

**Bile acid-induced expression of activation-induced cytidine deaminase
during the development of Barrett's oesophageal adenocarcinoma**

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Short Title: AID expression in Barrett's epithelium

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Abbreviations: AID, activation-induced cytidine deaminase; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; DCA, deoxycolic acid; *H.pylori*, *Helicobacter pylori*

Abstract

Activation-induced cytidine deaminase (AID) induces somatic mutations in various host genes of non-lymphoid tissues, thereby contributing to carcinogenesis. We recently demonstrated that *Helicobacter pylori* infection and/or proinflammatory cytokine stimulation triggers aberrant AID expression in gastric epithelial cells, causing mutations in the tumour-suppressor *TP53* gene. The findings of the present study provide evidence of ectopic AID expression in Barrett's oesophagus and Barrett's oesophageal adenocarcinoma, a cancer that develops under chronic inflammatory conditions. Immunoreactivity for endogenous AID was observed in 24 of 28 (85.7%) specimens of the columnar-cell lined Barrett's oesophagus and in 20 of 22 (90.9%) of Barrett's adenocarcinoma, while weak or no AID protein expression was detectable in normal squamous epithelial cells of the oesophagus. We validated these results by analyzing tissue specimens from another cohort comprising 16 cases with Barrett's oesophagus and 4 cases with Barrett's adenocarcinoma. *In vitro*, treatment of human non-neoplastic oesophageal squamous-derived cells with sodium salt deoxycholic acid induced ectopic AID expression via the NF- κ B activation pathway. These findings suggest that aberrant AID expression occurs in a substantial proportion of Barrett's epithelium, at least in part due to bile acid stimulation. Considering the genotoxic activity of AID, our current findings suggest that aberrant AID expression might enhance the susceptibility to genetic alterations in Barrett's columnar-lined epithelial cells, leading to cancer development.

Introduction

Chronic inflammation has a critical role in the development of many human cancers (1-4), such as hepatitis-associated hepatocellular carcinoma, colitis-associated colorectal cancers, cholangitis-related cholangiocarcinoma, and *Helicobacter pylori* (*H. pylori*) infection-related gastric cancers (3). Several studies have demonstrated various alterations in tumour-related genes in inflamed tissues before cancer onset (2, 3, 5). We previously demonstrated a substantial number of nucleotide alterations in the tumour suppressor *TP53* gene in nontumorous epithelial tissues of patients with chronic hepatitis (6) or *H. pylori* -related chronic gastritis (7). Although the molecular mechanisms underlying the transition from chronic inflammation to the accumulation of genetic mutations leading to tumorigenesis remain unknown, the discovery of nucleotide-editing enzymes that potentiate the genetic changes in DNA and/or RNA sequences (8, 9) has provided a breakthrough in this research.

Among the human nucleotide-editing enzymes identified, only activation-induced cytidine deaminase (AID) has been shown to induce mutations in human host DNA sequences (10). AID was originally identified as an inducer of somatic hypermutations, which diversifies the variable regions of immunoglobulin genes in activated B cells in germinal centres (11, 12). Animal models with constitutive and ubiquitous expression of this nucleotide-editing enzyme, however, revealed that AID induces somatic mutations in various tumour-related genes, leading to the development of both lymphoid and non-lymphoid tumours (13, 14). Moreover, we recently demonstrated that pro-inflammatory cytokine stimulation induces aberrant AID expression in various human epithelial cells, including colonic epithelium (15), hepatocytes (6, 16), biliary cells (17), and gastric columnar cells (7). These findings indicate that inflammatory response-mediated aberrant AID expression may be a mechanism of the mutational accumulation required for malignant transformation in human epithelial cells (18, 19).

Barrett's oesophagus is a metaplastic change from the normal stratified, squamous epithelium of the lower oesophagus to a columnar-lined epithelium with intestinal-type differentiation (20). Barrett's oesophagus is clinically significant because it is associated with high risk of oesophageal adenocarcinoma (21). It is well recognized that chronic duodenogastro-oesophageal reflux and the resultant inflammatory response play a critical role in the development of Barrett's oesophagus and adenocarcinoma (21-23). Several previous studies demonstrated the involvement of *TP53* mutations at an early stage during malignant transformation of Barrett's oesophagus (24, 25). In this regard, it is noteworthy that AID is aberrantly upregulated in response to inflammatory stimulation and AID activation induces the accumulation of multiple genetic alterations in *TP53* in the columnar cells of the stomach (7, 26). Thus, because aberrant expression of AID in gastrointestinal columnar cells can be genotoxic during inflammation, we speculated that AID might also be involved in the pathogenesis of Barrett's oesophageal adenocarcinoma. Therefore, here we investigated the expression of endogenous AID in human columnar epithelial cells lining Barrett's oesophagus and Barrett's oesophageal adenocarcinoma, a representative cancer that develops under chronic inflammatory conditions.

Materials and methods

Study population

The study group comprised patients who underwent endoscopic resection for Barrett's oesophageal adenocarcinoma, or endoscopic biopsy for Barrett's oesophagus at Kyoto University Hospital or Saku Central Hospital between 2003 and 2005. Selection of patients enrolled in this study was based on the availability of a sufficient amount of tissue for analysis. The study group comprised 22 patients with Barrett's adenocarcinoma (Table 1) and 6 patients with Barrett's oesophagus but no oesophageal adenocarcinoma (3 men and 3 women, with a median age at the time of endoscopic treatment of 61 [range 38-81] years). In addition to tumour tissues and the surrounding nontumorous tissues of the columnar-epithelium lined Barrett's oesophagus, the normal squamous cells surrounding Barrett's epithelium were obtained from 16 cases with Barrett's adenocarcinoma. To validate the results of the initial analysis, we performed replication analyses using an independent another cohort from a different region of Japan (Shimane University). This second cohort included 16 patients with Barrett's oesophagus but no oesophageal adenocarcinoma (7 men and 9 women, with a median age at the time of endoscopic treatment of 70 [range 45-80] years) and 4 patients with Barrett's oesophageal adenocarcinoma (3 men and 1 woman, with a median age at the time of endoscopic treatment of 80 [range 65-85] years). Barrett's oesophagus was defined by endoscopically recognizable columnar metaplasia of the oesophageal mucosa that was confirmed to have intestinal metaplasia by biopsy of the tubular oesophagus (27). Normal gastric mucosa and oesophageal squamous epithelium were obtained from biopsy samples of nontumorous tissues from patients with benign submucosal tumours of the stomach or the oesophagus, none of which showed evidence of *H. pylori* infection, and were used for AID immunostaining as a control. 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.

Immunohistochemistry

A polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen (28). Immunohistochemical staining for endogenous AID protein was performed as described previously (7). For semi-quantitative analysis of the immunostaining results, the slides were scored for AID staining independently by two evaluators (S.M. and Y.M.). Visual assessment of the degree and intensity of the immunoreactivity was classified as no staining (-), no appreciable staining; weak positive staining (+), less than 50% of the gastrointestinal epithelial cells or tumour cells stained with AID; and strong positive staining (++), more than 50% of the epithelial cells or tumour cells stained with AID.

Cell culture and transfection

Human non-neoplastic oesophageal squamous-derived Het-1A cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in Bronchial Epithelial Cell medium supplemented with the growth factors (BEGM Bulletkit, Lonza, Basel, Switzerland). To express the super-repressor form of the I κ B- α protein, pcDNA3-I κ B- α -del-N was made by inserting the cDNA fragment of human I κ B- α into the BamHI-EcoRI sites of pcDNA3 (Invitrogen, Carlsbad, CA). The cDNA fragment for pcDNA3-I κ B- α -del-N (amino acids 37-317) was synthesized by reverse-transcription (RT) - polymerase chain reaction (PCR) with the oligonucleotide primers 5'-CGCGGATCCATGAAAGACGAGGAGTACGA-3' (forward) and 5'-CCGGAATTCTCATAACGTCAGACGCTGGC-3' (reverse), as described previously (16). Lipofectamine 2000 reagent (Invitrogen) was used for transfection of the plasmids. The small interfering RNA (siRNA) duplex composed of 21-nucleotides against the endogenous

noncatalytic subunit I κ B kinase- γ (IKK γ ; also known as NEMO [NF- κ B essential modulator]) was obtained from Invitrogen (5'-UGCUCUUGAUGUGGUUGUCGUAUUC-3', Stealth RNAi) and cells were treated with 0.07 nmol/ml siRNA for IKK- γ /NEMO knockdown. A nonrelated control, Stealth RNAi Negative Control Duplex (Invitrogen), which lacks identity with known gene targets, was used as a control for nonsequence-specific effects. *TransIT*-TKO reagent (Mirus, Madison, WI) was used for siRNA transfection. Sodium salt deoxycholic acid (DCA) and the NF- κ B inhibitors MG132 and SN50 were purchased from Sigma (St. Louis, MO), Calbiochem (Darmstadt, Germany), and Biomol International LP (Plymouth Meeting, PA), respectively.

Generation of stable cell lines

Stable cell lines derived from Het-1A cells were established by a lentiviral vector-mediated AID expression system (15, 29). In brief, lentiviral stocks were produced in 293T cells in accordance with the manufacturer's protocol (Invitrogen). Virus-containing medium was collected 48 h post-transfection and filtered through a 0.45- μ m filter. Het-1A cells were cultured in virus-containing medium in the presence of Neomycin (Sigma, St. Louis, MO) until colonies of stably transfected clones grew. Genomic DNA was extracted from the cells derived from the three independent cultured colonies 8 weeks after viral transduction.

Subcloning and sequencing of tumour-related genes

The primer sets for amplification of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene were: 5'- GAGGCCGATCCAGGTCAT -3' (forward) and 5'- TTTACGGTAGTGGGGGAAGG -3' (reverse). The primer sets for amplification of the *TP53* gene were: 5'- CCCTTCCCAGAAAACCTACC -3' (forward) and 5'- CCTCATTGAGCTCTCGGAAC -3' (reverse). Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland), and the products were subcloned into a pGEM-T Easy

vector (Promega, Madison, WI). The resulting plasmids were subjected to sequence analysis using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Quantitative real-time RT-PCR

Human AID gene expression was determined by quantitative real-time RT-PCR using a Light Cycler 480 and Fast Start Universal Probe Master (Roche, Mannheim, Germany)(7). The 6-carboxyfluorescein (FAM)-labelled probe specific for human *AID* was 5'-TCGGCGTGAGACCTACCTGTGCTAC-3'. Standard curves for *AID* were generated for every target using a 10-fold serial dilution series of five independent transcripts derived from BL2-lymphoma cells that contain a high expression level of endogenous *AID*. To assess the quantity of isolated RNA as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene *18S ribosomal RNA* (*18S rRNA*) (15). For simplicity, ratios are represented as relative values compared with expression levels in a lysate from control cells. The PCR procedure was performed at least three times for each sample, and results are expressed as box plots.

Statistical analysis

Statistical differences in AID expression levels were analyzed using the Kruskal-Wallis H-test with Bonferroni correction. Statistical significance was evaluated using the χ^2 test for sequence analyses, and the χ^2 test with Bonferroni correction for immunostaining analyses. Values of $P < 0.05$ were considered statistically significant except when using the Bonferroni correction.

Results

Endogenous AID expression is upregulated in the columnar epithelial cells lining human Barrett's oesophagus and oesophageal adenocarcinoma cells.

To clarify the expression and localization of AID protein in human oesophageal epithelium under physiological and pathological conditions, immunohistochemistry was performed using a specific antibody against human AID. Specificity of the antibody in immunostaining was confirmed by control staining performed on germinal centres of human mesenteric lymph nodes containing mostly activated B cells (6). First, weak or no immunostaining for AID protein was observed in the columnar cells from the normal gastric mucosa that did not exhibit inflammatory changes, consistent with our previous findings (7) (Figure 1A and Table 2). Similarly, weak or no AID expression was observed in normal squamous epithelial cells of individuals who lacked histological evidence of Barrett's oesophageal changes (Figure 1A and Table 2). The normal squamous epithelial cells surrounding the columnar-lined Barrett's epithelial cells were weakly positive or negative for AID immunostaining (Figure 1B and Table 2). No AID immunostaining was observed in any of the tissue specimens when nonimmunized serum or phosphate-buffered saline was used instead of antibodies against AID (data not shown). In contrast, AID immunoreactivity was detected in 24 of 28 (85.7%) specimens and strong immunoreactivity for AID was detected in 17 of 28 (60.7%) specimens of the columnar epithelial cell-lined Barrett's oesophagus (Figure 1B and Table 2). In the cancer tissues, AID immunoreactivity was detected in 20 of 22 (90.9%) patients, and strong expression of AID protein was present in 12 of 22 (54.5 %) patients with Barrett's oesophageal adenocarcinoma (Figure 2 and Table 2). The frequency of positivity for AID expression in Barrett's epithelial cells was significantly higher than that in normal squamous epithelial cells of patients without Barrett's oesophagus ($P = 0.021$, Table 2). Although the frequency of positivity for AID expression in cancer cells tended to be

higher than that in normal squamous epithelial cells of patients without Barrett's oesophagus, the difference was not significant ($P = 0.038$ [a level of $P < 0.025$ was considered significant], Table 2). We validated the AID immunostaining results by analyzing the tissue specimens from another Japanese cohort comprising 10 subjects with normal squamous cells, 4 with Barrett's adenocarcinoma, and 16 with Barrett's columnar cells but no oesophageal adenocarcinoma (Supplementary Table 1). Strong AID protein expression was observed in the columnar epithelial cells lining Barrett's oesophagus in 15 of 16 (94.0%) patients who did not develop oesophageal adenocarcinoma, and in 4 of 4 (100%) patients with Barrett's adenocarcinoma (Supplementary Figure 1). The frequency of positivity for AID expression in Barrett's epithelial cells or in cancer cells was significantly higher than that in normal squamous epithelial cells ($P = 0.00025$, $P = 0.014$, respectively, Supplementary Table 1). These findings suggest that the inflammatory response related to the development of Barrett's oesophagus involves aberrant AID expression in the columnar epithelial cells lining Barrett's oesophagus. Regarding the effect of treatment with proton pump inhibitors (PPIs) on AID expression, we found no significant difference in the frequency of endogenous AID expression between patients with and without PPI medication (data not shown).

Aberrant AID expression is induced by DCA stimulation in both human oesophageal squamous and gastrointestinal columnar-lined cells.

Bile reflux, which is usually associated with acid reflux, plays a critical role in the pathogenesis of Barrett's oesophagus and the development of Barrett's oesophageal cancer (21, 30). Several studies have demonstrated that bile acid stimulation induces the activation of transcriptional factor NF- κ B in gastrointestinal epithelial cells (31-34). We previously found that AID expression in gastric epithelial cells is regulated by the NF- κ B pathway (7); therefore, we investigated whether bile acid stimulation could induce AID upregulation in oesophageal

squamous cell epithelium *in vitro*. Accordingly, expression of AID mRNA transcripts was analyzed by quantitative RT-PCR in human non-neoplastic oesophageal squamous epithelium-derived Het-1A cells in the absence or presence of DCA, a bile acid that has a putative role in the development of gastrointestinal cancer (35). Only small amounts of AID expression were detectable in the quiescent Het-1A cells without any stimulation. Endogenous AID expression, however, was induced in Het-1A cells in response to DCA treatment in a dose-dependent manner (Figure 3A, 3B). In addition, DCA induced a time-dependent transcriptional upregulation of AID mRNA in the cells, peaking at 10 hours (Figure 3C). Next we examined whether the DCA-mediated AID expression was achieved through the NF- κ B signalling pathway. The cells were pre-treated with the NF- κ B inhibitory reagents MG132 and SN50, followed by stimulation with DCA. Treatment with MG132 or SN50 significantly reduced the DCA-induced increase in AID transcripts ($P<0.01$, Figure 3D, 3E). Moreover, the enhanced AID expression induced by DCA was significantly reduced by coproduction of the negative regulator of NF- κ B, the dominant negative form of I κ B- α kinase, in Het-1A cells ($P<0.01$, Figure 3F). In addition, DCA significantly failed to elicit an increase in AID expression in cells in which the endogenous noncatalytic subunit I κ B kinase- γ (IKK γ /NEMO) was reduced by siRNA ($P<0.01$, Figure 3G). Taken together, these findings indicate that the induction of AID expression in human oesophageal squamous cell epithelium by bile acid stimulation is achieved through the activation of NF- κ B. We also tested whether DCA would be also involved in the aberrant AID expression in gastric columnar cell-lined epithelium. Quantitative RT-PCR analysis clearly showed that DCA stimulation in human gastric AGS cells substantially upregulated AID transcripts (Supplementary Figure 2). These findings suggest that aberrant AID expression in the oesophageal epithelium during the progression of Barrett's oesophagus is achieved by bile acid stimulation through the activation of NF- κ B.

AID activation resulted in the accumulation of nucleotide alterations in tumour-related genes of the human oesophageal squamous epithelium-derived cells.

We previously demonstrated that aberrant *AID* gene expression triggers the accumulation of genomic mutations in the *TP53* and *CDKN2A* genes of the cultured human gastric columnar epithelial cell lining (7, 36). To clarify whether DCA-induced aberrant *AID* gene expression is genotoxic in oesophageal squamous epithelial cells, we investigated whether *AID* caused somatic mutations in the tumour suppressor genes, *TP53* and *CDKN2A*, both of which have been reported to contain nucleotide alterations in human Barrett's oesophageal adenocarcinoma tissues (24, 25, 37, 38). For this purpose, we established cultures of human non-neoplastic oesophageal squamous epithelium-derived Het-1A cells with constitutive *AID* expression using a lentiviral system (Supplementary Figure 3), followed by sequencing analyses performed on DNA samples extracted from the cells with or without *AID* activation for 8 weeks. Accordingly, over 40 cultured colonies carrying the amplified tumour-related gene fragments were randomly picked and subjected to sequence analyses. Less than one substitution per 1×10^4 nucleotides was detected in the tumour-related genes subcloned from control cells without *AID* activation (Table 3). In contrast, more nucleotide alterations appeared in *CDKN2A* gene of the cells expressing *AID*, while 4 of the 6 mutations emerged in *CDKN2A* gene were identical silent mutations (Table 3 and Supplementary Table 2). The nucleotide alterations induced by *AID* gene activation were also observed in the *TP53* sequences, but the difference in the mutation frequency between the *AID*-expressing cells and control cells was not significant ($P=0.32$, Table 3 and Supplementary Table 2). Although these findings suggest the possibility that aberrant *AID* gene expression might act as a DNA mutator for some tumour suppressor genes in human oesophageal squamous epithelium cells, the evidence was insufficient to reach a clear conclusion.

Discussion

Barrett's oesophagus is the strongest known risk factor for the development of adenocarcinoma of the distal oesophagus (21). Various genetic alterations in relation to the dysregulation of cell growth and apoptosis occur during the development of Barrett's oesophageal cancers (24, 25, 39). For example, *TP53* is the most commonly mutated tumour suppressor gene and is implicated in oesophageal adenocarcinoma developing in patients with Barrett's oesophagus (39). How somatic mutations accumulate through the process of carcinogenesis under the background of Barrett's oesophagus, however, remains unknown. In the present study, we demonstrated that a recently identified DNA editing enzyme, AID, is upregulated in the columnar-cell epithelium of Barrett's oesophagus, while normal gastric columnar cells and oesophageal squamous cells showed weak or no expression of AID protein. Moreover, strong AID expression was frequently observed in the tumour cells of Barrett's adenocarcinoma. Considering the genotoxic activity of AID, our present findings suggest the aberrant AID expression enhances the susceptibility to genetic alterations in Barrett's columnar-lined epithelial cells.

Increased exposure of the oesophagus to refluxed gastric and duodenal contents has a critical role in the development of Barrett's oesophagus and tumour development. The concentration of bile acids in the oesophageal refluxate correlates with the degree of oesophageal mucosal injury (22, 40). More importantly, a secondary bile acid such as DCA is implicated in various cancers that develop in the gastrointestinal tract (41). In oesophageal adenocarcinoma, DCA is thought to contribute to carcinogenesis during reflux of the gastrointestinal contents (23, 42, 43). Several studies report that NF- κ B is activated by bile acid components, resulting in the upregulation of a variety of genes involved in the development of metaplasia of Barrett's oesophagus and cancer development (31-33, 39, 44). We previously demonstrated that NF- κ B enhances AID expression

in hepatocytes, and in colonic and gastric epithelial cells (7, 15, 16). In the present study, AID transcriptional upregulation was induced in response to bile acid stimulation in oesophageal epithelial cells via the NF- κ B signaling pathway. Consistent with our findings, a recent paper demonstrated a non-linear dose response to DCA for DNA damage and NF- κ B activation in oesophageal cells (45). Thus, bile acid-mediated aberrant AID expression provides a novel link between gastroduodenal reflux and the increased susceptibility to carcinogenesis in Barrett's oesophagus.

Several clinical and molecular features differ markedly between squamous cell carcinoma and Barrett's adenocarcinoma of the oesophagus. In Barrett's oesophagus, the tissue undergoes a variety of genetic alterations and a prospective follow-up of lesions biopsied by endoscopy indicated that *TP53* gene mutations usually occur as an early event in the molecular pathogenesis of Barrett's oesophageal adenocarcinoma (39). Previous studies also demonstrated the same *TP53* mutations in adenocarcinoma and adjacent non-cancerous Barrett's oesophageal epithelium, suggesting that the *TP53* mutation is an important step in the progression toward adenocarcinoma (25, 46). Moreover, somatic mutations of the *CDKN2A* gene are frequently observed in human Barrett's oesophageal adenocarcinoma tissues (37, 38). Our previous findings of constitutive AID expression in the columnar-cell lined gastric epithelium with nucleotide alterations in both the *TP53* and *CDKN2A* genes (7, 36) and submicroscopic deletions in the *CDKN2A* gene locus (36) support the hypothesis that AID expression contributes to the enhanced susceptibility of the columnar-cell lined epithelium in Barrett's oesophagus to tumourigenesis, potentially leading to adenocarcinoma. In the present study, we found that constitutive expression of AID in oesophageal squamous cells might also contribute to the accumulation of somatic mutations in both *CDKN2A* and *TP53* genes, however, the evidence was insufficient to conclude that AID was genotoxic in human oesophageal squamous cells.

In conclusion, the present findings indicate that aberrant AID expression occurs in a substantial proportion of human Barrett's epithelial and adenocarcinoma cells, suggesting that the genotoxicity of AID in columnar epithelial cells lining Barrett's oesophagus contributes to the accumulation of genetic alterations of tumour-related genes. Our findings also revealed that bile acid reflux plays a critical role in aberrant AID expression in the development of Barrett's oesophageal adenocarcinoma. A recent study suggested that Barrett's oesophagus is genetically heterogeneous where there are multiple independent clones (47), and a distinct clonal evolution from metaplasia to dysplasia was observed in the human stomach (48). Thus, further analyses are necessary to clarify whether aberrant AID expression in Barrett's oesophageal epithelium causes the genetic aberrations that contribute to expanding the clonal field of dysplasia, promoting the development of multiple independent subclones to enhance the risk of developing putative cancer cells.

Figure legend

Fig. 1. Aberrant expression of AID protein in Barrett's oesophagus tissue specimens.

Representative immunostaining for endogenous AID is shown. Immunohistochemistry was performed on normal columnar-cell lined gastric epithelium (A; upper panels), normal oesophageal squamous epithelium (A; lower panels), Barrett's oesophagus (case #1 and #2, B; top and middle panels), and squamous cells surrounding Barrett's epithelium (B; bottom panels). (original magnification $\times 100$)

Fig. 2. AID protein production in tumour cells of Barrett's oesophageal adenocarcinoma.

Representative moderate-to-strong AID immunostaining is shown in the tumour tissues of Barrett's oesophageal adenocarcinoma. (original magnification $\times 100$)

Fig. 3. DCA-mediated AID expression in human non-neoplastic oesophageal

squamous-epithelium derived Het-1A cells. (A) RT-PCR for the expression of *AID* by Het-1A cells at 10 h after stimulation with 150 μM of DCA (upper panel). *β -actin* was quantified in each sample as an internal control (lower panel). (B) Het-1A cells were treated with various concentrations of DCA (0-150 μM) for 10 hours. AID transcripts were measured by quantitative real-time RT-PCR. The expression levels were normalized to 18s ribosomal RNA (18s rRNA) as an endogenous control. The ratios are shown as relative value compared with the AID expression levels in non-stimulated Het-1A cells. (C) Time-dependent effects of DCA on AID gene expression. Het-1A cells were harvested and subjected to total RNA isolation immediately before (0) and 9, 10, 11, and 12 hours after stimulation by DCA (100 μM). (D, E) Het-1A cells were treated with MG132 (0.15 μM) (D) or SN50 (250ng/ml) (E) for 2 hours and treated with DCA (150 μM) for 10 hours. *AID* transcripts were analyzed by quantitative real-time RT-PCR.

(F) pcDNA3-I κ B- α -del-N (I κ B α [Mutant]) was transfected into Het-1A cells and then treated with DCA (150 μ M) for 10 hours. Empty vector was used as a control. Total RNA was isolated from each cell and the expression levels of *AID* mRNA were measured by quantitative real-time RT-PCR. (G) siRNA targeting IKK γ (IKK γ siRNA) or control siRNA (CTR siRNA) were transfected into Het-1A cells, followed by DCA stimulation for 10 hours. Total RNA was isolated from each cell and the expression levels of *AID* mRNA were measured by quantitative real-time RT-PCR. *, $P < 0.01$.

Supplementary material

Supplementary Figure 1.

Aberrant expression of AID protein in Barrett's oesophagus and Barrett's oesophageal adenocarcinoma tissue specimens of the second patient group. Representative immunostaining for endogenous AID is shown. Immunohistochemistry was performed on normal gastric columnar-lined epithelium (A; upper panels), normal oesophageal squamous epithelium (A; lower panels), and Barrett's oesophagus (case #1 and #2, B; upper and lower panels). (original magnification $\times 100$) (C) Representative moderate-to-strong AID immunostaining is shown in the tumour tissues of Barrett's oesophageal adenocarcinoma. (original magnification $\times 100$)

Supplementary Figure 2.

AID expression by DCA in human gastrointestinal columnar-lined epithelial cells. (A) Human gastric epithelium-derived AGS cells were treated with various concentrations of DCA (0-100 μ M) for 10 hours. AID transcripts were measured by quantitative real-time RT-PCR. The expression levels were normalized to 18s rRNA as an endogenous control. The ratios are shown as relative value compared with the AID expression levels in non-stimulated AGS cells. (B) Time-dependent effects of DCA on AID gene expression. AGS cells were harvested and

subjected to total RNA isolation immediately before (0) and 9, 10, 11, and 12 hours after stimulation by DCA (50 μ M).

Supplementary Figure 3.

RT-PCR for the expression of *AID* (upper panel) by control Het-1A cells (CTR), Het-1A cells at 10 hours after stimulation with DCA (DCA), and Het-1A cells with constitutive AID expression using a lentiviral system (AID). *β -actin* (lower panel) was quantified for each sample as an internal control.

Funding: This work was supported by Japan Society for the Promotion of Science (JSPS) Grants-in-aid for Scientific Research, Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan, and Takeda Science Foundation.

Acknowledgements: We thank Manabu Muto (Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan) for his advice on statistical analysis, and Yoko Endo (Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan) for her technical assistance.

Conflict of Interest Statement: None declared.

Reference

1. Balkwill, F., and Mantovani, A. (2001) Inflammation and cancer: back to Virchow? *Lancet* 357:539-545.
2. Grivennikov, S.I., Greten, F.R., and Karin, M. (2010) Immunity, inflammation, and cancer. *Cell* 140:883-899.
3. Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008) Cancer-related inflammation. *Nature* 454:436-444.
4. Thibodeau, S.N., Bren, G., and Schaid, D. (1993) Microsatellite instability in cancer of the proximal colon. *Science* 260:816-819.
5. Colotta, F., Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* 30:1073-1081.
6. Kou, T., Marusawa, H., Kinoshita, K., Endo, Y., Okazaki, I.M., Ueda, Y., Kodama, Y., Haga, H., Ikai, I., and Chiba, T. (2007) Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. *Int J Cancer* 120:469-476.
7. Matsumoto, Y., Marusawa, H., Kinoshita, K., Endo, Y., Kou, T., Morisawa, T., Azuma, T., Okazaki, I.M., Honjo, T., and Chiba, T. (2007) Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat Med* 13:470-476.
8. Harris, R.S., Petersen-Mahrt, S.K., and Neuberger, M.S. (2002) RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol Cell* 10:1247-1253.
9. Harris, R.S., and Liddament, M.T. (2004) Retroviral restriction by APOBEC proteins. *Nat Rev Immunol* 4:868-877.
10. Honjo, T., Kinoshita, K., and Muramatsu, M. (2002) Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu Rev Immunol* 20:165-196.
11. Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labeu, R., Gennery, A., et al. (2000) Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 102:565-575.
12. Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102:553-563.
13. Okazaki, I.M., Hiai, H., Kakazu, N., Yamada, S., Muramatsu, M., Kinoshita, K., and Honjo, T. (2003) Constitutive expression of AID leads to tumorigenesis. *J Exp Med*

197:1173-1181.

14. Morisawa, T., Marusawa, H., Ueda, Y., Iwai, A., Okazaki, I.M., Honjo, T., and Chiba, T. (2008) Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression. *Int J Cancer* 123:2735-2740.
15. Endo, Y., Marusawa, H., Kou, T., Nakase, H., Fujii, S., Fujimori, T., Kinoshita, K., Honjo, T., and Chiba, T. (2008) Activation-induced cytidine deaminase links between inflammation and the development of colitis-associated colorectal cancers. *Gastroenterology* 135:889-898, 898 e881-883.
16. Endo, Y., Marusawa, H., Kinoshita, K., Morisawa, T., Sakurai, T., Okazaki, I.M., Watashi, K., Shimotohno, K., Honjo, T., and Chiba, T. (2007) Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. *Oncogene* 26:5587-5595.
17. Komori, J., Marusawa, H., Machimoto, T., Endo, Y., Kinoshita, K., Kou, T., Haga, H., Ikai, I., Uemoto, S., and Chiba, T. (2008) Activation-induced cytidine deaminase links bile duct inflammation to human cholangiocarcinoma. *Hepatology* 47:888-896.
18. Marusawa, H. (2008) Aberrant AID expression and human cancer development. *Int J Biochem Cell Biol* 40:1399-1402.
19. Kinoshita, K., and Nonaka, T. (2006) The dark side of activation-induced cytidine deaminase: relationship with leukemia and beyond. *Int J Hematol* 83:201-207.
20. Reid, B.J., Li, X., Galipeau, P.C., and Vaughan, T.L. (2010) Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis. *Nat Rev Cancer* 10:87-101.
21. Shaheen, N.J., and Richter, J.E. (2009) Barrett's oesophagus. *Lancet* 373:850-861.
22. Nehra, D., Howell, P., Williams, C.P., Pye, J.K., and Beynon, J. (1999) Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. *Gut* 44:598-602.
23. Zhang, F., Altorki, N.K., Wu, Y.C., Soslow, R.A., Subbaramaiah, K., and Dannenberg, A.J. (2001) Duodenal reflux induces cyclooxygenase-2 in the esophageal mucosa of rats: evidence for involvement of bile acids. *Gastroenterology* 121:1391-1399.
24. Flejou, J.F. (2005) Barrett's oesophagus: from metaplasia to dysplasia and cancer. *Gut* 54 Suppl 1:i6-12.
25. Barrett, M.T., Sanchez, C.A., Prevo, L.J., Wong, D.J., Galipeau, P.C., Paulson, T.G., Rabinovitch, P.S., and Reid, B.J. (1999) Evolution of neoplastic cell lineages in Barrett oesophagus. *Nat Genet* 22:106-109.
26. Marusawa, H., and Chiba, T. (2010) Helicobacter pylori-induced activation-induced cytidine deaminase expression and carcinogenesis. *Curr Opin Immunol* 22:442-447.
27. Sampliner, R.E. (2002) Updated guidelines for the diagnosis, surveillance, and therapy of Barrett's esophagus. *Am J Gastroenterol* 97:1888-1895.

28. Ta, V.T., Nagaoka, H., Catalan, N., Durandy, A., Fischer, A., Imai, K., Nonoyama, S., Tashiro, J., Ikegawa, M., Ito, S., et al. (2003) AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* 4:843-848.
29. Horie, T., Ono, K., Horiguchi, M., Nishi, H., Nakamura, T., Nagao, K., Kinoshita, M., Kuwabara, Y., Marusawa, H., Iwanaga, Y., et al. (2010) MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. *Proc Natl Acad Sci U S A* 107:17321-17326.
30. Miwa, K., Sahara, H., Segawa, M., Kinami, S., Sato, T., Miyazaki, I., and Hattori, T. (1996) Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. *Int J Cancer* 67:269-274.
31. Jenkins, G.J., Harries, K., Doak, S.H., Wilmes, A., Griffiths, A.P., Baxter, J.N., and Parry, J.M. (2004) The bile acid deoxycholic acid (DCA) at neutral pH activates NF-kappaB and induces IL-8 expression in oesophageal cells in vitro. *Carcinogenesis* 25:317-323.
32. Debruyne, P.R., Witek, M., Gong, L., Birbe, R., Chervoneva, I., Jin, T., Domon-Cell, C., Palazzo, J.P., Freund, J.N., Li, P., et al. (2006) Bile acids induce ectopic expression of intestinal guanylyl cyclase C Through nuclear factor-kappaB and Cdx2 in human esophageal cells. *Gastroenterology* 130:1191-1206.
33. Payne, C.M., Weber, C., Crowley-Skillicorn, C., Dvorak, K., Bernstein, H., Bernstein, C., Holubec, H., Dvorakova, B., and Garewal, H. (2007) Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. *Carcinogenesis* 28:215-222.
34. Wu, J., Gong, J., Geng, J., and Song, Y. (2008) Deoxycholic acid induces the overexpression of intestinal mucin, MUC2, via NF-kB signaling pathway in human esophageal adenocarcinoma cells. *BMC Cancer* 8:333.
35. Cronin, J., Williams, L., McAdam, E., Eltahir, Z., Griffiths, P., Baxter, J., and Jenkins, G. (2010) The role of secondary bile acids in neoplastic development in the oesophagus. *Biochem Soc Trans* 38:337-342.
36. Matsumoto, Y., Marusawa, H., Kinoshita, K., Niwa, Y., Sakai, Y., and Chiba, T. (2010) Upregulation of activation-induced cytidine deaminase causes genetic aberrations at the CDKN2b-CDKN2a in gastric cancer. *Gastroenterology*.
37. Maley, C.C. (2007) Multistage carcinogenesis in Barrett's esophagus. *Cancer Lett* 245:22-32.
38. Paulson, T.G., Galipeau, P.C., Xu, L., Kissel, H.D., Li, X., Blount, P.L., Sanchez, C.A., Odze, R.D., and Reid, B.J. (2008) p16 mutation spectrum in the premalignant condition Barrett's esophagus. *PLoS One* 3:e3809.
39. Fitzgerald, R.C. (2006) Molecular basis of Barrett's oesophagus and oesophageal

adenocarcinoma. *Gut* 55:1810-1820.

40. Gillen, P., Keeling, P., Byrne, P.J., Healy, M., O'Moore, R.R., and Hennessy, T.P. (1988) Implication of duodenogastric reflux in the pathogenesis of Barrett's oesophagus. *Br J Surg* 75:540-543.
41. Nagengast, F.M., Grubben, M.J., and van Munster, I.P. (1995) Role of bile acids in colorectal carcinogenesis. *Eur J Cancer* 31A:1067-1070.
42. Tselepis, C., Morris, C.D., Wakelin, D., Hardy, R., Perry, I., Luong, Q.T., Harper, E., Harrison, R., Attwood, S.E., and Jankowski, J.A. (2003) Upregulation of the oncogene c-myc in Barrett's adenocarcinoma: induction of c-myc by acidified bile acid in vitro. *Gut* 52:174-180.
43. Looby, E., Abdel-Latif, M.M., Athie-Morales, V., Duggan, S., Long, A., and Kelleher, D. (2009) Deoxycholate induces COX-2 expression via Erk1/2-, p38-MAPK and AP-1-dependent mechanisms in esophageal cancer cells. *BMC Cancer* 9:190.
44. Yen, C.J., Izzo, J.G., Lee, D.F., Guha, S., Wei, Y., Wu, T.T., Chen, C.T., Kuo, H.P., Hsu, J.M., Sun, H.L., et al. (2008) Bile acid exposure up-regulates tuberous sclerosis complex 1/mammalian target of rapamycin pathway in Barrett's-associated esophageal adenocarcinoma. *Cancer Res* 68:2632-2640.
45. Jenkins, G.J., Cronin, J., Alhamdani, A., Rawat, N., D'Souza, F., Thomas, T., Eltahir, Z., Griffiths, A.P., and Baxter, J.N. (2008) The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS. *Mutagenesis* 23:399-405.
46. Neshat, K., Sanchez, C.A., Galipeau, P.C., Blount, P.L., Levine, D.S., Joslyn, G., and Reid, B.J. (1994) p53 mutations in Barrett's adenocarcinoma and high-grade dysplasia. *Gastroenterology* 106:1589-1595.
47. Leedham, S.J., Preston, S.L., McDonald, S.A., Elia, G., Bhandari, P., Poller, D., Harrison, R., Novelli, M.R., Jankowski, J.A., and Wright, N.A. (2008) Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. *Gut* 57:1041-1048.
48. Gutierrez-Gonzalez, L., Graham, T.A., Rodriguez-Justo, M., Leedham, S.J., Novelli, M.R., Gay, L.J., Ventayol-Garcia, T., Green, A., Mitchell, I., Stoker, D.L., et al. (2011) The clonal origins of dysplasia from intestinal metaplasia in the human stomach. *Gastroenterology* 140:1251-1260 e1251-1256.

Figure1. Morita et al.

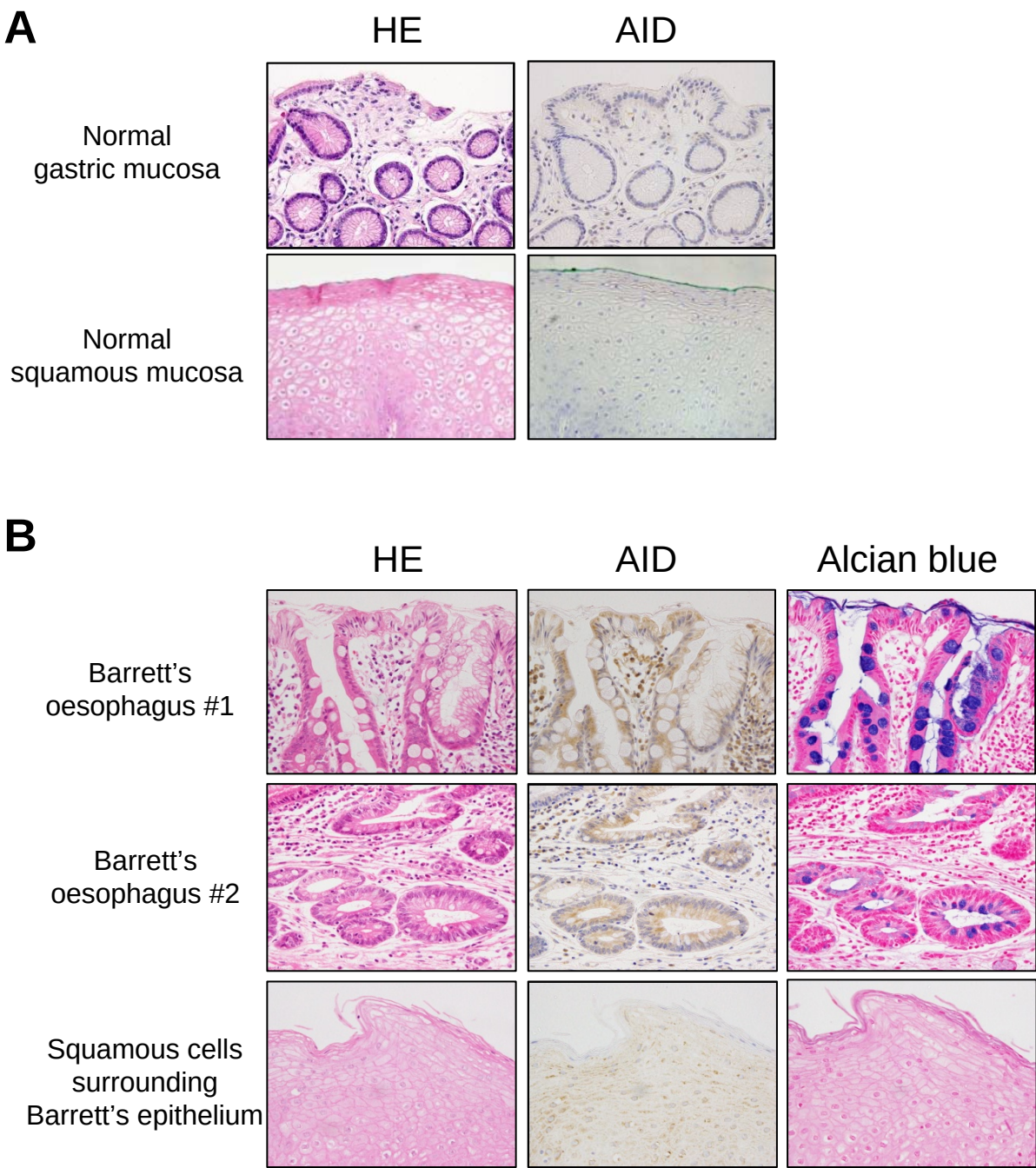


Figure2. Morita et al.

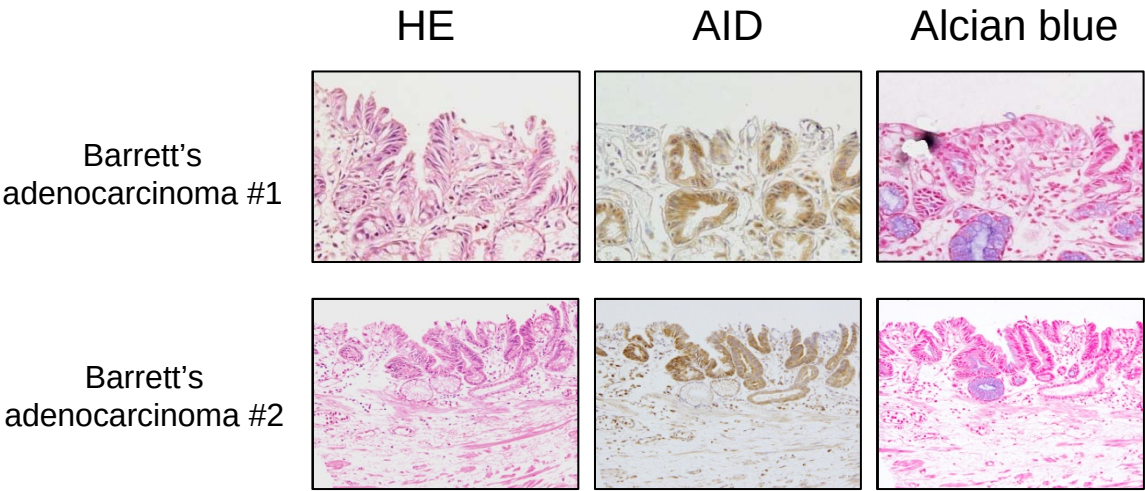
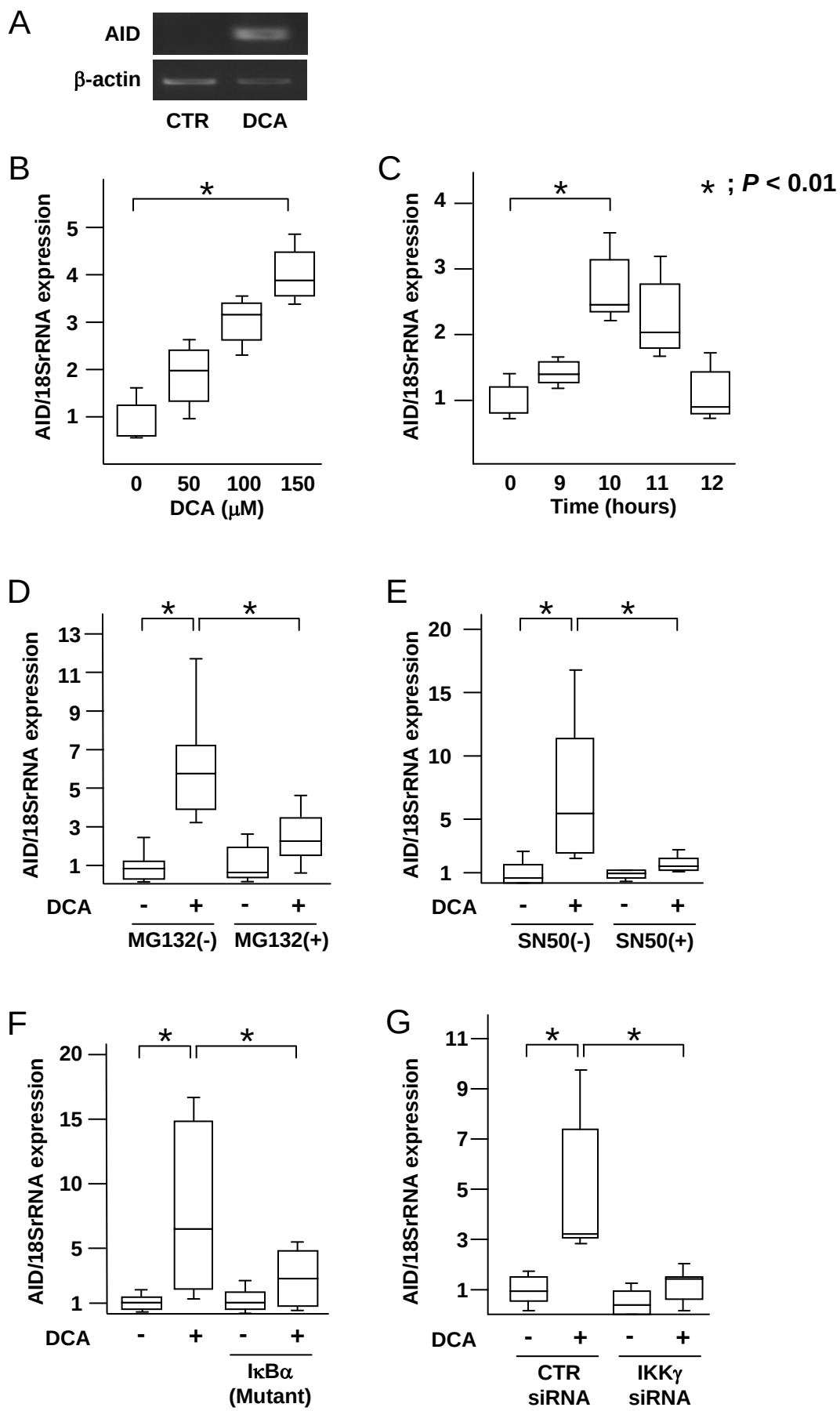


Figure 3. Morita et al.

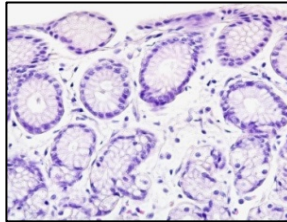


Supplementary Figure1. Morita et al.

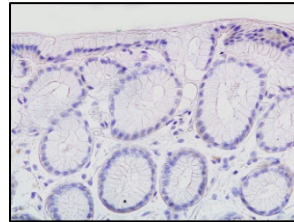
A

Normal
gastric mucosa

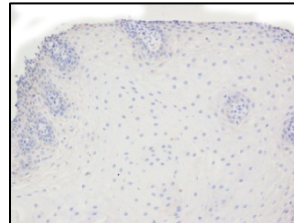
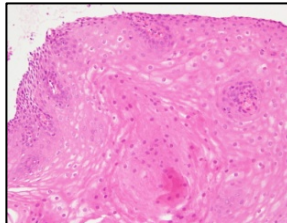
HE



AID



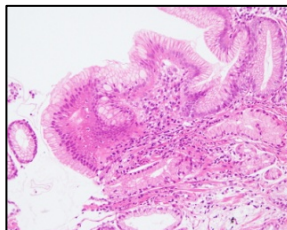
Normal
squamous mucosa



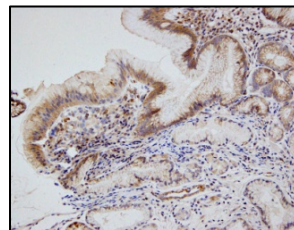
B

Barrett's
oesophagus #1

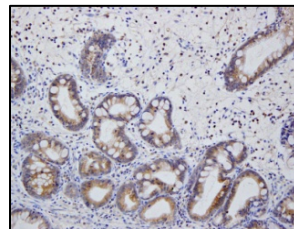
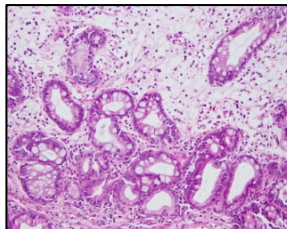
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AID



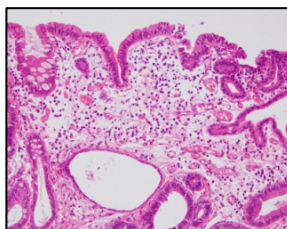
Barrett's
oesophagus #2



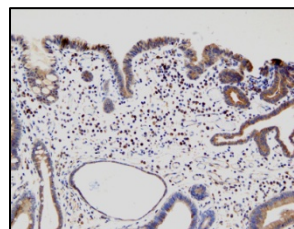
C

Barrett's
adenocarcinoma #1

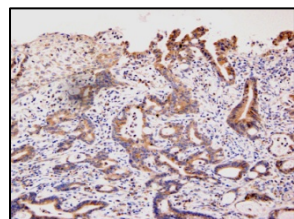
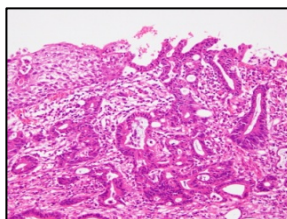
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AID

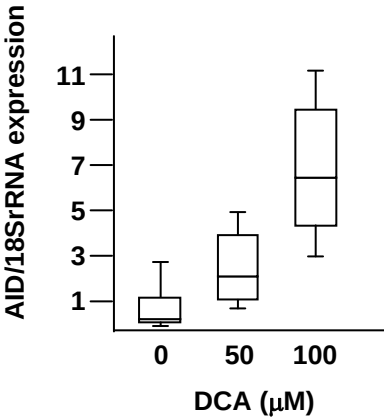


Barrett's
adenocarcinoma #2

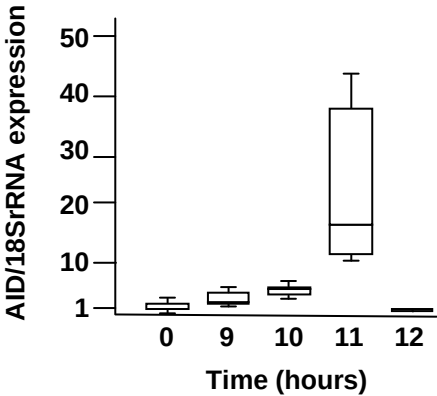


Supplementary Figure 2. Morita et al.

A



B



Supplementary Figure 3. Morita et al.

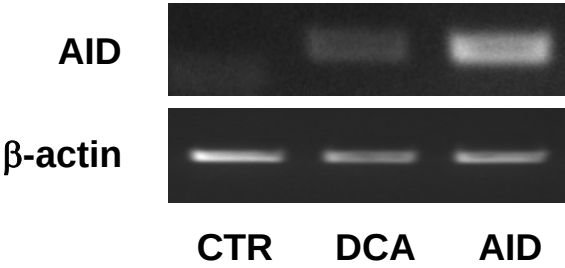


Table 1. Clinicopathological Features of Patients with Barrett's oesophageal adenocarcinoma

Age (years)	
Median age (range)	69 (30-81)
Gender	
Male	19
Female	3
Tumor size (cm)	
Median size (range)	2.0 (0.4-9.4)
Tumor differentiation	
differentiated	17
poorly	2
unkown	3
TNM staging	
0	2
I	13
II	3
III	0
IV	3
unknown	1

Table 2. Semiquantitation of AID immunoreactivity in normal columnar cells, normal squamous cells, Barrett's columnar cells and Barrett's adenocarcinoma.

Cell types	Specimens analyzed (n)	Specimens with AID Immunoreactivity			
		(-)	(+)	(++)	
Normal columnar cells	12	8	4	0	
Normal squamous cells	10	4	6	0	] P = 0.021
Normal squamous cells surrounding Barrett's epithelium	16	7	9	0	
Barrett's columnar cells	28	4	7	17	
Barrett's adenocarcinoma	22	2	8	12	] P = 0.038

(++) : Strongly positive, (+) : Weakly positive, (-) : Negative
n=number of the patients

Table 3. Mutation frequency of *CDKN2A* and *TP53* genes in Het-1A cells with or without AID activation.

	AID(+)			Control		
	Mean mutation frequency	(Mutated bases per total bases in the target gene sequenced)	Mutated clones/total clones	Mean mutation frequency	(Mutated bases per total bases in the target gene sequenced)	Mutated clones/total clones
<i>CDKN2A</i>	$2.70/10^4$	(6/22,231)	6/43	$0.41/10^4$	(1/24,299)	1/47
<i>TP53</i>	$1.52/10^4$	(5/32,956)	5/44	$0.74/10^4$	(3/40,446)	3/54

CDKN2A and *TP53* sequences were amplified from Het-1A cells with or without AID activation for 8 weeks, subcloned into the vector, and then the cultured colonies carrying the amplified tumor-related gene fragments were randomly picked and subjected to sequence analyses. Data represent mean mutation frequency (left), number of mutated bases per number of total base pairs in *CDKN2A* or *TP53* genes sequenced (middle, in parentheses), and number of mutated clones per number of total clones examined (right).

Supplementary Table1. Morita et al.

Semiquantitation of AID immunoreactivity in normal squamous cells, Barrett's columnar cells and Barrett's adenocarcinoma of the patients in the second cohort group.

Cell types	Specimens analyzed (n)	Specimens with AID Immunoreactivity			
		(-)	(+)	(++)	
Normal squamous cells	10	3	7	0	$P = 0.00025$
Barrett's columnar cells	16	0	1	15	
Barrett's adenocarcinoma	4	0	0	4	
					$P = 0.014$

(++) : Strongly positive, (+) : Weakly positive, (-) : Negative
n=number of the patients

Supplementary Table 2. Morita et al.

Mutation patterns of *CDKN2A* and *TP53* genes in Het-1A cells with or without AID activation.

		Cell clone #	mRNA position	cDNA position	Nucleotide change	Amino acid change
<i>CDKN2A</i>	AID(+)	#2	558	252	C to T	GAC (Asp) to GAT (Asp)
		#8	615	-	G to A	- *
		#11	806	-	G to C	-
		#23	806	-	G to C	-
		#34	806	-	G to C	-
		#39	806	-	G to C	-
	Control	#18	914	-	T to C	-
<i>TP53</i>	AID(+)	#5	523	326	T to C	TTC (Phe) to TCC (Ser)
		#26	642	445	T to C	TCC (Ser) to CCC (Pro)
		#31	729	532	del C	CAC (His) to AC (frame shift)
		#37	844	647	T to C	GTG (Val) to GCG (Ala)
		#43	884	687	T to C	TGT (Cys) to TGC (Cys)
	Control	#9	645	448	A to G	ACA (Thr) to GCA (Ala)
		#12	1019	822	T to A	GTT (Val) to GTA (Val)
		#25	1205	1008	G to A	GAG (Glu) to GAA (Glu)

* - ; silent mutations that did not alter amino acid sequences