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

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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.

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Abbreviations: AID, activation-induced cytidine deaminase; CCA, cholangiocarcinoma; GC, germinal center; GANP, germinal center-associated nuclear protein; *H. pylori*, *Helicobacter pylori*; Ig, immunoglobulin; IHC, immunohistochemistry; mAb, monoclonal antibody; SHM, somatic hypermutation

Key words: cholangiocarcinoma, activation-induced cytidine deaminase, germinal center-associated nuclear protein, chronic inflammation, oncogenesis, γH2AX, ionizing-irradiation, TNF-α

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 germinal 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 *leu2* 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 *ganp* 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 perineuron was reviewed retrospectively based upon medical records and pathologic records from the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University. Survival of patients was recorded from the date of surgery to the date of death or to March 13, 2007. Five patients were excluded from the survival analysis; two were lost to follow-up and three patients died within a month after surgery (perioperative death).

Cell lines. Human cell lines were maintained in suitable culture medium with 10% heat-inactivated fetal calf serum (Dainippon Pharmaceutical, Osaka, Japan), 2 mM L-glutamine (Cambrex, Charles City, IA), and $5x10^{-5}$ M 2-mercaptoethanol in a humidified incubator with 5% CO_2 at 37˚C: CCA lines [OCA17, M055, and KKU-100; established from patients with 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 MMNK-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). *AID* and *ganp* 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 HybProbe (Roche Diagnostics, Indianapolis, IN). Specific oligonucleotide primers were synthesized according to published information on the *AID* and *ganp* genes as follows: *AID* sense, 5'-TGGTGGGACGACAAACTG-3'; *AID* antisense, 5'-GTCCCAGTCCGAGATGTAG-3'; *ganp* sense, 5'-CGTGGAGCTGATGGAACG-3'; *gan*p anti-sense, 5'-GCA GAAGCACTGAAGCTCCT-3'. For *AID*, the donor probe 5'-CCGTTCTTATTGCGAAGATAACCAAAGTCCAG-3' was labeled with fluorescein at its 3'-end, whereas the acceptor probe 5'-GAAAAGGATGTAGCACTGTCACGCC TCTTCA-3' was labeled with LC Red 640 at its 5'-end. For *ganp*, the donor probe 5'-AGTGGGCACAGACATCCTCAC AGCAACG-3' and the acceptor probe 5'-GCCACACGGAC CCTCTGGTCTGTCTCTA-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'-GGCCATCCACAGTCTTCT-3'. The sequences of the *gapdh* probes used for real-time LightCycler PCR were 5'-GGTCATCCATGACAACTTTGGTATCGTGGAA-3' and 5'-GACTCATGACCACAGTCCATGCCATCACTG-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

72˚C for 5 sec; *ganp*, 95˚C for 1 min, then 50 cycles of 95˚C for 0 sec, 55˚C for 5 sec, and 72˚C for 5 sec; *gapdh*, 95˚C for 1 min, followed by 45 cycles of 95˚C for 0 sec, 55˚C for 5 sec, and 72˚C for 7 sec.

Immunohistochemistry (IHC). Paraffin sections (4-μm) were immunostained with rat anti-GANP monoclonal Ab (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 blindly by 2 investigators as IHC score by multiplication of positivity (0-100%) and the staining intensity (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 lyzed 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 centrifugation at 15,000 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). ß-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'-TTCCCATCAAGCCCTAGG GCT-3'; reverse, 5'-TTTTGAGAAGCTCAAAACTTTTA GC-3'. Transcribed, forward, 5'-TACTCCCCTGCCCTCAA CAAGATGTT-3'; reverse, 5'-CTCGCTTAGTGCTCCCT 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'-CCAGCGU CUUCUGGAGUAAGUCAUU-3' and anti-sense, 5'-AAUG ACUUACUCCAGAAGACGCUGG-3' were used and for scrambled control RNAi, sense, 5'-CCCACCUCAAGUGUU GGACCAACUU-3' and anti-sense, 5'-AAGUUGGUCCAA CACUUGAGGUGGG-3' were used.

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

Immunofluorescence of γH2AX. 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) cells, 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*

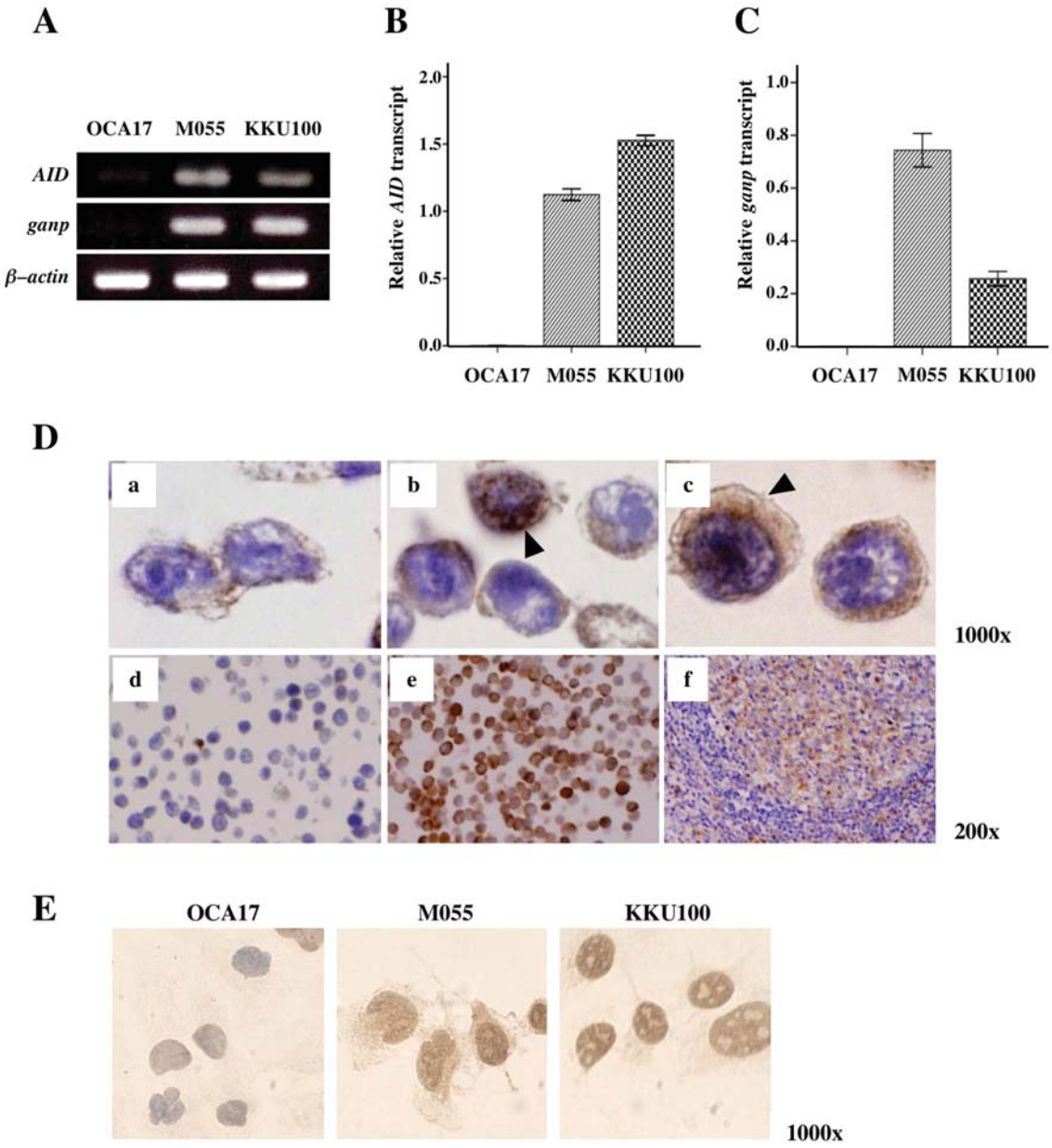

Figure 1. AID and GANP expression levels in CCA lines. (A) RT-PCR results for AID and *ganp* transcripts are shown with *β-actin* control. (B and C) *AID* (B) and *ganp* (C) transcripts were examined by real-time RT-PCR, and their levels are presented as the mean ± SE from 3 independent experiments. (D) Immunostaining of AID in CCA cell lines. AID was detected throughout the cytoplasm, and occasionally in the nucleus, of M055 (b) and KKU-100 (c) at a higher level than in OCA17 cells (a). Arrowheads indicate cells expressing AID in both nucleus and cytoplasm in comparison with the expression in Jurkat (d) and Ramos (e) or human tonsillar GCs (f). (E) Immunostaining of GANP in CCA cell lines. GANP expression was predominantly detected in nuclei of cells.

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 immunocytostaining (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-

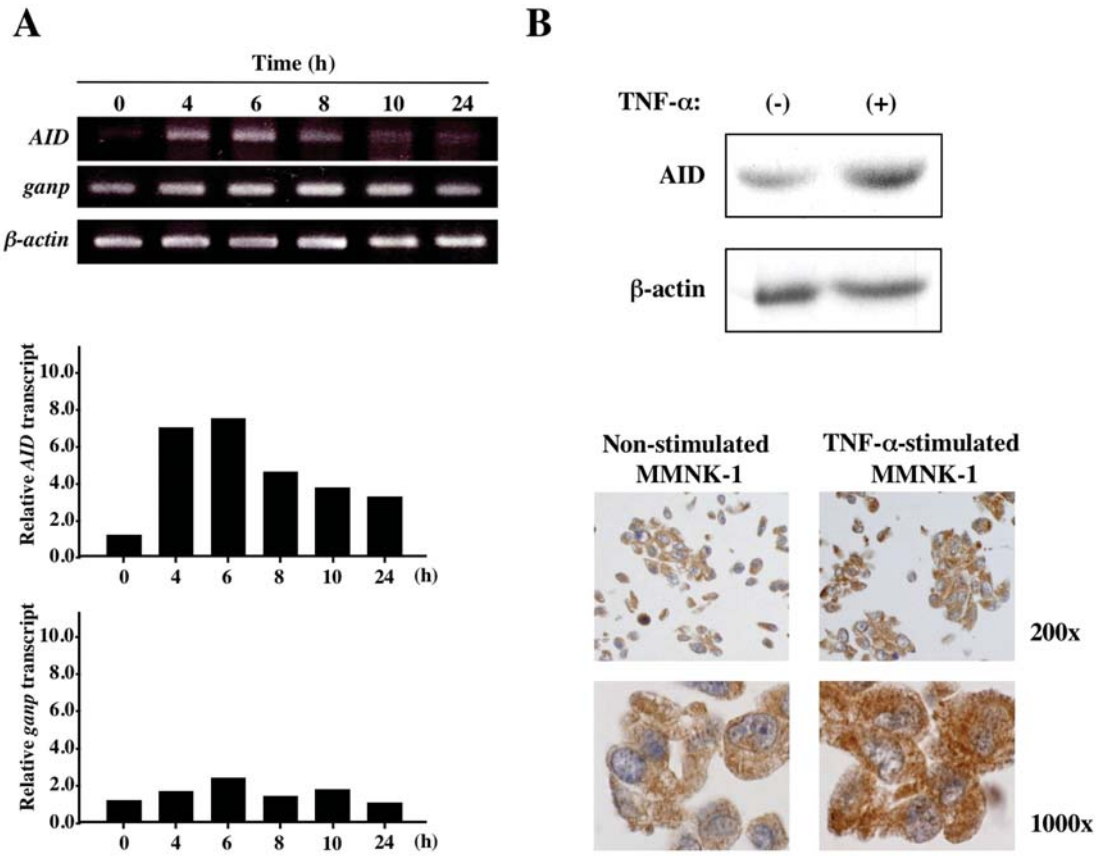

Figure 2. Induction of AID in normal cholangiocytes by TNF-α. (A) Transient induction of *AID*, but not *ganp*, in TNF-α-stimulated MMNK-1 cells. MMNK-1 cells were stimulated with TNF-α (100 ng/ml) for indicated times, and semi-quantitative RT-PCR was performed using specific primer for *AID, ganp*, and *β-actin* (upper panel). Real-time RT-PCR of *AID* and *ganp* transcripts was performed using same samples as (A) (lower panels). (B) Increased expression of AID in TNF-α-stimulated MMNK-1 cells. MMNK-1 cells were stimulated with or without TNF-α (100 ng/ml) for 8 h, and immunoblot (upper panel) and immunostaining (lower panel) of AID were performed.

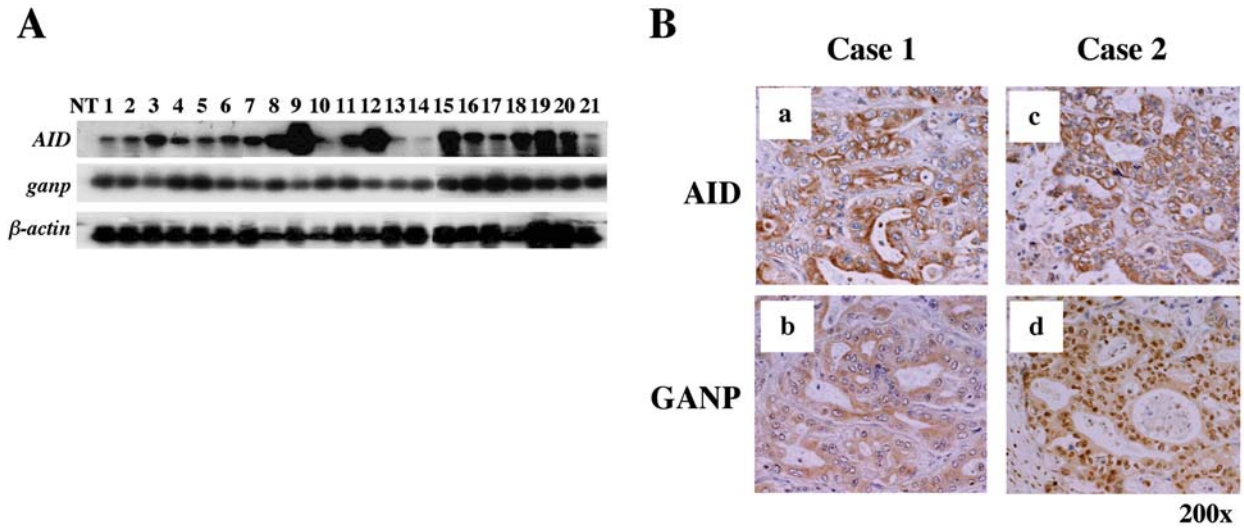

Figure 3. Expression of AID and GANP in liver fluke-related CCA tissues. (A) RT-PCR of *AID* and *ganp* transcripts in patients' samples. NT, non-template. (B) Immunostaining of AID and GANP in CCA samples. AID was exclusively detected in cytoplasms (a and c), whereas GANP was found in cells as a cytoplasm-dominant (b) or a nucleus-dominant type (d).

sions was evaluated by IHC in comparison with clinicopathological parameters (Table I). Levels of AID and GANP^{nucl} in CCA specimens were 133±44 and 44±47 (mean ± SD), respectively. AID was not associated with any parameters significantly. However, the expression of GANP^{nucl} showed a significant correlation with the histological types of CCA (p=0.037). GANP^{nucl} was up-regulated in less-differentiated CCAs (63±59; mean ± SD), the level was significantly higher

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

Parameters	N (65)	Nuclear GANP		Cytoplasmic AID	
		IHC score (mean ± SD)	p-value	IHC score (mean ± SD)	p-value
Age (year)			NS		NS
>50	48	38±42		138±47	
≤50	17	61±57		120±33	
Gender			NS		NS
Male	44	37±44		131±42	
Female	21	58±50		137±50	
Histological type			0.037[a]		NS
Well-differentiated	45	35±39		136±43	
Less-differentiated	20	63±59		127±47	
Lymph node metastasis			NS		NS
Positive	23	34±48		135±34	
Negative	42	49±46		132±49	
Neural invasion			NS		NS
Positive	3	27±22		140±99	
Negative	62	45±48		133±42	
Vascular invasion			NS		NS
Positive	14	68±66		144±70	
Negative	51	37±39		130±35	

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

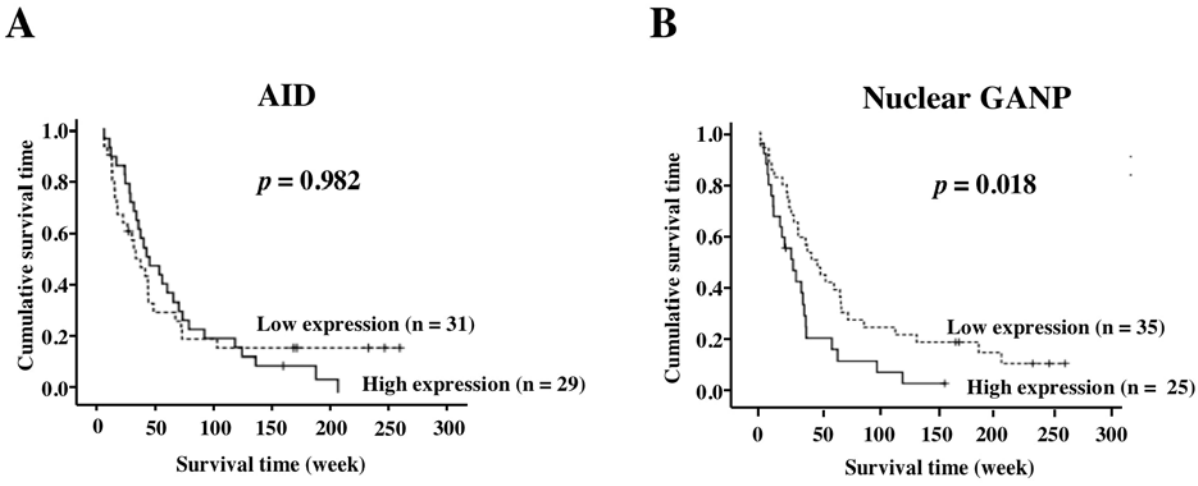

Figure 4. Survival of patients with GANP and AID expression. (A) The patient survival was compared with the expression levels of AID. (B) The survival rate of patients with high expression of $GANP^{nucl}$ was compared.

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.

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

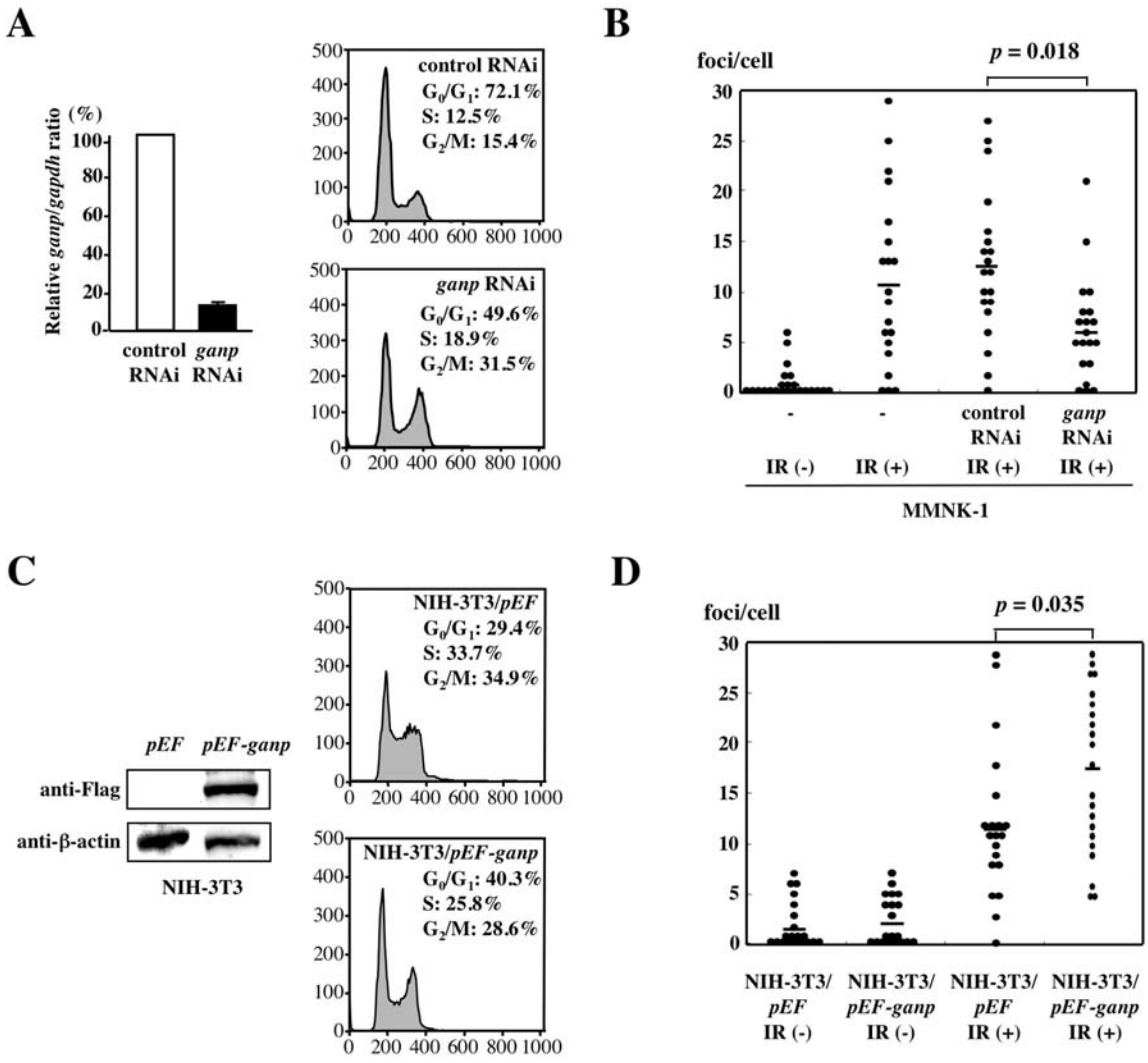

Figure 5. Regulation of DNA repair by GANP. (A) MMNK-1 cells with *ganp* RNAi-treatment. The knockdown efficiency of *ganp* transcripts was confirmed by real-time RT-PCR (left panel). The cell-cycle progressions in control and *ganp* RNAi-treated MMNK-1 were analyzed by PI staining (right panels). (B) The generation of γH2AX foci was examined in the *ganp* RNAi-treated cells (loss-of-function) after ionizing-irradiation (1 Gy). The average numbers of foci/cell are shown with the bars. (C) Establishment of NIH-3T3 cells over-expressing *ganp* cDNA. The introduced GANP was clearly detected in the cell line by anti-Flag immunoblot (left panel). The cell-cycle progressions in NIH-3T3/*pEF* and NIH-3T3/*pEF-ganp* were analyzed by PI staining (right panels). (D) The effects of *ganp* cDNA transfection (gain-of-function) were examined in NIH-3T3 cells (B). The average number of γH2AX foci indicated with the bar was compared.

(Fig. 4A). 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, 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, log-rank test; Fig. 4B). 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→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 AID^{+}GANP^{Lo} and AID^{+}GANP^{nucl} cases (Table II). This genetic analysis could not detect any obvious difference in the frequency of *TP53* mutations between the GANP^{Lo} and GANP^{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 *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), indicating that *ganp* is necessary for normal cell cycle progression of MMNK-1 cells (Fig. 5A, right panel); and these cells generated fewer γH2AX foci (6.1 foci/cell) compared with control cells

Table II. Mutations of *TP53* gene in CCA tumors.

CCA case with	Untranscribed region		Transcribed (exon 5-8) region	
	No. of mutation/ no. of sequence	Mutation frequency (/10^4 bp)	No. of mutation/ no. of sequence	Mutation frequency (/10^4 bp)
$AID^{+}GANP^{nucl}$	0/6	0.2	3/6	0.3
$AID^{+}GANP^{Lo}$	0/5	0.2	5/5	0.5

(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 γH2AX foci [17.5% of NIH-3T3/*pEF-ganp* (IR^{+}) vs. 11.7% of NIH-3T3/*pEF* (IR^{+})] (Fig. 5D). This increased number of γH2AX foci by the over-expression of *ganp* cDNA was not detected without ionizing-irradiation [2.2% of NIH-3T3/*pEF-ganp* (IR^{-}) vs. 1.7% of NIH-3T3/*pEF* (IR^{-})]. 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-α, suggesting that aberrant AID expression occurs under inflammatory conditions (Fig. 2), which is compatible to a previous report (20). On the contrary, GANP expression was already detected in MMNK-1 cells in the absence of TNF-α, 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 $GANP^{Lo}$ and $GANP^{nucl}$ CCA cases, both expressing AID (Table II). GANP over-expression increased the generation of γH2AX 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.

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