Journal of Pharmacopuncture

ISSN 2093-6966(Print), **ISSN 2234-6856**(Online)
Journal of Pharmacopuncture 2012;15(3):019-030
DOI: http://dx.doi.org/10.3831/KPI.2012.15.002

Original Article

Galectin-3-independent Down-regulation of GABABR1 due to Treatment with Korean Herbal Extract HAD-B Reduces Proliferation of Human Colon Cancer Cells

Kyung-Hee Kim[1,3], Yong-Kyun Kwon[2], Chong-Kwan Cho[2], Yeon-Weol Lee[2], So-Hyun Lee[1], Sang-Geun Jang[1,3], Byong-Chul Yoo[1], Hwa-Seong Yoo[2]*

[1] Research Institute, National Cancer Center, Goyang, Korea
[2] East-West Cancer Center, Dunsan Oriental Hospital of Daejeon University, Daejeon, Korea
[3] Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea

Key Words

HAD-B; GABABR1; galectin-3; human colon cancer; proliferation; 5-fluorouracil

Abstract

Objectives: Many efforts have shown multi-oncologic roles of galectin-3 for cell proliferation, angiogenesis, and apoptosis. However, the mechanisms by which galectin-3 is involved in cell proliferation are not yet fully understood, especially in human colon cancer cells.

Methods: To cluster genes showing positively or negatively correlated expression with galectin-3, we employed human colon cancer cell lines, SNU-61, SNU-81, SNU-769B, SNU-C4 and SNU-C5 in high-throughput gene expression profiling. Gene and protein expression levels were determined by using real-time quantitative polymerase chain reaction (PCR) and western blot analysis, respectively. The proliferation rate of human colon cancer cells was measured by using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Results: Expression of γ-aminobutyric acid B receptor 1 (GABABR1) showed a positive correlation with galectin-3 at both the transcriptional and the translational levels. Down-regulation of galectin-3 decreased not only GABABR1 expression but also the proliferation rate of human colon cancer cells. However, Korean herbal extract, HangAmDan-B (HAD-B), decreased expression of GABABR1 without any expressional change of galectin-3, and offset γ-aminobutyric acid (GABA)-enhanced human colon cancer cell proliferation.

Conclusions: Our present study confirmed that GABABR1 expression was regulated by galectin-3. HAD-B induced galectin-3-independent down-regulation of GABABR1, which resulted in a decreased proliferation of human colon cancer cells. The therapeutic effect of HAD-B for the treatment of human colon cancer needs to be further validated.

Received: July 4, 2012 **Accepted:** Sept 10, 2012

1. Introduction

Galectin-3 is a member of the family of β-galactoside-binding proteins that bind to the carbohydrate portion of cell-surface glycoproteins and glycolipids [1]. Galectin-3 has a chimera-type structure consisting of three different structural domains: a short NH2-terminal domain of 12 amino acids that contains a serine phosphorylation site; a repeated collagen-like sequence that rich in glycine, tyrosine, and proline amino acid residues, which serves as a substrate for matrix metalloproteinases (MMPs); and a COOH-terminal carbohydrate recognition domain [1-3]. Galectin-3 is a multifunctional oncogene [1], which regulates cell growth [4], adhesion [5], proliferation [6], angiogenesis [7], and apoptosis [8].

Many studies have shown that galectin-3 regulates cancer cell proliferation. Galectin-3-stimulated cell proliferation of IMR-90 human lung fibroblasts [6]; a decrease of galectin-3 expression in activated T lymphocytes paralleled a downregulation or even a blocking of proliferation [9]; and the introd-

*Corresponding author
Hwa-Seung Yoo. East-West Cancer Center, Dunsan Oriental Hospital of Daejeon University, 1136 Dunsan-dong, Seo-gu, Daejeon 302-122, Korea.
Tel: +82-42-470-9132 Fax: +82-42-470-9006
E-mail: altyhs@dju.kr

uction of galectin-3 cDNA caused human lymphoma Jurkat T cells to grow faster [10]. A recent report provided evidence that downregulation of galectin-3 led to diminished human colon cancer cell proliferation via modulation of the hete-rogeneous nuclear ribonucleoprotein Q (hnRNP Q) level [11].

Overexpression of galectin-3 has been reported in gastric cancer [12]. Positive galectin-3 expression was observed in 84% of gastric cancer cases. In enhanced cells of a cancerous lesion, 48% showed stronger nuclear immunoreactivity than a cytoplasmic one whereas adjacent epithelial cells showed little or weak nuclear immunoreactivity [12]. In addition, decreased galectin-3 expression was found in breast [13], ovary [14], prostate [15], epithelial skin cancer [16], and head-and-neck squamous cell carcinomas [17] than in corresponding normal tissue.

HangAmDan (HAD)-B consists of eight species of Korean medicinal plants and animals (Table 1), and is an upgraded version of HangAmDan (HAD) used traditionally for solid masses, which also shows anti-angiogenic activity [18]. A mixture of these plants has been shown to exert strong anti-cancer activity against solid tumors, including pancreatic, lung, colorectal, and stomach cancers. Additionally, anti-angiogenesis effects and inhibition of cancer cell proliferation and metastasis have been reported [19]. In particular, case reports observed with HAD have been selected as part of the National Cancer Institute's Best Case Series Program [20]. HAD-B has shown efficacy in inhibiting migration and proliferation of human umbilical vein endothelial cells and in limiting the formation of capillary tube structures [21]. Furthermore, a safety evaluation of HAD-B has revealed no side-effects in both healthy subjects and cancer patients [22].

Even though a number of studies have reported the functions of galectin-3 in many types of cancer, the mechanisms by which galectin-3 is involved in cell proliferation are not yet fully understood, especially in human colon cancer cells. In the present study we report that γ-aminobutyric acid B receptor 1 (GABABR1) expression is linked to galectin-3 in human colon cancer cell line, and we discuss the effect of galectin-3-independent down-regulation of GABABR1 by treatment with Korean herbal extract HAD-B in human colon cancer cells.

2. Materials and methods

2.1. Human colon cancer cell lines

Human colon cancer cell lines, SNU-61, SNU-81, SNU-769B, SNU-C4 and SNU-C5, were obtained from the Korean Cell Line Bank (Seoul, Korea).

2.2. Preparation of water extract of HAD-B

Table 1 Ingredients of HAD-B

Scientific name	Relative amount (mg)
Panax notoginseng Radix	84.0
Cordyceps Militaris	64.0
Santsigu Tuber	64.0
Ginseng Radix	64.0
Bovis Calculus	64.0
Margarita	64.0
Bostaurus var.domesticus Gmelin	48.0
Commiphora myrrha	48.0
Total amount (1 capsule)	500.0

HAD-B was provided from the East-West Cancer Center of Dunsan Oriental Medical Hospital, Daejeon University, Daejeon, Korea (Table 1). The water extract of HAD-B was prepared by extracting HAD-B powder with 10-times (v/w) the amount of distilled water at room temperature for 24 hrs. The extract was centrifuged at 1000×g for 30 mins and was then filtered and lyophilized. The extract powder was dissolved directly in distilled water.

2.3. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

A colorimetric assay using tetrazolium salt, MTT, was used to assess cell proliferation after galectin-3 suppression. MTT assays were performed as described in a previous report [11]. Briefly, equal numbers of cells were incubated in each well in 0.18 ml of culture medium to which 0.02 ml of 10 × 5-FU (Choongwae Pharma Corporation), HAD-B, GABA or PBS (for untreated 100% survival control) had been added. After 4 days of culture, 0.1 mg of MTT was added to each well and incubated at 37° C for a further 4 hrs. Plates were centrifuged at 450 × g for 5 mins at room temperature, and the medium was removed. Dimethyl sulfoxide (0.15 ml) was added to each well to solubilize the crystals, and plates were immediately read at 540 nm by using a scanning multiwell spectrometer (Bio-Tek Instruments Inc., Winooski, VT). All experiments were performed three times, and the IC50 (μg/ml) values are presented as means ± standard deviations.

2.4. Western blot analysis

Western blot analyses were performed as described in a previous report [11]. Primary antibodies against galectin-3 (Abcam, Cambridge, UK), γ-aminobutyric acid B receptor 1 (GABABR1) (Abcam) and actin (Abcam) (1:1,000) were used.

2.5. Immunoprecipitation

All procedures were performed at 4° C unless otherwise specified. Approximately 10^7 cells in 1 ml of cold 1 × radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Roche Diagnostics) were incubated on ice for 30 mins with occasional mixing. Cell lysates were centrifuged at 12,000 × g for 10 mins, and the supernatant was collected carefully without disturbing the pellet. The supernatant was mixed with primary antibody against either galectin-3 (Abcam) or GABABR1 (Abcam) and was incubated for 2 hrs on a rocking platform. Prepared protein G sepharose beads (GE Health Care Life Sciences) were added and further incubated on ice for 1 hrs on a rocking platform. The mixture was centrifuged at 10,000 g for 30 s, and the supernatant was removed completely. Protein G sepharose beads were washed 5 times with 1 ml of cold 1 × RIPA to minimize the background. Next, 100 μl of 2 × sodium dodecyl sulfate (SDS) sample buffer was added to the bead pellets and heated to 100° C for 10 mins. After boiling, immunoprecipitates were centrifuged at 10000 × g for 5 mins, and the supernatant was collected for the Western blot analysis.

2.6. Intracellular cAMP measurement

The intracellular cAMP for human colon cancer cells was determined by using a cAMP Direct Immunoassay Kit (Abcam), as recommended by the manufacturer.

2.7. RNA preparation and Affymetrix GeneChip hybridization

Total RNA was extracted using Trizol reagent (Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's instructions. Genes expressed in the chemosensitive and chemoresistant groups were analyzed on a high-density

oligonucleotide microarray (HG-U133A; Affymetrix, Santa Clara, CA) containing 22,283 transcripts. Target preparation and microarray processing procedures were performed, following the Affymetrix GeneChip Expression Analysis Manual (Affymetrix). Briefly, total RNA extracted was purified with an RNeasy kit (Qiagen). Double-stranded cDNA was synthesized from total RNA (20 μg) with SuperScript II reverse transcriptase (Life Technologies, Inc. Rockville, MD) and a T7-(dT)24 primer (Metabion, Germany). Biotinylated cRNA was synthesized from double-stranded cDNA by using a RNA Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY), purified, and fragmented. Fragmented cRNA was hybridized to the oligonucleotide microarray, which was washed and stained with streptavidin-phycoerythrin. Scanning was performed with an Agilent Microarray Scanner (Agilent Technologies, Santa Clara, CA).

2.8. Affymetrix GeneChip data analysis

A GeneChip analysis was performed based on the Affymetrix GeneChip Manual (Affymetrix) with Data Mining Tool (DMT) 2.0 and Microarray Database software. All genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 500. Fold changes were calculated by comparing transcripts between the cell lines tested. The DMT 2.0 software employed changed calls (increased or decreased) to analyze the expression of a particular transcript statistically and to determine whether it had been relatively increased, decreased or remained unchanged. After filtration through a "present" call ($p < 0.05$), a transcript was considered differentially expressed at a fold change of greater than 2.0.

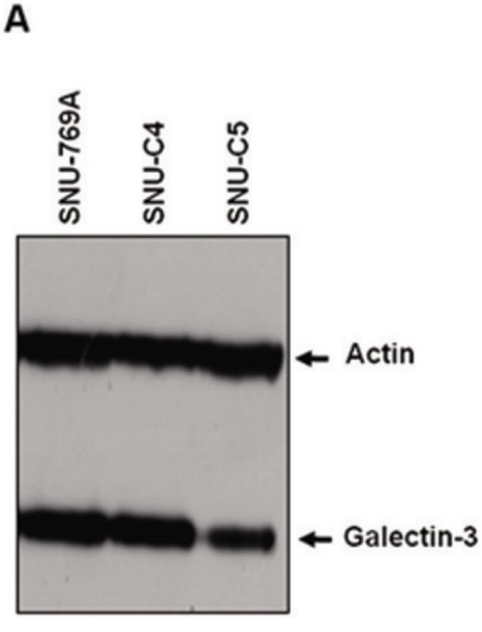
2.9. Real-time quantitative reverse transcription polymerase chain reaction

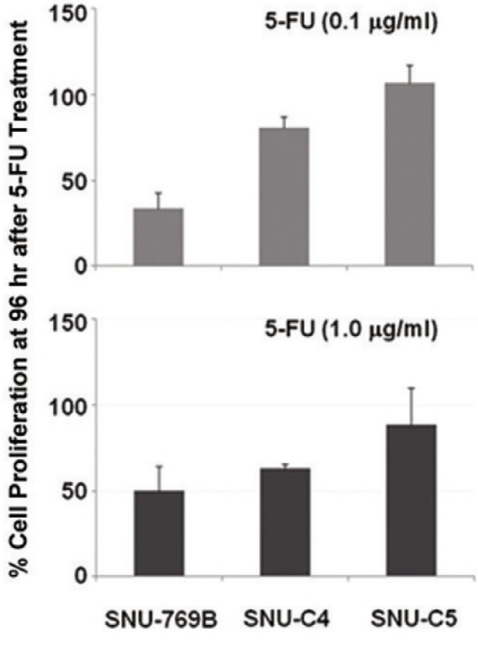
Four genes (ELF3, AXIN2, ENO2 and SACS) were selected for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) for validation of the microarray data. Using the SuperScript Pre-amplification System for first strand cDNA synthesis, 5 mg of total RNA was used for creation of single-stranded cDNA (Life Technologies). The cDNA was diluted and quantitatively equalized for PCR amplification. For real-time qRT-PCR, the ABI Prism 7900 sequence detection system (Applied Biosystems) was used. AccuPower GreenStar PCR Master Mix (Bioneer Corporation, Daejeon, Korea) was used for each PCR reaction, and the GAPDH gene was simultaneously run as a control and was used for normalization. Non-template-control wells without cDNA were included as negative controls. Each test sample was run in triplicate. The primer sets for PCR amplification were designed as follows: ELF3-F: 5' -TGAGCTGCTGGAGAAGGATG-3' , ELF3-R: 5' -CCCTTCTTGCAGTCACGAAA-3' , AXIN2-F: 5' -AATCATTCGGCCACTGTTCA-3' , AXIN2-R: 5' -CACAGGCAAACTCATCGCTT-3' , ENO2-F: 5' -CTGATGCTGGAGTTGGATGG-3' , ENO2-R: 5' -CCATTGATCACGTTGAAGGC-3' , SACS-F: 5' -CCATTTGTTGGCATTTTTGG-3' , and SACS-R: 5' -CGCTCATGTTTCAGTGCCTT-3' . Following the standard curve method, the expressed quantities of the examined genes were determined using the standard curves and the CT values and were normalized using the GAPDH expression quantities.

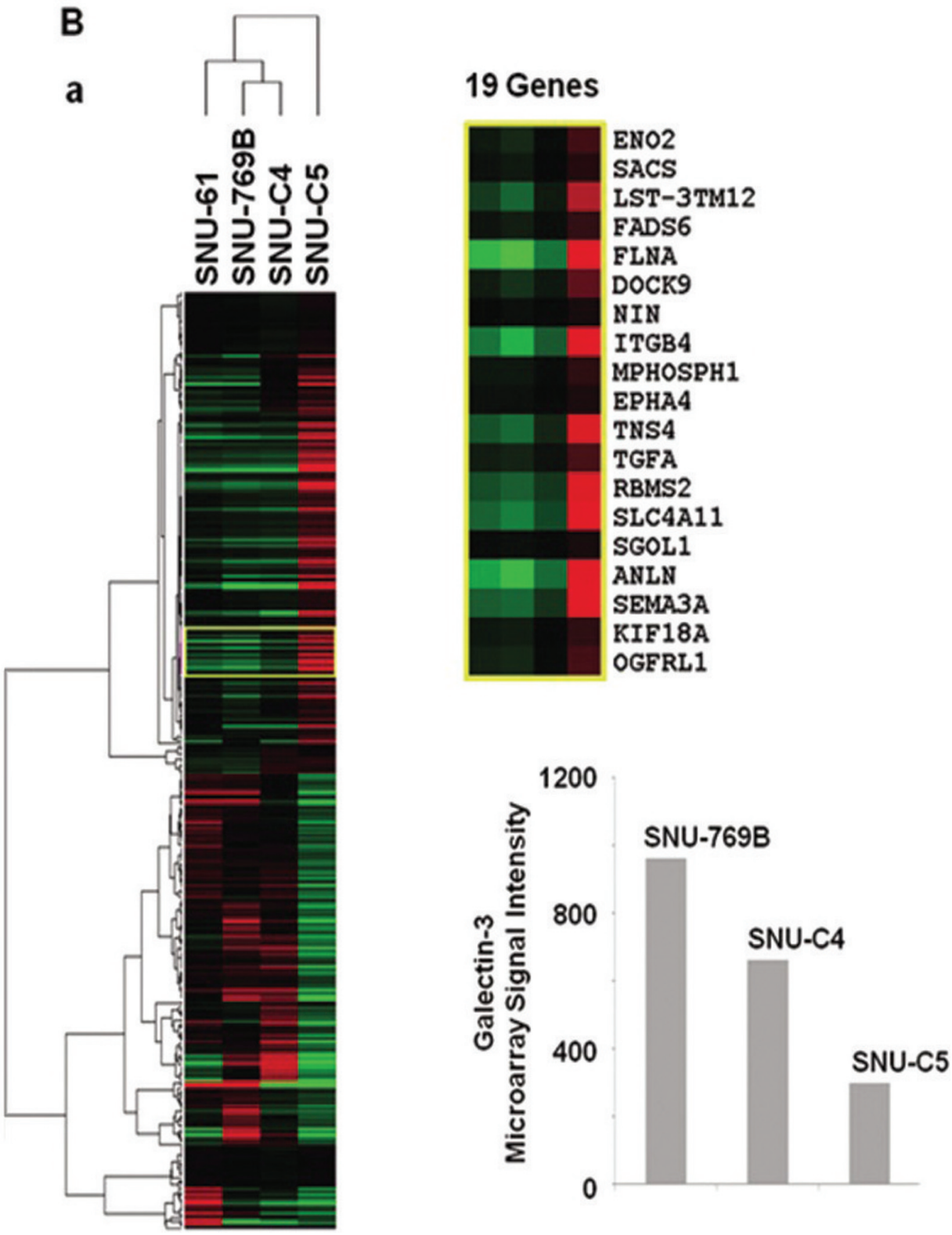
3. Results

3.1. Galectin-3 expression related to 5-FU susceptibility in human colon cancer cells

To confirm the correlation between galectin-3 expression and 5-FU susceptibility in human colon cancer cells, we performed Western blot and MTT analyses on three human colon cancer cell lines, SNU-769B, SNU-C4 and SNU-C5. 5-FU susceptibility showed a decreasing tendency that depended on both the transcriptional (Fig. 1A) and the translational (Fig. 1Ba) levels of galectin-3. To cluster the genes showing positively or negatively correlated expression with galectin-3, we employed SNU-61, which had almost the same 5-FU susceptibility as SNU-769B, in a high-throughput gene expression profiling experiment (Fig. 1B & Tables 2, 3). Figure 1Ba shows an example of 19 genes clustered in a galectin-3 expression pattern, which was confirmed by real-time PCR (Fig. 1Bb). The top 50 down- and up-regulated genes in SNU-C5, compared to SNU-769B, are listed in Tables 2 and 3, respectively.






B
a
SNU-61
SNU-769B
SNU-C4
SNU-C5
19 Genes
ENO2
SACS
LST-3TM12
FADS6
FLNA
DOCK9
NIN
ITGB4
MPHOSPH1
EPHA4
TNS4
TGFA
RBMS2
SLC4A11
SGOL1
ANLN
SEMA3A
KIF18A
OGFRL1
Galectin-3
Microarray Signal Intensity
1200
800
400
0
SNU-769B
SNU-C4
SNU-C5

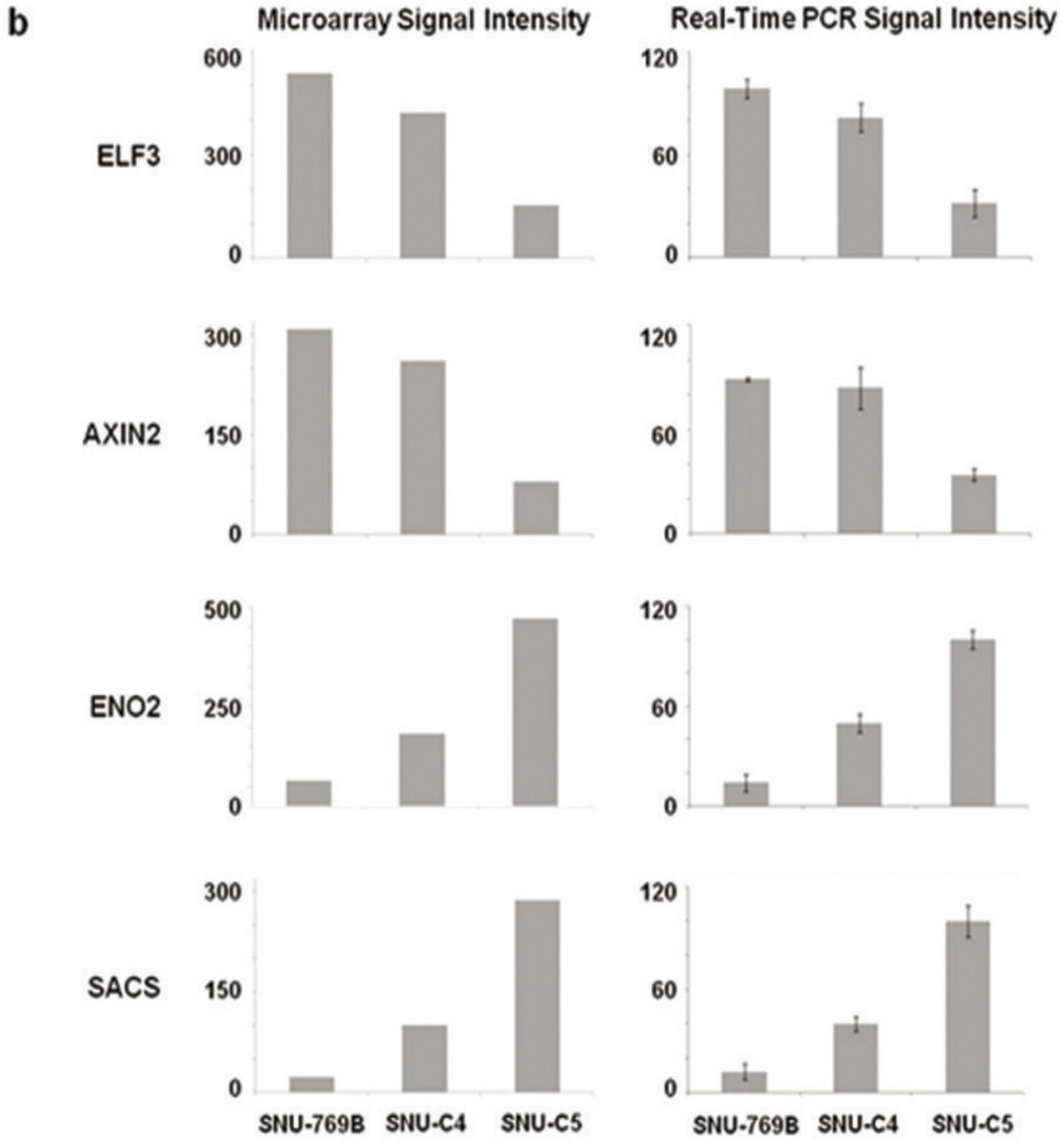


Figure 1 Galectin-3 expression correlated with 5-FU susceptibility in human colon cancer cell lines and gene expression profiling linked to galectin-3. (A) Galectin-3 protein expression correlated with 5-FU susceptibility in three human colon cancer cell lines, SNU-769B, SNU-C4 and SNU-C5. Whole proteomes obtained from the human colon cancer cell lines employed were subjected to SDS-PAGE and were electro-transferred to PVDF membranes for western blot analysis. When galectin-3 expression was higher, human colon cancer cell lines showed more 5-FU susceptibility. (B) Gene expression profiling liked to galectin-3. To satisfy minimum clustering sample size, we added SNU-61, which has almost the same 5-FU susceptibility as SNU-769B, and as shown in the enlarged yellow box, genes linked to galectin-3 expression were selected (a). The expressional profiling was further confirmed by using real-time PCR as shown in panel (b). All genes showing positive and negative expressional correlations with galectin-3 are listed in Tables 2 and 3, respectively.

3.2. Galectin-3-dependent γ-aminobutyric acid B receptor 1 (GABABR1) expression in human colon cancer cells

Although the genes listed in Tables 2 and 3 contain γ-aminobutyric acid B receptor 1 (GABABR1), its expression was positi-vely correlated with galectin-3 as previously reported (Fig. 2A) [11]. GABABR1 expression at the translational level was highest in SNU-769B among the three human colon cancer cell lines tested (Fig. 2B). To validate the interaction between galectin-3 and GABABR1, we performed reverse immunoprecipitation: however, galectin-3 did not form a complex with GABABR1 (Fig. 2C).

3.3. Galectin-3-independent down-regulation of GABABR1 protein by HAD-B in human colon cancer cells

To check the effect of HAD-B treatment on the expression level of galectin-3 and GABABR1, we cultured SNU-C4 with modest expression of galectin-3 and GABABR1 in the presence of HAD-B, and we performed a Western blot analysis. At 96 hrs after treatment with 1 mg/ml HAD-B, expression of GABABR1 was reduced, but galectin-3 did not show any expressional change (Fig. 3A).

A

Probe Set ID	Gene Symbol	SNU-769A	SNU-C4	SNU-C5	FC	GO Biological Process Term	GO Cellular Component Term	GO Molecular Function Term
8124654	GABBR1	68	72	247	1.9	signal transduction // negative regulation of adenylate cyclase activity // gamma-aminobutyric acid signaling pathway	integral to plasma membrane	receptor activity

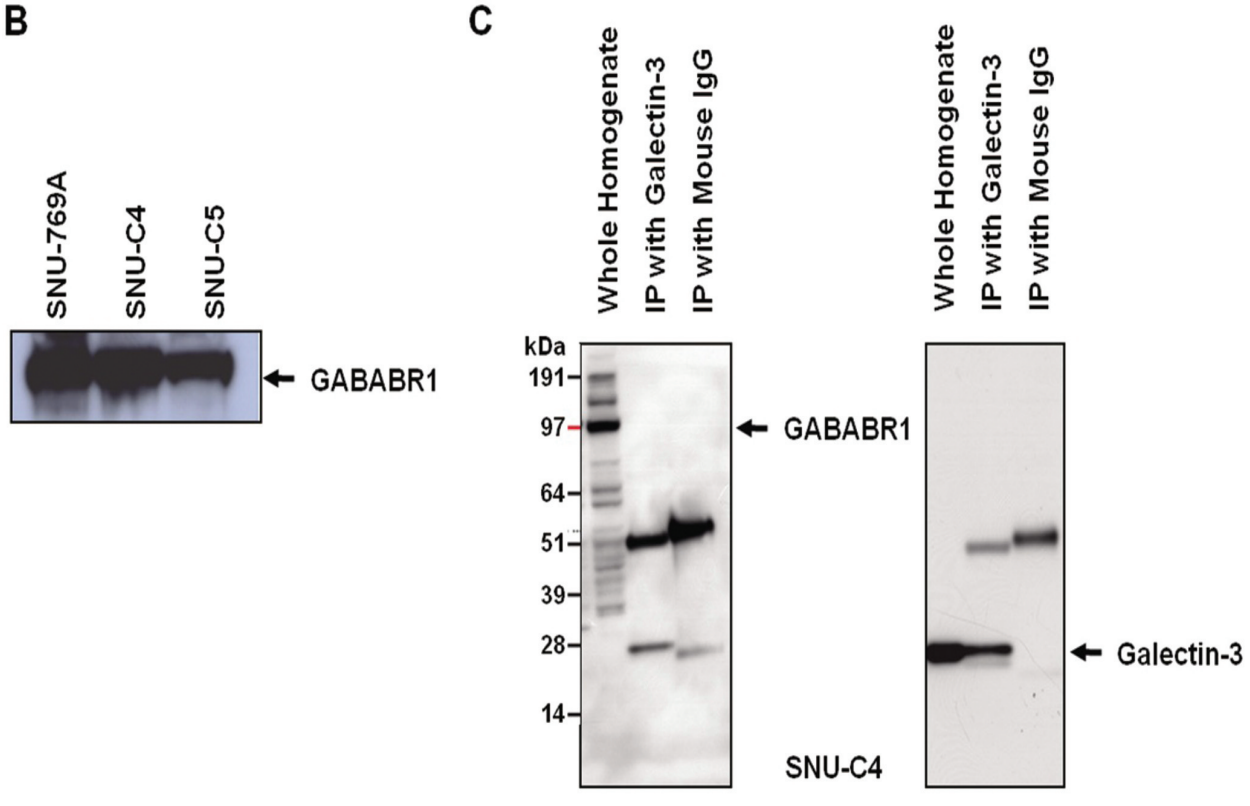


Figure 2 Gene and protein expressions of GABABR1 positively linked to galectin-3 expression. (A) GABABR1 in the list of the genes showing positive expressional correlation with galectin-3. (B) Protein expression of GABABR1 in the three human colon cancer cell lines tested. GABABR1 protein expression also showed positive correlation with galectin-3 expression. (C) Reverse immunoprecipitation using anti-galectin and GABABR1 antibody. Results demonstrated that two proteins did not interact to form a complex in SNU-C4 with modest expression of galectin-3 and GABABR1.

3.4. GABABR1-mediated proliferation of human colon cancer cells suppressed by HAD-B treatment

Treatment with γ-aminobutyric acid (GABA) in the culture medium promoted proliferation of the human colon cancer cell line SNU-C4 (Fig. 3B). At 48 hrs after treatment with GABA, cell proliferation was increased up to ~50% compared to none-treated controls, but rate of increase of proliferation was not maintained (Fig. 3B). HAD-B significantly decreased cell proliferation at 48 hrs after treatment compared to the control, but the suppressed proliferation had recovered at 96 hrs (Fig. 3B). Cells co-treated with GABA and HAD-B showed almost the same pattern of proliferation as that of the control (Fig. 3B). Either GABA or HAD-B treatment slightly increased the intracellular cAMP in SNU-C4 compared to that in the non-treated control (Fig. 3C).

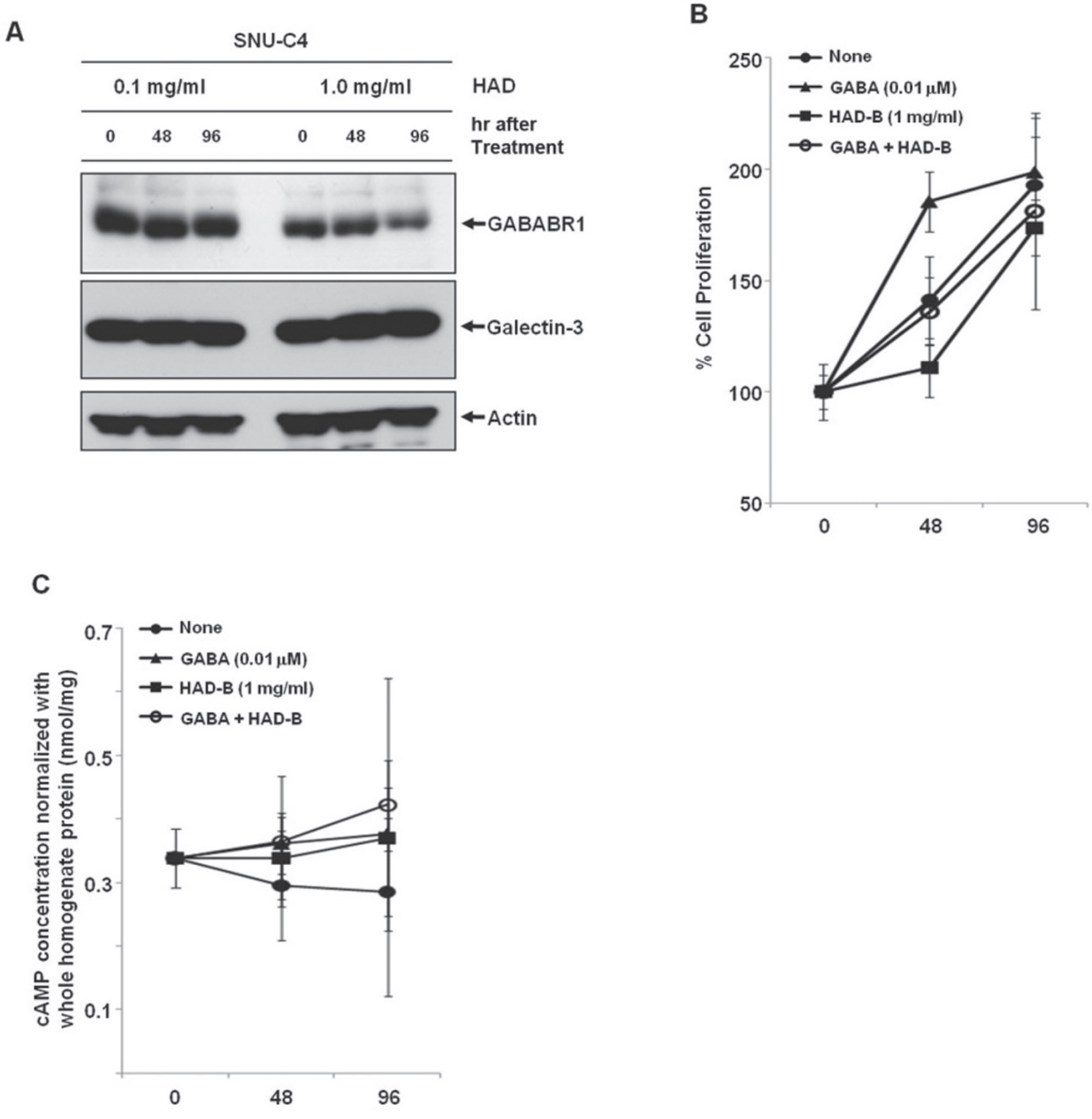


Figure 3 Reduced GABABR1 expression and suppressed cell proliferation of SNU-C4 by treatment with HAD-B. (A) Decreased GABABR1 expression by treatment with HAD-B. At 96 hrs after treatment of with 1 mg/ml HAD-B, protein expression of GABABR1 was decreased in SNU-C4. (B) Suppressed cell proliferation by treatment with HAD-B. GABA treatment recovered the rate of proliferation of SNU-C4 that had been suppressed by HAD-B treatment. (C) Increase in the intracellular cAMP by either GABA or HAD-B treatment. Treatment with GABA or HAD-B increased the basal level of intracellular cAMP.

Table 2 Top 50 down-regulated genes in SNU-C5 compared to SNU-769B

Probe Set ID	Gene Symbol	SNU-769A	SNU-C4	SNU-C5	FC	GO Biological Process Term	GO Cellular Component Term	GO Molecular Function Term
8101893	ADH1C	2263	1125	14	-7.3	alcohol metabolic process	cytoplasm	alcohol dehydrogenase activity, zinc-dependent
7989501	CA12	2428	789	26	-6.5	one-carbon compound metabolic process	membrane	carbonate dehydratase activity
8022692	DSC3	904	35	10	-6.5	cell adhesion	membrane fraction	calcium ion binding
7979658	GPX2	2129	1408	24	-6.5	response to oxidative stress	cytoplasm	glutathione peroxidase activity
7919055	HMGCS2	2091	1229	28	-6.2	acetyl-CoA metabolic process	mitochondrion	hydroxymethylglutaryl-CoA synthase activity
8036591	LGALS4	5079	4762	69	-6.2	cell adhesion	cytosol	sugar binding
7928770	PCDH21	1522	291	21	-6.2	homophilic cell adhesion	membrane	calcium ion binding
7953200	CCND2	2210	60	37	-5.9	regulation of progression through cell cycle	nucleus	protein binding
7928766	C10orf99	1970	493	33	-5.9			
8138392	AGR3	292	174	6	-5.7			
7919984	SELENBP1	2409	1499	54	-5.5			selenium binding
8174654	KLHL13	650	164	16	-5.3			protein binding
7967107	C12orf27	367	229	9	-5.3			
8161884	PRUNE2	396	230	12	-5.1			
8106354	IQGAP2	704	95	22	-5.0	signal transduction	intracellular	actin binding
8134339	PEG10	658	28	21	-5.0	negative regulation of transforming growth factor beta receptor signaling pathway	cytoplasm	nucleic acid binding
8135378	PRKAR2B	543	85	18	-4.9	protein amino acid phosphorylation	cAMP-dependent protein kinase complex	nucleotide binding
8091283	PLOD2	358	98	13	-4.8	protein modification process	endoplasmic reticulum	iron ion binding
8128123	RRAGD	311	58	12	-4.7		nucleus	nucleotide binding
7983606	EID1	759	568	30	-4.7	negative regulation of transcription from RNA polymerase II promoter	cellular component	protein binding
8100734	UGT2B17	125	6	5	-4.6	metabolic process	membrane fraction	glucuronosyltransferase activity
8080964	PPP4R2	293	130	12	-4.6	protein modification process	centrosome	protein binding
8151592	CA1	256	23	11	-4.6	one-carbon compound metabolic process	cytoplasm	carbonate dehydratase activity
8101757	GPRIN3	711	256	31	-4.5			
7926545	PLXDC2	499	87	22	-4.5	multicellular organismal development	membrane	receptor activity
7916185	ZCCHC11	266	262	12	-4.5		intracellular	nucleic acid binding
8008172	B4GALNT2	693	38	30	-4.5	UDP-N-acetylgalactosamine metabolic process	membrane	acetylgalactosaminyltransferase activity
8040374	FAM84A	998	492	44	-4.5			
8168589	ZNF711	378	106	19	-4.4	regulation of transcription, DNA-dependent	intracellular	DNA binding
8043981	IL1R2	663	54	33	-4.3	immune response	membrane	receptor activity
7923578	FMOD	359	74	18	-4.3	transforming growth factor beta receptor complex assembly	proteinaceous extracellular matrix	protein binding

Probe Set ID	Gene Symbol	SNU-769A	SNU-C4	SNU-C5	FC	GO Biological Process Term	GO Cellular Component Term	GO Molecular Function Term
8138553	FAM126A	210	69	11	-4.3	biological process	cellular component	signal transducer activity
8077323	CNTN4	168	11	10	-4.1	cell adhesion	plasma membrane	protein binding
7999553	FLJ11151	337	207	20	-4.1			hydrolase activity
7940565	FADS2	502	380	31	-4.0	lipid metabolic process	membrane fraction	iron ion binding
7951554	RDX	259	67	16	-4.0	cytoskeletal anchoring	cytoplasm	actin binding
8044212	SULT1C2	215	39	13	-4.0	amine metabolic process	cytoplasm	sulfotransferase activity
7903742	GSTM2	946	183	59	-4.0	metabolic process		glutathione transferase activity
7937335	IFITM1	402	343	25	-4.0	regulation of progression through cell cycle	plasma membrane	receptor signaling protein activity
8041383	LTBP1	470	213	30	-4.0	biological process	proteinaceous extracellular matrix	transforming growth factor beta receptor activity
8142171	SLC26A3	185	18	12	-4.0	transport	membrane fraction	transcription factor activity
7951789	FAM55D	318	209	21	-3.9			
8078544	MLH1	155	121	10	-3.9	mismatch repair	nucleus	single-stranded DNA binding
8111772	DAB2	344	72	23	-3.9	cellular morphogenesis during differentiation	coated pit	protein C-terminus binding
8094988	FLJ21511	270	75	18	-3.9			
7918223	C1orf59	122	101	8	-3.9			
8095110	KIT	160	29	11	-3.9	protein amino acid dephosphorylation	external side of plasma membrane	nucleotide binding
8125149	SLC44A4	1306	1163	88	-3.9		membrane	
8178653	NEU1	1306	1163	88	-3.9	metabolic process	lysosome	exo-alpha-sialidase activity
8179861	NEU1	1306	1163	88	-3.9	metabolic process	lysosome	exo-alpha-sialidase activity

FC: Fold-change was calculated from the signal Log ratio value.

Table 3 Top 50 up-regulated genes in SNU-C5, compared to SNU-769B

Probe Set ID	Gene Symbol	SNU-769A	SNU-C4	SNU-C5	FC	GO Biological Process Term	GO Cellular Component Term	GO Molecular Function Term
7954330	SLCO1B3	6	551	921	7.3	ion transport	integral to plasma membrane	transporter activity
7959856	PIWIL1	11	17	834	6.3	multicellular organismal development	cytoplasm	single-stranded RNA binding
7954090	EMP1	23	51	1597	6.1	multicellular organismal development	membrane fraction	
7954344	LST-3TM12	14	236	875	6.0	transport	membrane	transporter activity
8026490	LOC729642	29	34	1815	5.9			
8108217	TGFBI	36	105	2089	5.9	cell adhesion	proteinaceous extracellular matrix	integrin binding
8176026	FLNA	67	699	2852	5.4	cell motility	nucleus	actin binding
7954356	SLCO1B1	10	55	327	5.1	ion transport	membrane fraction	transporter activity
8124437	HIST1H3F	45	49	1482	5.0	nucleosome assembly	nucleosome	DNA binding
7997139	CALB2	33	35	1020	5.0			calcium ion binding
8102950	INPP4B	25	131	786	5.0	signal transduction		phosphatidylinositol-3, 4-bisphosphate 4-phosphatase activity

Probe Set ID	Gene Symbol	SNU-769A	SNU-C4	SNU-C5	FC	GO Biological Process Term	GO Cellular Component Term	GO Molecular Function Term
8095728	EREG	62	230	1896	4.9	regulation of progression through cell cycle	extracellular space	epidermal growth factor receptor binding
8155849	ANXA1	65	980	1942	4.9	lipid metabolic process	cornified envelope	phospholipase inhibitor activity
8089082	DCBLD2	131	308	3841	4.9	cell adhesion	integral to plasma membrane	protein binding
8140668	SEMA3A	36	190	1067	4.9	multicellular organismal development	extracellular region	chemorepellant activity
7920128	S100A11	41	806	1209	4.9	signal transduction	ruffle	calcium ion binding
8098470	WWC2	13	20	358	4.8			
8067233	TMEPAI	50	115	1311	4.7	androgen receptor signaling pathway	membrane	molecular function
7909789	TGFB2	18	22	440	4.6	cell morphogenesis	extracellular region	beta-amyloid binding
8015016	TNS4	42	240	1027	4.6	apoptosis	cytoskeleton	actin binding
8095744	AREG	44	240	1080	4.6	cell-cell signaling	extracellular space	cytokine activity
8021442	ZNF532	28	73	673	4.6		intracellular	nucleic acid binding
7933312	LOC653110	27	29	648	4.6			
7981514	AHNAK2	22	42	532	4.6			protein binding
8027778	FXYD5	65	433	1459	4.5	ion transport	membrane	actin binding
7908072	LAMC2	73	317	1591	4.4	cell adhesion	basement membrane	protein binding
8075310	LIF	54	56	1139	4.4	immune response	extracellular region	cytokine activity
8138466	7A5	61	316	1226	4.3			
7986446	ALDH1A3	88	114	1741	4.3	alcohol metabolic process		3-chloroallyl aldehyde dehydrogenase activity
8041179	CLIP4	18	20	356	4.3			
8124413	HIST1H4D	63	338	1234	4.3			
7924029	LAMB3	77	169	1473	4.3	electron transport	proteinaceous extracellular matrix	structural molecule activity
8129379	ECHDC1	38	685	717	4.3	metabolic process		catalytic activity
7945321	LOC89944	33	510	625	4.2	carbohydrate metabolic process	beta-galactosidase complex	catalytic activity
8179731	HLA-C	153	616	2862	4.2	ciliary or flagellar motility	axonemal dynein complex	microtubule motor activity
8064613	SLC4A11	72	239	1300	4.2	anion transport	membrane	inorganic anion exchanger activity
8167185	TIMP1	215	857	3816	4.1	multicellular organismal development	extracellular region	enzyme inhibitor activity
8120602	OGFRL1	28	141	438	4.0		membrane	receptor activity
8178489	HLA-C	190	730	2990	4.0	ciliary or flagellar motility	axonemal dynein complex	microtubule motor activity
8060758	PRNP	108	486	1632	3.9	copper ion homeostasis	cytoplasm	copper ion binding
8178498	HLA-B	164	517	2463	3.9	antigen processing and presentation of peptide antigen via MHC class I	cellular component	molecular function
8124911	HLA-B	131	408	1955	3.9	antigen processing and presentation of peptide antigen via MHC class I	cellular component	molecular function
7973985	MIPOL1	9	92	134	3.9			
7944722	STS-1	32	58	473	3.9		nucleus	

Probe Set ID	Gene Symbol	SNU-769A	SNU-C4	SNU-C5	FC	GO Biological Process Term	GO Cellular Component Term	GO Molecular Function Term
8124901	HLA-C	205	724	2974	3.9	ciliary or flagellar motility	axonemal dynein complex	microtubule motor activity
8095736	LOC727738	43	170	615	3.8			
8091411	TM4SF1	38	220	538	3.8	biological process	integral to plasma membrane	molecular function
7917875	F3	78	87	1078	3.8	immune response	plasma membrane	transmembrane receptor activity
8092726	CLDN1	57	195	772	3.8	cell adhesion	integral to plasma membrane	structural molecule activity
8126820	GPR110	16	20	217	3.8	signal transduction	membrane	receptor activity

FC: Fold-change was calculated from the signal Log ratio value.

4. Discussion

Colon cancer causes almost a half million deaths every year [23]. In the past 3 decades, 5-fluorouracil (5-FU) chemotherapy and 5-FU-based chemotherapy have been the mainstream in adjuvant treatment of colon cancer [24]; however, partial or complete responses of colon cancer to 5-FU are generally followed by eventual tumor re-growth [25]. Numerous studies have focused on identifying the mechanisms and key molecules involved in natural or acquired 5-FU resistance. Nevertheless, conclusive and consistent results have not been obtained so far. A recent proteome approach identified galectin-3 as a protein affecting 5-FU resistance and the proliferation rate of human colon cancer cells [11]. Our present study confirmed the correlation between galectin-3 expression and 5-FU susceptibility in three human colon cancer cell lines. 5-FU susceptibility of human colon cancer cells was different depending on both the transcriptional and the translational levels of galectin-3 (Figs. 1A and B). Because the identification of genes showing positively or negatively correlated expression with galectin-3 can provide further information on how galectin-3 regulates proliferation of human colon cancer cells, a high-density oligonucleotide microarray was performed. From this transcriptional analysis, we were able to list the genes down- and up-regulated based on the level of galectin-3 expression (Fig. 1B, Tables 2 and 3). Though γ-aminobutyric acid B receptor 1 (GABABR1) was not in the top 50 genes linked to galectin-3 (Tables 2 and 3), interestingly we found that both the transcriptional and the translational levels of GABABR1 were positively correlated with galectin-3 (Fig. 2A and 2B). Even though the biological functions of each individual protein have been well studied, we could not find a report describing the relation between galectin-3 and GABABR1.

GABABRs have been found to play a key role in regulating membrane excitability and synaptic transmission in the brain [26]. GABABRs are G-protein coupled receptors that associate with a subset of G-proteins that trigger cAMP cascades [26]. GABABR subtypes exist; two GABAB-receptor splice variants, GABABR1a and GABABR1b, have been cloned [27], and a new GABABR subtype, GABABR2, does not bind with available GABAB antagonists with measurable potency [28]. GABABR1a, GABABR1b and GABABR2 alone do not activate Kir3-type potassium channels efficiently, but co-expression of these receptors yields a robust coupling to activation of Kir3 channels. GABABR2 and GABABR1a/b proteins immunoprecipitate and localize together at dendritic spines [28]. The heteromeric receptor complexes exhibit a significant increase in agonist- and partial agonist-binding potencies as compared with individual receptors and probably represent the predominant native GABAB receptor [28]. As a previous report also showed that the transcriptional level of GABABR1 was decreased by transfection of galectin-3 small interfering RNA (siRNA) [11], expression of GABABR1 could be regulated by galectin-3. However, reverse immunoprecipitation to validate the interaction between two proteins revealed that galectin-3 did not affect the protein stability of GABABR1 because it formed a complex with GABABR1 (Fig. 2C).

Gamma-aminobutyric acid (GABA) has been reported to affect cancer development. For example, GABA can be a potential tumor suppressor for small airway-derived lung adenocarcinomas [29]. The GABA agonist nembutal has been reported to be a potent inhibitor of primary colon cancer and metastasis [30]. The GABABR agonist baclofen induced G(0)/G(1) phase arrest of human hepatocellular carcinomas (HCCs), which suggested the possibility of developing baclofen as a therapeutic drug for the treatment of HCCs [31]. Furthermore, stimulation of GABABR signaling has been suggested as a novel target for the treatment and the prevention of pancreatic cancer [32]. However, in our present study, treatment with GABA promoted proliferation of the human colon cancer cell line SNU-C4 (Fig. 3B). The Korean herbal extract HAD-B not only decreased GABABR1 expression but also reduced proliferation of human colon cancer cells without any expressional change of galectin-3 (Figs. 3A and 3B). GABABR activation can lead to down-regulation of the intracellular cAMP level in human cancer cells [30,32]. Down-regulation of GABABR1 by HAD-B treatment increased the basal level of intracellular cAMP in SNU-C4 (Fig. 3C). However, such an increased cAMP was also observed after GABA treatment (Fig. 3C). The overall findings in the present study were inconsistent with those in previous reports describing the activation of GABABR1 to prevent the progression of a human carcinoma. Nevertheless, our present results showed a link between galectin-3 and GABABR1 in human colon cancer cell proliferation. Galectin-3 regulated GABABR1 expression [11]. Decreased galectin-3 expression reduced not only GABABR1 expression but also the proliferation rate of human colon cancer cells [11]. Even GABA promoted human colon cancer cell proliferation by activating GABABR1 signaling, and the increased proliferation was offset by HAD-B treatment because HAD-B led to galectin-3-independent down-regulation of GABABR1 (Fig. 3).

5. Conclusion

Our present study confirmed that GABABR1 expression was regulated by galectin-3. Korean herbal extract HAD-B induced galectin-3-independent down-regulation of GABABR1, which resulted in a decreased proliferation of human colon cancer cells. The therapeutic effect of HAD-B for the treatment of human colon cancer needs to be further validated.

Acknowledgment

This work was supported by a research grant (NCC-1010050) from the National Cancer Center, Korea.

References

1. Barondes SH, Cooper DN, Gitt MA, Leffler H. Galectins. Structure and function of a large family of animal lectins. J Biol Chem. 1994;269(33):20807-10.
2. Gong HC, Honjo Y, Nangia-Makker P, Hogan V, Mazurak N, Bresalier RS, et al. The NH2 terminus of galectin-3 governs cellular compartmentalization and functions in cancer cells. Cancer Res. 1999;59(24):6239-45.
3. Herrmann J, Turck CW, Atchison RE, Huflejt ME, Poulter L, Gitt MA, et al. Primary structure of the soluble lactose binding lectin L-29 from rat and dog and interaction of its non-collagenous proline-, glycine-, tyrosine-rich sequence with bacterial and tissue collagenase. J Biol Chem. 1993;268(35):26704-11.
4. Yang RY, Liu FT. Galectins in cell growth and apoptosis. Cell Mol Life Sci. 2003;60(2):267-76.
5. Inohara H, Raz A. Functional evidence that cell surface galectin-3 mediates homotypic cell adhesion. Cancer Res. 1995;55(15):3267-71.
6. Inohara H, Akahani S, Raz A. Galectin-3 stimulates cell proliferation. Exp Cell Res. 1998;245(2):294-302.
7. Nangia-Makker P, Honjo Y, Sarvis R, Akahani S, Hogan V, Pienta KJ, et al. Galectin-3 induces endothelial cell morphogenesis and angiogenesis. Am J Pathol. 2000;156(3):899-909.
8. Fukumori T, Oka N, Takenaka Y, Nangia-Makker P, Elsammaan E, Kasai T, et al. Galectin-3 regulates mitochondrial stability and antiapoptotic function in response to anticancer drug in prostate cancer. Cancer Res. 2006;66(6):3114-9.
9. Joo HG, Goedegebuure PS, Sadanaga N, Nagoshi M, Von Bernstorff W, Eberlein TJ. Expression and function of galectin-3, a beta-galactoside-binding protein in activated T lymphocytes. J Leukoc Biol. 2001;69(4):555-64.
10. Yoshii T, Inohara H, Takenaka Y, Honjo Y, Akahani S, Nomura T, et al. Galectin-3 maintains the transformed phenotype of thyroid papillary carcinoma cells. Int J Oncol. 2001;18(4):787-92.
11. Yoo BC, Hong SH, Ku JL, Kim YH, Shin YK, Jang SG, et al. Galectin-3 stabilizes heterogeneous nuclear ribonucleoprotein Q to maintain proliferation of human colon cancer cells. Cell Mol Life Sci. 2009;66(2):350-64.
12. Miyazaki J, Hokari R, Kato S, Tsuzuki Y, Kawaguchi A, Nagao S, et al. Increased expression of galectin-3 in primary gastric cancer and the metastatic lymph nodes. Oncol Rep. 2002;9(6):1307-12.
13. Castronovo V, Van Den Brûle FA, Jackers P, Clausse N, Liu FT, Gillet C, et al. Decreased expression of galectin-3 is associated with progression of human breast cancer. J Pathol. 1996;179(1):43-8.
14. Van den Brûle FA, Berchuck A, Bast RC, Liu FT, Gillet C, Sobel ME, et al. Differential expression of the 67-kD laminin receptor and 31-kD human laminin-binding protein in human ovarian carcinomas. Eur J Cancer. 1994;30(8):1096-9.
15. Pacis RA, Pilat MJP, Pienta KJ, Wojno KJ, Raz A, Hogan V, et al. Decreased galectin-3 expression in prostate cancer. Prostate. 2000;44(2):118-23.
16. Mollenhauer J, Deichmann M, Helmke B, Müller H, Kollender G, Holmskov U, et al. Frequent down-regulation of DMBT1 and galectin-3 in epithelial skin cancer. Int J Cancer. 2003;105(2):149-57.
17. Choufani G, Nagy N, Saussez S, Marchant H, Bisschop P, Burchert M, et al. The levels of expression of galectin-1, galectin-3, and the Thomsen-Friedenreich antigen and their binding sites decrease as clinical aggressiveness increases in head and neck cancers. Cancer. 1999;86(11):2353-63.
18. Choi YJ, Shin DY, Lee YW, Cho CK, Kim GY, Kim WJ, et al. Inhibition of cell motility and invasion by HangAmDan-B in NCI-H460 human non-small cell lung cancer cells. Oncol Rep. 2011;26(6):1601-8.
19. Bang JY, Kim KS, Kim EY, Yoo HS, Lee YW, Cho CK, et al. Anti-angiogenic effects of the water extract of HangAmDan (WEHAD), a Korean traditional medicine. Sci China Life Sci. 2011;54(3):248-54.
20. Park HM, Kim SY, Jung IC, Lee YW, Cho CK, Yoo HS. Integrative tumor board: a case report and discussion from East-West Cancer Center. Integr Cancer Ther. 2010;9(2):236-45.
21. Bang JY, Kim EY, Shim TK, Yoo HS, Lee YW, Kim YS, et al. Analysis of anti-angiogenic mechanism of HangAmDan-B (HAD-B), a Korean traditional medicine, using antibody microarray chip. BioChip J. 2010;4(4):350-5.
22. Yoo HS, Lee HJ, Kim JS, Yoon J, Lee GH, Lee YW, et al. A toxicological study of HangAmDan-B in mice. J Acupunct Meridian Stud. 2011;4(1):54-60.
23. Xu R, Zhou B, Fung PCW, Li X. Recent advances in the treatment of colon cancer. Histol Histopathol. 2006;21(8):867-72.
24. Nordman IC, Iyer S, Joshua AM, Clarke SJ. Advances in the adjuvant treatment of colorectal cancer. ANZ J Surg. 2006;76(5):373-80.
25. Li J, Hou N, Faried A, Tsutsumi S, Takeuchi T, Kuwano H. Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. Ann Surg Oncol. 2009;16(3):761-71.
26. Padgett CL, Slesinger PA. GABAB receptor coupling to G-proteins and ion channels. Adv Pharmacol. 2010;58:123-47.
27. Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, et al. Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. Nature. 1997;386(6622):239-46.
28. Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, et al. GABA(B)-receptor subtypes assemble into functional heteromeric complexes. Nature. 1998;396(6712):683-7.
29. Schuller HM, Al-Wadei HA, Majidi M. Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. Carcinogenesis. 2008;29(10):1979-85.
30. Thaker PH, Yokoi K, Jennings NB, Li Y, Rebhun RB, Rousseau DL Jr, et al. Inhibition of experimental colon cancer metastasis by the GABA-receptor agonist nembutal. Cancer Biol Ther. 2005;4(7):753-8.
31. Wan T, Huang W, Chen F. Baclofen, a GABAB receptor agonist, inhibits human hepatocellular carcinoma cell growth in vitro and in vivo. Life Sci. 2008;82(9-10):536-41.
32. Schuller HM, Al-Wadei HA, Majidi M. GABA B receptor is a novel drug target for pancreatic cancer. Cancer. 2008;112(4):767-78.