SPANDIDOS PUBLICATIONS

Sp1 is involved in 8-chloro-adenosine-upregulated death receptor 5 expression in human hepatoma cells

MINMIN SUN, JINCHUN ZHANG, SHILIAN LIU, YANXIN LIU and DEXIAN ZHENG

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, P.R. China

Received April 20, 2007; Accepted August 2, 2007

Abstract. 8-Chloro-adenosine (8-Cl-Ado) is an adenosine derivative, which inhibits proliferation and induces apoptosis in various tumor cells. Subtoxic concentration of 8-Cl-Ado sensitizes human hepatoma cells to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-triggered apoptosis. However, the molecular mechanism by which TRAIL cytotoxicity is amplified by 8-Cl-Ado is unknown. In the present study, we demonstrated by Western blot and real-time PCR that 8-Cl-Ado selectively up-regulated death receptor 5 (DR5), but not death receptor 4 (DR4), at both protein and RNA levels in human hepatoma cell line BEL-7402. Analysis of the transcriptional regulation of DR5 expression by using Dual-Luciferase reporter assay system demonstrated that the 5'-flanking fragment -207 to -145 upstream to the ATG site within the *DR5* promoter region was responsible for the 8-Cl-Ado-upregulated DR5 expression. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) confirmed that 8-Cl-Ado treatment facilitated transcription factor Sp1 binding to its cis-element -198/-189 in the *DR5* promoter, suggesting that Sp1 is at least one of the 8-Cl-Ado-responsive transcription factors. However, we observed that nuclear factor κB (NF-κB) activity remained invariable in the cells treated with 8-Cl-Ado. These data allowed us to draw a conclusion that 8-Cl-Ado-enhanced *DR5* expression is regulated by Sp1 binding to the -198/-189 cis-element in *DR5* promoter without affecting NF-κB activity in the hepatoma cells. This study may shed light on further screening the regulators of DR5 expression and developing novel therapeutic drugs for liver cancer.

Correspondence to: Professors Dexian Zheng or Yanxin Liu, Institute of Basic Medical Sciences, CAMS and PUMC, 5 Dong Dan San Tiao, Beijing 100005, P.R. China
E-mail: zhengdx@pumc.edu.cn or zhengdx@tom.com

Abbreviations: 8-Cl-Ado, 8-chloro-adenosine; 8-Cl-cAMP, 8-chloro-cyclic AMP; ChIP, chromatin immunoprecipitation; cons., consensus sequence; DR5, death receptor 5; DR4, death receptor 4; DD, death domain; EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase; NF-κB, nuclear factor κB; OPG, osteoprotegerin; rsTRAIL, recombinant soluble TRAIL; Sp1, specificity protein 1; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; TNF, tumor necrosis factor

Key words: Sp1, 8-chloro-adenosine, NF-κB, death receptor 5, hepatoma cells

Introduction

8-Chloro-adenosine (8-Cl-Ado), a dephosphorylated metabolite of anti-neoplastic agent 8-chloro-cyclic AMP (8-Cl-cAMP), has been reported to inhibit proliferation and induce apoptosis in various tumor cells (1,2). Langeveld *et al* showed that 8-Cl-Ado blocks cell cycle progression and induces mitotic catastrophe (1,3). Furthermore, signalling cascade such as PKA or PKC activation in the cells treated with 8-Cl-Ado might be triggered (4,5). The cDNA microarray assay revealed that the expression levels of ~100 cDNAs were altered in the 8-Cl-Ado-treated human cells (6). The genes involved in cell differentiation, development, and immune response were up-regulated, while those involved in cell proliferation and transformation were down-regulated. However, the molecular mechanism by which 8-Cl-Ado regulates gene expression remaines to be clarified.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF super-family, which selectively induces apoptosis in a variety of tumor cells, but not in most normal cells (7). Moreover, the combinations of recombinant soluble TRAIL with chemotherapy or radiotherapy have been reported to exert synergistic anti-tumor effects in mice-bearing human tumor xenografts (8,9). TRAIL exerts its cytotoxic activity via engaging its receptors on the surfaces of target cells. Five receptors specific for TRAIL have been identified. Two of them, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), are capable of transducing an apoptosis signal (10,11), whereas the other three, DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG), serve as decoy receptors to block TRAIL-mediated apoptosis (12-14). DR4 and DR5 share a common intracellular death domain (DD), which is indispensable for initiation of the intracellular signalling cascade leading to cell death (15,16). DR5 is a highly inducible receptor, whose expression is regulated by both p53-dependent and -independent pathway (17-21). Our previous study (22) showed that subtoxic concentration of 8-Cl-Ado sensitized the human hepatoma cell line BEL-7402

to recombinant soluble TRAIL (rsTRAIL)-induced apoptosis, and facilitated DR5 expression at protein level, but not DR4, in the cells treated with 8-Cl-Ado plus rsTRAIL in a p53-independent manner, suggesting that there might be unknown factors in the death signalling pathway regulating DR5 expression.

To further explore the molecular mechanism of DR5 expression, we investigated the transcriptional regulation of DR5 expression in the human hepatoma BEL-7402 cells treated with 8-Cl-Ado in the present study. The data showed that 8-Cl-Ado enhanced the binding of the transcription factor specificity protein 1 (Sp1) with its cis-element -198/-189 in *DR5* promoter without influencing nuclear factor-κB (NF-κB) transcriptional activity, indicating that Sp1 appears to be one of 8-Cl-Ado-responsive transcription factors and contributes to the transcriptional regulation of DR5 expression.

Materials and methods

Materials. 8-Cl-Ado was a kind gift from Lihe Zhang (2,23), Peking University Medical Center. The recombinant soluble TRAIL (rsTRAIL, 95-281 a.a.) was prepared as described by Shi *et al* (24). Polyclonal rabbit anti-human DR5 antibody was purchased from MBL International Corporation (Woburn, MA). Rabbit anti-Sp1 polyclonal antibody, mouse anti-Sp1 monoclonal antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-ß-actin monoclonal antibody and protein A-Sepharose beads were purchased from Sigma Chemicals Co. (St. Louis, MO). Anti-DR4 polyclonal antibody was prepared by immunizing rabbits with the recombinant soluble DR4 (extracellular domain) in the laboratory.

Cell culture. The human hepatoma cell line BEL-7402 was purchased from the Committee on Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 media (Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2.

Western blot analysis. The cell lysates were prepared by suspending 1x10^7 cells in 100 μl of lysis buffer (Roche Applied Science, Mannheim, Germany) and incubated on ice for 1 h. The lysates were collected by centrifugation at 12000 rpm and 4˚C for 15 min. Lysates (20 μl) containing 50 μg proteins were boiled immediately and subjected to 10% SDS-PAGE. The proteins in the gel were subsequently electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with 5% (w/v) nonfat dry milk in TBS-T [20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% (v/v) Tween-20] for 1-2 h at room temperature. The membrane was then incubated with the primary specific antibodies in TBS-T containing 5% (w/v) nonfat dry milk at 4˚C overnight and followed by washing 3 times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1.5-2 h. After washing 3 times with TBS-T, the targeted proteins were visualized by using the ECL Plus Western blotting detection system (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's instructions.

Quantitative real-time PCR. The total RNAs were isolated from BEL-7402 cells using TRIzol reagent (Invitrogen), and the cDNAs were synthesized from 2 μg of total RNAs using an oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). For the relative quantitation of the mRNA expression, real-time PCR was performed by using the cDNA samples and specific primers on ABI real-time PCR system and SYBR green as the fluorophore. The sequences of the sense and anti-sense primers for *DR5* were 5'-TGG TTCCAGCAAATGAAGGTG-3' and 5'-CCGCTGCCT CAGCTTTAGC-3', respectively. The primers of ß-actin as internal controls were sense 5'-ATGGTGGGAATGGG TCAGAAG-3' and anti-sense 5'-CACGCAGCTCATTGTA GAAGG-3'. All PCR conditions and primers were optimized to produce a single product with the correct base pair size. The Ct value of DR5 mRNA in each sample was normalized by using the Ct value of ß-actin.

Cloning of DR5 promoter region. The genomic DNA of BEL-7402 cells were prepared by using phenol extraction protocol (25). The 5'-flanking fragment of *DR5* gene corresponding to the nucleotide residue of -1152 to -2 (numbering from the ATG site) was amplified by PCR using the BEL-7402 genomic DNA as template and the following primers: sense 5'-GAG CTCAGGAACAAACTCCAGACACG-3' and antisense 5'-CTCGAGGCGGTAGGGAACGCTCTTAT-3'. The PCR product was purified by 1% (w/v) agarose gel electrophoresis and subcloned into the firefly luciferase-based pGL3-Basic vector (Promega, Madison, WI) between Sac I and Xho I sites. The consequent plasmid was designated as pGL3-Basic/-1152. The nucleotide sequence was identified by sequence analysis and compared with the reported *DR5* gene promoter in human peripheral blood leukocytes (GenBank accession no. AB054004) (26).

Construction of reporter plasmid. The deletion mutant series of *DR5* promoter were generated by PCR amplification using pGL3-Basic/-1152 plasmid as template and the following primers and subcloned into the pGL3-Basic vector to generate the reporter plasmid series of pGL3-Basic/-949, pGL3-Basic/-744, pGL3-Basic/-552, pGL3-Basic/-488, pGL3-Basic/-377, pGL3-Basic/-270, pGL3-Basic/-207, and pGL3-Basic/-144, respectively. The sense primer 5'-GAGCTCCAGGGACACA GGCACATTTG-3' was for pGL3-Basic/-949, 5'-GAGCTC CACACCACCGTTCACCAATC-3' for pGL3-Basic/-744, 5'-GAGCTCAGAGAACAGAAGGGGCAGGT-3' for pGL3-Basic/-552, 5'-GAGCTCCTCTGAACCTCAAGACCCTG-3' for pGL3-Basic/-488, 5'-GAGCTCCGGACAGGACCCAG AAACAA-3' for pGL3-Basic/-377, 5'-GAGCTCTGCGTT GACGAGACTCTTATT-3' for pGL3-Basic/-270, 5'-GAG CTCATCTGATTCGCCCCGCC-3' for pGL3-Basic/-207, and 5'-GAGCTCCCAAGTCAGCCTGGACACAT-3' for pGL3-Basic/-144, and the anti-sense primer 5'-CTCGAGG CGGTAGGGAACGCTCTTAT-3' for all the PCR reactions. The sequences of all constructed reporter plasmids were confirmed by nucleotide-sequencing analysis.

tional activity assay. The BEL-7402 cells cultured l plates were co-transfected with 4 ng of phRL-TK, which encodes *Renilla* luciferase as internal control (Promega), and 200 ng of the various reporter plasmids by using Lipofectamine 2000 (Invitrogen). In case of NF-κB activity assay, pNF-κB-luc reporter plasmid was constructed as previously described (22), which expresses firefly luciferase after NF-κB binds with its cis-element in the plasmid. After 24 h of transfection, the cells were incubated with 8-Cl-Ado (6 μg/ml) or media (control) for desired time. The cells were lysed with protein lysis buffer (Promega). Both firefly and *Renilla* luciferase activities in the cell lysates were quantitated using Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized by *Renilla* luciferase activity.

Electrophoretic mobility shift assay (EMSA). Nuclear protein extracts were prepared from the BEL-7402 cells treated with or without 8-Cl-Ado by using high salt extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 25% (v/v) glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) as described by Lim *et al* (27). The double-stranded oligonucleotide probes and specific competitors used were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, China) and the sequences were: Sp1 consensus sequence (Sp1 cons.), 5'-ATTCGATCGGGGCGGGGCGAGC-3'; CREB cons., 5'-AGAGATTGCCTGACGTCAGAGAGC TAG-3'; p300 cons., 5'-GGGAGTGGGGAGTGGGGA GTG-3'; GATA-2 cons., 5'-GAGGATAGCGGAGGATA GCGGAGGATAGCG-3', and NF-κB cons., 5'-AGTTGAG GGGACTTTCCCAGGC-3'. The electrophoretic mobility shift assay (EMSA) was carried out according to the study by Zhang *et al* (28). Briefly, the double-stranded oligonucleotides were end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol) at the presence of T4 polynucleotide kinase. Nuclear protein extracts (10 μl) containing ≤10 μg of proteins from the BEL-7402 cells were incubated with ^{32}P-labelled probes in the binding buffer containing 50% (v/v) glycerol, 100 mM HEPES (pH 7.9), 5 mM EDTA, 2.5 mM dithiothreitol, 250 mM KCl, 10 mM $MgCl_2$, 20% (v/v) Ficoll 400, and 0.5 mg/ml salmon DNA at room temperature for 20 min. Unlabelled oligonucleotides, competitor oligonucleotides, or antibody for supershifting were used as controls. The reaction mixtures were resolved by electrophoresis with 5% (w/v) polyacrylamide gel containing 0.5 X Tris borate/EDTA at 120 V for 2 h. The gel was dried and subjected to autoradiography.

Chromatin immunoprecipitation (ChIP). The 2x10^7 BEL-7402 cells were treated with or without 8-Cl-Ado (6 μg/ml) for 4-8 h. The cells were incubated with formaldehyde to cross-link DNA with DNA-bound proteins and then collected in 1 ml cell collection buffer (100 mM Tris-HCl, pH 9.4, 10 mM DTT) containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin) (Boehringer Mannheim, Mannheim, Germany). The DNA-protein complexes in the cells were extracted as described by Reid G. (http://fellowsnet.embo.org) and dissolved into 2 ml of lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 0.5% (v/v) Empigen BB, 1% (w/v) SDS, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin) followed by sonicating on Sonic dismembrator 550 (Fisher Scientific, Pittsburgh, PA) for 20 sec by 15 times to shear the DNA into 200 to 1000 bp fragments. The chromatin immunoprecipitation (ChIP) was carried out as described by Reid G. Briefly, 500 μl sheared DNA-protein complex were subjected to immunoprecipitation by adding rabbit anti-Sp1 polyclonal antibody and protein A-agarose beads at 4˚C for 2.5 h. Each DNA-protein complex (50 μl) were used as nonimmunoprecipitation controls (inputs). After washing successively with washing buffer I, II, III, and IV, the immune complex on the protein A-agarose beads were eluted by using extraction buffer [1% (w/v) SDS, 0.1 M $NaHCO_3$] at room temperature for 1.5 h and collected by centrifugation. The inputs and eluates were incubated with NaCl (0.3 M) and proteinase K (0.05 mg/ml) at 65˚C to perform reverse cross-linking. The DNA fragments in the immune complex were purified by phenol/chloroform extraction and ethanol precipitation. PCR

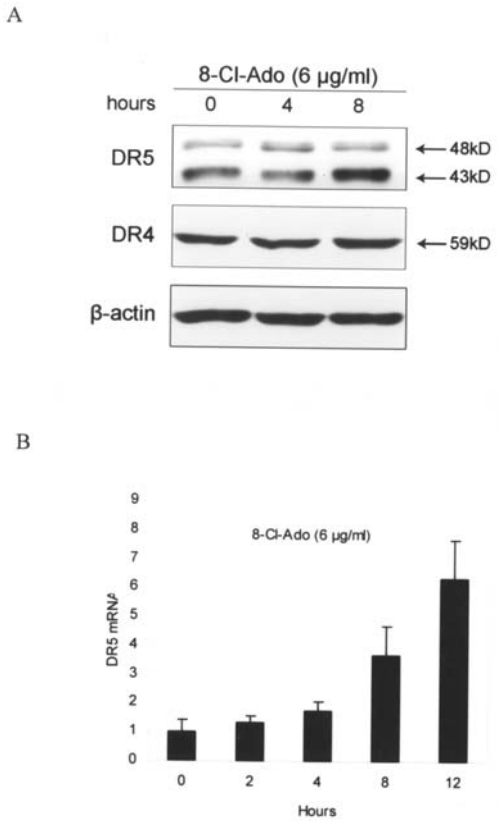


Figure 1. 8-Cl-Ado up-regulates DR5 expression in BEL-7402 cells. (A) Western blot analysis of the expression of DR4 and DR5 in BEL-7402 liver cancer cells treated with or without 8-Cl-Ado (6 μg/ml) for the indicated time course. Equal amounts of the cell lysates containing 50 μg proteins were subjected to SDS-PAGE. ß-actin was used as loading control. The proteins in the gel were transferred on the PVDF membranes. The membranes were blotted with specific antibody against DR4 or DR5 and probed with HRP-conjugated goat anti-rabbit IgG complex successively. The protein bands were visualized with ECL system and exposed to X-ray film. (B) Real-time PCR analysis of the expression of DR5 mRNA in the cells treated with or without 8-Cl-Ado (6 μg/ml) for the indicated time course. Total RNAs were isolated and the cDNAs were synthesized by using an oligo-dT primer and M-MLV reverse transcriptase. The real-time PCR was performed by using the cDNAs as template and specific primers. ß-actin mRNA served as a loading control. Each column represents the mean ± SD in three individual experiments.

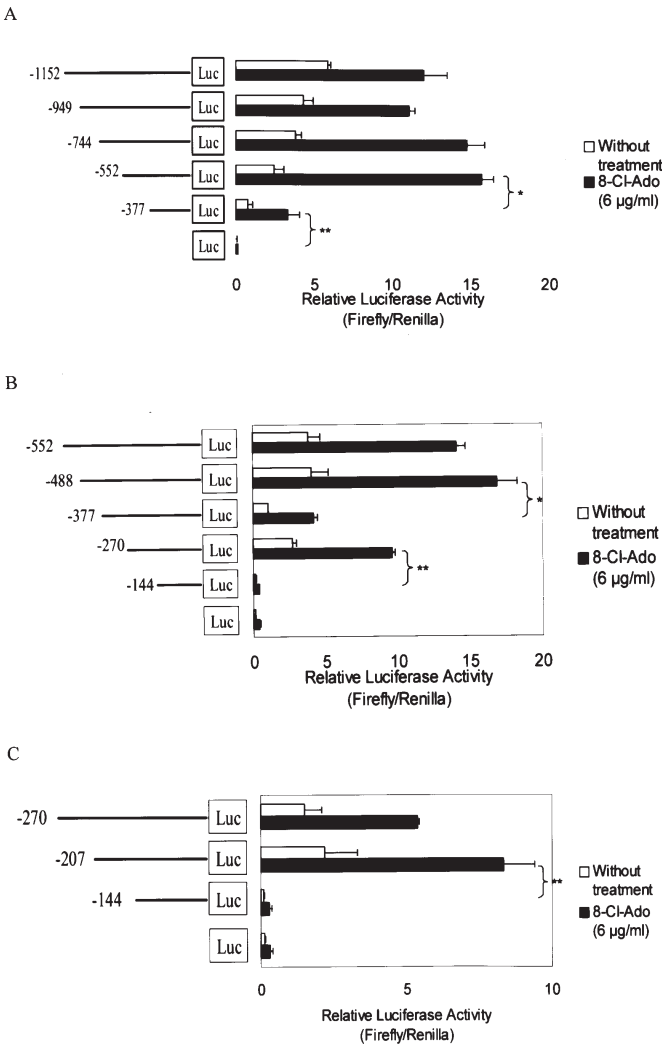


Figure 2. Identification of the responsible element in *DR5* promoter for the 8-Cl-Ado-enhanced DR5 expression. The BEL-7402 cells were transfected with the indicated luciferase reporter plasmids and subsequently incubated in the presence or absence of 8-Cl-Ado (6 μg/ml). The cells were lysed and the luciferase activities were analyzed. The panels of (A), (B), and (C) show that the 5' fragments of *DR5* promoter in the luciferase reporter plasmids were deleted gradually. Each column represents the mean ± SD in three individual experiments ($^{*}p<0.05$, $^{**}p<0.01$).

were carried out to amplify 276-bp fragments of the *DR5* promoter including Sp1 cis-element of -198/-189 by using desired volumes of the purified DNA as templates and the following oligo-nucleotides as primers, sense 5'-GCCAGG GCGAAGGTTA-3' and antisense 5'-GGGCATCGTC GGTGTAT-3'. Since PCR products amplified from the input (non-IP sample) at various dilutions are correlated with the concentrations of the template DNA used in the ChIP assay, so that, 1:5, 1:25, and 1:125 diluted inputs were used to optimize the dilution of template DNA for internal control in the PCR reaction. Pre-immune rabbit IgG, without adding antibody, or without adding chromatin served as negative controls.

Statistical analysis. The data represent at least three independent experiments and are expressed as the mean ± SD. Statistical differences were evaluated by using Student's t-test and considered significant at the $p<0.05$ level.

Results

8-Cl-Ado augmented DR5 expression at both translational and transcriptional levels in the hepatoma BEL-7402 cells. It is well known that TRAIL triggers apoptotic signals via the interaction with the two death receptors, DR4 and DR5. Our previous study showed that subtoxic concentration of 8-Cl-Ado sensitized the human hepatoma cells BEL-7402 to rsTRAIL-induced apoptosis and the DR5 expression on the cell surfaces was enhanced in a p53-independent manner (22). To explore the molecular mechanisms of 8-Cl-Ado-enhanced DR5 expression, the BEL-7402 cells were treated with or without the subtoxic concentration (6 μg/ml) of 8-Cl-Ado for a time course, and then the protein and mRNA expressions of DR4 and DR5 were determined by Western blot and real-time PCR analysis, respectively. As shown in Fig. 1A, the expression of DR5 protein, but not DR4, was augmented significantly by 8-Cl-Ado treatment for 8 h, confirming that 8-Cl-Ado may sensitize the hepatoma cells to TRAIL-induced apoptosis via up-regulation of DR5. Real-time PCR analysis demonstrated that *DR5* mRNA expression was also elevated gradually in the time course and increased up to 6.3-fold in the cells treated with 8-Cl-Ado for 12 h than that without treatment (Fig. 1B), suggesting that 8-Cl-Ado enhanced DR5 expression at both translational and transcriptional levels in a time-dependent manner.

8-Cl-Ado facilitated DR5 promoter activity via specific sequence in the 5'-flanking region. To further understand the transcriptional regulation of DR5 expression, the luciferase reporter gene system was used to determine the specific element responsible for the 8-Cl-Ado-augmented DR5 expression. The 8-Cl-Ado-responsive element in the *DR5* promoter was evaluated by determining the luciferase activity after transfection with pGL3-Basic/-1152, pGL3-Basic/-949, pGL3-Basic/-744, pGL3-Basic/-552, and pGL3-Basic/-377, respectively. As shown in Fig. 2A, 8-Cl-Ado significantly facilitated the luciferase activity in the pGL3-Basic/-1152-transfected cells, indicating that there is at least one cis-element in the 5'-flanking region of *DR5* gene responsible for 8-Cl-Ado-enhanced DR5 expression. The 5'-deletion to -552 did not change the luciferase activity in the cells treated with 8-Cl-Ado, but further deletion to -377 reduced the luciferase activity significantly, suggesting that the responsible element for 8-Cl-Ado-enhanced DR5 expression might exist in the fragment spanning from -552 to -2 in the *DR5* promoter region.

By more detailed deletion analysis, we observed that the deletion of the -488/-378 fragment reduced some of the luciferase activity, however, similar ratios (4-fold) of the luciferase activities were kept in the cells treated with or without 8-Cl-Ado (Fig. 2B), suggesting that 8-Cl-Ado could still regulate *DR5* gene expression in the cells with the deletion of -488/-378 in *DR5* promoter. No luciferase activities were observed in the cells with the deletion of -270/ -145 and treated with 8-Cl-Ado, indicating that the cis-element responsible for 8-Cl-Ado-enhanced DR5 expression may exist in the -270/-145 fragment. The deletion of -377/-271 promoted the luciferase activity, suggesting that a cis-element inhibiting *DR5* transcription might be present in the fragment of -377/-271.

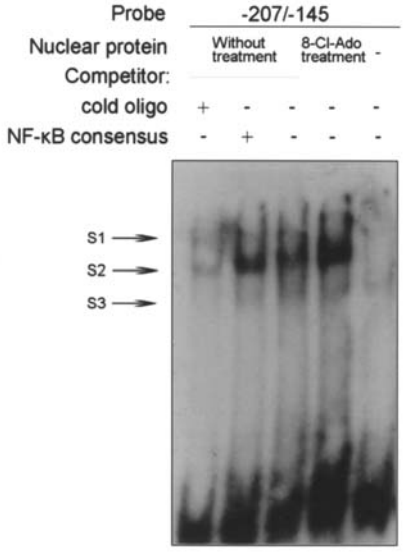


Figure 3. 8-Cl-Ado enhances Sp1 binding to the specific fragment in *DR5* promoter. (A) The BEL-7402 cells were treated with or without 8-Cl-Ado (6 μg/ml) for 4 h. Nuclear proteins were extracted and EMSA was performed by using γ-^{32}P-labelled double strand -207/-145 probe. Equal amounts of nuclear proteins (10 μg) from the cells were incubated with ^{32}P-labelled probes in the binding buffer at room temperature for 20 min (left panel). Binding specificity was determined by using the unlabelled probe (cold oligo) or NF-κB binding consensus element to compete with the labelled oligonucleotide (right panel). The reaction mixtures were resolved in 5% (w/v) polyacrylamide gel electrophoresis. The gel was dried and exposed to the X-ray film. S1, S2, and S3 represent oligonucleotide-protein complexes. (B) Potential transcription factor-binding elements in -207/-145 fragment of *DR5* promoter were analyzed by searching the TFSearch transcription factor database. (C) Competition analysis of 8-Cl-Ado-responsive transcription factor. EMSA was performed by using γ-^{32}P-labelled double strand -207/-145 probe and unlabelled Sp1, CREB, p300, or GATA-2 consensus elements as the competitors (100-fold more than the labelled probe). (D) Super-shift analysis of Sp1 in the oligonucleotide-protein complex using anti-Sp1 monoclonal antibody.

B

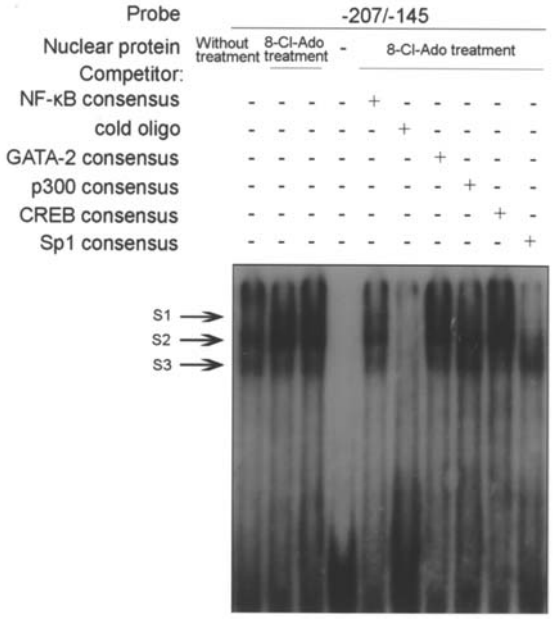
TFMATRIX entries with High-scoring:

1 ATCGGATTCG CCCCGCCCCG AATGACGCCT GCCCGGAGGC AGTGAAAGTA	entry	score
<----------	M00008 Sp1	94.5
---------->	M00032 c-Ets-1	85.3
---------->	M00033 p300	83.2
---------->	M00113 CREB	82.8
---------->	M00039 CREB	82.2
---------->	M00076 GATA-2	81.4
----------	M00148 SRY	80.9
<----------	M00002 E47	80.8

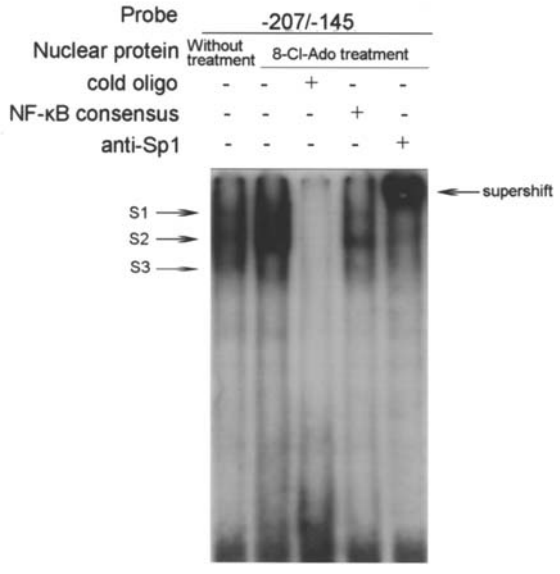
51 CAGCCGCGCC GCCC	entry	score
>	M00148 SRY	80.9

Total 8 high-scoring sites found.
Max score: 94.5 point, Min score: 80.8 point

C



D



Further deletion demonstrated that lack of the fragment of -207/-145 blocked *DR5* promoter activity significantly, confirming that 8-Cl-Ado responsive element is located in the fragment of -207/-145 (Fig. 2C).

8-Cl-Ado enhanced Sp1 binding to the DR5 promoter. To further explore whether there is a binding site for a transcription factor within the -207/-145 fragment, the electrophoretic mobility shift assay (EMSA) was performed by using γ-^{32}P-labelled -207/-145 fragment, the unlabelled fragment and NF-κB consensus element as competitors. As shown in Fig. 3A, 2 out of 3 DNA-protein binding complexes (S1 and S2) were increased in the 8-Cl-Ado-treated cells, suggesting that the two proteins (S1 and S2) binding with -207/-145 fragment in *DR5* promoter are responsible for 8-Cl-Ado-enhanced DR5 expression.

To identify the proteins binding with -207/-145 fragment in *DR5* promoter, the TFSearch transcription factor database (48) was searched. As shown in Fig. 3B, the transcription factors, Sp1, c-Ets-1, p300, CREB, GATA-2, SRY, and E47 were able to bind to the fragment -207/-145. It is reported in the literatures that c-Ets-1 is a transcription factor in mouse and chicken (29), SRY is involved in sex determination (30), and E47 is related to the regulation of IgH expression (31), suggesting that any one of these three transcription factors is unlikely responsible for the 8-Cl-Ado-enhanced DR5 expression in the BEL-7402 cells. Therefore, competitive EMSA was performed by using the cis-elements binding with Sp1, p300, CREB, and GATA-2 to determine which one is involved in the 8-Cl-Ado-enhanced DR5 expression in the BEL-7402 cells. As shown in Fig. 3C, the DNA-protein complexes, S1 and S2, were dramatically competed out by 100-X unlabelled competitor oligo-nucleotide of Sp1 consensus binding element (Sp1 cons.), and the competitive elements of p300 cons., CREB cons., and GATA-2 cons., however, had little affect on the binding of nuclear proteins with the labelled probes. These data indicate that the transcription factor binding to the -207/-145 fragment and responsible for 8-Cl-Ado-enhanced DR5 expression is likely Sp1-related.

To confirm that Sp1 binds to the -207/-145 fragment in the *DR5* promoter, super-shift assay was carried out by using the monoclonal antibody against Sp1. As shown in Fig. 3D, in the presence of anti-Sp1 antibody, the shift bands (S1, S2, and S3) were completely abolished, and an additional super-shift band with a slower mobility was observed in the cells treated with 8-Cl-Ado, indicating that 8-Cl-Ado enhanced the formation of Sp1-DNA complex and Sp1 does bind to the -207/-145 fragment. The shift band S3, which represents a Sp1-DNA complex in the super-shift assay, however, was not affected by the Sp1 oligo competitor (Fig. 3C).

8-Cl-Ado enhanced Sp1 binding to the DR5 promoter in vivo. Sequence analysis revealed that Sp1 cis-element in the *DR5* promoter was located at -198 to -189. To confirm that Sp1 does bind to the Sp1 cis-element -198/-189 in the *DR5* promoter in the 8-Cl-Ado-treated cells, the chromatin immunoprecipitation (ChIP) assay was performed. The PCR primers were designed to amplify a 276-bp fragment consisting of Sp1 binding element -198/-189 in the *DR5*

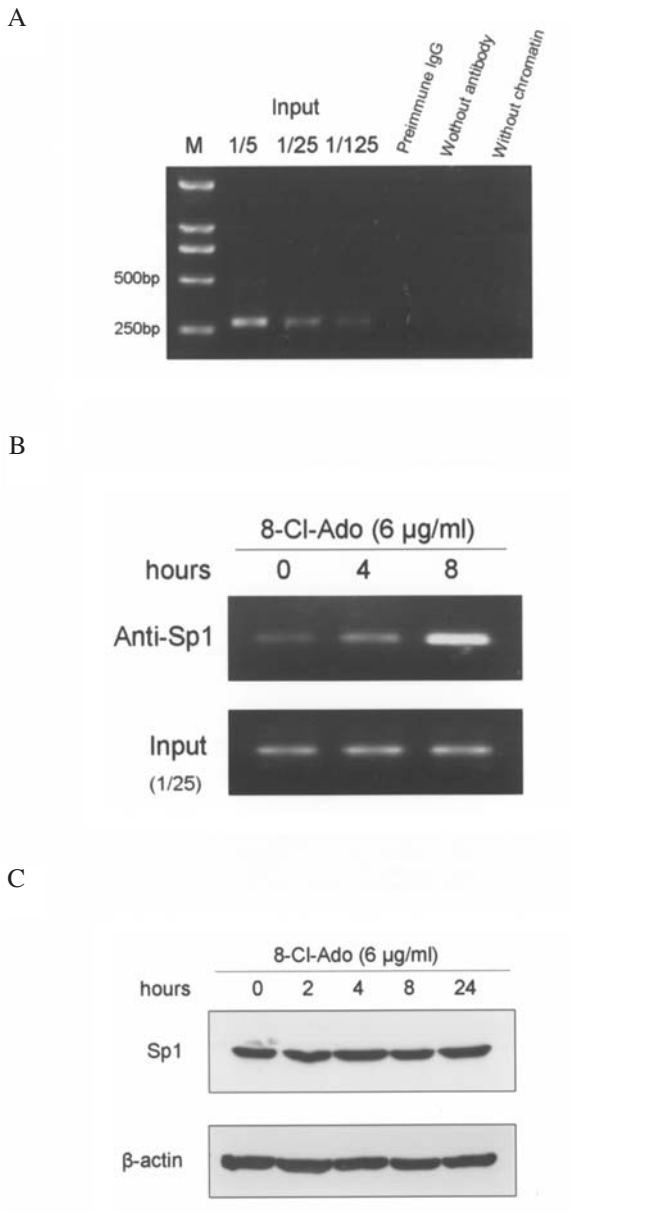
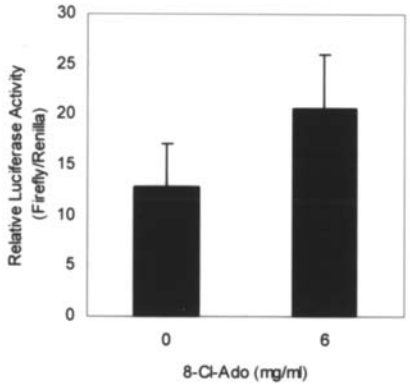


Figure 4. 8-Cl-Ado promotes Sp1 binding to its cis-element in *DR5* promoter. The BEL-7402 cells were treated with or without 8-Cl-Ado (6 μg/ml) for the indicated time. The cells were incubated with formaldehyde to cross-link DNA with DNA-bound proteins and then collected. The DNA-protein complex were extracted and sonicated to shear the DNA into 200 to 1000 bp fragments. The chromatin immunoprecipitation (ChIP) was carried out and the anti-Sp1 immunoprecipitated complexes were subjected to PCR. The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis. (A) The PCR products amplified from the non-immunoprecipitation complexes (Inputs) and the negative controls with pre-immune rabbit IgG, without antibody, or without chromatin. (B) The PCR products from the immunoprecipitated complexes. Input (1:25 dilution) was used as loading control. (C) Western blot analysis of Sp1 protein in the 8-Cl-Ado-treated cells by using specific polyclonal antibody.

promoter. As shown in Fig. 4A and B, the 276-bp fragment consisting of Sp1 binding element -198/-189 was markedly amplified in the cells treated with 8-Cl-Ado. However, immunoblot assay (Fig. 4C) with the specific antibody against Sp1 showed that the level of Sp1 protein expression was not altered by 8-Cl-Ado treatment. Thus, these data allowed us to draw a conclusion that 8-Cl-Ado treatment



B

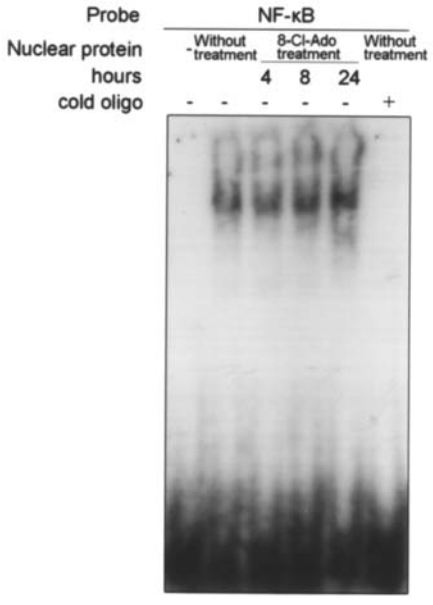


Figure 5. 8-Cl-Ado has no effect on NF-κB transcriptional activity. (A) The BEL-7402 cells were transfected with pNF-κB-luc luciferase reporter plasmid consisting of NF-κB DNA binding element. The phRL-TK was used as the control of transfection efficiency. The cells were then treated with or without 8-Cl-Ado (6 μg/ml) for 8 h and the luciferase activities were measured. NF-κB transcriptional activity is represented by firefly luciferase activity normalized by *Renilla* luciferase activity. The data in each column represent the mean ± SD in three individual experiments. (B) BEL-7402 cells were treated with or without 8-Cl-Ado (6 μg/ml) for the indicated time course. Nuclear proteins were extracted and EMSA was performed by using γ-^{32}P-labelled double strand NF-κB binding consensus element. Equal amounts (10 μg) of nuclear proteins were loaded in each lane. Binding specificity was determined by using the unlabelled probe (cold oligo) to compete with the labelled oligonucleotide.

facilitates the binding affinity of Sp1 to the Sp1 binding element -198/-189, but does not augment the level of Sp1 protein expression in the BEL-7402 cells.

8-Cl-Ado treatment does not affect NF-κB activity. It is reported that the transcription factor NF-κB and p53 also bind to the specific sequences within *DR5* gene locus at intron 1 (19,32). Our unpublished data (49) showed that p53 was not involved in the induction of apoptosis in the BEL-7402 cells

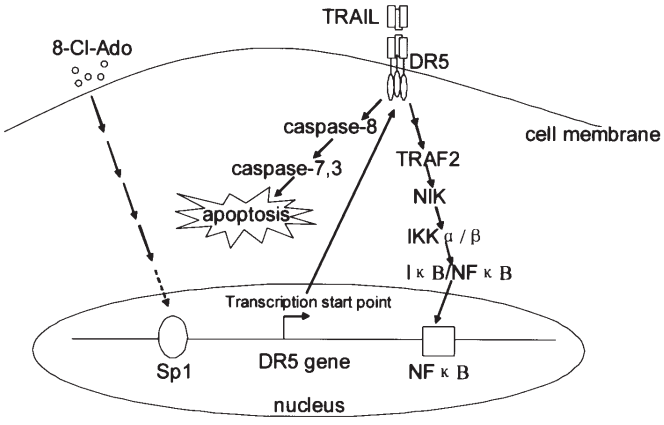


Figure 6. Hypothesis on the mechanism of 8-Cl-Ado sensitizing TRAIL-triggered apoptosis.

treated with the combination of rsTRAIL and 8-Cl-Ado. Therefore, it is essential to examine whether NF-κB transcriptional activity in the 8-Cl-Ado-treated cells was affected or not. As shown in Fig. 5A by pNF-κB-luc reporter gene assay, 8-Cl-Ado treatment did not have notable effects on the luciferase activity in the cells. EMSA with γ-^{32}P-labelled NF-κB consensus binding element further demonstrated that no strengthened band shift of DNA-protein complex was observed in the cells treated with 8-Cl-Ado for a time course compared with that without treatment (Fig. 5B), indicating that 8-Cl-Ado does not affect NF-κB-DNA binding activity. Collectively, 8-Cl-Ado-upregulated *DR5* gene expression is independent of NF-κB transcriptional activity.

Discussion

Our previous report demonstrated that 8-Cl-Ado sensitizing human hepatoma cells BEL-7402 to rsTRAIL cytotoxicity was due to DR5 protein expression, but not DR4 (22). In the present study, we further confirmed that 8-Cl-Ado facilitated DR5 expression at both protein and mRNA levels. Analysis of the transcriptional regulation of DR5 expression showed that the transcription factor Sp1 binding with the -198/-189 cis-element in the *DR5* promoter was greatly strengthened, but without influence on NF-κB transcriptional activity, in the BEL-7402 liver cancer cells. These data indicated that the 8-Cl-Ado-elevated sensitivity of BEL-7402 cells to rsTRAIL cytotoxicity is contributed at least in part by facilitating DR5 expression via Sp1 binding to the -198/-189 cis-element in the *DR5* promoter.

By using reporter gene assay, we observed that the deletion of the -488/-378 fragment caused some decreases in *DR5* promoter activity, and the relative luciferase activities were correspondingly reduced in the cells treated with or without 8-Cl-Ado. The mechanism by which -488/-378 fragment partially contributes to *DR5* promoter activity remains unknown. It is also notable that the deletion of the -377/-271 fragment promoted *DR5* promoter activity, suggesting that there might be a cis-element with negative transcriptional activity in this region, which is valuable to explore the underlying mechanism.

The EMSA and ChIP assay showed that 8-Cl-Ado appeared to activate *DR5* gene transcription by facilitating the binding of Sp1 to the -198/-189 cis-element in *DR5* promoter, but not altering the level of Sp1 protein expression. These data provide insight into the regulation of DR5 expression in the 8-Cl-Ado treated BEL-7402 liver cancer cells (Fig. 6). The transcription factor Sp1 binds to G-rich cis-elements, such as GC-box and GT-box (33), and is involved in basal and inducible transcription of genes (33-37). Yoshida *et al* (26) reported that Sp1 was responsible for the basal activity of *DR5* promoter. In fact, Sp1 is the only identified regulatory factor for *DR5* expression (38,39). Sp1 regulates the inducible gene expression usually via post-translational modifications, such as phosphorylation and interaction with other co-activating proteins (33,34,40,41). Our data that 8-Cl-Ado enhanced DR5 expression by facilitating Sp1 binding with its consensus cis-element -198/-189 in the *DR5* promoter, but did not alter the Sp1 protein expression level, provide novel evidence for the pattern that Sp1 participates in the regulation of the inducible gene expression via post-translational modifications. How does 8-Cl-Ado affect the protein-protein interaction in the signalling pathway eventually leading to the augment of Sp1 transcriptional activity remains to be further clarified.

8-Cl-Ado is an adenosine derivative and an active metabolite of chemotherapeutics 8-Cl-cAMP responsible for its anti-proliferative effects (42). 8-Cl-Ado could regulate both differentiation and apoptosis in tumor cells via PKA signalling pathway (5). Furthermore, signalling cascades, such as PKC activation and cyclin B down-regulation might be involved in the 8-Cl-Ado-treated cells (4). To clarify whether Sp1 is phosphorylated by PKA or PKC, or activated by other co-activating proteins is valuable for further understanding the mechanism of DR5 up-regulation in the 8-Cl-Ado-treated BEL-7402 liver cancer cells.

The NF-κB is an extremely important family of dimeric transcription factors in modulating cell survival during stress and immune responses (43). It has been reported that NF-κB induced DR5 expression and then mediated apoptosis in certain tumor cells (32). However, in the present study, neither the transcriptional activity nor DNA binding activity of NF-κB was elevated in the BEL-7402 liver cancer cells treated with 8-Cl-Ado. Thus, we proposed that NF-κB's transcriptional activity for DR5 expression is stimulator-specific and it does not contribute to 8-Cl-Ado-triggered DR5 expression in the BEL-7402 cells.

Studies on the regulation of DR5 and DR4 expression demonstrated that the death receptors play a critical role in the synergistic effect of the combination of rsTRAIL and chemotherapeutic agents on inducing apoptosis of cancer cells (44,45). However, there are no common potential transcription factor binding sites reported in the highly homologous regions of *DR4* and *DR5* promoters (46), suggesting that the distinct regulation mechanisms of the two receptors are involved. Our results that while 8-Cl-Ado upregulated DR5 expression, DR4 expression remained unchanged, provide further evidence for the distinct regulation mechanisms of DR4 and DR5 expression.

DNA damaging agents and chemotherapeutic drugs have been shown to induce DR5 expression in a p53-dependent or -independent manner (17-21). TRAIL itself could also upregulate DR5 expression via NF-κB activation pathway (47). There are limited reports on understanding the molecular mechanisms of *DR5* gene expression and regulation, especially on the cis-elements within *DR5* promoter and transcription factors, our data may shed light on screening the regulators of DR5 expression and developing novel therapeutic drugs for liver cancer treatment.

Acknowledgements

We are grateful to Professor Lihe Zhang (Peking University Medical Center) for the kind gift of 8-Cl-Ado. This study was partially supported by grants of National Natural Science Foundation of China (No. 30571687) and State Key Basic Research Program of China (No. 2007CB507404).

References

1. Langeveld CH, Jongenelen CA, Theeuwes JW, Baak JP, Heimans JJ, Stoof JC and Peters GJ: The antiproliferative effect of 8-chloro-adenosine, an active metabolite of 8-chloro-cyclic adenosine monophosphate, and disturbances in nucleic acid synthesis and cell cycle kinetics. Biochem Pharmacol 53: 141-148, 1997.
2. Wang XT, Zheng DX and Liu R: 8-chloroadenosine induces apoptosis in human MOLT-4 cell line. China Sci Bull 42: 592-597, 1997.
3. Zhang HY, Gu YY, Li ZG, Jia YH, Yuan L, Li SY, An GS, Ni JH and Jia HT: Exposure of human lung cancer cells to 8-chloro-adenosine induces G2/M arrest and mitotic catastrophe. Neoplasia 6: 802-812, 2004.
4. Ahn Y, Jung JM and Hong SH: 8-Cl-cAMP and its metabolite, 8-Cl-adenosine induce growth inhibition in mouse fibroblast DT cells through the same pathways: protein kinase C activation and cyclin B down-regulation. J Cell Physiol 201: 277-285, 2004.
5. Lange-Carter CA, Vuillequez JJ and Malkinson AM: 8-Chloro-adenosine mediates 8-chloro-cyclic AMP-induced down-regulation of cyclic AMP-dependent protein kinase in normal and neoplastic mouse lung epithelial cells by a cyclic AMP-independent mechanism. Cancer Res 53: 393-400, 1993.
6. Park GH, Choe J, Choo HJ, Park YG, Sohn J and Kim MK: Genome-wide expression profiling of 8-chloroadenosine- and 8-cholro-cAMP-treated human neuroblastoma cells using radioactive human cDNA microarray. Exp Mol Med 34: 184-193, 2002.
7. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C and Smith CA: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673-682, 1995.
8. Gliniak B and Le T: Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity *in vivo* is enhanced by the chemotherapeutic agent CPT-11. Cancer Res 59: 6153-6158, 1999.
9. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD and Rehemtulla A: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci USA 97: 1754-1759, 2000.
10. Pan G, Rorke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J and Dixit VM: The receptor for the cytotoxic ligand TRAIL. Science 276: 111-113, 1997.
11. Screaton GR, Mongkolsapaya AM, Xu XN, Cowper AE, McMichael AJ and Bell JI: TRICK2: a new alternatively spliced receptor that transduces the cytotoxic signaling from TRAIL. Curr Biol 7: 693-696, 1997.
12. Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF, Goodwin RG and Smith CA: Cloning and characterization of TRAIL-R3: a novel member of the emerging TRAIL receptor family. J Exp Med 186: 1165-1170, 1997.
13. Marsters SA, Scheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P and Ashkenazi A: A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol 7: 1003-1006, 1997.

JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC and Young PR: Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem 273: 14363-14367, 1998.
15. Wajant J, Pfizenmaier K and Scheurich P: TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance. Apoptosis 7: 449-459, 2002.
16. Gura T: How TRAIL kills cancer cell, but normal cells. Science 277: 768, 1997.
17. Higuchi H, Bronk SF, Takikawa Y, Werneburg N, Takimoto R, El-Deiry W and Gores GJ: The bile acid glycochenodeoxycholate induces trail-receptor 2/DR5 expression and apoptosis. J Biol Chem 276: 38610-38618, 2001.
18. Sheikh MS, Burns TF, Huang Y, Wu GS, Amundson S, Brooks KS, Fornace AJ Jr and El-Deiry WS: p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. Cancer Res 58: 1593-1598, 1998.
19. Takimoto R and El-Deiry WS: Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. Oncogene 19: 1735-1743, 2000.
20. Meng RD and El-Deiry WS: p53-independent upregulation of KILL/DR5 TRAIL receptor expression by glucocorticoids and interferon-γ. Exp Cell Res 262: 154-169, 2001.
21. Wu GS, Kim K and El-Deiry WS: KILLER/DR5, a novel DNA-damage inducible death receptor gene, links the p53-tumor suppressor to caspase activation and apoptotic death. Adv Exp Med Biol 465: 143-151, 2000.
22. Wang MJ, Liu YX, Liu SL and Zheng DX: 8-chloro-adenosine sensitizes the human hepatoma cell line to TRAIL-induced apoptosis by caspase-dependent and -independent pathways. Oncol Rep 12: 193-199, 2004.
23. Zhang ZH, Cui JR and Zhang LH: Effect of 8-chloroadenosine on proliferation related signal proteins in gastric carcinoma cells. Chin J Pharmacol Toxicol 4: 46-49, 2005.
24. Shi J, Liu YX and Li XL: Expression and biological activity of rsTRAIL on willing cancer cells. China J Bioeng 23: 46-49, 2003.
25. Li YX: Isolation and purification of nucleic acid. In: Current Protocols for Molecular Biology. Lu SD (ed). Peking Union Medical College Press, Beijing, pp102-104, 1999.
26. Yoshida T, Maeda A, Tani N and Sakai T: Promoter structure and transcription initiation sites of the human death receptor 5/TRAIL-R2 gene. FEBS Lett 507: 381-385, 2001.
27. Lim JW, Kim H and Kim KH: Expression of Ku70 and Ku80 mediated by NF-κB and cyclooxygenase-2 is related to proliferation of human gastric cancer cells. J Biol Chem 277: 46093-46100, 2002.
28. Zhang JD, Liu N, Zhang JC, Liu SL, Liu YX and Zheng DX: PKCδ protects human breast tumor MCF-7 cells against tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis. J. Cell Biochem 96: 522-532, 2005.
29. Nye JA, Petersen JM, Gunther CV, Jonsen MD and Graves BJ: Interaction of murine Ets-1 with GGA-binding sites establishes the Ets domain as a new DNA-binding motif. Genes Dev 6: 975-990, 1992.
30. Nasrin N, Buggs C, Kong XF, Carnazza J, Goebl M and Alexander-Bridges M: DNA-binding properties of the product of the testis-determining gene and a related protein. Nature 354: 317-320, 1991.
31. Schlissel M, Voronova A and Baltimore D: Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. Genes Dev 5: 1367-1376, 1991.
32. Ravi R, Bedi GC, Engstrom LW, Zeng Q, Mookerjee B, Gelinas C, Fuchs EJ and Bedi A: Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. Nat Cell Biol 3: 409-416, 2001.
33. Bouwman P and Philipsen S: Regulation of the activity of Sp1-related transcription factors. Mol Cell Endocrinol 195: 27-38, 2002.
34. Black AR, Black JD and Azizkhan-Clifford J: Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. J Cell Physiol 188: 143-160, 2001.
35. Rafty LA and Khachigian LM: Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta. Nucleic Acids Res 29: 1027-1033, 2001.
36. Tanaka T, Kanai H, Sekiguchi K, Aihara Y, Yokoyama T, Arai M, Kanda T, Nagai R and Kurabayashi M: Induction of VEGF gene transcription by IL-1 beta is mediated through stress-activated MAP kinases and Sp1 sites in cardiac myocytes. J Mol Cell Cardiol 32: 1955-1967, 2000.
37. Haidweger E, Novy M and Rotheneder H: Modulation of Sp1 activity by a cyclin A/CDK complex. J Mol Biol 306: 201-212, 2001.
38. Higuchi H, Grambihler A, Canbay A, Bronk SF and Gores GJ: Bile acids upregulate death receptor 5/TRAIL receptor 2 expression via a c-Jun N-terminal kinase-dependent pathway involving Sp1. J Biol Chem 279: 51-60, 2004.
39. Kim Y, Park J, Lee J and Kwon TK: Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells. Carcinogenesis 25: 1-8, 2004.
40. Jackson SP and Tjian R: O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell 55: 125-133, 1988.
41. Jackson SP, MacDonald JJ, Lees-Miller S and Tjian R: GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell 63: 155-165, 1990.
42. Robbins SK, Houlbrook S, Priddle JD and Harris AL: 8-Cl-adenosine is an active metabolite of 8-Cl-cAMP responsible for its *in vitro* antiproliferative effects on CHO mutants hypersensitive to cytostatic drugs. Cancer Chemother Pharmacol 48: 451-458, 2001.
43. Baeuerle PA and Baltimore D: NF-κB: ten years after. Cell 87: 13-20, 1996.
44. Tomasetti M, Andera L, Alleva R, Borghi B, Neuzil J and Procopio A: Alpha-tocopheryl succinate induces DR4 and DR5 expression by a p53-dependent route: Implication for sensitisation of resistant cancer cells to TRAIL apoptosis. FEBS Lett 580: 1925-1931, 2006.
45. Choi SY, Kim MJ, Chung HY, Lee SJ and Jang YJ: Phytosphingosine in combination with TRAIL sensitizes cancer cells to TRAIL through synergistic up-regulation of DR4 and DR5. Oncol Rep 17: 175-184, 2007.
46. Yoshida T and Sakai T: Promoter of TRAIL-R2 gene. Vitam Horm 67: 35-49, 2004.
47. Shetty S, Gladden JB, Henson ES, Hu X, Villanueva J, Haney N and Gibson SB: Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) up-regulates death receptor 5 (DR5) mediated by NF-κB activation in epithelial derived cell lines. Apoptosis 7: 413-420, 2002.
48. Akiyama Y: TFSEARCH: Searching Transcription Factor Binding Sites. http://www.cbrc.jp/research/db/TFSEARCH.html
49. Wang MJ, Liu YX and Zheng DX: 8-Chloro-adenosine sensitizes a human hepatoma cell line to TRAIL-induced apoptosis by caspase-dependent and -independent pathways. Oncol Rep 12: 193-199, 2004.