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Silencing of galectin-3 changes the gene expression and augments the sensitivity of gastric cancer cells to chemotherapeutic agents

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Galectin-3 is known to modulate cell proliferation and apoptosis and is highly expressed in human cancers, but its function in gastric cancer is still controversial. Here, we examined the role of galectin-3 in gastric cancer cells by silencing it with synthetic double-stranded siRNA. After silencing of galectin-3, cell numbers decreased and cell shape changed. Galectin-3 siRNA treatment also induced G_1 arrest. DNA microarray analysis was used to assess changes in gene expression following galectin-3 silencing. We found that silencing of galectin-3 caused changes in gene expression. RT-PCR and real-time PCR were utilized for validation of the changes found in microarray studies. Western blot analysis confirmed changes in the expression of proteins of interest: cyclin D1, survivin, XIAP, XAF, PUMA, and GADD45α. Generally, it tended to increase the expression of several pro-apoptotic genes, and to decrease the expression of cell cycle progressive genes. We also confirmed that changes in the expression of these genes were caused by galectin-3 overexpression. Finally, we demonstrated that silencing of galectin-3 enhanced apoptosis induction with chemotherapeutic agents by further reducing the expression of anti-apoptotic and/or cell survival molecules such as survivin, cyclin D1, and XIAP, and increasing the expression of pro-apoptotic XAF-1. We conclude that galectin-3 is involved in cancer progression and malignancy by modulating the expression of several relevant genes, and inhibition of galectin-3 may be an approach to improve chemotherapy of gastric cancers. (*Cancer Sci* 2010; 101: 94–102)

Galectin-3 is a member of the carbohydrate-binding protein family, which are characterized by their affinity for β-galactosides.[1] It is the only chimera-type galectin, containing one CRD connected to an N-terminal proline- and glycine-rich domain. Galectin-3 is known to modulate a large number of cellular processes, especially inhibition of apoptosis and promotion of cell proliferation.[2,3] Galectin-3 contains an Asp-Trp-Gly-Arg (NWGR) motif in its C-terminal domain. The NWGR motif is also found in the BH-1 domain of Bcl-2 protein.[3] The NWGR motif in galectin-3 functions in the mitochondria, and exerts its anti-apoptotic activity by interacting with other apoptosis regulators and is thus crucial for its apoptotic function. Galectin-3 is also found in the nucleus as a nuclear matrix protein involved in pre-mRNA splicing, the Hedgehog or WNT signal-transduction pathway, mainly interacting with gemin4 and sufu.[4–7] These findings suggest that galectin-3 could be one of the essential factors for normal cell proliferation and/or development in the nucleus.

In previous studies, a high level of cellular expression of galectin-3 was detected in many cancer types, including gastric cancer.[8–11] For example, knocked-down galectin-3 in human prostate cancer PC3 cells showed G_1 phase arrest, p21 upregulation, and hypophosphorylation of Rb, without influence on cyclin D1 or p27 protein expression levels.[12] Overexpressed galectin-3 inhibited ROS generation by 4HPR to block inhibit apoptosis induction in breast BT549 cancer cells.[13] In addition, tumors in which galectin-3 was cleaved by MMP showed more aggressive tumor progression.[14]

In gastric cancer, galectin-3 was detected in both primary gastric cancer tissue and the metastatic lymph nodes.[15,16] In particular, strong nuclear immunoreactivity of galectin-3 was observed in cells of cancerous lesions, whereas adjacent epithelial cells showed little or weak nuclear immunoreactivity.[16] However, whether galectin-3 is involved in gastric cancer tumorigenesis is unclear and the role of galectin-3 in gastric cancer remains controversial.[17]

In order to define the role of galectin-3 in gastric cancer cells, we silenced galectin-3 in AGS cells with synthetic double-stranded siRNA, and using microarray analysis, attempted to map the changes in gene expression. We also examined how the silencing of galectin-3 influences classical chemotherapeutic approaches to gastric cancer.

Materials and Methods

Cell culture and siRNA transfection. The AGS and SNU638 human gastric cancer cell lines were cultured in RPMI-1640 medium (Gibco Invitrogen, Grand Island, NY, USA) containing 5% FBS (Gibco Invitrogen) and 1% antibiotic solution (Gibco Invitrogen) at 37°C in 5% CO_2. The cells were trypsinized with Trypsin/EDTA solution (Gibco Invitrogen), washed with PBS, and counted with a hemocytometer through the exclusion of trypan blue. Three types of galectin-3 siRNA duplex (type I, 5′-UCCAGACCCAGAUAACGCAUCAUGG-3′; type II, 5′-UAAAGUGGAAGGCAACAUCAUUCCC-3′; and type III, 5′-AUAUGAAGCACUGGUGAGGUCUAUG-3′) and a stealth RNAi as a negative control, were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). AGS cells were cultured in complete medium without antibiotic solution 1 day before transfection, and were then transfected with the oligonucleotide duplexes 100 nM (final concentration in a transfection mixture) premixed with RNAiMAX reagent (Invitrogen) in Opti-MEM for 20 min. The inhibition efficiency was determined by collecting cells after 48 h and analyzing the levels of galectin-3 mRNA and protein expression.

RNA preparation and Affymetrix genechip hybridization. Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Gene expression was analyzed in eight cell lines on a high-density oligonucleotide microarray (HG-U133 Plus 2.0; Affymetrix, Santa Clara, CA, USA) containing 54 675 transcripts. Target preparation and microarray processing procedures

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were carried out as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix). Briefly, the extracted total RNA was purified with an RNeasy kit (Qiagen, Valencia, CA, USA). Twenty μg of total RNA was used to synthesize cDNA with SuperScript II reverse transcriptase (Life Technologies, Inc. Rockville, MD) and a T7-(dT)24 primer (Metabion, Lena-christ, Planegg, Germany). The synthesized cDNA was purified with a DNA purification kit (Qiagen) and ethanol precipitated with 1 mL glycogen, 20 mL 7.5 M ammonium acetate, and 100 mL of 100% ethanol. Biotinylated cRNA was synthesized from the double-stranded cDNA using the GeneChip Expression 30-Amplification Reagents, and then purified and fragmented. The fragmented cRNA was quantified, and 10 mg of cRNA was hybridized to the oligonucleotide microarray, which was subsequently washed and stained with streptavidin–phycoerythrin. Scanning was carried out with a scanner.

Microarray data analysis. GeneChip analysis was carried out based on the Affymetrix GeneChip Manual with Microarray Analysis Suite 5.0, Data Mining Tool 2.0, and Microarray Database software (Santa Clara, CA, USA). All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 500. Fold changes were calculated by comparing transcripts between parents and acquired drug-resistant cell lines. The Microarray Analysis Suite software used the Wilcoxon's test to determine present or absent and increased or decreased calls, and used the calls to statistically determine whether a transcript was expressed or not, and whether it was relatively increased, decreased, or unchanged. A transcript was considered differentially expressed when transcripts increased or decreased greater than two-fold. The NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) allows the correlation of the microarray results with the specific array design and with annotation tools. The Gene Ontology Mining Tool, used in the NetAffx Analysis Center, matches GeneChip probe sets to annotated genes within the biological process, molecular function, or cellular component to allow for biological interpretation of microarray results.

Semiquantitative and quantitative RT-PCR analysis. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen). First-strand complementary DNA synthesis was carried out using the Reverse Transcription system (Promega, Madison, WI, USA). PCR was carried out using ExTaq polymerase (Takara, Otsu, Shiga, Japan). Quantitative RT-PCR was then carried out for 17 selected genes using ABI PRISM (Applied Biosystems) according to the manufacturer's instructions. cDNA was used as a template and it was detected with SYBR green I dye as an intercalating agent. Primer sequences are listed in Table 1.

Construction of the galectin-3 overexpression plasmid and transfection. To amplify the galectin-3 cDNA from human cells by PCR the following primer pair was used: 5′-ATGGC AGACAATTTTTCGCT-3′ and 5′-TTATATCATGGTATATG AAGCACTGGT-3′. The PCR products were digested with *Bst*X1 and *Eco*RV (New England BioLabs, Ipswich, MA, USA) and ligated into the *Bst*X1 and *Eco*RV sites of the pcDNA3.1/NT-GFP vector (Invitrogen) to create pcDNA3.1/NT-GFP-Gal3. SNU638 cells grown in six-well plates were transfected with 2 μg pcDNA3.1/NT-GFP-Gal3 using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h post-transfection and western blot analyses were carried out.

Western blot analysis. The cells were washed in PBS and lysed in RIPA buffer containing 10 mM NaF, 5 mM VO_4, and protease inhibitors (Sigma, St Louis, MO, USA). The cells were then incubated on ice and centrifuged. The supernatants were collected and protein concentration was determined using a quant-it protein assay kit (Invitrogen). Twenty μg of extracted proteins from AGS and SNU638 cells were separated on 10% or 12% SDS-polyacrylamide gels, and the gels were transferred to PVDF membranes (Amersham Life Science, Pittsburgh, PA, USA) by electroblotting. Membranes were probed with primary antibodies for the following proteins: galectin-3, XAF-1, and β-actin (from Santa Cruz Biotechnology) and cyclin D1, XIAP, PUMA, GADD45α, Caspase-3, PARP, and survivin (from Cell Signaling Biotechnology). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulins purchased from Amersham were used as secondary antibodies and signals were detected using an enhanced chemiluminescence (ECL) plus kit (Amersham Life Science) according to the manufacturer's directions.

MTT assay. Inhibition of cell proliferation by siRNA of galectin-3 was measured by MTT assay. The cells were plated in 96-well culture plates (3×10^3 per well). After incubation for 24 h, the cells were treated with galectin-3 siRNA (0, 1, 5, 10, and 20 nM) for 24, 48, and 72 h. AGS cells were treated with cisplatin and doxorubicin after treatment of galectin-3 siRNA for 24 h. MTT solution (Sigma) was subsequently added to each well. After 4 h of additional incubation, the MTT solution was discarded and 200 μL of DMSO (Amresco, Solon, OH, USA) added and the plate was shaken gently. The absorbance was measured on an ELISA reader at a test wavelength of 570 nm.

Flow cytometric analysis. The cells were plated in 6-cm culture plates and treated with galectin-3 siRNA (10 nM). The cells were harvested after trypsinization and fixed in ice-cold 70% ethanol for at least 2 h. Cell pellets were washed twice with cold PBS and incubated for 30 min at room temperature in 1 mL PBS containing 50 μg PI (Sigma) and 50 μg RNaseA (Sigma). After staining, samples were analyzed with a FACScan (BD Biosciences, San Jose, CA, USA) of 10 000 events per sample. Data from flow cytometry were analyzed using Cell Quest software (BD Bioscience, San Jose, CA, USA).

Assessment of apoptosis. Quantitative assessment of apoptosis was carried out using an Annexin V assay kit (BD Biosciences PharMingen, San Jose, CA, USA). Briefly, cells grown in 10-cm Petri dishes were treated with a trypsin/EDTA solution and washed in PBS. Cells were then resuspended in binding buffer, and stained with Annexin V–FITC and PI at room temperature for 15 min in the dark. Cells were then analyzed in a FACScan within 1 h after staining. Data from 10 000 cells were collected for each data file and analyzed using Cell Quest software. Annexin V-positive, PI-negative cells were defined as apoptotic cells.

Results

Silencing of galectin-3 by its siRNA in human gastric cancer AGS cells. AGS cells were transfected with three different kinds of siRNA and harvested after 48 h for detection of galectin-3 mRNA and protein levels (Fig. 1A,B). All three siRNA treatments completely diminished the expression levels of both galectin-3 mRNA and protein. The inhibitory role on cell growth of silencing of galectin-3 in human gastric cancer cells was assessed by MTT assay. All three kinds of siRNA reduced AGS cell proliferation in a dose-dependent manner and changed the shape of cells to round (Fig 1C). Representative data are shown in Figure 1(D).

Cell cycle distribution was also detected by PI staining (Fig. 2). The G_1 population of cells increased up to 77% in siRNA-treated cells compared with control or untreated cells. These findings indicate that the expression of galectin-3 in gastric cancer increases gastric cancer cell proliferation.

DNA microarray analysis. To demonstrate the effect of galectin-3 on gene expression, affymetrix DNA microarray was carried out in galectin-3-silenced cells after siRNA treatment. The affected genes are listed according to their function in Tables 2 and 3. In general, the expression of apoptosis induction-related genes, such as those related to the TNF family, interferon-β, PHLDA1, PDCD4, and caspase recruitment domain 6, were

Table 1. The primer sequences used in this study

Gene name	Sequence	Gene name	Sequence	Gene name	Sequence	Gene name	Sequence
LGALS-3	5′-TAA TAA CTG GGG AAG GGA AG-3′ 5′-AGC ACT GGT GAG GTC TAT GT-3′	TNFRSF19	5′-CTA TGG GGA GGA TGC ACA GT-3′ 5′-CAT GTC TTG AAA GCC GAC AA-3′	ID3	5′-TCC AAC GAC AAA AGG AGC TT-3′ 5′-GCT CCT GAG CAC CAG GTT TA-3′	CCNA2	5′-GCA CCC CTT AAG GAT CTT CC-3′ 5′-ATG GTT TTC TGG GTC CAG GT-3′
TNFSF10	5′-GGA ACC CAA GGT GGG TAG AT-3′ 5′-TCT CAC CAC ACT GCA ACC TC-3′	CDKN2B	5′-CGC CCA CAA CGA CTT TAT TT-3′ 5′-TTC GCT TCA TGG TGA GTG TC-3′	EGFR	5′-GCA CGA GTA ACA AGC TCA CG-3′ 5′-GGA ATT CGC TCC ACT GTG TT-3′	BCCIP	5′-GGA TGA AGA GGA CGA GGT CA-3′ 5′-TCT TCT GAA ACA TCC GTT TGC-3′
IFNB1	5′-AAT TGA ATG GGA GGC TTG AA-3′ 5′-AGC CAG GAG GTT CTC AAC AA-3′	ANXA9	5′-GAG CAA AGG CAG CTC ATC TC-3′ 5′-AGA ATT TCA ATG GCC ACG TC-3′	CDC25C	5′-GGC ACC TGA TTG GTG ATT TT-3′ 5′-TTT AAG GCT CCC TGG ATG TG-3′	PAK3	5′-AGC TTT TGC AGC ATC CAT TT-3′ 5′-CCA CCC CAT TTG ACT GTC TT-3′
CDC42	5′-TTC TTG CTT GTT GGG ACT CA-3′ 5′-CAG CCA ATA TTG CTT CGT CA-3′	PHLDA1	5′-GGG ACA AAG GAA GGA AGG AC-3′ 5′-CCC ATA GAG CAC ACC CAG AT-3′	STS-1	5′-CAA GTG TGC CAC ATC AAT CC-3′ 5′-TGG GGT GAA GCA ATA AGC TC-3′	BCL2L12	5′-TCT TCC GCG TTA CCT ACG AT-3′ 5′-TCG GCT TTT GGG TCT GTT AC-3′
BIRC4BP	5′-ATC ATG TCC TGT GCA GCA AC-3′ 5′-GAT CCT CAC CCT CCT TCC AT-3′	MMP7	5′-CAG ATG TGG AGT GCC AGA TG-3′ 5′-TGT CAG CAG TTC CCC ATA CA-3′	PIM2	5′-GTT AAG CCA AGG GCA ATG AA-3′ 5′-TTT TTG GCT CAG CTT TCA CA-3′	BNIP2	5′-AGC TCG AAA TTC AGC CAA AA-3′ 5′-CAC TGC TCC ATC AGC ACA TT-3′
CTSS	5′-GAA ACA AAG GCT GCA ATG GT-3′ 5′-GCC ACA GCT TCT TTC AGG AC-3′	PLAUR	5′-CTG GAG CTG GTG GAG AAA AG-3′ 5′-CAT GTC TGA TGA GCC ACA GG-3′	DLG7	5′-AAG AAG CTG ACC CAG TCC AA-3′ 5′-GAA TCC TTT GCG GTC ACA AT-3′	CCND1	5′-AAC TAC CTG GAC CGC TTC CT-3′ 5′-CCA CTT GAG CTT GTT CAC CA-3′
SOX13	5′-AGT ATG GGA ACC CCA AAT CC-3′ 5′-CCC CGA GTG TAA ACA GGA GA-3′	PDCD4	5′-GAA AAT GCT GGG ACT GAG GA-3′ 5′-TGG ACT GGT TGG CAC AGT TA-3′	IGFBP5	5′-CTT CCG TGA CAG CAA GTT GA-3′ 5′-TCC TTG ATG AAC GAG TGC AG-3′	GPX2	5′-CAA GCG CCT CCT TAA AGT TG-3′ 5′-GAG GGT TGG GAG AGG AAA AG-3′
IFI16	5′-ACC TTC CCT GAG AGC CAT CT-3′ 5′-ATC TGA GGA GTG TGG GGA TG-3′	CCND3	5′-AGA CCT TTT TGG CCC TCT GT-3′ 5′-AGC TTC GAT CTG CTC CTG AC-3′	KIF20A	5′-AAG AGA TCC CGG AGG TCC TA-3′ 5′-GCT GCG GTG AAT GGA TAT TT-3′	TFDP1	5′-GAG TTC ATT TTT GCC CCT GA-3′ 5′-TTG GAC ACC GTA CCA CTG AA-3′
TXNRD3	5′-GGC CTG CTG CTG TTT AGT TC-3′ 5′-AGC AAG ACC ACA AGG CAG AT-3′	CARD6	5′-CCT CAC CCT CAG TCC TTT CA-3′ 5′-AGG GTT TAG GCT GGG AGT GT-3′	PLK1	5′-TAA TGC CGC ACT CTA TGC AC-3′ 5′-GCG TGT TCT GGT CCT CAA AT-3′	BIRC5	5′-TTT TCA TCG TCG TCC CTA GC-3′ 5′-CGA CTC AGA TGT GGC AGA AA-3′
MAP1B	5′-GAA GGA AAC TGA GCC AGT CG-3′ 5′-AAA ACC GGC TCC TTC ATC TT-3′	HIST1H1B	5′-AGA AAT CCC CGG CTA AGA AG-3′ 5′-GCG GCT GTT ATT CTT CTC CA-3′	CSE1L	5′-GGG TTC CTC TGC AGA CAT TC-3′ 5′-TGT AAT GGG GAG ACC AGA GG-3′	HDAC4	5′-TGA AGA ATG GCT TTG CTG TG-3′ 5′-ACG CTA GGG TCG CTG TAG AA-3′
TGFBI	5′-TGC GAC TAG CCC CTG TCT AT-3′ 5′-CAT GCA CAA GGC TCA CAT CT-3′	RGS5	5′-AGC CAA GAC CCA GAA AAC CT-3′ 5′-TTT GCC TTC TCA GCC ATC TT-3′	CDC20	5′-AGG ACA AGC GCT ATG GAG AA-3′ 5′-CTG CCT CTG TCT TCC TGG AG-3′	BAG1	5′-CCC TCT AGC TCC CAC TTC CT-3′ 5′-GAC AGC AGA CAG CCA ACA AA-3′
HSPB8	5′-GCA AGA AGG TGG CAT TGT TT-3′ 5′-ACA GGT GAC TTC CTG GCT GT-3′	MMP1	5′-GAT GTG GAG TGC CTG ATG TG-3′ 5′-TGC TTG ACC CTC AGA GAC CT-3′	CCNF	5′-TCA ACC CAC CAA AAC AAC AA-3′ 5′-AGG GTT CTC CCA ATC TTC GT-3′	b-*actin*	5′-AGC CTC GCC TTT GCC GA-3′ 5′-CTG GTG CCT GGG GCG-3′
CFLAR	5′-CTA AAT TTG GTT GCC CCA GA-3′ 5′-CTC CCA TTA TGG AGC CTG AA-3′	HSPD1	5′-CAC AGT CTT TCG CCA GAT GA-3′ 5′-ACT TCC CCA ACT CTG CTC AA-3′	CCNB2	5′-CTA AAT TTG GTT GCC CCA GA-3′ 5′-CTC CCA TTA TGG AGC CTG AA-3′	GAPDH	5′-GGC TGC TTT TAA CTC TGG TA-3′ 5′-CTC CCA TTA TGG AGC CTG AA-3′

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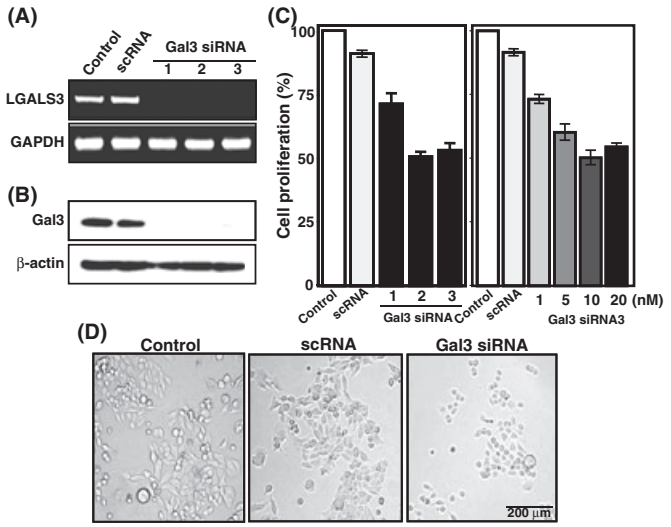

Fig. 1. Effect of galectin-3 silencing on cell proliferation and morphology in AGS cells. (A) Gene and (B) protein expression levels of galectin-3 in AGS cells after treatment with scramble siRNA (scRNA) and three kinds of galectin-3 siRNA (Gal3 siRNA). AGS cells treated with scRNA or galectin-3 siRNA were harvested after 48 h and then these levels were detected by RT-PCR and western blotting. GAPDH and β-actin were used as loading controls. (C) Cell proliferation was detected in AGS cells transfected with scRNA or three kinds of galectin-3 siRNA by MTT assay. (D) Morphological changes were detected by microscopy after AGS cell transfection with siRNA. Scale bar = 200 μm. LGALS, The gene of galetin-3.

increased significantly in galectin-3-silenced cells. Interestingly, proteolysis-related genes, such as ubiquitin D and cathepsin S, were upregulated (Table 2). On the other hand, apoptosis inhibitor, Bcl-2 family genes decreased in galectin-3-silenced cells. A large number of cell cycle progression-related genes, such as cdc25, DLG7, PLK1, and cyclin D1, were downregulated, and protein amino acid phosphorylation-related proteins, such as

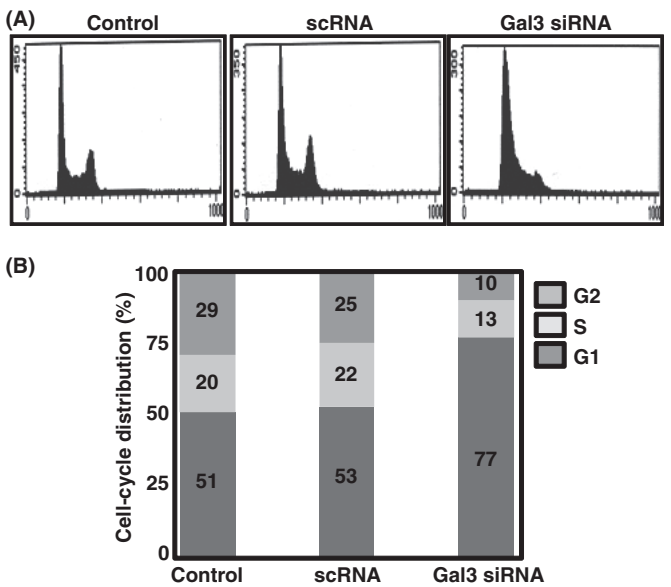

Fig. 2. Effect of galectin-3 silencing on cell cycle distribution in AGS cells. Cell cycle distribution was analyzed and presented as (A) diagrams and (B) a graph. After transfection of galectin-3 siRNA, AGS cells were harvested, fixed with 70%, and then stained with propidium iodide. scRNA, scramble siRNA.

Table 2. Up-regulated genes by galectin-3 silencing in AGS cells

Gene symbol	Accession number	Gene name	Fold change (2^n)
Apoptosis			
TNFSF10	NM_003810	Tumor necrosis factor (ligand) superfamily, member 10	5.14
IFNB1	NM_002176	Interferon, beta 1, fibroblast	4.76
PHLDA1	NM_007350	Pleckstrin homology-like domain, family A, member 1	2.53
PDCD4	NM_014456	Programmed cell death 4 (neoplastic transformation inhibitor)	1.80
CARD6	NM_032587	Caspase recruitment domain family, member 6	1.48
TNFRSF19	NM_018647	Tumor necrosis factor receptor superfamily, member 19	1.11
Cell cycle			
CDC42	NM_001039802	Cell division cycle 42 (GTP binding protein, 25kDa)	3.58
CCND3	NM_001760	Cyclin D3	1.58
CDKN2B	NM_004936	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.03
Immune response			
GBP1	NM_002053	Guanylate binding protein 1, interferon-inducible, 67kDa	5.61
IL26	NM_018402	Interleukin 26	5.47
Metabolism			
MMP7	NM_002423	Matrix metallopeptidase 7 (matrilysin, uterine)	2.23
Proteolysis			
UBD	NM_006398	Ubiquitin D	5.00
CTSS	NM_004079	Cathepsin S	3.14
CFLAR	NM_003879	CASP8 and FADD-like apoptosis regulator	1.25
Transcription			
IFI16	NM_005531	Interferon, gamma-inducible protein 16	2.84
SOX13	NM_005686	SRY (sex determining region Y)-box 13	2.96
Cell adhesion			
TGFBI	NM_000358	Transforming growth factor, beta-induced, 68kDa	2.77
ANXA9	NM_003568	Annexin A9	2.46
Protein folding			
HSPB8	NM_014365	Heat shock 22 kDa protein 8	2.71
Cell motility			
PLAUR	NM_002659	Plasminogen activator, urokinase receptor	2.21
Microtubule bundle formation			
MAP1B	NM_005909	Microtubule-associated protein 1B	2.79

Table 3. Down-regulated genes by galectin-3 silencing in AGS cells

Gene symbol	Accession number	Gene name	Fold change (2^n)
Apoptosis			
CSE1L	NM_001316	CSE1 chromosome segregation 1-like (yeast)	−1.33
BCL2L12	NM_001040668	BCL2-like 12 (proline rich)	−1.00
BNIP2	NM_004330	BCL2/adenovirus E1B 19kDa interacting protein 2	−1.00
Cell cycle			
CDC25C	NM_001790	Cell division cycle 25 homolog C (S. cerevisiae)	−2.00
SKP2	NM_005983	S-phase kinase-associated protein 2 (p45)	−1.84
DLG7	NM_014750	Discs, large homolog 7 (Drosophila)	−1.84
PLK1	NM_005030	Polo-like kinase 1 (Drosophila)	−1.76
BIRC5	NM_001168	Baculoviral IAP repeat-containing 5 (survivin)	−1.42
CCNF	NM_001761	Cyclin F	−1.30
CDC20	NM_001255	Cell division cycle 20 homolog (*S. cerevisiae*)	−1.30
CCNB2	NM_004701	Cyclin B2	−1.28
CCNA2	NM_001237	Cyclin A2	−1.26
BCCIP	NM_016567	BRCA2 and CDKN1A interacting protein	−1.15
CCND1	NM_053056	Cyclin D1	−0.99
TFDP1	NM_007111	Transcription factor Dp-1	−0.96
Protein amino acid phosphorylation			
PIM2	NM_006875	pim-2 oncogene	−.88
PAK3	NM_002578	p21 (CDKN1A)-activated kinase 3	−1.04
Protein folding			
HSPD1	NM_002156	Heat shock 60 kDa protein 1 (chaperonin)	−2.31
BAG1	NM_004323	BCL2-associated athanogene	−1.37
Metabolism			
MMP1	NM_002421	Matrix metallopeptidase 1 (interstitial collagenase)	−2.33
STS-1	NM_032873	Cbl-interacting protein Sts-1	−1.96
Transcription			
ID3	NM_002167	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	−2.19
HDAC4	NM_006037	Histone deacetylase 4	−1.39
Cell morphogenesis			
EGFR	NM_005228	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	−2.12
Regulation of cell growth			
IGFBP5	NM_000599	Insulin-like growth factor binding protein 5	−1.77
Response to oxidative stress			
GPX2	NM_002083	Glutathione peroxidase 2 (gastrointestinal)	−1.76
Nucleosome assembly			
HIST1H1B	NM_005322	Histone cluster 1, H1b	−3.09
Microtubule-based movement			
KIF20A	NM_005733	Kinesin family member 20A	−1.77
Signaling pathway			
RGS5	NM_003617	Regulator of G-protein signalling 5	−2.87

pim-2 oncogene and p21-activated kinase 3, were also reduced by galectin-3 silencing (Table 3).

Of the above genes, we choose 19 upregulated and 28 downregulated genes for a secondary validation by RT-PCR (Fig. 3). We also validated the microarray data on gene expression by real time RT-PCR (Fig. 4). Ten downregulated genes (PIM2, DLG7, KIF20A, PLK1, GPX2, BIRC5, BAG1, BCCIP, BNIP2, and TFDP1) and seven upregulated genes (PDCD4, CDKN2B, TNFRSF19, CFLAR, BIRC4BP, IFI16, and PHLDA1) were selected from the RT-PCR results for the real-time PCR. The expression patterns of these genes in RT-PCR and real-time RT-PCR results corresponded to those from the microarray results (Fig. 4).

Effect of silencing galectin-3 by its siRNA on the protein expression level, cell cycle progression, and apoptosis in AGS cells. To confirm the changes protein in expression genes, we determined the protein levels of the cell cycle progression-related and apoptosis-related genes cyclin D1, survivin, XIAP, XAF-1, PUMA, and GADD45α by western blot analysis (Fig. 5). Cyclin D1 is well known to be regulated by galectin-3, and its protein level decreased after galectin-3 silencing. Survivin and XIAP are members of the IAP family, which are negative regulators of apoptosis that function by inhibiting the executioners of cell death (caspases), or by blocking the pathways that activate them.[18] Interestingly, downregulation of galectin-3 diminished the protein level of survivin and XIAP. XAF-1 is a unique control protein of IAP function,[18,19] and its protein expression was upregulated by galectin-3 silencing. PUMA and GADD45α are known to be regulated by p53 and/or DNA damage;[20,21] however, knock down of galectin-3 also increased their protein expression levels in gastric cancer cells (Fig. 5A).

Effect of galectin-3 overexpression on the protein expression level of cell cycle progression-related and apoptosis-related genes in AGS cells. We found that overexpression of galectin-3 also changed the level of protein expression, which was detected by galectin-3 silencing (Fig. 5B). The upregulation of cyclin D1, survivin, and XIAP protein level of by transfection of the galectin-3 construct was confirmed by western blotting. The level of XAF-1 protein was not decreased after transfection of the galectin-3 construct. These results suggest that galectin-3 regulates the expression level of cell cycle progression-related and/or apoptosis-related proteins.

Effect of galectin-3 silencing by its siRNA on the therapeutic effect of cisplatin or doxorubicin in AGS cells. We also found that galectin-3 has effects on chemotherapy by changing the expression level of cell cycle progression-related and/or apoptosis-related proteins. AGS cells were transfected with siRNA for 24 h and then treated with cisplatin and doxorubicin. After 48 h, cell proliferation was determined by MTT assay (Fig. 6A,B). Treatment with cisplatin alone or cisplatin and scrambled RNA had little effect on cell growth inhibition up to a concentration of 100 ng/mL. Interestingly, the combination treatment of cisplatin and galectin-3 siRNA significantly augmented the growth inhibition of gastric cancer cells with galectin-3 siRNA in a dose-dependent manner, whereas treatment with 2 nM galectin-3 siRNA alone had little effect on cell growth inhibition (Fig. 6A). The combination treatment with doxorubicin and galectin-3 siRNA also had a similar effect on the inhibition of cell survival. Compared to doxorubicin treatment alone or scrambled RNA treatment, these combinations augmented the growth inhibition of gastric cancer cells in a dose-dependent manner (Fig. 6B).

Apoptosis induction was demonstrated by the combined treatment with cisplatin and galectin-3 siRNA (Fig. 6C). The treatment of cisplatin at 25 ng/mL or scrambled RNA alone did not induce apoptosis, and 5 nM of galectin-3 siRNA alone induced 47.2% of apoptosis. However, the combination treatment with cisplatin and galectin-3 siRNA increased apoptosis

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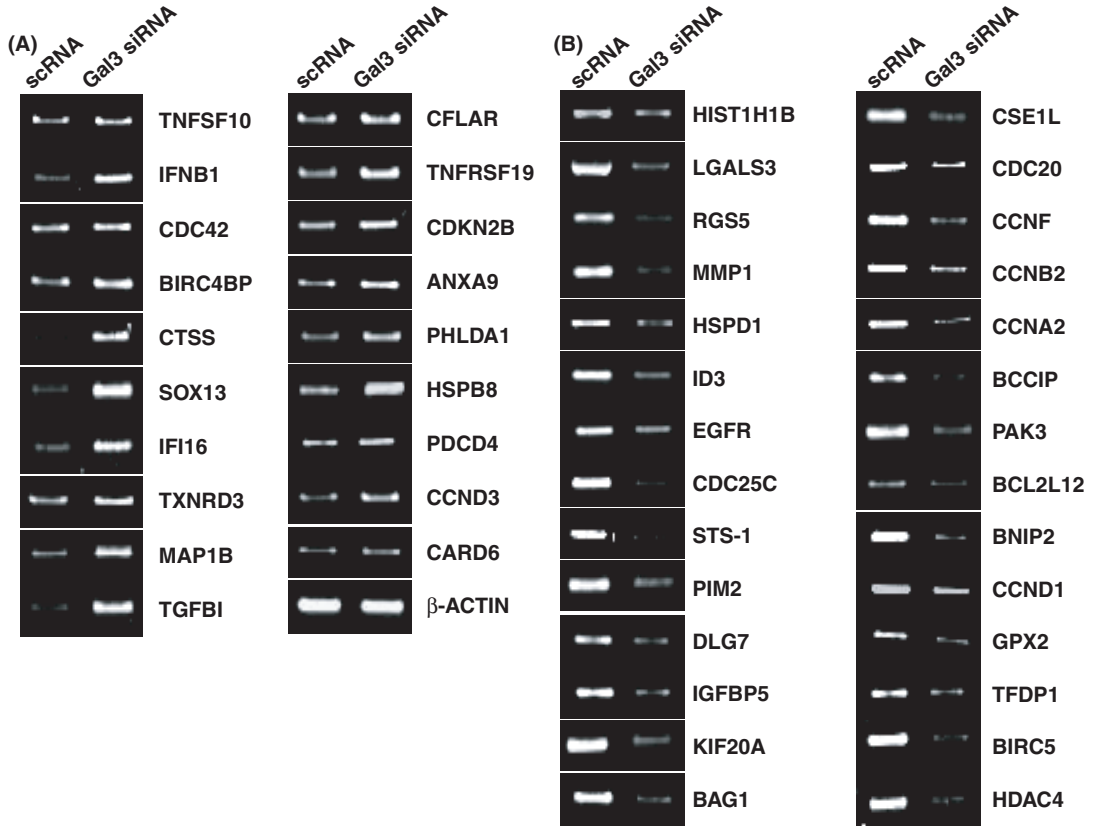

Fig. 3. Effect of galectin-3 silencing on gene expression detected by RT-PCR in AGS cells. After galectin-3 silencing, (A) 19 upregulated and (B) 28 downregulated genes were selected from the microarray and analyzed by RT-PCR. β-Actin was used as a loading control. scRNA, scramble siRNA.

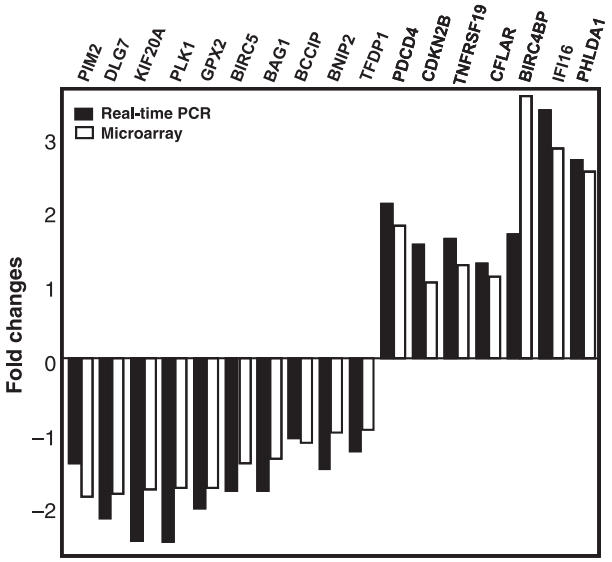

Fig. 4. Effect of galecin-3 silencing on gene expression detected by real time RT-PCR in AGS cells. The results of microarray and selected RT-PCR data were confirmed by real time RT-PCR. β-Actin was used as a loading control.

induction (up to 78%) significantly more than the additional effect. We also determined the expression level of apoptosis-related proteins after the combination treatment (Fig. 7). We detected downregulation of galectin-3 with galectin-3 siRNA treatment. The expression levels of cyclin D1 and XIAP

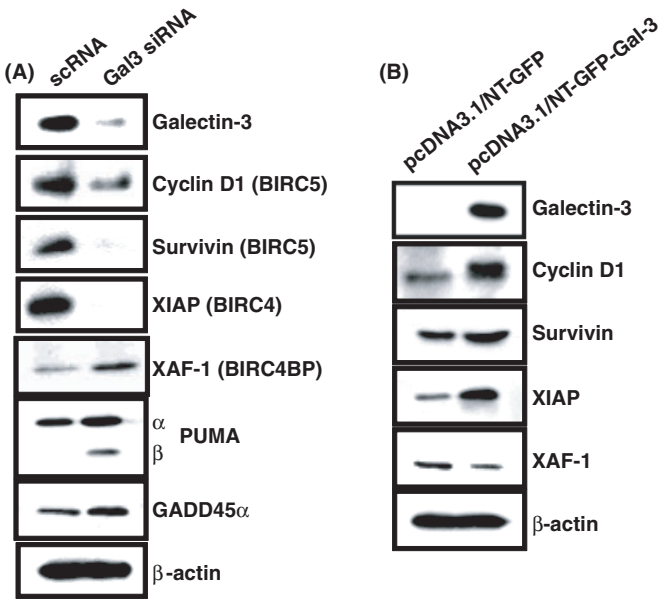

Fig. 5. Effects of galectin-3 silencing or overexpression on protein expression levels in AGS cells. (A) Protein expression levels after galectin-3 silencing were detected by western blotting. AGS cells were transfected with galectin-3 siRNA and harvested 48 h later. Total protein was isolated and then detected with antibodies. (B) Protein expression levels after overexpression of galetin-3 were detected by western blotting. SNU638 cells were transfected with pcDNA3.1-GFP-Galectin3 and harvested after 48 h. Total protein was prepared and detected with antibodies. β-Actin was used as a loading control. scRNA, scramble siRNA.

decreased with galectin-3 downregulation, and this decrease was augmented by the combination of galectin-3 siRNA and cisplatin treatment. The expression of survivin also decreased with galectin-3 downregulation, and cisplatin treatment alone slightly increased the expression. However, this increasing expression of survivin with cisplatin was diminished by galectin-3 siRNA treatment. The expression of XAF-1 increased with galectin-3 downregulation, and this increase was enhanced by combination treatment. The cleaved forms of apoptosis executive protein procaspase-3 and PARP were also clearly detected after the combination treatment.

Discussion

Galectin-3 is involved in multiple cellular processing steps. Among them, anti-apoptotic activity in response to various apoptotic stimuli, such as chemotherapeutic agents, on cells is accepted as a major galectin-3 function.[22,23] Overexpression of galectin-3 is known to induce resistance to chemotherapy.[24] For example, it was found that galectin-3-transfected cells were more resistant to apoptosis induced by anti-Fas antibodies or staurosporine compared to the non-transfected control cells.[3] Galectin-3-overexpressing BT549 cells were more resistant to apoptosis induced by cisplatin, nitric oxide, and anoikis than NWGR motif-mutated galectin-3-transfected or galectin-3 low-expressing control cells.[3,13,25] However, the molecular mechanisms by which galectin-3 can inhibit apoptosis induced by different stimuli remains to be elucidated.

In the present study, we carried out microarray and assessed the changes in gene expression after galectin-3 knock down in gastric cancer cells. We were interested in changes in the expression of cell survival-related and/or apoptosis-related genes. It was determined that the protein levels of these genes, such as cyclin D1, survivin, and XIAP, were reduced and those of XAF-1, PUMA, and GADD45α were increased. We also found that the regulation of protein expression by galectin-3 affected the sensitivity of cells to chemotherapy. Galectin-3 siRNA treatment with cisplatin or doxorubicin significantly reduced

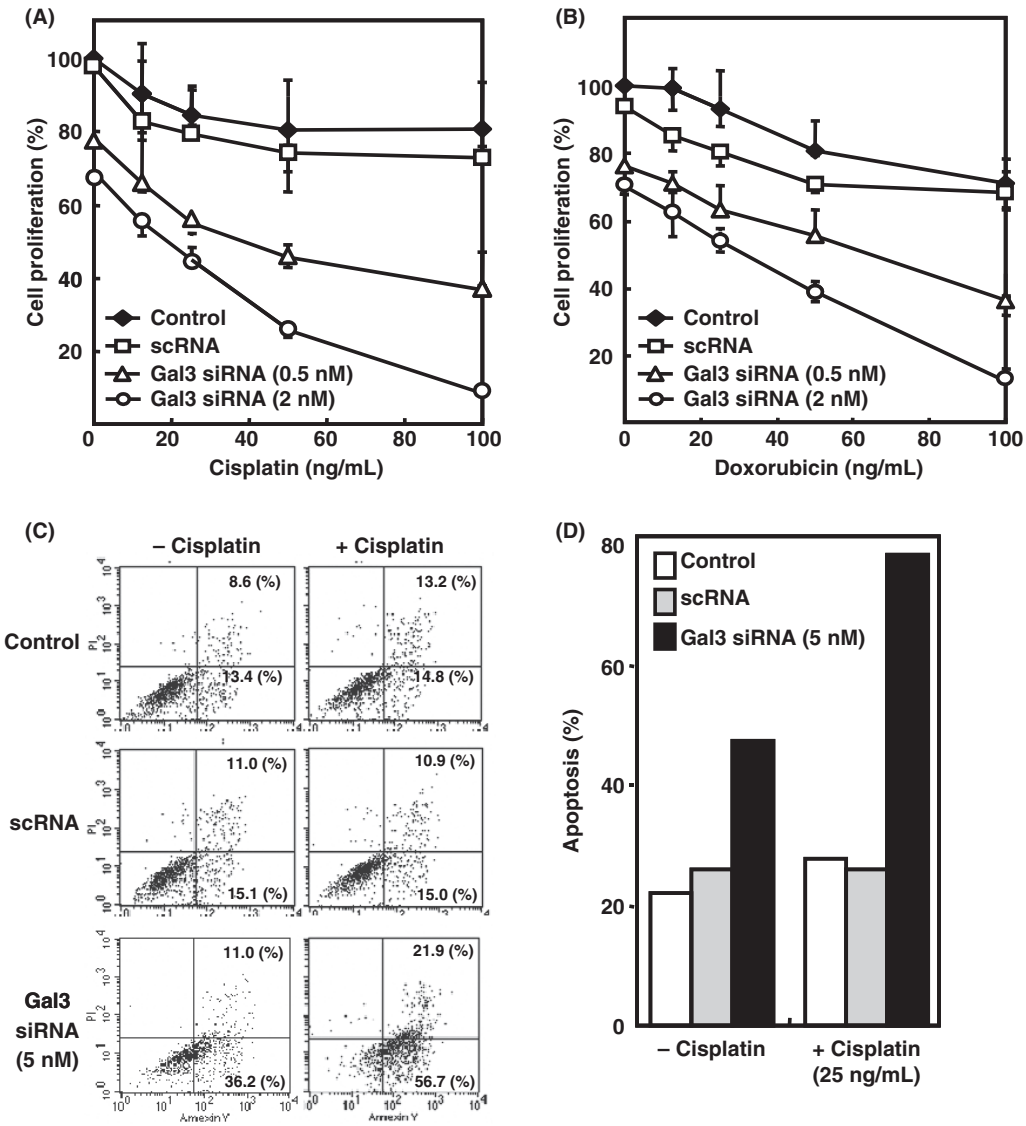

Fig. 6. Effects of combination treatment with galectin-3 siRNA and cisplatin or doxorubicin on cell proliferation and apoptosis. AGS cells were transfected with galectin-3 siRNA and then treated with or without (A) cisplatin or (B) doxorubicin. Cell proliferation was detected by MTT assay. (C) Apoptosis induction by combination treatment with galectin-3 siRNA and cisplatin was dectected with Annexin IV.(D) The percentage of apoptosis induction was calculated as the sum of the percentages in the right lower panel (early apoptosis) and right upper panel (late apoptosis). scRNA, scramble siRNA.

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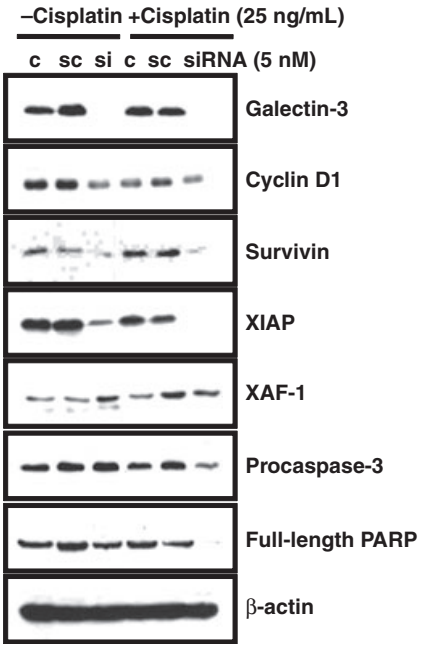

Fig. 7. Effect of combination treatment with galectin-3 siRNA and cisplatin on protein expression in AGS cells. Expression levels of apoptosis-related proteins were detected by western blotting. AGS cells were transfected with galectin-3 siRNA and then treated with or without cisplatin. Total protein was prepared and detected with antibodies. β-Actin was used as a loading control. C, control; Sc, scramble RNA; Si, siRNA.

the survival of gastric cancer cells compared with cisplatin or doxorubicin treatment alone. The protein levels of cyclin D1, survivin, and XIAP were decreased and the XAF-1 level was increased more with this combination treatment. The levels of apoptosis executive proteins, caspase-3, and PARP were enhanced after combination treatment. Previously, it has been demonstrated that galectin-3 regulates apoptosis induction in the cytosol.[3,13,26] Galectin-3 contains the NWGR motif in the C-terminal domain, which has been designated as the anti-death motif and shown to provide the anti-apoptotic function of galectin-3, but it cannot completely explain how galectin-3 shows or influences anti-apoptotic activity or the promotion of cell proliferation.

This is the first study to show that galectin-3 regulates the expression of IAP family members. They have a BIR protein domain and are known to regulate apoptosis and cell survival signal transduction through direct interaction of their BIR domains, or through their ubiquitin ligase activity of the RING domain.[18,27,28] Among these genes, we determined the expression levels of survivin, XIAP, and XAF-1. Survivin and XIAP are highly expressed in many adult malignances and their expression levels correlate with aggressiveness and poor clinical diagnosis in many types of cancers, including human gastric cancer.[28–30] For example, survivin and XIAP showed inhibition of caspase-3 through binding of their BIR domains and blocking of the active binding site of caspase-3.[31,32] We found that XAF-1, which is an interferon-inducible IAP antagonist,[19,33] was upregulated by galectin-3 silencing. XAF-1 binds directly to XIAP and antagonizes XIAP-mediated caspase-3 inhibition. It also reverses the sensitivity of XIAP-mediated chemotherapeutic resistance against cisplatin and etoposide. Moreover, there are several reports showing that the upregulation of survivin and XIAP mediates the increase in resistance to chemotherapy and radiation.[34–36] Specially, there are convincing data that the expression of survivin and/or XIAP correlates with chemotherapeutic sensitivity in human gastric cancer cells.[37,38]

Therefore, we studied whether galectin-3, as a regulator of survivin, XIAP, and XAF-1, can be a critical target for enhancing chemotherapeutic effects in gastric cancer. However, we still have a question of how galectin-3 regulates the expression level of these genes. One possible mechanism is that galectin-3 binds transcription factors directly and translocates into the nucleus, and regulates the expression of these genes. We detected the downregulation of cyclin D1 with galectin-3 silencing. There are previous reports showing that galectin-3 upregulates the expression of cyclin D1 by translocation into the nucleus.[39,40] Furthermore, galectin-3 directly interacts with β-catenin/TCF-4, which regulates the expression of cyclin D1.[7,41] This strongly suggests that galectin-3 regulates the expression of cyclin D1 by direct interaction with β-catenin/TCF-4. However, the regulation of cyclin D1 expression is detected in prostate cancer,[12] suggesting that it is cell type of organ type dependent manner.

Taken together, our study demonstrates that galectin-3 is involved in cancer progression, malignancy, and chemotherapeutic resistance by modulating the expression of apoptosis-related and/or cell survival-related genes such as cyclin D1, survivin, XIAP, and XAF-1. Therefore, inhibition of galectin-3 enhances the current chemotherapeutic approaches in gastric cancers. Further studies to test this hypothesis should help to advance the development of improved gastric cancer therapies.

Acknowledgments

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Disclosure Statement

No conflicts of interest exist for any of the authors.

Abbreviations

BAG1	BCL2-associated athanogene
BCCIP	BRCA2 and CDKN1A interacting protein
BIR	baculovirus inhibitor of apoptosis protein repeat
BIRC	Baculoviral IAP repeat containing protein
cdc	cell division cycle
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CFLAR	CASP8 and FADD-like apoptosis regulator
CRD	Carbohydrate recognition domain
DLG	discs, large homolog
GADD	growth arrest and DNA-damage-inducible
GPX2	Glutathione peroxidase 2
HPR	Haptoglobin-related protein
IAP	inhibitor of apoptosis protein
IFI16	Interferon, gamma-inducible protein 16
KIF20A	Kinesin family member 20A
PARP	Poly ADP ribose polymerase
PDCD	programmed cell death
PHLDA	pleckstrin homolog-like domain A
PI	propidium iodide
PIM	pim oncogene
PLK	polo-like kinase
PUMA	p53-upregulated modulator of apoptosis
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
TCF	Transcription factor
TFDP1	Transcription factor Dp-1
TNF	Tumor-necrosis factor
TNFRSF	Tumor necrosis factor receptor superfamily
XAF	X-linked inhibitor of apoptosis protein

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