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

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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, whereas weak or no AID protein expression was detectable in normal squamous epithelial cells of the oesophagus. We validated these results by analysing tissue specimens from another cohort comprising 16 cases with Barrett’s oesophagus and four 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 nuclear factor-kappaB 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 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 non-tumourous 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 tumourgenesis 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 the 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 proinflammatory 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 [three men and three 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 non-tumourous 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 [seven men and nine 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 [three men and one 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 non-tumourous
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 re-
combinant 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 were
classified as no staining (−/C0), no appreciable staining; weak positive staining
(+), 50% of the gastrointestinal epithelial cells or tumour cells stained with
AID and strong positive staining (++), >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 ob-
tained 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 IκB-α protein, pcDNA3-IκB-α-del-N was made by inserting the
complementary DNA (cDNA) fragment of human IκB-α into the BamHI–EcoRI
sites of pcDNA3 (Invitrogen, Carlsbad, CA). The cDNA fragment for pcDNA3-
IκB-α-del-N (amino acids 37-317) was synthesized by reverse transcription
(RT)–polymerase chain reaction (PCR) with the oligonucleotide primers

Fig. 1. Aberrant expression of AID protein in Barrett’s oesophagus tissue specimens. Representative immunostaining for endogenous AID is shown.

Table I. Clinicopathological features of patients with Barrett’s oesophageal adenocarcinoma

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Median age (range)</th>
<th>Gender</th>
<th>Tumour size (cm)</th>
<th>Tumour differentiation</th>
<th>TNM staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>69 (30–81)</td>
<td>Male</td>
<td>Female</td>
<td>3</td>
<td>Differentiated</td>
</tr>
<tr>
<td>Tumour size (cm)</td>
<td>2.0 (0.4–9.4)</td>
<td>Poorly</td>
<td>Unknown</td>
<td>3</td>
<td>Tumour size (cm)</td>
</tr>
<tr>
<td>Tumour differentiation</td>
<td>Differentiated</td>
<td>Poorly</td>
<td>Unknown</td>
<td>3</td>
<td>TNM staging</td>
</tr>
<tr>
<td>Differentiated</td>
<td>17</td>
<td>Poorly</td>
<td>2</td>
<td>Unknown</td>
<td>3</td>
</tr>
<tr>
<td>Poorly</td>
<td>2</td>
<td>Unknown</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*TNM staging is a cancer staging system that describes the extent of cancer in
a patient’s body.

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 non-tumourous
TP53 obtained from Invitrogen (5′ also known as NEMO, nuclear factor-kappaB (NF-κB) gene were 5′-GAGGCCGATCCAGGTCAT-3′ (forward) and 5′-TCGGCGTGAGACCTACCTGTGC-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 non-catalytic subunit IκB kinase-γ [IKKγ; also known as NEMO, nuclear factor-kappaB essential modulator (NF-κB)] essential modulator] was obtained from Invitrogen (5′-UGCUCUGAUUGUGUUGCUAUC-3′, Stealth RNAi) and cells were treated with 0.07 nmol/ml siRNA for IKKγ/NEMO knockdown. A non-related control, Stealth RNAi Negative Control Duplex (Invitrogen), which lacks identity with known gene targets, was used as a control for non-sequence-specific effects. TransIT-TKO reagent (Mirus, Madison, WI) was used for siRNA transfection. Sodium salt deoxycholic acid (DCA) and the IT-TKO reagent (Mirus, Madison, WI) 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 μm filter. Het-1A cells were cultured in virus-containing medium in the presence of Neomycin (Sigma) 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′-TTAATGAGTGGGGAAGG-3′ (reverse). The primer sets for amplification of the IPP53 gene were 5′-CCTTCCTCCAGGAAACCTACC-3′ (forward) and 5′-CCTTCATCCAGCTCTCAGA-3′ (reverse). Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finzymes, 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).

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

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Specimens analysed (n)</th>
<th>Specimens with AID immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>Normal columnar cells</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Normal squamous cells</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Normal squamous cells</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Barrett’s columnar cells</td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td>Barrett’s adenocarcinoma</td>
<td>22</td>
<td>2</td>
</tr>
</tbody>
</table>

n = number of the patients; (+++), strongly positive; (+), weakly positive; (-), negative.

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 ×100).

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′-TCGGCGTGAGCCTACCTGTGCGTAC-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 messenger RNA 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 the results are expressed as box plots.

Statistical analysis

Statistical differences in AID expression levels were analysed using the Kruskal–Wallis H-test with Bonferroni correction. Statistical significance was evaluated using the χ² test for sequence analyses, and the χ² 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). Firstly, weak or no immunostaining for AID protein was observed.
Total RNA was isolated from each cell and the expression levels of AID siRNA-targeting IKK\(\beta\) RNA was isolated from each cell and the expression levels of transcripts were analysed by quantitative real-time RT–PCR. (F) pcDNA3-SN50 (250 ng/ml) (E) for 2 h and treated with DCA (150 \(\mu\)M) immediately before (0) and 9, 10, 11 and 12 h after stimulation by DCA (100 \(\mu\)M). (D and E) Het-1A cells were treated with MG132 (0.15 \(\mu\)M) for 10 h. AID transcripts were measured by quantitative real-time RT–PCR. (G) 1709

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 \(\mu\)M of DCA (upper panel). \(\beta\)-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 \(\mu\)M) for 10 h. AID transcripts were measured by quantitative real-time RT–PCR. The expression levels were normalized to 18s ribosomal RNA (18s RNA) 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 h after stimulation by DCA (100 \(\mu\)M). (D and E) Het-1A cells were treated with MG132 (0.15 \(\mu\)M) (D) or SN50 (250 ng/ml) (E) for 2 h and treated with DCA (150 \(\mu\)M) for 10 h. AID transcripts were analysed by quantitative real-time RT–PCR. (F) pcDNA3-IkB\(\alpha\)del-N [IkB\(\alpha\) (mutant)] was transfected into Het-1A cells and then treated with DCA (150 \(\mu\)M) for 10 h. Empty vector was used as a control. Total RNA was isolated from each cell and the expression levels of AID messenger RNA were measured by quantitative real-time RT–PCR. * \(P < 0.01\).

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 II). 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 II). 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 II). No AID immunostaining was observed in any of the tissue specimens when non-immunized 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 II). 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 II). 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 II). 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 II). We validated the AID immunostaining results by analysing 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 is available at Carcinogenesis Online). 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 is available at Carcinogenesis Online). 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 is available at Carcinogenesis Online). 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 on AID expression, we found no significant difference in the frequency of endogenous AID expression between patients with and without proton pump inhibitor 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-\(\kappa\)B in gastrointestinal epithelial cells (31–34). We previously found that AID expression in gastric epithelial cells is regulated by the NF-\(\kappa\)B inhibitory reagents MG132 and SN50, respectively (7); therefore, we investigated whether bile acid stimulation could induce AID upregulation in oesophageal squamous cell epithelium in vitro. Accordingly, expression of AID messenger RNA transcripts was analysed 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 and 3B). In addition, DCA induced a time-dependent transcriptional upregulation of AID messenger RNA in the cells, peaking at 10 h (Figure 3C). Next, we examined whether the DCA-mediated AID expression was achieved through the NF-\(\kappa\)B signalling pathway. The cells were pre-treated with the NF-\(\kappa\)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 and 3E). Moreover, the enhanced AID expression induced by DCA was significantly reduced by coproduction of the negative regulator of NF-κB, the dominant negative form of IκB-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 non-catalytic subunit Iκκ/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-κB. 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 is available at Carcinogenesis Online). 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-κB.

**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 is available at Carcinogenesis Online), 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. 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).

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

**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 tumour-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).**

**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, whereas 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 that 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-κB 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-κB 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-κB signalling pathway. Consistent with our findings, a recent paper demonstrated a non-linear dose–response to DCA for DNA damage and NF-κB 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 towards adenocarcinoma (25,46). Moreover, somatic mutations of the CDKNA2A 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 the CDKNA2A genes (7,36) and submicroscopic deletions in the CDKNA2A 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 tumourgenesis, 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 CDKNA2A 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.

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Conflict of Interest Statement: None declared.

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