Functional cell surface expression of Toll-like receptor 9 promotes cell proliferation and survival in human hepatocellular carcinomas

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Abstract. Toll-like receptor 9 (TLR9) is a pattern-recognition receptor that is involved in immune signaling and plays a crucial role in cell survival through recognition of various bacterial and viral components including unmethylated CpG-DNA. TLR9 expression and function in cancer cells are not well understood. We investigated the expression of TLR9, and the function of TLR9 signaling, in hepatocellular carcinoma (HCC) cells following stimulation with CpG-oligodeoxynucleotides (ODNs). Positive immunohistochemical staining for TLR9 was observed in 85.7% of HCC tissues. Western blot analysis revealed that TLR9 was expressed both on the cell membrane and in the cytoplasm of HCC cell lines. Full-length TLR9 was predominantly expressed on the membrane rather than in the cytoplasm, whereas multiple cleaved forms of TLR9 were predominantly expressed in the cytoplasm rather than on the membrane. Cell surface stimulation of TLR9 promoted cell proliferation, and, furthermore, the TLR9 agonists, CpG-ODNs, reduced the cytotoxicity of the anti-cancer drug adriamycin (ADM) via up-regulation of apoptosis inhibitors such as survivin, Bcl-xL, XIAP and cFLIP, in HCC cell lines. Although cell surface stimulation of TLR9 did not activate either the NF-κB signaling pathway or the type-I IFN secretion pathway, gene chip microarray analysis indicated that TLR9 agonists closely regulated multiple oncology-related genes and transcription factors involved in tumorigenesis and cancer progression. In conclusion, our results indicate that functional cell surface expression of TLR9 in human HCC may play an important role in tumorigenesis and cancer progression.

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Introduction

Toll-like receptors (TLRs) are involved in innate immunity against microbial pathogens such as bacteria, protozoa, fungi or viruses. In mammals, the TLR family is currently known to consist of 11 members, which exhibit specificity for pathogen-derived ligands. These receptors are type I transmembrane proteins and play a critical role in the subsequent induction of adaptive immune responses (1-5). Stimulation of TLRs can induce a range of innate and adaptive immune responses through cytokines, interferons, chemokines and cell surface molecules, as well as increase cellular effector functions.

TLRs are broadly distributed in various cell types of the immune system, including polymorphonuclear phagocytes, monocytes, dendritic cells and natural killer cells, as well as in some epithelial and endothelial cells (6-9). However, to date, the specific subcellular localization of TLRs remains to be determined. TLR1, 2, 4, 5 and 6 are present in the plasma membrane whereas TLR3, 7, 8 and 9 are postulated to be present in endosomes. All TLRs are most likely involved in signal pathways (10). Via the TLR signaling pathways, the liver is continuously exposed to a large variety of antigens, such as dietary antigens, bacterial toxins and several proinflammatory cytokines through TLR signaling (11). Hepatocytes express low levels of TLR2 and 4, which are responsive to lipopolysaccharide (LPS). Kupffer cells, as well as hepatic stellate cells, biliary epithelial cells and sinusoidal endothelial cells, which express TLR4, produce several proinflammatory cytokines in response to LPS (11-13). In humans, TLR9 is expressed in B-lymphocytes, monocytes and plasmacytoid dendritic cells. TLR9 recognizes specific oligodeoxynucleotide (ODN) sequences consisting of unmethylated CpG-ODNs, which are frequently present in bacterial and viral DNA (14). It has recently been reported that treatment of chronic hepatitis C infection with the TLR9 agonist CpG 10101, a synthetic ODN, is associated with dose-dependent increases in markers of immune activation (interferon (IFN)-γ-inducible protein 10 (IP-10), IFN-α and 2'5'-oligoadenylate synthetase (OAS)) and with a decrease in HCV-RNA levels (15).

However, the exact mechanism responsible for the expression and function of TLR9 in liver diseases has yet to be fully elucidated.

Besides TLR expression in immune cells, recent evidence suggests that functional TLRs are also expressed by a wide variety of cancer cells (16,17), and that TLR signaling may contribute to tumor cell proliferation, survival and chemosensitivity (18-20). In particular, the expression of TLR9 has been detected in various normal epithelial and cancer cells, including breast, brain, lung, gastric and prostate cancers (21-25). These data suggest that functional TLR9 signaling in tumor cells is associated with the progression of cancer and the evasion of host defenses. However, little is known regarding the significance of TLR9 in human hepatocellular carcinoma (HCC) cells.

We therefore investigated the expression and role of TLR9 in HCC cells and tissues by examination of the function of the TLR9 signaling which occurs after stimulation of HCC cells with CpG-ODNs. Surprisingly, we found TLR9 was expressed not only in the cytoplasm, but also on the membrane, and that TLR9 signaling pathway promotes the proliferation and survival of HCC cells.

Materials and methods

Cells and HCC tissues. The human HCC cell lines HepG2 (JCRB 1054), HLE (JCRB 0404) and Huh7 (JCRB 0403) were purchased from the Human Science Research Resource Bank (Osaka, Japan). The human HCC cell lines SK-Hep1 and the colon adenocarcinoma cells Colo 320 were purchased from the American Type Culture Collection (Rockville, MD). All cells were cultured in DMEM at 37˚C, supplemented with 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY) and 10% heat-inactivated fetal calf serum (Gibco BRL).

A total of 42 HCC tissues (9 non-tumor tissues, which included 4 from patients with cirrhotic liver and 5 from patients with chronic hepatitis, and 8 tissues with metastasis from HCC tissues) were obtained from tissue array slides (SuperBioChips Laboratories, Seoul, Korea). We obtained informed consent from all patients prior to the subsequent use of their resected tissues. Resected tissues were frozen immediately at -80˚C or were fixed in 10% formalin.

Reagents. The type C CpG oligonucleotide (ODN M362: 5'-tcg tcg tcg ttc gaa cga cgt tga t-3') used as a human TLR9 ligand, and the control, non-stimulatory oligonucleotide ODN M362 (ODN M362 Control: 5'-tgc tgc tgc ttg caa gca gct tga t-3'), were purchased from InvivoGen (San Diego, CA). Cisplatin (CDDP) was purchased from Sigma (St. Louis, MO). Adriamycin (ADM) was purchased from Wako (Osaka, Japan), and Lipofectamine LTX (Lipo) was purchased from Invitrogen (Carlsbad, CA).

Flow cytometric analysis. Non-permeabilized live HCC cell lines which had been cultured for 48 h, were washed with PBS and then incubated with FITC-anti-TLR9 antibody (Imgenex, San Diego, CA) or control IgG1. Cells were analyzed by FACScan using CellQuest software (Becton-Dickinson, Tokyo, Japan).

Immunohistochemical staining. Immunohistochemical staining of TLR9 was performed on HCC- and non-HCC-tissues using a labeled streptavidin-biotin method. Deparaffinized sections were heated for 5 min at 120˚C in a pressure cooker to reactivate the antigen. Sections were blocked and incubated with an anti-TLR9 antibody (Imgenex) overnight at 4˚C. Sections were then incubated with a second biotinylated antibody, followed by the avidin-biotin-peroxidase complex. The peroxidase signal was then developed in a substrate solution of 0.01% 3,3'-diaminobenzidene-hydrogen peroxidase and the tissue was counterstained with 10% hematoxylin.

Cell proliferation assay. HCC cells were seeded at a density of $1.0x10^4$ cells/well in 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, NY) and were incubated at 37˚C in 5% CO_2. After incubation for 24 h, various reagents were added, and the plates were incubated for 48 h. To assess the viability of HCC cells, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed using a Cell Titer 96-assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Immunoblotting. The expression of TLR9, and the loading control α-tubulin, in colon adenocarcinoma cell line (Colo 320) and in human HCC cell lines (SK-Hep1, HepG2, HLE, Huh7) was analyzed by immunoblotting. Briefly, after incubation on 6-well plates (Nunc™ Brand Products, Denmark) for 48 h, these cells were washed twice with phosphate-buffered saline (PBS) and were then lysed by the addition of SDS sample buffer (50 mmol/l Tris-HCl, pH 6.8, 2.5% SDS, 5% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue). Equal amounts of extracted proteins were separated by SDS-PAGE and were then transferred to PVDF membranes (Millipore, Billerica, MA). Blots were blocked by incubation in Tris-HCl (pH 7.5) containing 5% milk and 0.1% Tween-20 for 30 min at room temperature, and were probed overnight at 4˚C with primary antibodies. The following primary antibodies were used: anti-TLR9 monoclonal antibody (Imgenex), anti-α-tubulin monoclonal antibody (Oncogene Research Products, San Diego, CA). Antibodies were diluted in 5% milk or in BSA in Tris-HCl (pH 7.5) containing 0.1% Tween-20. Immunoblots were then probed with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (diluted 1:1000 in 1% milk or in BSA in Tris-HCl, pH 7.5) (Amersham Biosciences, Buckinghamshire, UK). After the final wash, signals were detected using an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

To elucidate the subcellular localization of TLR9 in HepG2 cells, cell lysates were separated into cytosolic (F1), membrane/organelle (F2), nuclear (F3) and cytoskeletal matrix (F4) protein fractions using a ProteoExtract™ Subcellular Proteome Extraction kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The expression of calpain and pan-cadherin were also assayed as markers of cytosolic and membrane/organelle fractions. The following primary antibodies were used: anti-TLR9 monoclonal antibody (Imgenex), anti-calpain monoclonal antibody (Calbiochem, San Diego, CA) and anti-pan cadherin monoclonal antibody (Sigma).

The expression of apoptosis inhibitors (survivin, XIAP, cFLIP and Bcl-xL) in HCC cell lines was also analyzed by

immunoblotting. After stimulation of HCC cells for 48 h with 1 μM CpG, 1 μg/ml ADM or 1 μM CpG + 1 μg/ml ADM, cell lysates were separated by SDS-PAGE and were transferred to nitrocellulose membranes. Immunoblots were probed with anti-survivin monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-cFLIP polyclonal antibody (MBL, Nagoya, Japan), anti-hILP/XIAP monoclonal antibody, anti-Bcl-xL polyclonal antibody (both from Transduction Laboratories, Lexington, KY) and anti-α-tubulin monoclonal antibody (Oncogene Research Products). Immunoblots were then probed with horseradish peroxidase-conjugated anti-mouse IgG or horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences).

NF-κB activity assays and expression analysis of IRF-7. The NF-κB activity assay was performed using the Dual-Glo™ Luciferase Assay System (Promega) and the pGL4.32 (*luc*2P/NF-κB-RE/Hygro) Vector (Promega) according to the manufacturer's instructions.

The expression of interferon regulatory factor 7 (IRF-7) in CpG-stimulated or non-stimulated, HCC cells was analyzed by immunoblotting using an anti-IRF-7 antibody (Abnova, Taipei, Taiwan).

RNA extraction and oligonucleotide microarray. Gene expression profiles were examined to determine the effects of stimulation of TLR9 with its agonist CpG-ODNs on gene expression in HCC cell lines. Briefly, HepG2 and Huh7 cells were incubated without stimulation, or with 1 μM CpG ODNs, for 48 h. Total RNA was extracted from both CpG-stimulated and non-stimulated HCC cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity and purity were verified using the Agilent Bioanalyzer 2100 (Agilent Technologies, Rockville, MD). Total RNA (10 μg) was reverse transcribed into single-stranded cDNA, which was then converted to double-stranded cDNA. The double-stranded cDNA was extracted using the GeneChip Sample Cleanup Module (Qiagen, Hilden, Germany). To synthesize biotinylated cRNA, the purified cDNA was used as a template in an *in vitro* transcription reaction using the Enzo BioArray HighYield RNA Transcript Labeling kit (Enzo Life Science, Farmingdale, NY). The biotinylated target cRNA was purified using the GeneChip Sample Cleanup Module (Qiagen). The purified biotinylated cRNA (20 μg) was then fragmented. The hybridization cocktail consisted of 15 μg of fragmented biotin-labeled cRNA spiked with a eukaryotic hybridization control. This hybridization cocktail (200 μl) was directly loaded onto the human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) and was then hybridized at 45˚C for 16 h, with rotation at 60 rpm using a rotisserie motor. After hybridization, the array was washed, stained with streptavidin phycoerythrin using the Affymetrix GeneChip Fluidics Workstation 450, and was scanned on an Affymetrix GeneChip Scanner 3000. The preparation of cRNA, hybridization and scanning of the microarrays were carried out according to the manufacturer's protocols.

Microarray data analysis. The Affymetrix Microarray Suite 5.1 program was used to monitor specific hybridization to the microarray and to monitor gene expression. Data from individual arrays were analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA), which was used to identify genes uniquely up- or down-regulated at single or multiple time-points. Clustering by QT-Clust algorithm, and calculation of significant functional genes, were also performed using this software.

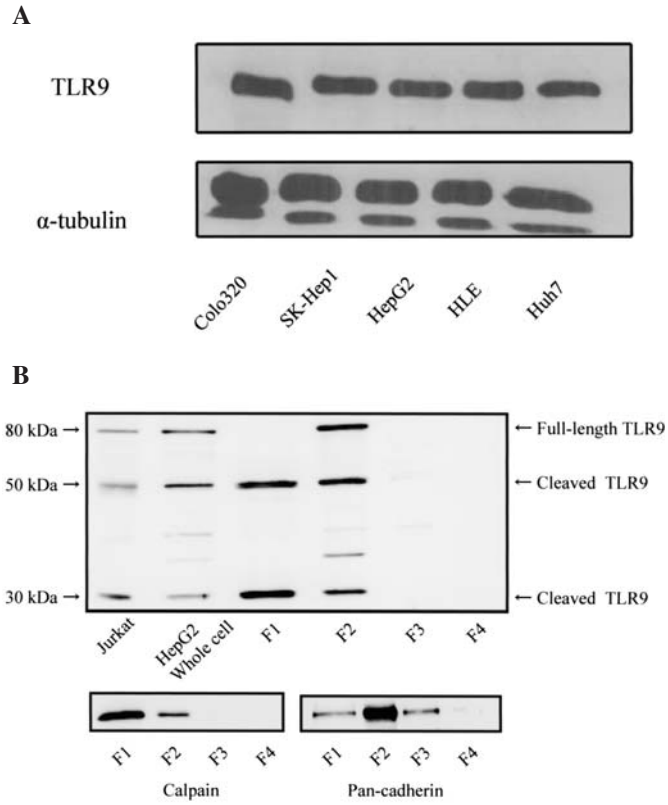

Figure 1. Western blot analysis of TLR9 expression in cancer cells and in subcellular fractions. (A) Western blot analysis of TLR9, and the loading control α-tubulin, in colon adenocarcinoma cells (Colo 320) and in human HCC cells (SK-Hep1, HepG2, HLE, Huh7). Cell lysates were separated by SDS-PAGE and were transferred to nitrocellulose prior to blotting. (B) Western blot analysis of TLR9 in subcellular fractions of HepG2 cells. Cell lysates were analyzed by Western blotting for TLR9 expression prior to, and following, separation of the lysate into cytosolic (F1), membrane/organelle (F2), nuclear (F3) and cytoskeletal matrix (F4) protein fractions using a ProteoExtract™ Subcellular Proteome Extraction kit. The expression of TLR9 on Jurkat cells was assayed as a positive control. Western blots of calpain and pan-cadherin, markers of cytosolic and membrane/organelle fractions respectively, were also shown. Full-length TLR9 was preferentially expressed in the membrane fraction rather than in the cytoplasm. In contrast, multiple cleaved forms of TLR9 were preferentially expressed in the cytoplasmic rather than in the membrane fraction. Cell lysates were separated by SDS-PAGE followed by transfer to nitrocellulose prior to blotting.

Results

TLR9 is expressed on both the cell membrane and in the cytoplasm of HCC cell lines and liver tissues. TLR9 protein expression levels were analyzed by Western blot analysis in cell lysates of cancer cell lines. As shown in Fig. 1A, TLR9 was detected in all HCC cell lines and in colonic adenocarcinoma cells, indicating a high prevalence of TLR9 expression in human HCC. Additionally, TLR9 was expressed not only in the cytoplasm, but also on the membrane of HepG2 cells. Full-length TLR9 was predominantly expressed on the

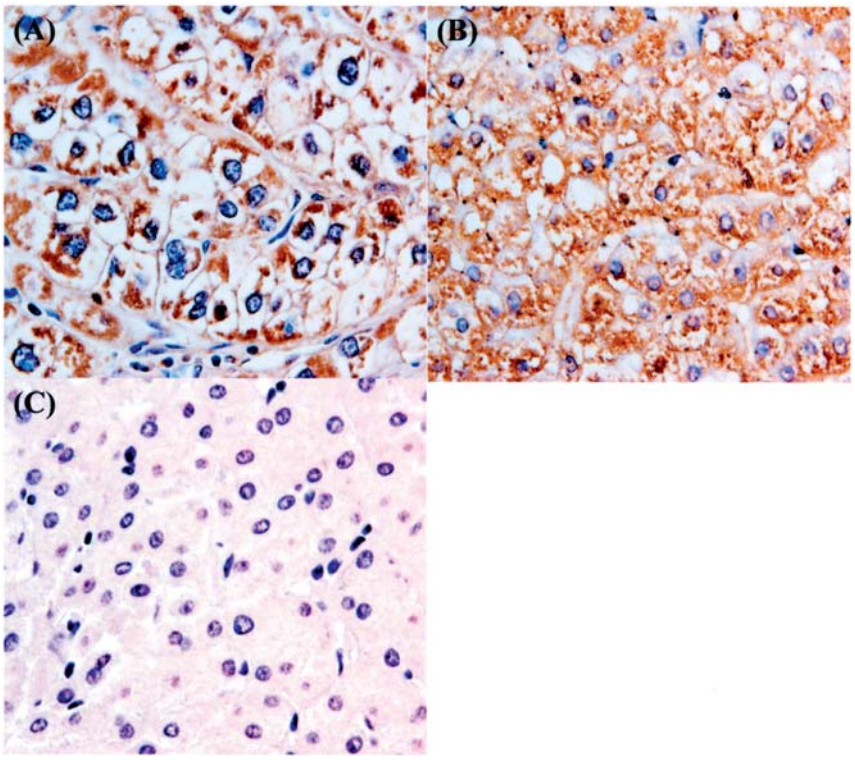

Figure 2. Immunohistochemical analysis of TLR9 expression in human tissue. Immunohistochemical staining of a human HCC (A) and non-HCC (B) tissue specimen using an anti-TLR9 antibody, and control immunohistochemical staining of a human HCC specimen (C) using an iso-type matched mouse IgG. Staining was carried out using a labeled streptavidin-biotin method. Note TLR9 staining is observed not only in the cytoplasm, but also in the membrane (original magnification, x400).

Table I. Expression of TLR9 in HCC and non-tumor tissues.

Histology	Staining		
	No or weak (%)	Moderate (%)	Strong (%)
Non-tumor tissues	2 (22.2)	6 (66.7)	1 (11.1)
HCC			
Poorly differentiated	1 (12.5)	4 (50.0)	3 (37.5)
Moderately differentiated	4 (17.4)	14 (60.9)	9 (21.7)
Well differentiated	1 (14.3)	3 (42.9)	3 (42.9)
Metastasis from HCC	1 (12.5)	6 (75.0)	1 (12.5)

membrane rather than in the cytoplasm. In contrast, multiple cleaved forms of TLR9 were predominantly expressed in the cytoplasm rather than on the membrane in the absence of stimulation with CpG-ODNs (Fig. 1B).

TLR9 expression was further investigated in liver tissue by immunohistochemical staining of both non-HCC and HCC lesions (Fig. 2). While in 36 of 42 HCC cases (85.7%) there was clear TLR9 positivity, no significant differences were observed between different histological grades. TLR9 staining was detected not only in the cytoplasm, but also on the membrane. Few differences were noted in the TLR9 staining patterns between non-tumor and tumor tissues. In metastases from HCC, 7 of 8 cases (87.5%) showed TLR9 positivity (Table I).

Since TLR9 receptor localization depends upon cell type, we further investigated the cell surface expression of TLR9 in HCC cells using flow cytometric analysis. As shown in Fig. 3, significant detectable surface staining of TLR9 was observed in all HCC cell lines tested. These results suggest that TLR9 is frequently expressed not only in the cytoplasm, but also on the membrane, of both normal and cancerous liver cells.

CpG-ODNs affect cell proliferation. In order to determine the biological significance of the signaling that occurs via the cell surface TLR9 in HCC cells, we investigated the cytotoxicity of cell treatment with the TLR9 ligand CpG-ODNs. As shown in Fig. 4A, stimulation with CpG-ODNs for 48 h

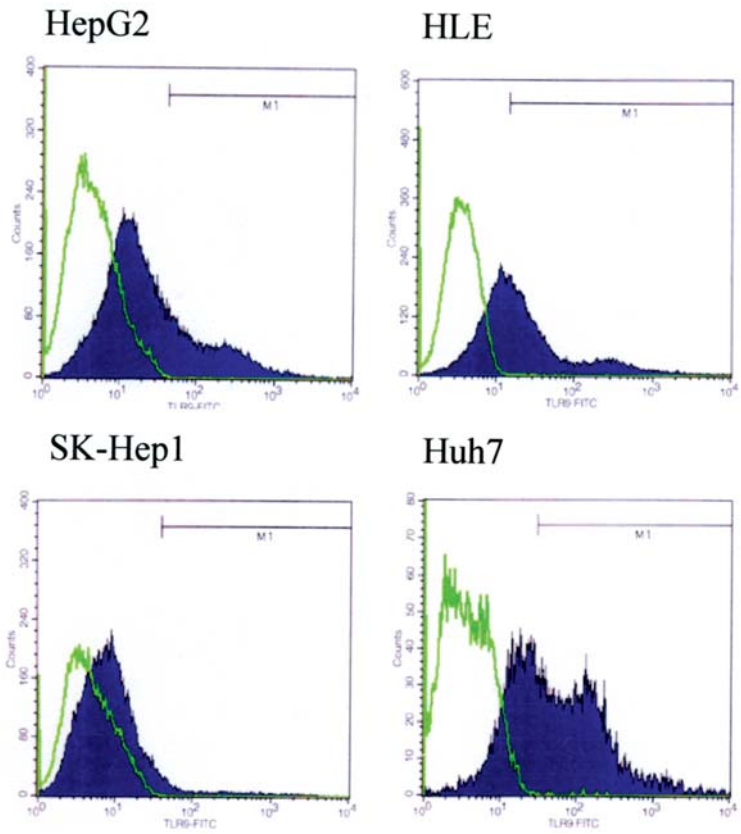

Figure 3. Cell surface expression of TLR9 in HCC cell lines. The cell surface expression of TLR9 in the HCC cell lines SK-Hep1, HepG2, Huh7 and HLE, was assayed by incubation of the cells for 48 h followed by FACS analysis using a FITC-anti-TLR9 antibody. Autofluorescence was determined using cells incubated with control, FITC-mouse IgG1 (green lines).

increased the cell viability of Huh7 and SK-Hep1 cells in a dose-dependent manner. We next determined the biological significance of the signaling that occurs via the cytoplasmic TLR9 in HCC cells, by investigation of the cytotoxicity of transfected CpG-ODNs. As shown in Fig. 4B, stimulation with transfected CpG-ODNs, which stimulates intracellular TLR9 receptors, did not affect cell viability compared to stimulation with non-stimulatory oligonucleotides (control oligo). These results suggest that cell surface stimulation with CpG-ODNs might affect cell proliferation and survival in HCC cells.

CpG-ODNs reduce the cytotoxicity of ADM via up-regulation of apoptosis inhibitors in HCC cells. We next examined possible interactions between the effect of CpG-ODNs and that of the anti-cancer reagents, adriamycin (ADM) or cisplatin (CDDP), on the viability of HCC cell lines. Cell surface stimulation of HepG2 cells with a combination of 1 μM CpG-ODNs and a subtoxic level of ADM for 48 h resulted in an increase in cell viability of about 56% compared to stimulation with ADM alone. Similarly, in Huh7 cells, cell surface stimulation with a combination of 1 μM CpG-ODNs and a subtoxic level of ADM for 48 h resulted in an increase in cell viability of about 69% compared to stimulation with ADM alone (Fig. 5A). In contrast, intracellular TLR9 stimulation with a combination of transfected CpG-ODNs and a subtoxic level of ADM for 48h did not increase the cell viability compared to stimulation with ADM alone (Fig. 5B). To investigate the molecular mechanisms underlying the observed increase in cell viability which occurred following cell surface stimulation with 1 μM CpG-ODNs and a subtoxic level of ADM, we analyzed the expression of apoptosis inhibitors by immunoblotting analysis (Fig. 5C). Stimulation with 1 μg/ml ADM down-regulated the expression levels of survivin, Bcl-xL, XIAP and cFLIP, whereas stimulation with 1 μg/ml ADM in combination with 1 μM CpG-ODNs significantly inhibited the ADM-mediated down-regulation of the expression levels of these proteins. These results suggest that CpG-ODNs might contribute to a reduction in the cytotoxicity of ADM via up-regulation of apoptosis inhibitors in HepG2 cells.

CpG-ODNs do not activate either the NF-κB signaling pathway or the type I-IFN secretion pathway. We further examined TLR9-mediated induction of NF-κB and type I IFNs, since TLR9 signaling is known to modulate these signaling pathways. Induction of NF-κB activity following cell surface stimulation with or without CpG-ODNs was assayed using a luciferase reporter assay. No significant differences were noted in NF-κB activity between the CpG-stimulated and CpG-unstimulated group (data not shown). Regulation of the expression level of interferon regulatory factor 7 (IRF-7), by cell surface stimulation with or without 1 μM CpG-ODNs, was assayed using immunoblotting. However, the expression levels of IRF-7 were also not increased by treatment with or without 1 μM CpG-ODNs in

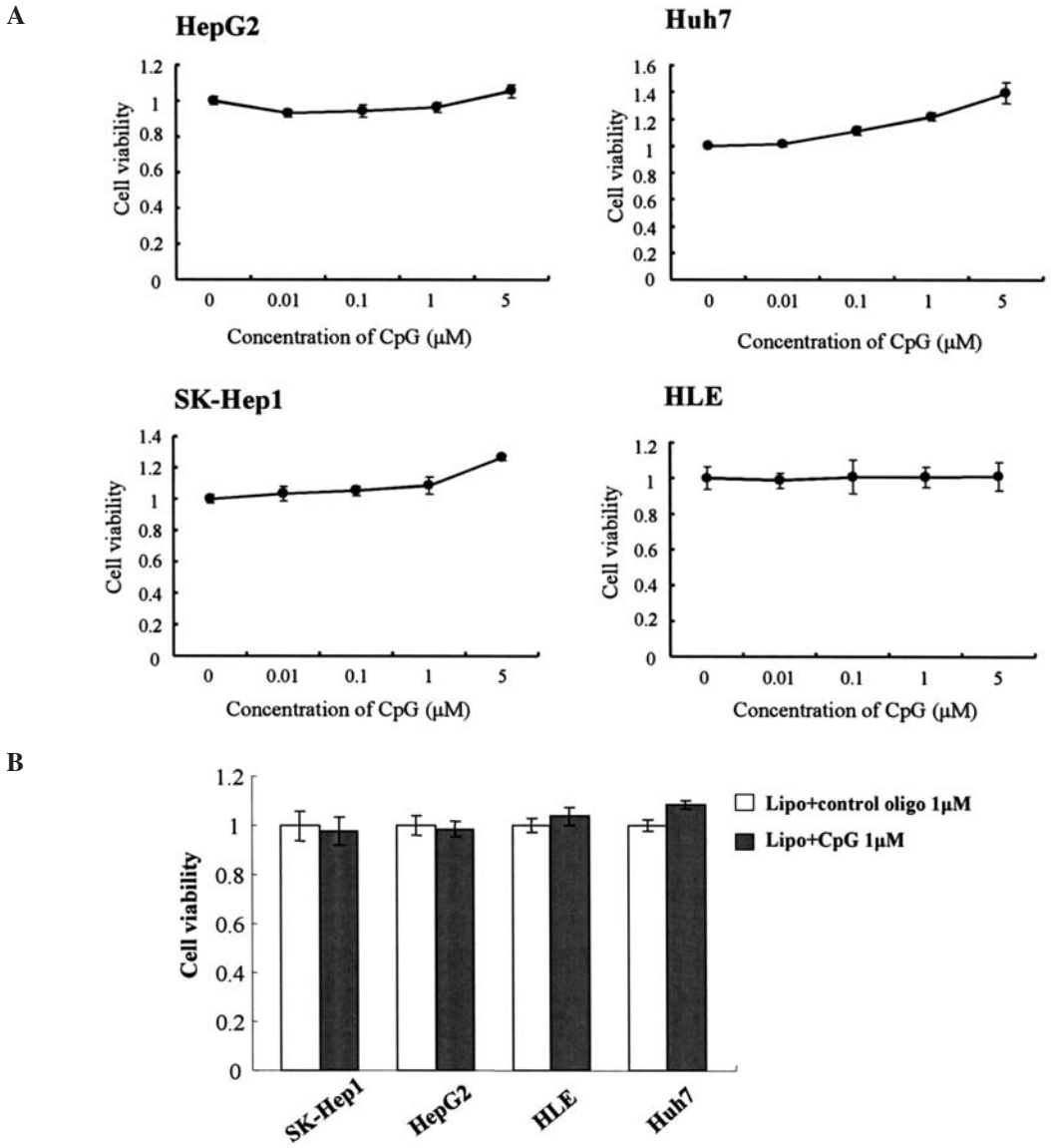

Figure 4. Effects of CpG on HCC cell viability. (A) Effect of exogenous CpG on HCC cell viability. The indicated HCC cells were incubated with various concentrations of CpG for 48 h following which, cell viability was assessed using the MTT assay in which cell viability in the absence of treatment was set at 1. Data shown are the means ± SD of six independent experiments. (B) Effect of transfected CpG on HCC cell viability. The indicated HCC cells were incubated with 2.5 μl/ml Lipofectamine LTX + 1 μM of a non-stimulatory oligonucleotide (control oligo; open columns) or + 1 μM CpG (closed columns) for 48 h. Cell viability was assessed using the MTT assay in which viability of the cells transfected with the control oligo was set at 1. Data shown are the means ± SD of six independent experiments.

HCC cell lines (data not shown). These results indicate that cell surface stimulation with CpG-ODNs did not activate either the NF-κB signaling pathway or the type I-IFN secretion pathway.

CpG-ODN regulates multiple oncology-related genes. In order to determine potential signaling events underlying the effect of CpG-ODNs on HCC cell proliferation and survival, we investigated gene regulation following cell surface CpG-ODN-treatment of HepG2 and Huh7 cells using GeneChip microarray analysis. Several oncology-related genes and transcription factors, which were up- or down-regulated by cell surface CpG-ODN-treatment, were identified as shown in Tables II and III. These genes included *SNRPN*, *SMG1*, *MALAT1*, *SETBP1* and *NDRG* that are considered to be closely related with oncogenesis and tumor progression. The *GRAP* (GRB2-related adaptor protein) gene is also known to be regulated by oncogenic signaling (26). We found that the gene Kruppel-like factor 5 (*KLF5*), was the most highly down-regulated gene. *KLF5* is a member of the kruppel C2H2-type zinc-finger protein family which contains three C2H2-type zinc fingers. The expression of *KLF5* in TLR9-stimulated cells was reduced to about 50% of that in non-treated cells. The mRNA levels of the genes *GDF5OS*, *ANKRD20B* and *ANKRD6* were also reduced by TLR9 stimulation to about 70% of the levels in non-stimulated cells.

Discussion

In an earlier study, we demonstrated for the first time that several TLRs, including TLR9, are expressed in human HCC tissues (27). TLRs are known to be expressed by various

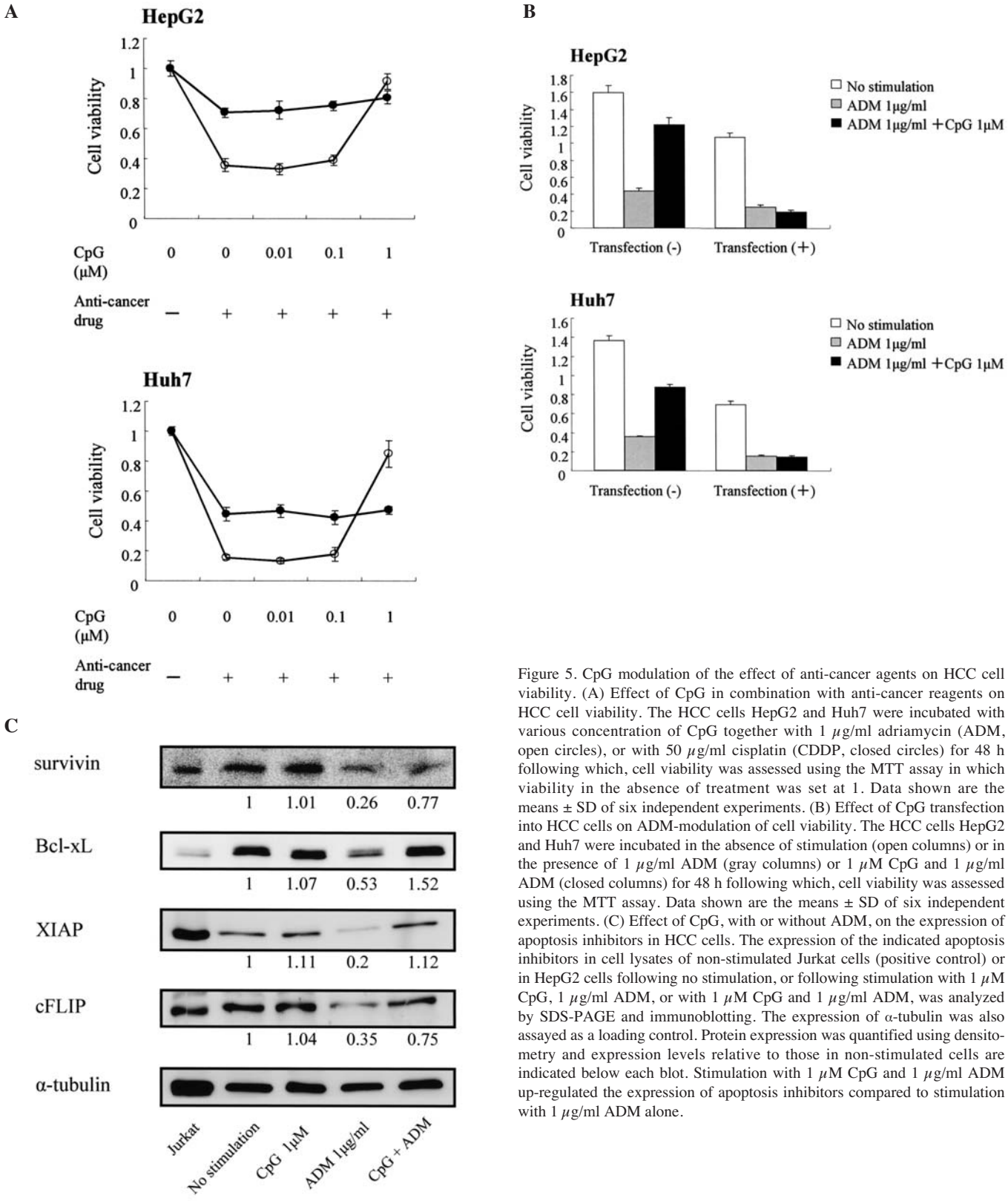

Figure 5. CpG modulation of the effect of anti-cancer agents on HCC cell viability. (A) Effect of CpG in combination with anti-cancer reagents on HCC cell viability. The HCC cells HepG2 and Huh7 were incubated with various concentration of CpG together with 1 μg/ml adriamycin (ADM, open circles), or with 50 μg/ml cisplatin (CDDP, closed circles) for 48 h following which, cell viability was assessed using the MTT assay in which viability in the absence of treatment was set at 1. Data shown are the means ± SD of six independent experiments. (B) Effect of CpG transfection into HCC cells on ADM-modulation of cell viability. The HCC cells HepG2 and Huh7 were incubated in the absence of stimulation (open columns) or in the presence of 1 μg/ml ADM (gray columns) or 1 μM CpG and 1 μg/ml ADM (closed columns) for 48 h following which, cell viability was assessed using the MTT assay. Data shown are the means ± SD of six independent experiments. (C) Effect of CpG, with or without ADM, on the expression of apoptosis inhibitors in HCC cells. The expression of the indicated apoptosis inhibitors in cell lysates of non-stimulated Jurkat cells (positive control) or in HepG2 cells following no stimulation, or following stimulation with 1 μM CpG, 1 μg/ml ADM, or with 1 μM CpG and 1 μg/ml ADM, was analyzed by SDS-PAGE and immunoblotting. The expression of α-tubulin was also assayed as a loading control. Protein expression was quantified using densitometry and expression levels relative to those in non-stimulated cells are indicated below each blot. Stimulation with 1 μM CpG and 1 μg/ml ADM up-regulated the expression of apoptosis inhibitors compared to stimulation with 1 μg/ml ADM alone.

immune cells, as well as by non-immune cells (1-4). Furthermore, TLRs, including TLR9, are expressed by a wide variety of cancer cells (16,17,21-25) and may contribute to tumor cell proliferation, survival and chemosensitivity (18-20). These data suggest that TLR9 signaling in tumor cells is associated with the progression of cancer and the evasion of host defenses. However, little is known regarding the significance of TLR9 for the function of human HCC cells. We therefore attempted to elucidate the functional significance of TLR9 expression in HCC cells and tissues.

The immunohistochemical analysis of the present study revealed that, in addition to non-tumor tissues such as cirrhotic or normal tissues, 85.7% of HCC tissues expressed TLR9. We also detected the TLR9 protein in a panel of HCC

Table II. Oncology-related genes regulated by TLR9 in HepG2 cells.

Gene ID	Gene symbol	Gene name	Change in Log2 ratio	Fold change
Up-regulated oncology-related genes				
BC010353	PTPLA	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	0.71	1.63
-	SNRPN/ SNORD116-2	Small nuclear ribonucleoprotein polypeptide N/ Small nuclear RNA, C/D box 116-2	0.57	1.48
U47077	PRKDC	Protein kinase, DNA-activated, catalytic polypeptide	0.56	1.48
DQ097177	HUWE1	HECT, UBA and WWE domain containing 1	0.56	1.47
AF395444	SMG1	PI-3-kinase-related kinase SMG-1	0.54	1.45
-	MALAT1	Metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)	0.52	1.43
AF265555	BIRC6	Baculoviral IAP repeat-containing 6 (apollon)	0.51	1.43
U79716	RELN	Reelin	0.51	1.42
Down-regulated oncology-related genes				
AF132818	KLF5	Kruppel-like factor 5 (intestinal)	-0.93	0.52
BC085019	GDF5OS	Growth differentiation factor 5 opposite strand	-0.50	0.71
-	ANKRD20B/ CCDC29	Ankyrin repeat domain 20B/ Coiled-coil domain containing 29	-0.46	0.73
BC042173	ANKRD6	Ankyrin repeat domain 6	-0.45	0.73

Table III. Oncology-related genes regulated by TLR9 in Huh7 cells.

Gene ID	Gene symbol	Gene name	Change in Log2 ratio	Fold change
Up-regulated oncology-related genes				
U05569	CRYAA	Crystallin, alpha A	0.77	1.71
BC035856	GRAP	GRB2-related adaptor protein	0.70	1.62
AY189690	NXN	Nucleoredoxin	0.62	1.54
BC146776	SETBP1	SET-binding protein 1	0.59	1.51
AF495523	REX01L1	REX1, RNA exonuclease 1 homolog (*S.cerevisiae*)-like 1	0.55	1.46
AF287261	SETD8	SET domain containing (lysine methyltransferase) 8	0.55	1.46
AF040962	PSPN	Persephin	0.54	1.46
AB021172	NDRG4	NDRG family member 4	0.54	1.45
Down-regulated oncology-related genes				
-	SCARNA7	Small Cajal body-specific RNA 7	-0.72	0.61
BC002673	HSPB8	Heat shock 22 kDa protein 8	-0.57	0.67

cell lines by Western blot analysis. Thus, our results indicate that there is high expression of TLR9 in human HCC.

In the present study, the results of Western blot analysis of subcellular fractions, and flow cytometric analysis of intact cells, revealed that TLR9 is clearly expressed on both the cell surface and in the cytoplasm of human HCC cells. TLR1, 2, 4, 5, 6 and 10 are usually reported as being expressed on the cell surface, while TLR3, 7, 8 and 9, which can recognize

nucleic acid ligands, are usually expressed in the endosomes and the endoplasmic reticulum. Specifically, TLR9 has been localized to the endoplasmic reticulum(ER) together with another ER-resident protein, UNC93B1. Following stimulation with CpG-DNA, TLR9 is translocated to the endosomal/lysosomal compartment in an UNC93B1-dependent manner, thereby allowing recruitment of the adaptor molecule, MyD88, and subsequent signaling (28-30). The localization of TLR9 both at the membrane and in intracellular compartments suggested the possibility that TLR9 may induce different signals at each location. Indeed we have previously demonstrated that functional TLR3 is expressed on both the cell surface and in the cytoplasm of HCC cells, and that intracellular TLR3 signaling is involved in cell death, while, in contrast, cell surface TLR3 signaling is responsible for activation of NF-κB (27). In the present study, stimulation of cell surface TLR9 promotes cellular proliferation and survival. However, direct stimulation of intracellular, cleaved, TLR9 had little effect on cell proliferation and apoptosis. In addition, full-length TLR9 is predominantly expressed on the membrane rather than in the cytoplasm. In contrast, multiple cleaved forms of TLR9 are predominantly expressed in the cytoplasm rather than on the membrane in the absence of CpG-ODN stimulation. It has been recently demonstrated in macrophages that, after stimulation, TLR9 is cleaved into a C-terminal fragment and that this cleaved form alone, translocated to the endosomal/lysosomal compartment, is competent for MyD88-dependent signaling (31). Although the real significance of the subcellular localization of TLR9 in human HCC cells remains uncertain, our findings strongly suggest that HCC cells may be able to respond to various nucleic acids using both the cell surface and endosomal TLR9. Therefore, further study will be needed to clarify the molecular function and regulation of endosomal TLR9.

We next attempted to elucidate the biological significance of the functional TLR9 signaling in HCC cells. In lung cancer cells, stimulation of TLR9 with its agonists CpG-ODNs promotes a reduction in tumor necrosis factor (TNF)-α induced apoptosis (23). The present study is the first report that cell surface stimulation with CpG-ODNs contributes to enhanced cell proliferation and survival in HCC cells. However this result is consistent with the fact that CpG-ODNs induce cancer cell proliferation via secretion of IL-10 which is also related to induction/enhancement of tumor angiogenesis (32).

This study also suggested that cell surface CpG-ODNs stimulation reduced toxicity of ADM via up-regulation of apoptosis inhibitor proteins. Thus, although subtoxic levels of ADM decreased cell viability, cell surface stimulation with a combination of CpG-ODNs and ADM resulted in an increase in cell viability. By Western blot analysis, we found that cell surface stimulation with ADM down-regulated the expression levels of apoptosis inhibitors such as survivin, Bcl-xL, XIAP and cFLIP, but surprisingly, cell surface stimulation with a combination of CpG-ODNs and ADM significantly inhibited the ADM-mediated down-regulation of the expression levels of these proteins. These results suggest that cell surface stimulation with CpG-ODNs might contribute to a reduction in the cytotoxicity of ADM towards HepG2 cells via up-regulation of apoptosis inhibitors.

Engagement of the TLR9 signaling pathway leads to the activation of two major transcription factors that have central roles in innate immunity, i.e., NF-κB and IRF-7. TLR9 requires the adaptor molecule MyD88 for initiation of these signals, and MyD88 can directly associate with and activate IRF-7, leading to type I-IFN production (33,34). Additionally, the MyD88-dependent signaling pathway leads to NF-κB nuclear translocation and activation of NF-κB-dependent genes via an interaction between IRAK1 and TRAF6 (2,35). NF-κB usually plays an important role in regulating immune and inflammatory responses, apoptosis and oncogenes (36-38). In cancers, once NF-κB is activated, NF-κB dimers typically enter the nucleus and induce the expression of cytokines, growth factors and anti-apoptotic proteins. However, activation of these signals by cell surface TLR9 was not detected in the present study, suggesting that cell surface TLR9 signaling in HCC cells may modulate a novel, MyD88-independent signaling pathway for the TLR9-dependent cancer progression.

To investigate which signals may play a role in this proposed novel signaling pathway, we carried out a microarray analysis of genes regulated by CpG-ODN in HepG2 cells. This microarray analysis found that CpG-ODNs regulated multiple oncology-related genes known to be involved in oncogene-signaling, cell proliferation and anti-apoptosis. For example, the metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*) gene was up-regulated in HepG2 cells stimulated with CpG-ODNs. This gene is associated with increased risk of metastasis and poorer prognosis in non-small cell lung cancer (39). *MALAT1* has also been shown to be overexpressed in hepatocellular carcinoma and, more broadly, 50-80% of cases of colon, lung, pancreas and breast cancer that were analyzed exhibited significantly increased *in situ* hybridization intensity for *MALAT1* compared with the surrounding normal tissues (40,41). Conversely, *KLF5* is down-regulated by CpG-ODN treatment and down-regulation of *KLF5* may be an early event in intestinal tumorigenesis. Although expression of *KLF5* in non-transformed intestinal epithelial cells enhances cell growth, *KLF5* expression in colon cancer cell lines inhibits cell growth (42). Also, ankyrin repeat domains regulate apoptosis through interaction with p53, and its family members play a role in cytokine signaling and hepatocarcinogenesis (43). These findings suggest that TLR9 agonists closely regulated oncogenic genes, which may contribute towards tumorigenesis and cancer progression.

In conclusion, TLR9 is expressed on both the cell surface and in the cytoplasm of HCC cells. Cell surface TLR9 signaling may promote the proliferation and survival of HCC cells by a novel, MyD88-independent signaling pathway. Furthermore, TLR9 agonists closely regulated the expression of oncogenic genes, which may contribute towards tumorigenesis and cancer progression.

The current findings may help to define clinical perspectives for inhibition of cell surface TLR9 agonists which can regulate cell proliferation and survival of HCC cells. Further evaluation of the potential physiological roles, and the type of regulation associated with TLR9, needs to be undertaken.

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