Cholangiocarcinomas associated with long-term inflammation express the activation-induced cytidine deaminase and germinatal center-associated nuclear protein involved in immunoglobulin V-region diversification

WARAPORN CHAN-ON1-3, KAZUHIKO KUWAHARA1, NAOYA KOBAYASHI4, KAZUTAKA OHTA1, TATSUYA SHIMASAKI5, BANCHOB SRIPA6, CHANVIT LEELAYUWAT2 and NOBUO SAKAGUCHI1,7

1Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; 2Department of Clinical Immunology, The Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, 3Biomedical Sciences Program, Khon Kaen University, Khon Kaen, Thailand; 4Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama; 5Institute of Resource Development and Analysis (IRDA), Kumamoto University, Kumamoto, Japan; 6Department of Pathology, Faculty of Medicine and Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen, Thailand; 7Core Research for Evolutional Science and Technology Program (CREST), Japan Science and Technology Agency (JST), Japan

Received March 20, 2009; Accepted May 12, 2009

DOI: 10.3892/ijo_00000339

Abstract. Cholangiocarcinoma (CCA) represents a model of tumor development after long-term inflammation which causes DNA damage or impairs DNA repair mechanism. AID and GANP, both appearing in antigen-driven B cells, are involved in affinity maturation of the immunoglobulin V-region with increased somatic mutation. A normal cholangiocyte line showed the induction of AID transcripts after stimulation with TNF-α, whereas ganp transcripts appeared constitutively in this cell line. Next, we examined the expression of AID and GANP in clinical CCA specimens to obtain information whether their expression levels are associated with the malignant grade of CCA. AID expression was similarly detected in the clinical cases of both well-differentiated and poorly-differentiated CCAs. On the contrary, GANP expression was detected in CCA cells at a higher level in the nucleus of poorly-differentiated CCAs with shorter survivals than in that of well-differentiated CCAs. The high and low cases of nuclear GANP expression showed no change in the frequency of the TP53 mutations, however, further investigation by in vitro experiment demonstrated that the high GANP expression caused the increased number of γH2AX foci after DNA damage by ionizing-irradiation. These results suggest that GANP is involved in regulation of DNA repair mechanism and the abnormal over-expression of GANP together with AID might be associated with rigorous DNA damage, potentially causing the malignant development of CCAs during long-term inflammation.

Introduction

Chronic infection with microorganisms including viruses, bacteria, and parasites is a serious risk factor of cancer (1). The high carcinogenic risk to humans infected with liver fluke or the bacterium Helicobacter (H.) pylori represents a peculiar clinical evidence prompting evaluation of the basis of the linkage between microbial infection and cancer development in humans. In Northeast Thailand, cholangiocarcinoma (CCA) occurs at a high incidence in patients infected with the liver fluke, Opisthorchis viverrini, implying that chronic inflammation of the hepatobiliary tract is associated with tumor development (2-5). The long-term inflammatory responses with the production of various pro-inflammatory cytokines presumably cause DNA damage or...
impair the DNA repair mechanism, thus contributing to carcinogenic transformation.

Activation-induced cytidine deaminase (AID) has been proposed to be a causative factor for TP53 mutations in patients infected with *H. pylori*, which indicates a close linkage of inflammation with gastric cancer (6). AID is induced specifically in germlinal center (GC)-B cells and is an initiator of somatic hypermutation (SHM) of immunoglobulin (Ig) V-region genes and class switch recombination in vivo (7). AID catalyzes deamination of cytidine, generating the C→U alteration that is a key DNA change, and eventually causing C→T and G→A mutations in both DNA strands (8).

GC-associated nuclear protein (GANP) is also required for affinity maturation with increased SHM at the IgV-region after immunization (9,10). GANP is a 210-kDa nuclear protein expressed ubiquitously but up-regulated in GC-B cells (11-13). The middle portion of GANP is homologous to *Saccharomyces cerevisiae* SAC3, which is involved in mRNA export from the nucleus to the cytoplasm. SAC3-deficient cells demonstrate DNA hyper-recombination, as measured by using a tandem-repeat *lea2* reporter gene (14); and similar activity has been demonstrated in GANP-deficient mouse cells (15). GANP is suggested to be involved in generation of SHM or modifying/repairing DNA injuries caused by cytidine deamination. Previously, we demonstrated that GANP expression is up-regulated in various hematological diseases and that the transgenic mice of *gan* gene under the control of Ig promoter/enhancer developed lymphomas (16). However, it remained to investigate whether the increased GANP expression is associated with tumorigenesis and tumor development in clinical cancer cases. Here, we studied the expression of AID and GANP in CCAs, and examined whether their expressions change among the clinical grades of CCA development.

**Materials and methods**

**CCA specimens.** Surgically resected specimens were obtained from 65 patients with intrahepatic cholangiocarcinoma who were admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. Informed consents were obtained from all patients who participated in the project approved by Human Research Ethics Committee of Khon Kaen University. The data including age, gender, histological grading, and the involvement of lymph node, vascular and liver fluke-related CCA in Department of Pathology, Faculty of Medicine, Khon Kaen University (17) in Ham-F12 (Invitrogen, Carlsbad, CA), the immortalized cholangiocyte line MMN-1 (18) in DMEM (Invitrogen), and lymphoid cell lines (Ramos and Jurkat) in RPMI-1640 (Invitrogen). NIH-3T3/pEF and NIH-3T3/pEF-ganp transfectants were established by using PolyFect (Qiagen, Valencia, CA) and cultured in DMEM containing hygromycin B (Invitrogen).

**Reverse transcriptase (RT)-PCR.** The cDNA was prepared by SuperScript III reverse transcriptase (Invitrogen) with total RNAs from cells cultured in vitro or from frozen CCA tissues. *AID* transcripts were amplified by oligonucleotide primers based on the information of a previous study (19). RT-PCR for *AID* was carried out using Z-Taq DNA polymerase (Takara Bio, Otsu, Japan), the amplification conditions were 35 cycles of 98°C for 10 sec, 58°C for 10 sec, and 72°C for 40 sec. *Ganp* was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) at 95°C for 9 min followed by 35 cycles of 94°C for 20 sec, 57°C for 20 sec, 72°C for 30 sec and final extension at 72°C for 5 min (12). *ß-actin* transcripts were amplified as internal controls with 23 cycles of 98°C for 5 sec, 55°C for 10 sec, and 72°C for 10 sec (12). and *gan* transcripts were verified by blot transfer and hybridization with human *AID*, *ganp*, and *ß-actin* cDNA probes.

**Real-time RT-PCR.** Real-time RT-PCR was conducted by using a LightCycler 2.0 Instrument (Roche Applied Science, Indianapolis, IN) and a pair of Hybridization Probes labeled either at the 5’-end with a LightCycler Red fluorophore or at the 3’-end with fluorescein and LightCycler DNA Master HybridProbe (Roche Diagnostics, Indianapolis, IN). Specific oligonucleotide primers were synthesized according to published information on the *AID* and *ganp* genes as follows: *AID* sense, 5’-TGGTTGGGACGACAAACTG-3’; *AID* antisense, 5’-GTCCCAAGTCGGAGATAG-3’; *ganp* sense, 5’-CTGGGAGGCGATGAGACG-3’; *ganp* anti-sense, 5’-GCA GAAGCAGTAAAGCCT-3’. For *AID*, the donor probe 5’-CCGGTTTATTTTGCAAGATACAAAGTTCCAG-3’ was labeled with fluorescein at its 3’-end, whereas the acceptor probe 5’-GAAAAAGATGACGACTGTCAGGCC TCTTCA-3’ was labeled with LC Red 640 at its 5’-end. For *ganp*, the donor probe 5’-AGTGGGCAAGACATCCTCAGCAGAACC-3’ and the acceptor probe 5’-GCCACACCGAC CCTTGTGCTGCTGCT-3’ were prepared. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers and probes specific for the constitutively expressed gene *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and normalized. The *gapdh* primers used were as follows: forward, 5’-CAGCCTCAAGATCATCAGC-3’; reverse, 5’-GCCATCCAGCTCTCT-3’. The sequences of the *ganp* probes used for real-time LightCycler PCR were 5’-GGTATCCATGACAACTTCTTGGTGGAA-3’ and 5’-GACTCATGACCACAGTCCATGCATCCTG-3’. All primers and probes were purchased from Nihon Gene Research Laboratories Inc. (Sendai, Japan). The cycle conditions for real-time RT-PCR were: *AID*, 95°C for 1 min, followed by 50 cycles of 95°C for 0 sec, 56°C for 5 sec, and...
Immunostaining by anti-GANP mAb
(11) or rat anti-AID mAb (EK2 5G9; Cell Signaling Technology, Danvers, MA) in combination with biotinylated anti-rat Ig Ab with Vectastain ABC complex (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). Human tonsil tissue was used as a positive control. The expression level was evaluated as (0, no; 1, weak; 2, moderate; and 3, strong).

Immunostaining by anti-GANP mAb. Three CCA lines were cultured in Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) at 37°C overnight. After fixation with 3.7% paraformaldehyde/ PBS and permeabilization with 0.2% Triton X-100, cells were incubated with anti-GANP mAb as a primary Ab. Histofine Simple Stain Mouse MAX PO (Nichirei Biosciences Inc., Tokyo, Japan) was used as a secondary Ab. After the development by 3,3′-diaminobenzidine tetrahydrochloride, cells were lightly counterstained by hematoxylin.

Western blot analysis. MMNK-1 cells with ganp RNAi-treatment or NIH-3T3/pEF-ganp transfectants were harvested by trypsinization, washed twice in cold PBS, and lysed with TNE buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 1 mM EDTA, supplemented with protease inhibitor cocktails and 1 mM PMSF). After clearing by 10,000 rpm for 15 min at 4°C, lysates in SDS sample buffer were heated for 5 min at 98°C, separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were washed in PBS containing 0.1% Tween-20 and then blocked with 5% non-fat dry milk in PBS/0.1% Tween-20. The anti-AID and anti-Flag (M2; Stratagene, La Jolla, CA) mAbs were used in combination with horseradish peroxidase-conjugated secondary Ab and an enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). β-actin was used as a loading control.

Subcloning and sequencing of human TP53 gene. Human TP53 was amplified from the genomic DNA in non-transcribed (nucleotide positions 1-842) and transcribed (exons 5-8) regions by using Pfu-Turbo DNA polymerase (Stratagene), subcloned into pENTR/D-TOPO (Invitrogen), and sequenced using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The TP53-specific primers used were as follows: untranscribed, forward, 5′-TCCCATCAAGCCTTACGG GCT-3′; reverse, 5′-TTTGGAGACTCAAAACCTTTA GC-3′. Transcribed, forward, 5′-TACCTCCTGCGCTC AATAAGTGT-3′; reverse, 5′-TCCTCGCTAGTGCTCCCT GGG-3′.

Ganp RNAi-treatment. MMNK-1 cells were transfected with ganp RNAi (10 nM) in the presence of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols. The following Stealth RNAi duplexes were synthesized by Invitrogen: for ganp RNAi-1, sense, 5′-CCAGCGCU UCCUGGAGAUGCUAU-3′ and anti-sense, 5′-AAUG ACUUAUCCCAGAACGCGUG-3′ were used and for scrambled control RNAi, sense, 5′-CCCACCUCAAGGUU GGACCAACAUU-3′ and anti-sense, 5′-AAGUUGGUC ACAAUCAGGG-3′ were used.

Cell cycle analysis. Cell cycle was analyzed with PI by using a FACS Calibur (Becton-Dickinson, San Jose, CA) as described previously (13).

Immunofluorescence of H2AX. After 3 h of 1-Gy irradiation, γH2AX foci were detected by using anti-phospho-histone H2AX (Ser139) mAb (Millipore, Temecula, CA) and Alexa Fluor 488-conjugated anti-mouse IgG Ab (Invitrogen) with 4,6-diamidino-2-phenylindole staining. The number of foci in 20 cells was counted under a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

Statistical analysis. AID and GANP were compared between the clinical groups classified with various parameters by statistical analysis using SPSS15.0 software for Mann-Whitney’s U test (Chicago, IL). Patient survival was calculated from the time of resection to either death or the last follow-up. The survival curves were assessed by the Kaplan-Meier analysis using a log-rank test. P<0.05 was considered statistically significant.

Results

Expression of AID and GANP at various CCA stages. CCA lines of well-differentiated (OCA17), moderately-differentiated (M055), and poorly-differentiated (KKU-100) tumors (17) were examined for their expression of AID and ganp transcripts. AID was not detected in OCA17; however, it was transcribed highly in M055 and KKU-100 cells (Fig. 1A). The ganp transcript level also increased with the progression of the malignancy grade (Fig. 1A). The AID transcript level was 10- and 15-fold higher in M055 and KKU-100 cells, respectively, than in well-differentiated OCA17 cells, as assessed by real-time RT-PCR (Fig. 1B). The ganp transcript level was increased in M055 (70-fold) and KKU-100 (22-fold) cells, but was not detectable in OCA17 cells (Fig. 1C).

Next, we examined the expression of AID and GANP proteins by immunostaining (Fig. 1D). AID expression was strong in M055 and KKU-100 (b and c) and moderate in OCA17 (a), appearing in the cytoplasm but occasionally also in the nucleus (arrowheads) in comparison with that in Jurkat (d, negative control), Ramos (e, positive control), and tonsillar GC-B (f, positive control) cells. GANP was seen mostly in the nuclei of CCA lines (Fig. 1E). The expression levels of GANP in M055 and KKU-100 were much stronger than that observed in OCA17. Thus AID and GANP appeared in the development of CCA, and their expression levels were high in less-differentiated CCAs.

AID transcription in normal cholangiocytes by TNF-α stimulation. We analyzed the transcripts of AID and ganp
after treatment of a normal cholangiocyte line, MMNK-1, with TNF-α. The AID transcript level was low in MMNK-1 cells but became elevated after stimulation with TNF-α (Fig. 2A), whereas ganp transcription was almost constitutive in the cells as shown in the relative fold expression (Fig. 2A, lower panel). AID expression at the protein level was markedly increased in MMNK-1 after TNF-α stimulation by Western blot analysis (Fig. 2B, upper panel) and immunocytochemistry (Fig. 2B, lower panel), suggesting that an aberrant increase of AID expression occurred after TNF-α stimulation in cholangiocytes that constitutively express GANP.

Expression of AID and GANP in liver fluke-related CCA samples. Next we studied whether AID and GANP were expressed in clinical cases of liver fluke-associated CCA by RT-PCR. AID and ganp transcripts were detected in all of CCA samples from 21 cases (Fig. 3A). CCA specimens (65 cases) showed higher AID expression similarly in tumors than in the surrounding stromal cells as shown in the representative cases (Fig. 3B, a and c). GANP was expressed in CCAs but with the different expression profiles of two types, i.e., as a cytoplasm-dominant (GANP cyto) type (Fig. 3B, b) and as a nucleus-dominant (GANP nucl) one (Fig. 3B, d). These results demonstrated that AID was aberrantly expressed in non-lymphoid tumor CCAs as reported previously (6,20) and additionally GANP was co-expressed, but with different expression levels in the CCA cells.

Correlation of AID and GANP with clinicopathological findings. The clinical significance of AID and GANP expres-
Levels of AID and GANPnucl in CCA specimens were 133±44 and 44±47 (mean ± SD), respectively. AID was not associated with any parameters significantly. However, the expression of GANPnucl showed a significant correlation with the histological types of CCA (p=0.037). GANPnucl was up-regulated in less-differentiated CCAs (63±59; mean ± SD), the level was significantly higher.
than that in well-differentiated type (35±39; mean ± SD). The increased expression of nuclear GANP might be associated with genetic and cellular changes in CCA.

### Table I. Correlation between the staining of GANP and AID and the clinicopathological factors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nuclear GANP</th>
<th>Cytoplasmic AID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IHC score (mean ± SD)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>38±42</td>
<td>NS</td>
</tr>
<tr>
<td>≤50</td>
<td>61±57</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37±44</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>58±50</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated</td>
<td>35±39</td>
<td>0.037*</td>
</tr>
<tr>
<td>Less-differentiated</td>
<td>63±59</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>34±48</td>
<td>NS</td>
</tr>
<tr>
<td>Negative</td>
<td>49±46</td>
<td></td>
</tr>
<tr>
<td>Neural invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>27±22</td>
<td>NS</td>
</tr>
<tr>
<td>Negative</td>
<td>45±48</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>68±66</td>
<td>NS</td>
</tr>
<tr>
<td>Negative</td>
<td>37±39</td>
<td></td>
</tr>
</tbody>
</table>

Well-differentiated, papillary and well-differentiated adenocarcinoma; less-differentiated, moderately- and poorly-differentiated adenocarcinoma. Abbreviation: NS, not significant. *Statistically significant, p<0.05.

Figure 4. Survival of patients with GANP and AID expression. (A) The patient survival was compared with the expression levels of AID. (B) The survival rate of patients with high expression of GANP was compared.

Expression of AID and GANP, and cumulative survival. CCA patients with high AID expression showed shorter survival times, but the difference was not statistically significant.
Aberrant expression of AID was observed in CCA specimens, but the difference in AID level may not be directly associated with the progress and clinical course\( (p=0.982, \text{log-rank test}) \). However, the patients with high GANP nucl expression showed a significantly shorter survival time than those with low GANP nucl expression\( (p=0.018, \text{log-rank test}) \). Thus, high expression of GANP nucl may be a useful prognostic indicator for malignant grades of CCAs.

Mutations of TP53 in CCA. CCA showed nuclear accumulation of TP53 in 25-75% of cases, and also displayed mutation hot-spots in exons 5-8\( (21) \). In liver fluke-associated CCAs (55 cases), the loss of heterozygosity and the microsatellite instability of\( TP53 \) gene were reported to occur in 32%\( (22) \). The predominant mutation pattern was G:C\( \rightarrow \)A:T (at CpG) in 29% of those cases. We examined whether expression of AID and GANP augmented the generation of\( TP53 \) mutations. The mutation frequency of transcribed and non-transcribed regions of the\( TP53 \) gene was compared between the\( \text{AID}^{\text{GAP}} \) and\( \text{AID}^{\text{GAP}} \) cases (Table II). This genetic analysis could not detect any obvious difference in the frequency of\( TP53 \) mutations between the GANP\( ^{\text{Lo}} \) and GANP\( ^{\text{nucl}} \) groups, which might suggest that the effect of GANP over-expression is not limited to\( TP53 \) gene.

Effect of GANP on DNA damage caused by ionizing-irradiation. To explore the effect of GANP over-expression on cell proliferation in response to the genetic change that might have been caused by AID, we examined the cell cycle progression and the DNA damage caused by the change of GANP expression using the\textit{in vitro} experimental systems. The\( ganp \) RNAi-treated cells showed a marked increase in the proportion of G2/M phase cells (31.5% of\( ganp \) RNAi cells vs. 15.4% of control RNAi cells). The cell-cycle progressions in\( NIH-3T3/pEF \) and\( NIH-3T3/pEF-ganp \) were analyzed by PI staining (right panels). The effects of\( ganp \) cDNA transfection (gain-of-function) were examined in\( NIH-3T3 \) cells (B). The average number of\( \gamma H2AX \) foci indicated with the bar was compared.
with no. of sequence (/10^4 bp) no. of sequence (/10^4 bp)

CCA case No. of mutation/ Mutation frequency No. of mutation/ Mutation frequency

<table>
<thead>
<tr>
<th>CCA case with</th>
<th>Untranscribed region</th>
<th>Transcribed (exon 5-8) region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mutation/ no. of sequence</td>
<td>Mutation frequency (/10^4 bp)</td>
</tr>
<tr>
<td>AID+GANP(\text{pos})</td>
<td>0/6 0.2</td>
<td>3/6 0.3</td>
</tr>
<tr>
<td>AID+GANP(\text{lo})</td>
<td>0/5 0.2</td>
<td>5/5 0.5</td>
</tr>
</tbody>
</table>

(12.6 foci/cell) upon the DNA damage caused by ionizing-irradiation (Fig. 5B). These results suggest that GANP plays a role to accelerate the DNA damage response after the DNA injuries induced by ionizing-irradiation or AID cytidine deaminase activity. Thus, the effect of GANP over-expression was further investigated in NIH-3T3 cells in the absence of AID expression. The \(ganp\) over-expression in NIH-3T3 cells did not cause an increase of G2/M phase cells compared with mock-transfected cells (Fig. 5C, right panel), however, it caused the increase in the number of \(\gamma\)H2AX foci [17.5% of NIH-3T3/pEF-ganp (IR\textsuperscript{+}) vs. 11.7% of NIH-3T3/pEF (IR\textsuperscript{-})] (Fig. 5D). This increased number of \(\gamma\)H2AX foci by the over-expression of \(ganp\) cDNA was not detected without ionizing-irradiation [2.2% of NIH-3T3/pEF-ganp (IR\textsuperscript{+}) vs. 1.7% of NIH-3T3/pEF (IR\textsuperscript{-})]. Collectively, GANP is necessary for cell cycle progression, presumably through regulation of the DNA repair pathway after DNA double-strand breaks.

**Discussion**

AID and GANP, both involved in Ig diversification (7,9), are expressed in liver fluke-related CCA. AID has been characterized as an activation-induced enzyme expressed specifically in B cells (7). AID was expressed and its expression was enhanced in MMNK-1 cells after stimulation with TNF-\(\alpha\), suggesting that aberrant AID expression occurs under inflammatory conditions (Fig. 2), which is compatible to a previous report (20). On the contrary, GANP over-expression was already detected in MMNK-1 cells in the absence of TNF-\(\alpha\), but we could not detect the enhancement of GANP expression in MMNK-1 cells in vitro (Fig. 2).

AID is expressed after \(H. pylori\) infection in gastric adenomatous cells (6), implying that AID might generate mutations of oncogenes and tumor suppressor genes. The fact that AID was expressed in cancerous cells suggested that the aberrant expression of AID unfavorably causes the cytidine deamination leading to accumulation of mutations in various critical genes of cell survival and cell cycle progression. Likewise, GANP was expressed in CCA at a particularly high level in less-differentiated CCA, which further suggested that both AID and GANP might play a part in tumor development.

AID is induced by \(H. pylori\) and is involved in the generation of TP53 mutations (6). Nevertheless, the mutation analysis of TP53 gene did not show any marked differences in the frequencies or mutation profiles between the GANP\(\text{lo}\) and GANP\(\text{pos}\) and CCA cases, both expressing AID (Table II). GANP over-expression increased the generation of \(\gamma\)H2AX foci (Fig. 5D), which might be associated with rigorous alteration of the genome rather than the increased accumulation of TP53 mutation. These observations were supported by the molecular properties of a GANP-homologue SAC3 in *Saccharomyces cerevisiae* (23). SAC3 is involved in mRNA export from the nucleus, and the lack of SAC3 causes DNA hyper-recombination by a homologous recombination mechanism at DNA injuries generated in the artificial reporter gene (14). The mechanism of DNA hyper-recombination triggered by the lack of SAC3 has not yet been elucidated, but it is thought that the lack of SAC3, causing impaired mRNA export from the nucleus as the hrRNP complex, generated DNA injuries (24). The decreased expression of mammalian GANP also caused homology-mediated DNA hyper-recombination generated by the ectopic expression of AID in NIH-3T3 cells (15), suggesting that GANP coordinately functions in the regulation of DNA injuries caused by AID.

Here, our results support a model of carcinogenesis associated with chronic infection of pathogenic microorganisms as a high-risk factor for cancer development. CCA might represent clinical cases of inflammation-associated oncogenesis. GANP, which regulates the process of the DNA-modifying/repairing mechanism after DNA injury, is closely associated with AID in tumorigenesis and tumor development of cholangiocytes during the long-term inflammation caused by a liver fluke infection.

**Acknowledgements**

We thank Ms. Y. Fukushima and Ms. Y. Kumamoto for excellent assistance. This work was supported by a Grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology (K.K. and N.S.), JSPS Asia-Africa Science Platform Program (N.S.), CREST of Japan Science and Technology Agency (N.S.) in Japan, and the Royal Golden Jubilee PhD Program (PHD/0257/2546) in Thailand (W.C. and C.L.).

**References**


