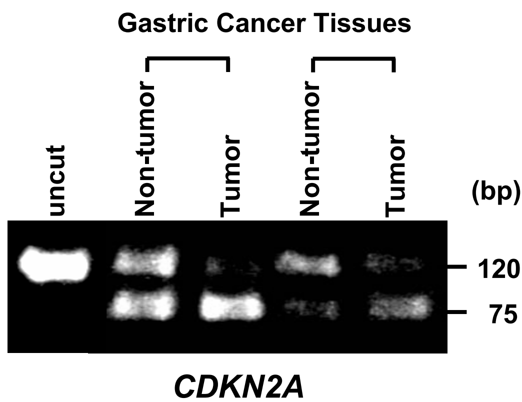

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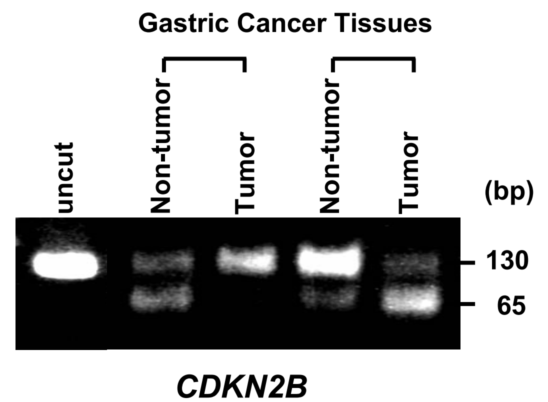


Table 1. Frequency of chromosomes with deleted signals identified in AID-expressing AGS cells using *CDKN2b-CDKN2a*/*ELAVL2* dual-color FISH analyses.

Deleted genes	AID(+)		Control	
	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) ***	**6.9%**	**(24/347)**
ELAVL2	**11.9%**	**(42/352)**	**11.2%**	**(39/347)**

Data represent the frequency of chromosomes with the deletions of targeted genes, and values in parentheses indicate number of chromosomes with deleted genes per number of total chromosomes examined. *, $P < 0.001$, vs. control.

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	**$3.01/10^4$**	**(19/63121)**	**16/199 ***	**$0.15/10^4$**	**(1/67726)**	**1/214**
CDKN2B	**$1.70/10^4$**	**(11/64842)**	**11/202 ***	**$0.29/10^4$**	**(2/68052)**	**2/212**
BCL6	**$0.46/10^4$**	**(5/109203)**	**5/178**	**$0.082/10^4$**	**(1/121473)**	**1/198**

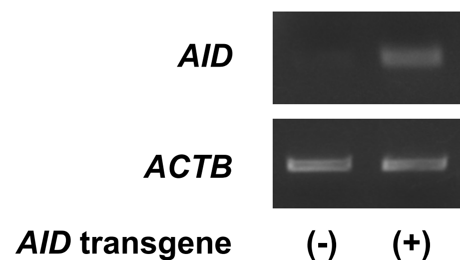
CDKN2A, *CDKN2B* and *BCL6* sequences were amplified from AGS cells with or without AID activation for 21 days, followed by analysis of nucleotide sequences in randomly selected clones. Data represent mean mutation frequency (left), number of mutated bases per number of total base pairs sequenced (middle, in parentheses), and number of mutated clones per number of total clones examined (right). *, $P < 0.01$, vs. control.

Table 3. Mutation frequency of *Cdkn2a* and *Cdkn2b* genes in normal gastric mucosa of a 1-year-old wild-type mouse (Control) and gastric cancer (GC) and non-cancerous mucosa (NC) of a 1-year-old AID transgenic (Tg) mouse.

	AIDTg(GC)			**AIDTg(NC)**			**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	**$0.89/10^4$**	**(14/157300)**	**14/270 ***	**$0.72/10^4$**	**(11/152347)**	**11/274 ***	**$0.077/10^4$**	**(1/130482)**	**1/319**
Cdkn2b	**$1.55/10^4$**	**(14/90479)**	**14/106 ***	**$1.05/10^4$**	**(10/95186)**	**10/182 ***	**$0.18/10^4$**	**(2/112968)**	**2/216**

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). *, $P < 0.01$, vs. control.

Supplementary Figure 1 Matsumoto et al. RT-PCR analyses showing the *AID* expression level in the cell system established by stable transformation of AGS cells with the plasmid encoding the conditionally active form of AID fused with the hormone-binding domain of the human estrogen receptor. The expression of *AID* (upper panel) and *ACTB*, encoding *β-actin*, as an internal control (lower panel), in AGS cells with (+) or without (-) the *AID* transgene is shown.



Supplementary Figure 2 Matsumoto et al. Chromosomal profiles of human gastric epithelial AGS cells with AID activation for 21 days based on CGH analysis. Ideograms of chromosomes are positioned horizontally and flanked by a copy number plot, which defines 0 as the normal copy number. Red and green dots represent copy number amplification and reduction, respectively.

Chr. 1 9 17

2 10 18

3 11 19

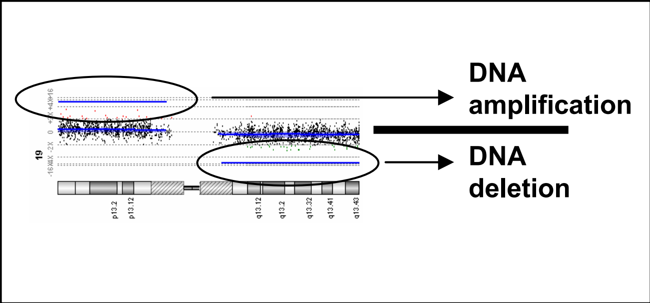
4 12 20

5 13 21

6 14 22

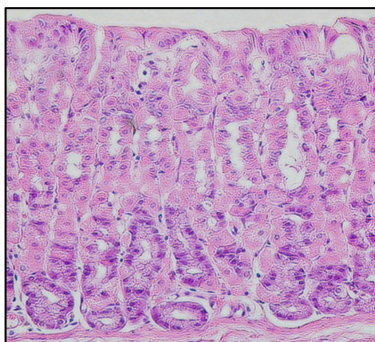
7 15

8 16

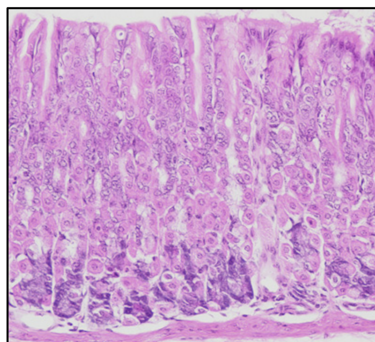


Supplementary Figure 3 Matsumoto et al. Histologic analysis of the gastric mucosa of the *H.pylori*-infected WT C57BL/6 (left panel) and AID-deficient (right panel) mice that developed chronic gastritis.

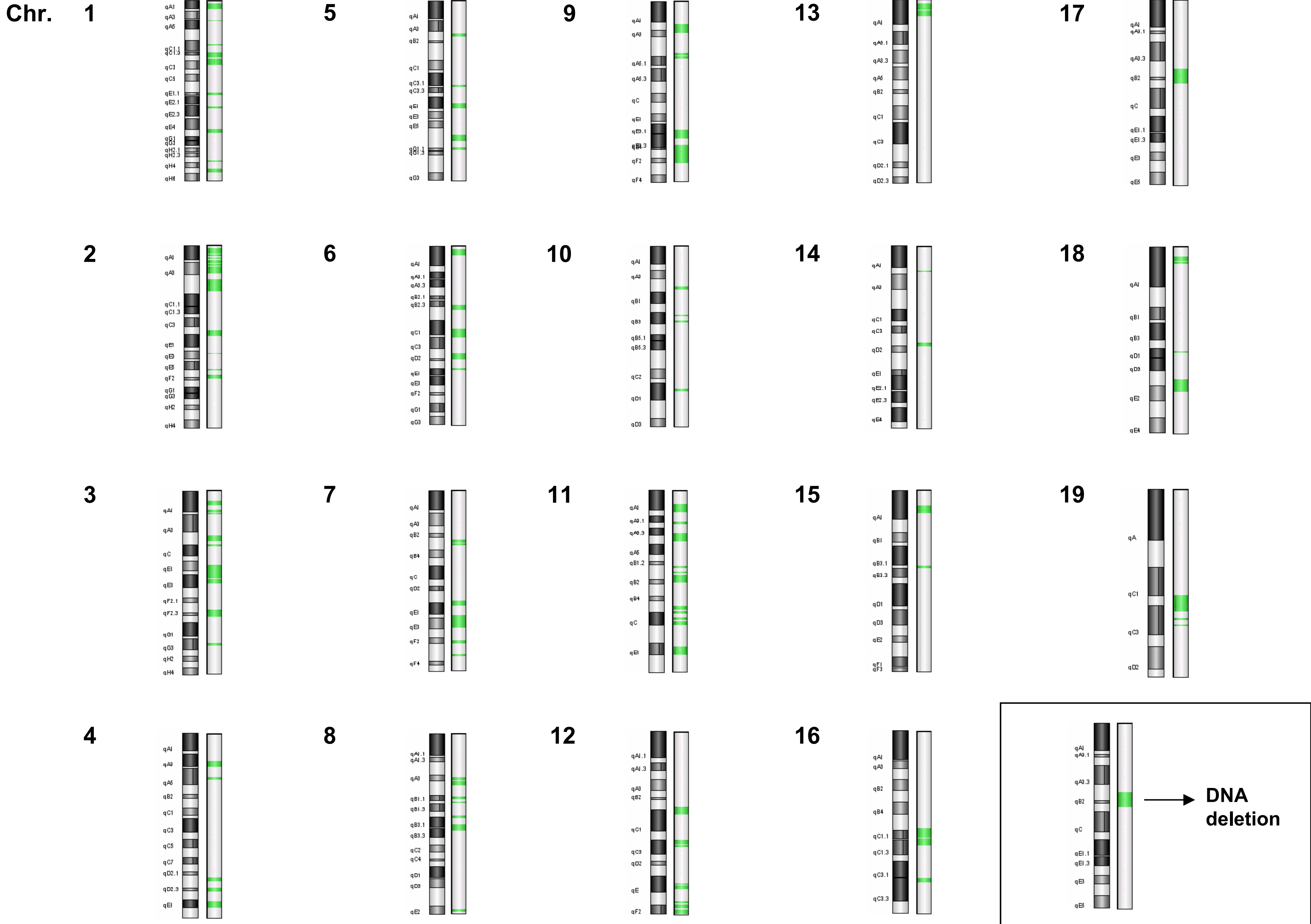
WT mouse



AID-deficient mouse

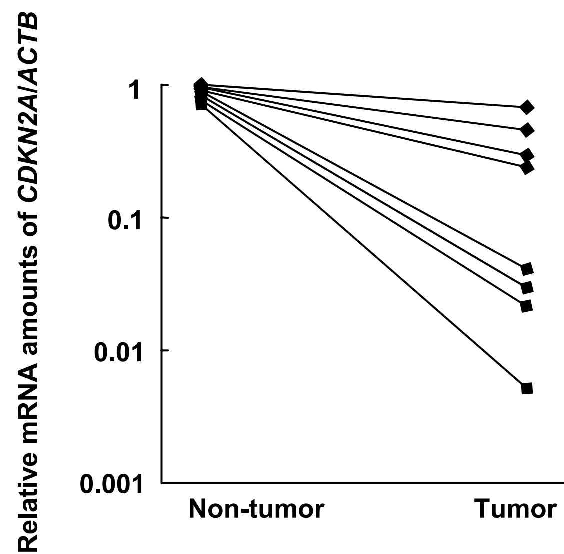


Supplementary Figure 4 Matsumoto et al. Chromosomal profiles of gastric epithelial cells of WT mouse infected with *H.pylori* for 2 years based on CGH analysis. Ideograms of chromosomes are positioned vertically. Green areas represent copy number reduction.



Supplementary Figure 5 Matsumoto et al. *CDKN2A* and *CDKN2B* mRNA expression in human gastric cancer and surrounding non-cancerous stomach tissues. Comparison of *CDKN2A* (*A*) and *CDKN2B* (*B*) transcript expression by quantitative real-time RT-PCR analyses in non-tumor and tumor tissues from the clinical specimens showing copy number reductions of the *CDKN2A* and *CDKN2B* genes.

A



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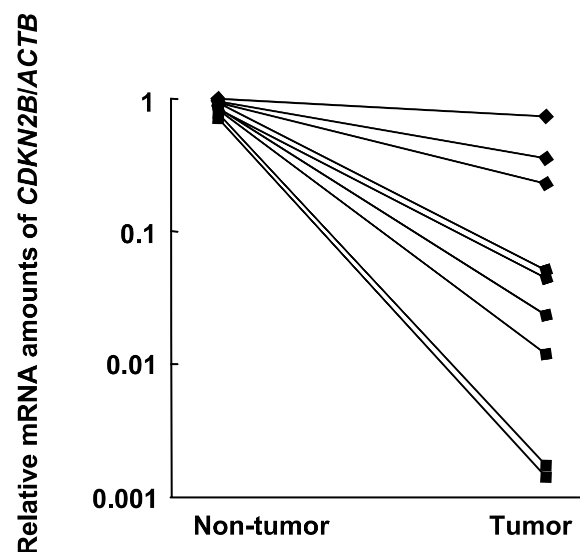

Supplementary Table 1 Matsumoto et al. Oligonucleotide primers used in this study.

Primers	Orientation	Sequence (5' – 3')
human *CDKN2A*	Sense	CACCTCAGAAGTCAGTGAGT
	Antisense	GTGCTTGAAATACACCTTTCC
human *CDKN2B*	Sense	AGACTACACAGGATGAGGTG
	Antisense	GCAAGTCATAAGGGGATTTC
human *CDKN2A*_1	Sense	CCGGAATTCAGCAGCATGGAGCCGGCGGCG
	Antisense	CCGCTCGAGCTGGATCGGCCTCCGACCGTA
human *CDKN2A*_2	Sense	CCGGAATTCGTCATGATGATGGGCAGCGCC
	Antisense	CCGCTCGAGCTGAGGGACCTTCCGCGGCAT
human *CDKN2B*	Sense	CCGGAATTCAGAGTGTCGTTAAGTTTACGG
	Antisense	CCGCTCGAGCAAATCTACATCGGCGATCT
human *CDKN2A*_SouthernBlot	Sense	CTGTCACCCAGGCTGGAGTG
	Antisense	GCCTCAGTTAAGATGGGAGG
human *BCL6*	Sense	CCGGAATTCAGCCTCGAACCGGAACCT
	Antisense	CCGCTCGAGTTCTTCTTTGCTGGCCTTGT
human *ACTB*	Sense	GCCCATCTACGAGGGGTATG
	Antisense	GGCCATCTCTTGCTCGAAGT
human *ACTB*_SouthernBlot	Sense	GCGCCCTTTCTCACTGGTTC
	Antisense	GCCAGACAGCACTGTGTTGG
human *CDKN2AB*_FISH	Sense	GGCTAGAGACGAATTATCTGTTTACGAA
	Antisense	GGACAATGAGGCAAAGAAATATCAAGTAAC
human *ELAVL2*_FISH	Sense	TGAGTAGAATTAAATTGGCTGAATGTAACA
	Antisense	ACCATATTGTATGCGAAGGACGTAG
mouse *Cdkn2a*_exon1a	Sense	AAGGAGGGACCCACTGGTCAC
	Antisense	CTGAATCGGGGTACGA
mouse *Cdkn2a*_exon1b	Sense	AGTACAGCAGCGGGAGCATG
	Antisense	TGGTCCAGGATTCCGGTGC
mouse *Cdkn2a*_exon2	Sense	TGATCCGAGTAGTTAACAGCG
	Antisense	GGGTTGCTTCTTCTTGTTCTG
mouse *Cdkn2b*_exon1	Sense	TGCCACAGACCGGGGACAAGG
	Antisense	CGCCCTCTGCCGGTAAGGCCT
mouse *Cdkn2b*_exon2	Sense	GATTGAGTAAGCAAATGAG
	Antisense	GAGAAAGAAGTCCTTGCGGAA
mouse *Cdkn2a*_1	Sense	CCGGAATTCCAAATCCTTCGCAAAGATTCG
	Antisense	CCGCTCGAGTACCTGAATCGGGGTACGAC
mouse *Cdkn2a*_2	Sense	CCGGAATTCTCCTGACTGTGGATGTCTGAT
	Antisense	CCGCTCGAGCTCCAAGAAGTGGGACGTTTA
mouse *Cdkn2b*	Sense	CCGGAATTCCCAGGCTGTAGCAATCTCAC
	Antisense	CCGCTCGAGGCTTGCAGTCTTCCTAGATGG
mouse *Cdkn2a*_SouthernBlot	Sense	CCGATGACTTCACCCCGTCA
	Antisense	GGGAAGGCAGTGGACGTAGG
mouse *Bcl6*	Sense	CACACCCGTCCATCATTGAA
	Antisense	TGTCCTCACGGTGCCTTTTT
mouse *Acot7*	Sense	CCTGCTGGACACCTGTTCTC
	Antisense	GGATTTCCTACATCGAGTGTCCT
mouse *Actb*	Sense	GTGTGATGGTGGGAATGGGT
	Antisense	CTGGGTCATCTTTTCACGGTT
mouse *Actb*_SouthernBlot	Sense	TCTGAAGAAAGCAAGACAAG
	Antisense	TGAGAGTACACAGTATTGGG
mouse *AID*	Sense	CGTGGTGAAGAGGAGAGATAGTG
	Antisense	CAGTCTGAGATGTAGCGTAGGAA

Supplementary Table 2 Matsumoto et al. Genes included in the chromosomal loci frequently deleted in AID-activated human gastric AGS cells.

	Deleted loci and genes	
	AID activation for 1 week	**AID activation for 3 weeks**
Chromosome 3	**3q25-28;** *MLF1, GFM1, RARRES1, KCNMB2, WIG1, PIK3CA, MFN1, GNB4, USP13, PEX5L,* ***BCL6****, TP73L, LEPREL1, CLDN1, 16, IL1RAP, FGF12, HRASLS, OPA1*	**3q25-28;** *MBNL1, MLF1, GFM1, RARRES1, SCHIP1, IMP-2, ETV5, DGKG, RFC4, SIAT1, MASP1, IFRG28,* ***BCL6****, LPP, TP73L, LEPREL1, CLDN1, 16, IL1RAP, OSTN, FGF12, HRASLS, OPA1, GP5, CENTB2, MUC20, 4, TNK2, PAK2, SENP5, NCBP2, SMP3, DLG1, BDH, LRCH3, IQCG, LMLN*
Chromosome 9	**9p21-23;** *NFIB, ZDHHC21, FREM1, SNAPC3, PSIP1, BNC2, SH3GL2, MLLT3, IFNB1, W1, A5, A2, A8, E1, MTAP,* ***CDKN2A****,* ***CDKN2B***	**9p21-23;** *PTPRD, MPDZ, NFIB, ZDHHC21, FREM1, SNAPC3, PSIP1, BNC2, SH3GL2, ADAMTSL1, FAM29A, SLC24A2, MLLT3, IFNB1, W1, A5, A2, A8, E1, MTAP,* ***CDKN2A****,* ***CDKN2B***