Altered mRNA expression levels of the major components of sphingolipid metabolism, ceramide synthases and their clinical implication in colorectal cancer

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Abstract. Ceramide synthases (CerSs) synthesize various ceramides of different acyl chain lengths and serve important roles in the proliferation and death of cancer cells by regulating sphingolipid metabolism-related signaling pathways. The present study investigated the mRNA expression levels of various CerS genes using mRNA expression data from six independent colorectal cancer (CRC) cohorts and a Korean CRC dataset. Expression levels of *CERS2*, *CERS5* and *CERS6* mRNA were significantly increased in the majority of the studied groups. However, *CERS4* expression was only significantly altered in two groups. Additionally, a positive correlation was observed between altered *CERS4* and *CERS5* mRNA levels in The Cancer Genome Atlas Colon and Rectal Cancer dataset. Notably, *CERS2* and *CERS4*, as well as *CERS5* and *CERS6* levels, were positively correlated with each other in Korean patients with CRC. However, the mRNA expression levels of these four CerS genes were not associated with any clinicopathological characteristics in Korean patients with CRC. Finally, overexpressing *CERS2* or *CERS6* inhibited the *in vitro* viability of various CRC cells. Taken together, these findings indicated that *CERS2*, *CERS4*, *CERS5*, and *CERS6* are significantly dysregulated in CRC, suggesting they may serve important roles in the pathophysiology of this malignancy.

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Abbreviations: CRC, colorectal cancer; CerS, ceramide synthase; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer; qPCR, quantitative polymerase chain reaction; NST, non-neoplastic surrounding colon tissues; LPAR1, lysophosphatidic acid receptor 1; NAAA, N-acylethanolamine acid amidase; SPHK1, sphingosine kinase 1; HPGD, 15-hydroxyprostaglandin dehydrogenase; SMPD1, sphingomyelin phosphodiesterase 1; SMPDL3A, sphingomyelin phosphodiesterase acid-like 3A; UGGT2, UDP-glucose glycoprotein glucosyltransferase 2; HA-*CERS2*, HA-tagged form of *CERS2*; HA-*CERS6*, HA-tagged form of *CERS6*

Key words: ceramide synthase, colorectal cancer, The Cancer Genome Atlas, sphingolipid metabolism, cBioPortal

Introduction

Colorectal cancer (CRC) is a malignancy derived from the colorectal epithelium and is the third most commonly diagnosed cancer type worldwide (1). Although the mortality rates of CRC have been decreasing due to screening, reduced risk factor prevalence and/or improved therapies (2,3), CRC remains a global health burden in terms of morbidity and mortality, with ~700,000 estimated mortalities annually (1). It has been reported that the complicated and complex pathogenetic mechanisms of CRC involve genomic rearrangements, chromatin remodeling, genetic mutations and epigenetic changes (4,5).

The sphingolipid rheostat is a proposed concept that may regulate cell fate decisions (6). The two major components of the sphingolipid rheostat are ceramide and sphinogosine-1 phosphate, which are interconvertible sphingolipid metabolites that regulate cell growth and survival by modulating sphingolipid rheostat-related signaling (6,7). Ceramide has tumor suppressive anticancer properties, including potentiating signaling networks that drive apoptosis, autophagy and cell cycle arrest (8). Ceramide synthases (CerSs) are integral membrane proteins of the endoplasmic reticulum that synthesize ceramides of different acyl chain lengths. To date, six CerS families have been identified in mammals (9). Dysregulation

of CerS activity has been reported to be associated with tumor cell invasion (10), proliferation (11), apoptosis (12) and epithelial-mesenchymal transition (13), as well as with the prognosis of patients with cancer (14). For example, in head and neck squamous cell carcinoma, downregulation of *CERS1* leads to apoptotic resistance (15), while *CERS1* overexpression enhances growth-inhibitory effects (16). Additionally, *CERS2*, *CERS4* and *CERS6* mRNA expression levels are increased in breast cancer (17), and the upregulation of *CERS4* and *CERS6* leads to reduced cell proliferation and the induction of apoptosis (18). Given these results and the association of altered CerS expression with malignant transformation, the present study aimed to characterize the mRNA expression of various CerS genes in CRC and non-neoplastic adjacent tissues (NST).

The present study investigated the mRNA expression levels of various CerS genes using mRNA expression data from six independent CRC cohorts and a Korean CRC dataset. Furthermore, the clinical significance of altered CerS genes expression was evaluated in the Korean CRC dataset.

Table I. Primer sequences used in quantitative polymerase chain reaction.

Primer	Sequence
CERS2 sense	5'-ATCGTCTTCGCCATTGTTTT-3'
CERS2 antisense	5'-GGCAGGATAGAGCTCCAGTG-3'
CERS4 sense	5'-GGAGGCCTGTAAGATGGTCA-3'
CERS4 antisense	5'-GAGGACCAGTCGGGTGTAGA-3'
CERS5 sense	5'-TGGAATTGGCCTTCTATTGG-3'
CERS5 antisense	5'-CAATGGTGACCAAGTGATGC-3'
CERS6 sense	5'-TGCCATTCTGGAAAAGGTCT-3'
CERS6 antisense	5'-ATGCTTCGAACATCCCAGTC-3'
β-actin sense	5'-CAGCCATGTACGTTGCTATCCAGG-3'
β-actin antisense	5'-AGGTCCAGACGCAGGATGGCATG-3'

CERS, ceramide synthase mRNA.

Materials and methods

Gene expression databases and cluster analysis. Gene expression RNAseq dataset (Level 3) and clinical data for The Cancer Genome Atlas Colon and Rectal Cancer (TCGA-COADREAD) cohort (19) were downloaded from the UCSC Xena (https://xena.ucsc.edu). CRC gene expression microarray data used in this study were downloaded from the publicly available GEO databases (http://www.ncbi.nlm.nih.gov/geo/): GSE21815 (20), GSE44076 (21), GSE44861 (22), GSE41258 (23) and GSE33113 (24). The GEO datasets used in this study include 562 CRC tissues and 222 NST from respective same patient groups. The downloaded raw data of GEO databases were normalized at the transcript and gene level using the Robust Multichip Average method (25). Cluster analysis was performed using Cluster 3.0 to classify the samples into statistically similar groups, and the resulting heatmaps were visualized in TreeView 1.6 (www.eisenlab.org/eisen). The four CerS genes present in the TCGA COADREAD, GSE44076 and GSE44861 cohorts were LASS2, LASS4, LASS5 and LASS6. The present study meets the publication guidelines provided by TCGA.

Patients and tissues. A total of 59 patients (mean age, 64.83±9.48; age range, 38-83; 34 males and 25 females) diagnosed with CRC were included in the present study. CRC and NSTs were obtained from patients undergoing surgery in Keimyung University Dongsan Medical Center (Daegu, Korea) between April 2008 and January 2010. Enrolled patients with CRC were classified according to the AJCC Tumor-Node Metastasis (TNM) staging criteria (26). Tissue samples were immediately frozen in liquid nitrogen and stored at -196˚C until RNA isolation. Tissue samples were provided by Keimyung Human Bio-Resource Bank (Daegu, Korea). Written informed consent was obtained from each study participant and the protocols were approved by the Institutional Review Board of Keimyung University Dongsan Medical Center (approval no. 2015-11-059-001).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted from tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was quantified using NanoDrop 1000 (Thermo Fisher Scientific, Inc.). Each cDNA was synthesized from 2 μg total RNA using MMLV reverse transcriptase (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. qPCR was performed on the LightCycler® 480 Real-Time PCR system (Roche Diagnostics GmbH, Mannheim, Germany) using the specific primer pairs presented in Table I and SYBR-Green Premix (Toyobo Life Science, Osaka, Japan). The qPCR was performed using the following thermocycling conditions: 95˚C for 10 min; followed by 45 cycles of 95˚C for 10 sec, 60˚C for 10 sec, and 72˚C for 12 sec. Melting curve was analyzed to determine primer specificity. b-actin was used as a housekeeping gene for normalization, and a no-template sample was used as a negative control. qPCR data were analyzed using the $2^{-\Delta\Delta Cq}$ method (27). Each experiment was performed three times.

Statistical analysis. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). The cell viability data were analyzed using one-way analysis of variance and the Student-Newman-Keuls post hoc test. Differences between the groups were analyzed statistically using Student's t-test or Mann Whitney U test. The co-expression of the mRNAs of various CerS genes in TCGA-COADREAD cohort were searched using cBioPortal (http://cbioportal.org) (28). The association between inter-individual mRNA expression levels of CerS genes in Korean patients with CRC was assessed using Pearson's correlation coefficient analysis for continuous variables. Clinicopathological associations with the mRNA expression levels of various CerS genes in Korean CRC were analyzed using the Linear by linear association, the Pearson's Chi-square test and the Fisher's exact test for categorical variables. The mean value was used as the cut-off value (low and high) for categorical variables. $P<0.05$ was considered to indicate a statistically significant difference.

Transient transfection. Various human colorectal adenocarcinoma cell lines, HCT116, HT29, SW403 and SW480 cells,

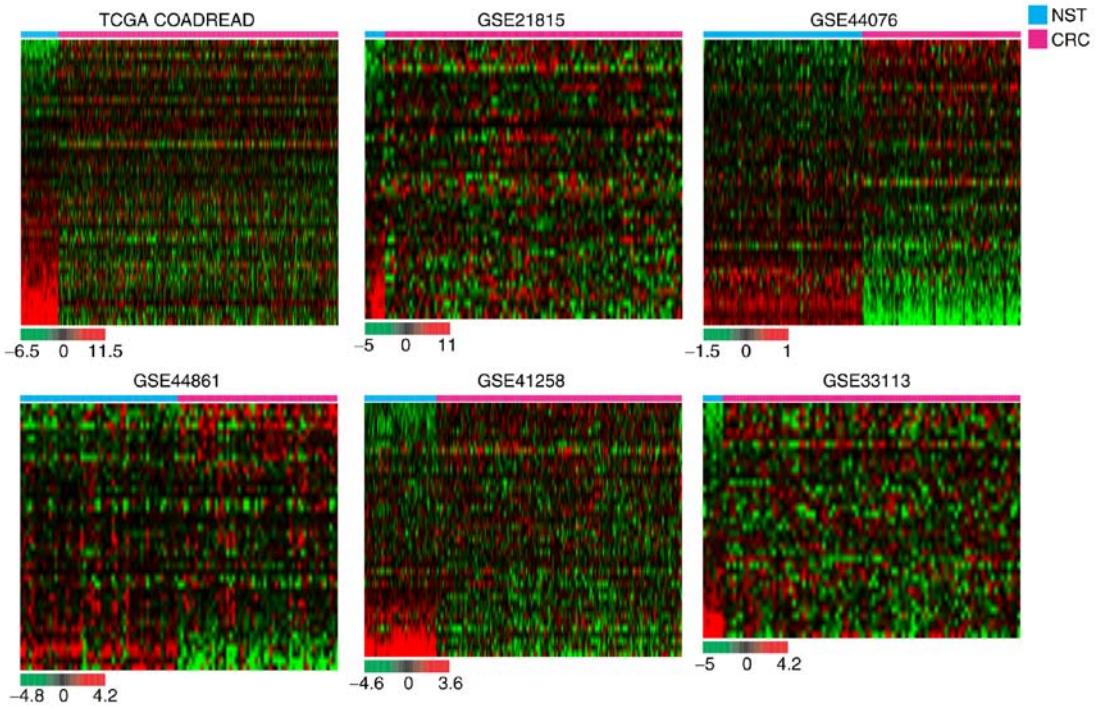

Figure 1. Heat-map showing the sphingolipid metabolism-related genes in CRC tissues compared with NST of 6 independent CRC cohorts. The data are presented in matrix format in which rows represent individual genes and columns represent each tissue. Each cell in the matrix represents the expression level of a gene feature in an individual tissue. Expression levels have been standardized (centered and scaled) within columns for visualization. The red and green colors in cells reflect relative high and low expression levels, respectively, as indicated by the scale bar. Images were obtained by re-analysis of the raw data of respective cohort. CRC, colorectal cancer; NST, non-neoplastic surrounding colon tissues; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer.

were plated onto 6-well plates at a density $7x10^5$ cells/well and cultured overnight. pcDNA3.1-empty vector was used for plasmid constructs, including HA-tagged form of CERS2 (HA-CERS2) and HA-tagged form of CERS6 (HA-CERS6) constructs. All plasmids, including pcDNA3.1-empty vector, HA-CERS2 and HA-CERS6 were provided by Professor Anthony H. Futerman (Weizmann Institute of Science, Rehovot, Israel). The CRC cells were transfected with pcDNA3.1-empty vector, 2 μg HA-CERS2 and HA-CERS6 plasmid in 6-well plates using Lipofectamine reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. At 24 h after plasmid transfection, the subsequent experiments were conducted.

Western blot analysis. The transient transfected CRC cells were collected and washed twice with cold PBS, and cell pellets were prepared by suspending in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4 and 1 mM NaF) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 2 mM EDTA). The lysates were centrifuged at 10,000 x g for 10 min at 4˚C, and the supernatant fractions were collected. The total protein concentration was measured using Micro BCA™ Protein assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cellular proteins (60 mg) were mixed with protein 5X sample buffer (Elpis Biotech., Inc., Daejeon, Korea) and heated at 95˚C for 5 min. The proteins were separated by 10% SDS-PAGE and then electrotransferred to Immobilon-P membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked at room temperature with 5% skimmed dried milk in PBS/0.1% Tween-20 for 1 h, and incubated overnight at 4˚C with anti-HA (1:2,000; mouse monoclonal; cat. no. SAB1411737) and anti-β-actin (1:2,000; mouse monoclonal; cat. no. A5441; both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The membranes were then washed six times with PBS/0.1% Tween-20 (30 min each) and incubated with the corresponding secondary antibodies (horseradish peroxidase-conjugated, horse antibodies to mouse IgG; 1:2,000; cat. no. 7076; Cell Signaling Technology, Inc.) for 1 h at room temperature. Following washing six times in PBS/0.1% Tween-20, the specific protein bands were detected using an enhanced chemiluminescence western blotting kit (EMD Millipore), according to the manufacturer's protocol.

Results

Altered expression levels of sphingolipid metabolism-related genes in six independent CRC cohorts. To investigate whether the sphingolipid metabolism-related genes (29) are dysregulated in CRC tissues, the present study re-analyzed the raw data of six independent CRC cohorts. To begin with, the cancer gene expression RNAseq datasets of 380 CRC patients were taken from the TCGA-COADREAD cohort through UCSC Xena. Next, CRC gene expression microarray data were downloaded from the publicly available Gene Expression Omnibus databases. The CRC gene expression microarrays, GSE21815, GSE33113, GSE41258, GSE44076 and GSE44861, were analyzed for potential transcriptome changes. Hierarchical clustering revealed that various sphingolipid metabolism-related genes were dysregulated in carcinomatous tissues compared with NST of patients

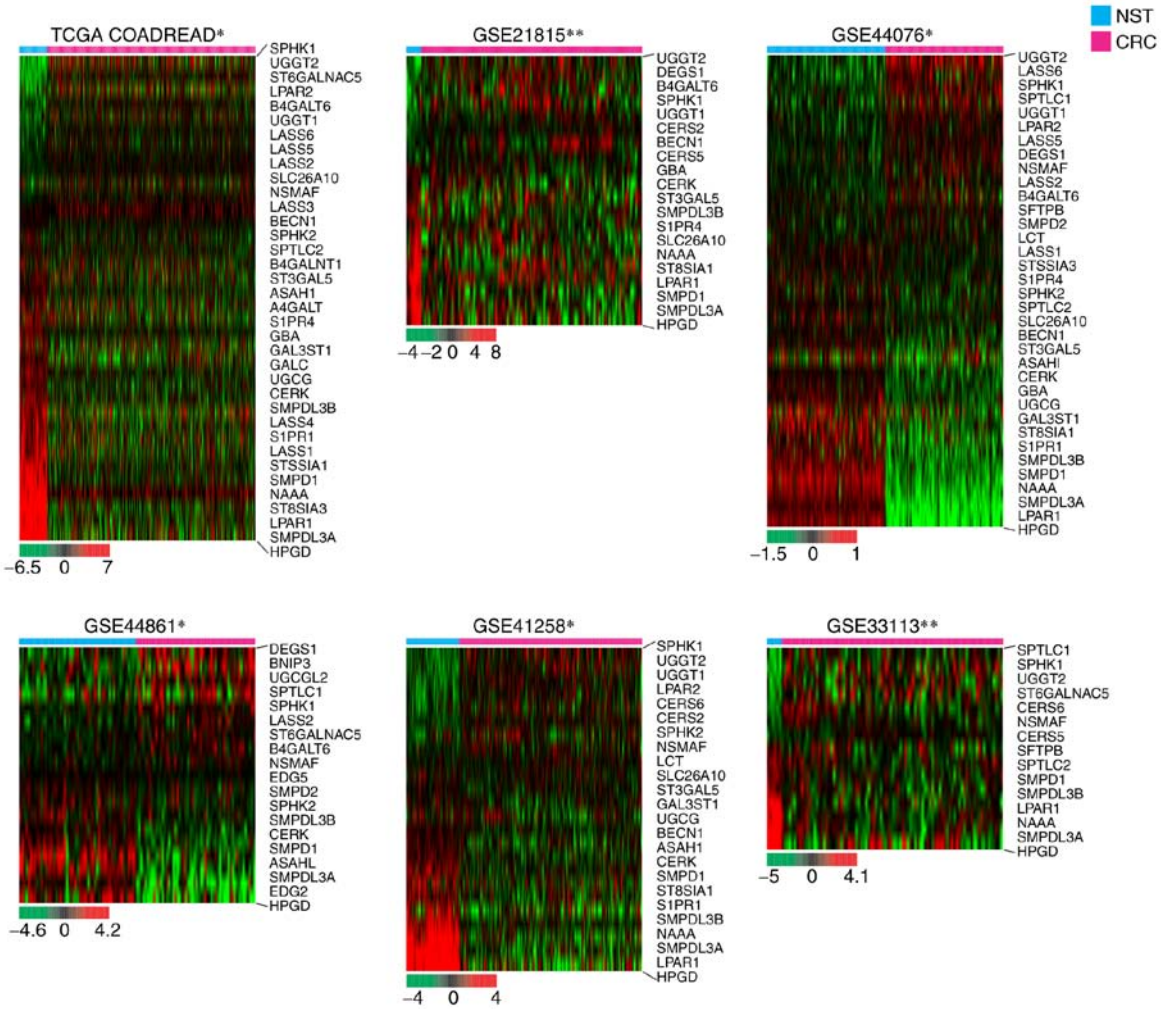

Figure 2. Heat-map showing significant differential expression of the sphingolipid metabolism-related genes in CRC tissues compared with NST of 6 independent CRC cohorts. The data are presented in matrix format in which rows represent individual genes and columns represent each tissue. Each cell in the matrix represents the expression level of a gene feature in an individual tissue. Expression levels have been standardized (centered and scaled) within columns for visualization. The red and green color in cells reflect relative high and low expression levels, respectively, as indicated by the scale bar. Images were obtained by re-analysis of the raw data of the respective cohort. Samples were sorted from left to right in the order of NSTs and CRC tissues according to the standardized expression level of each gene as indicated. *Student's t-test and **Mann Whitney U test; P<0.05. CRC, colorectal cancer; NST, non-neoplastic surrounding colon tissues; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer.

with CRC (Fig. 1). To identify the significance of altered mRNA expression levels between CRC and NST, Student's t-test or Mann Whitney U test were performed (P<0.05). As demonstrated in Fig. 2, hierarchical clustering revealed that various sphingolipid metabolism-related genes were significantly dysregulated in CRC tissues compared with NST from the same patient groups. The list of analyzed sphingolipid metabolism-related genes is presented in Table II. Sphingosine kinase 1 (*SPHK1*) and UDP-glucose glycoprotein glucosyltransferase 2 (*UGGT2*) were significantly upregulated in the CRC tissues of all cohorts, while 15-hydroxyprostaglandin dehydrogenase (*HPGD*), lysophosphatidic acid receptor 1 (*LPAR1*), N-acylethanolamine acid amidase (*NAAA*), sphingomyelin phosphodiesterase 1 (*SMPD1*) and sphingomyelin phosphodiesterase acid-like 3A (*SMPDL3A*) were significantly downregulated in the CRC tissues of all cohorts (Fig. 2 and Table II).

Dysregulation of various CerSs in six independent CRC cohorts. Next, the present study evaluated whether the mRNA expression levels of the four CerS genes, which are abundant in colorectal tissues (30), are dysregulated in human CRC specimens with respect to NST. As demonstrated in Fig. 3, among six cohorts, *CERS2* mRNA levels were significantly increased in five independent cohorts, while *CERS5* and *CERS6* were significantly upregulated in four independent cohorts. The specific platforms of each cohort and their associated studies are listed in Table III.

Altered CerS genes mRNA expression in Korean patients with CRC. To determine whether there is altered *CERS2*, *CERS4*, *CERS5* and/or *CERS6* mRNA expression in Korean patients with CRC, the expression levels of these four CerSs were measured using qPCR in 59 paired CRC and NST specimens from Korean patients. Following exclusion of unqualified results, the qPCR data were analyzed. The present study revealed that mRNA expression levels of all four CerS genes were significantly upregulated in CRC tissues compared with corresponding NSTs (*CERS2*, P<0.001; *CERS4*, P=0.006; *CERS5,* P<0.001; *CERS6*, P<0.001; Fig. 4; Table IV).

Table II. List of the analyzed genes involved in sphingolipid metabolism (Student's t-test, Mann Whitney U test; P<0.05).

Dataset	TCGA-COADREAD[a]	GSE21815[b]	GSE44076[a]	GSE44861[a]	GSE41258[a]	GSE33113[b]
No. analyzed genes	36	20	35	19	24	15
Gene symbol	A4GALT	B4GALT6	ASAH1	ASAHL (NAAA)	ASAH1	CERS5
	ASAH1	BECN1	B4GALT6	B4GALT6	BECN1	CERS6
	B4GALNT1	CERK	BECN1	BNIP3	CERK	HPGD
	B4GALT6	CERS2	CERK	CERK	CERS2	LPAR1
	BECN1	CERS5	DEGS1	DEGS1	CERS6	NAAA
	CERK	DEGS1	GAL3ST1	EDG2 (LPAR1)	GAL3ST1	NSMAF
	GAL3ST1	GBA	GBA	EDG5 (S1PR2)	HPGD	SFTPB
	GALC	HPGD	HPGD	HPGD	LCT	SMPD1
	GBA	LPAR1	LASS1	CERS2	LPAR1	SMPDL3A
	HPGD	NAAA	LASS2	NSMAF	LPAR2	SMPDL3B
	LASS1	S1PR4	LASS5	SMPD1	NAAA	SPHK1
	LASS2	SLC26A10	LASS6	SMPD2	NSMAF	SPTLC1
	LASS3	SMPD1	LCT	SMPDL3A	S1PR1	SPTLC2
	LASS4	SMPDL3A	LPAR1	SMPDL3B	SLC26A10	ST6GALNAC5
	LASS5	SMPDL3B	LPAR2	SPHK1	SMPD1	UGGT2
	LASS6	SPHK1	NAAA	SPHK2	SMPDL3A	
	LPAR1	ST3GAL5	NSMAF	SPTLC1	SMPDL3B	
	LPAR2	ST8SIA1	S1PR1	ST6GALNAC5	SPHK1	
	NAAA	UGGT1	S1PR4	UGCGL2 (UGGT2)	SPHK2	
	NSMAF	UGGT2	SFTPB		ST3GAL5	
	S1PR1		SLC26A10		ST8SIA1	
	S1PR4		SMPD1		UGCG	
	SLC26A10		SMPD2		UGGT1	
	SMPD1		SMPDL3A		UGGT2	
	SMPDL3A		SMPDL3B			
	SMPLL3B		SPHK1			
	SPHK1		SPHK2			
	SPHK2		SPTLC1			
	SPTLC2		SPTLC2			
	ST3GAL5		ST3GAL5			
	ST6GALNAC5		ST8SIA1			
	ST8SIA1		ST8SIA3			
	ST8SIA3		UGCG			
	UGCG		UGGT1			
	UGGT1		UGGT2			
	UGGT2					

[a]Student's t-test, [b]Mann Whitney U test. TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer.

Table III. mRNA expression levels of CerS gene in colorectal cancer tissues of patients from various datasets used in the present study.

Dataset	Platform	*CERS2*	*CERS4*	*CERS5*	*CERS6*	(Refs.)
TCGA (COADREAD)	RNA sequencing	Up	Down	Up	Up	(19)
GSE21815	Human Whole Genome Microarray 4x4K G4112F	Up	N/A	Up	N/A	(20)
GSE44076	Affymetrix Human Genome U219 Array	Up	N/A	Up	Up	(21)
GSE44861	Affymetrix HT Human Genome U133A Array	Up	N/A	N/A	N/A	(22)
GSE41258	Affymetrix U133A Array	Up	N/A	N/A	Up	(23)
GSE33113	Affymetrix Human Genome U133 Plus 2.0 Array	N/A	N/A	Up	Up	(24)

CERS, ceramide synthase; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer.

Table IV. mRNA expression levels of CerS gene in CRC tissues as compared with NST of Korean patients with CRC.

CERS family	*CERS2*		*CERS4*		*CERS5*		*CERS6*	
No. patients	59		55		49		55	
Type of tissue	NST	CRC	NST	CRC	NST	CRC	NST	CRC
Mean (ΔCq value)	5.85	4.26	5.68	4.43	8.89	7.85	7.26	4.51
Regulation	Up		Up		Up		Up	
P-value	<0.001		0.06		<0.001		<0.001	

Student t-test. CERS, ceramide synthase; NST, non-neoplastic surrounding colon tissues; CRC, colorectal cancer.

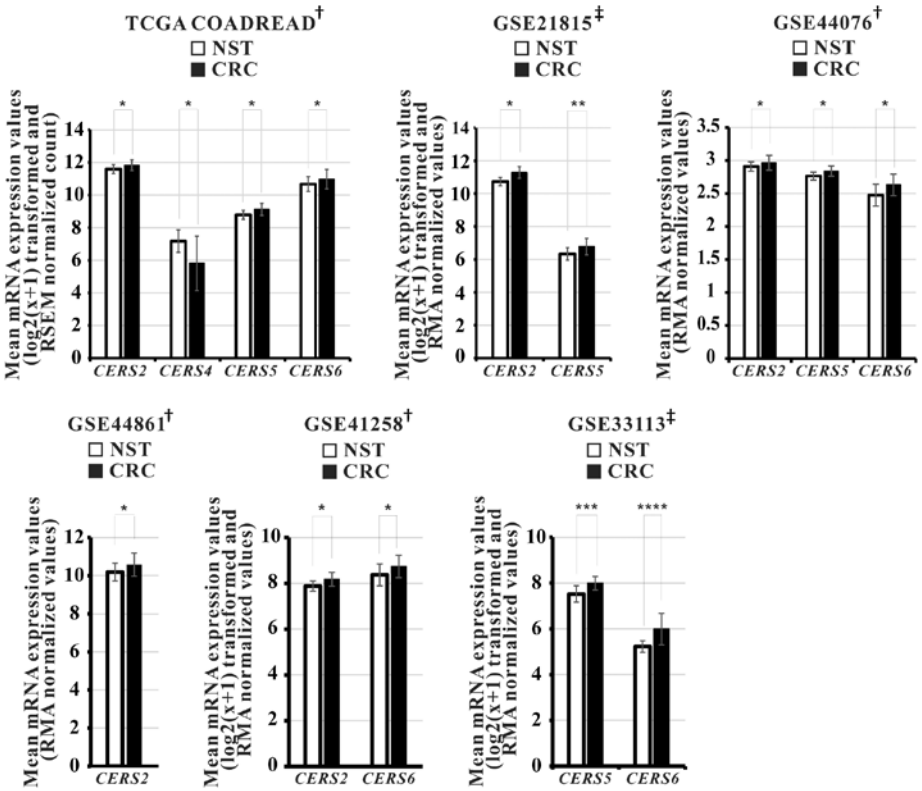

Figure 3. Relative mRNA expression levels of various ceramide synthases in the 6 independent CRC cohorts. Gene expression profiling datasets TCGA-COADREAD (NST, n=51; CRC, n=380), GSE21815 (NST, n=9; CRC, n=132), GSE44076 (NST, n=98; CRC, n=98), GSE44861 (NST, n=55; CRC, n=56), GSE41258 (NST, n=54; CRC, n=186) and GSE33113 (NST, n=6; CRC, n=90). P-values were calculated using †Student's t-test and ‡Mann Whitney U test, *P<0.001, **P=0.005, ***P=0.002, ****P=0.006. CRC, colorectal cancer; NST, non-neoplastic surrounding colon tissues; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer.

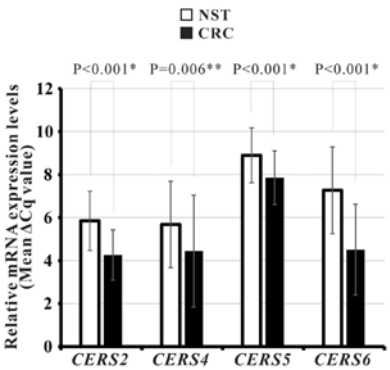

Figure 4. Relative mRNA expression levels of various ceramide synthases in CRC tissues and their corresponding NST of Korean patients. *Student's t-test. The graph is representative of three independent experiments. CRC, colorectal cancer; NST, non-neoplastic surrounding colon tissues; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer.

Exogenous CERS2 and CERS6 expression decreases the viability of human CRC cells. It has previously been observed that *CERS6*-overexpression reduces the proliferation of CRC cells and induces apoptosis, whereas *CERS2*-overexpression increases the proliferation of CRC cells (18). To confirm the effect of overexpressing CerSs in CRC cells, HCT116, HT29, SW403 and SW480 cells were transiently transfected with constructs to overexpress HA-*CERS2* and HA-*CERS6*, respectively. After 48 and 72 h, the numbers of viable cells were counted using a hemocytometer. As demonstrated in Fig. 5, overexpression of *CERS2* and *CERS6* decreased the viability of this panel of CRC cell lines.

Inter-individual associations between mRNA expression levels of CerS genes in patients with CRC. Combinational

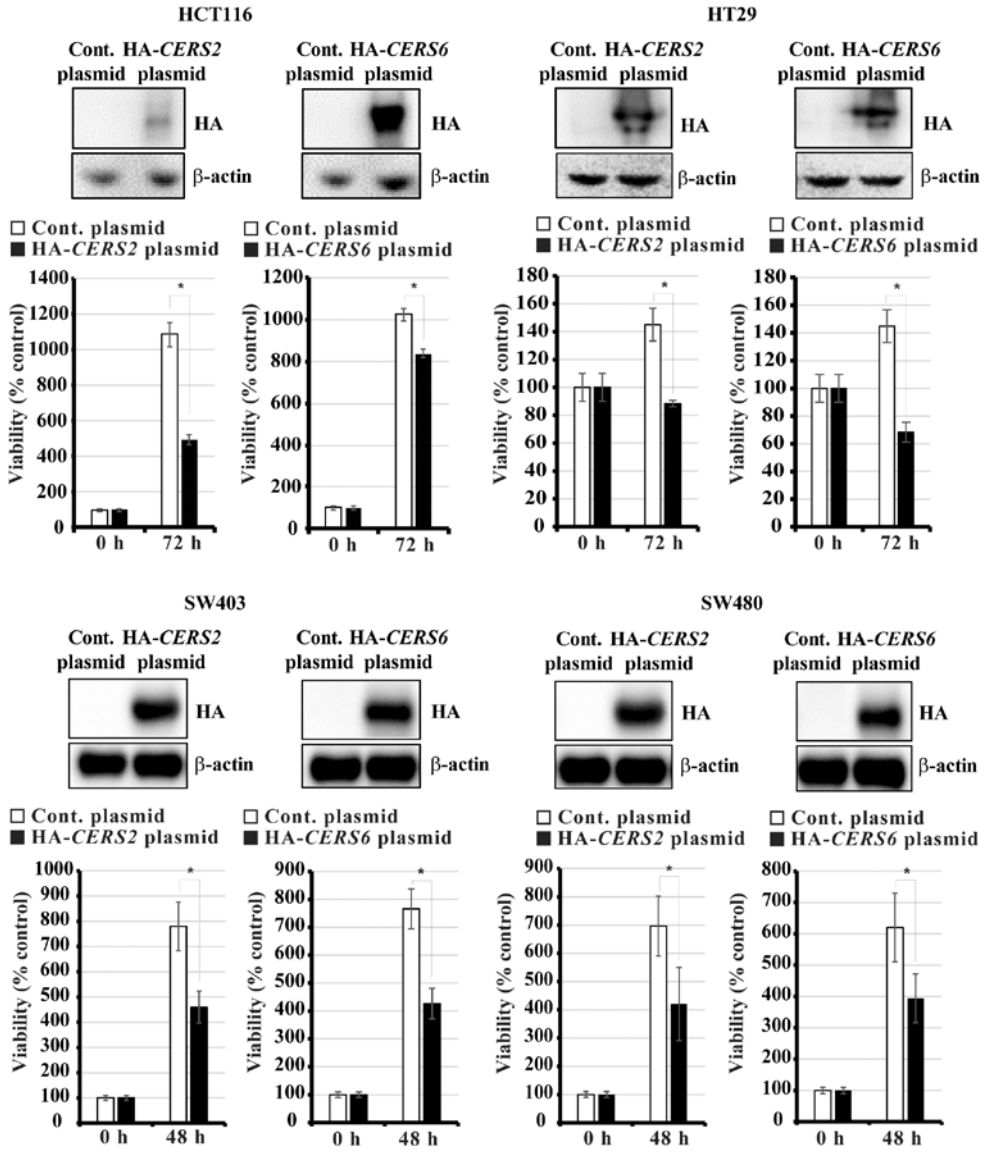

Figure 5. Effect of *CERS2*- or *CERS6*-overexpression on the viability of various colorectal cancer cell lines. HCT116, HT29, SW403 and SW480 cells were transiently transfected with control, HA-*CERS2* and HA-*CERS6* DNA constructs. At 24 h after transfection, cells were harvested and analyzed by western blot analysis with HA and β-actin antibodies. β-actin was used as a protein loading control. The viability of the cells were counted using a hemocytometer. Data are the mean values of three independent experiments and bars represent standard deviations. *P<0.05. CERS, ceramide synthase; Cont., control.

patterns of CerS gene expression, including CerS hetero-complexes and co-expression of CerS genes, serve important roles in sphingolipid metabolism (31,32). Therefore, associations between the mRNA levels of each CerS gene were identified in the TCGA-COADREAD cohort and in the Korean CRC cohort. Using cBioPortal to analyze the TCGA-COADREAD cohort, co-expression analysis revealed that *CERS4* and *CERS5* had high correlation coefficients (Pearson's correlation=0.36; Spearman's correlation=0.48; Fig. 6A). Next, these correlations were assessed using Pearson's correlation coefficient analysis in the 59 Korean patients with CRC. There were significant correlations between *CERS2* and *CERS4*, and also between *CERS5* and *CERS6,* with a Pearson's correlation coefficient value of 0.532 (P<0.001; Fig. 6B) and 0.439 (P=0.003; Fig. 6C), respectively. Furthermore, significant correlations between the mRNA expression levels of *CERS2* and *CERS4* (P=0.009) and of *CERS5* and *CERS6* (P<0.001) were identified using Fisher's exact test (Table V).

Association between mRNA expression levels of CerS genes and clinicopathological parameters of Korean patients with CRC. To determine the clinicopathological implications of dysregulated expression of specific CerS genes in CRC, the association between CerS gene mRNA level and clinicopathological characteristics, which are used to represent progression and aggressiveness, were evaluated. Prior to the statistical analysis, the 44 patients, whose clinical data were available, were classified according to each clinicopathological characteristic (Table V). The results obtained from the statistical analysis of the Korean cohort revealed that altered mRNA expression levels of CerS genes were not significantly associated with any clinical parameters, including sex, age, Tumor-Node-Metastasis stage, body mass index or carcinoembryonic antigen titer.

Table V. Association between mRNA expression levels of various CerS genes and clinicopathological parameters in Korean patients with colorectal cancer.

	CERS2 expression			*CERS4* expression			*CERS5* expression			*CERS6* expression		
Parameter	Low	High	P-value	Low	High	P-value	Low	High	P-value	Low	High	P-value
Sex			1.000[b]			0.195[b]			0.719[b]			1.000[b]
Male	24	4		17	11		20	8		22	6	
Female	14	2		13	3		13	3		13	3	
Age, years			0.606[b]			1.000[b]			0.408[b]			0.659[b]
≤50	8	2		7		3	9	1		9	1	
>50	30	4		23	11		24	10		26	8	
T stage			0.609[c]			0.675[c]			0.457[c]			0.140[c]
T1	2	0		2	0		0	2		0	2	
T2	7	1		4	4		8	0		7	1	
T3	24	4		21	7		20	8		23	5	
T4	5	1		3	3		5	1		5	1	
N stage			0.063[c]			0.061[c]			0.055[c]			0.288[c]
N0	25	2		21	6		18	9		21	6	
N1	7	1		5	3		6	2		5	3	
N2	6	3		4	5		9	0		9	0	
N3	0	0		0	0		0	0		0	0	
M stage			0.456[b]			0.581[b]			0.558[b]			0.566[b]
Negative	35	5		28	12		29	11		31	9	
Positive	3	1		2	2		4	0		4	0	
BMI			0.653[c]			0.320[c]			0.593[c]			0.566[c]
≤18.5	0	0		0	0		0	0		0	0	
18.5-24.9	1	0		1	0		1	0		1	0	
25-29.9	27	4		19	12		22	9		25	6	
>30	10	2		10	2		10	2		9	3	
CEA			1.000[b]			0.540[a]			0.706[b]			0.703[b]
>5	11	2		8	5		9	4		11	2	
≤5	27	4		22	9		24	7		24	7	
CERS2						0.009[b]			0.630[b]			1.000[b]
Low				29	9		29	9		30	8	
High				1	5		4	2		5	1	
CERS4			0.009[b]						0.456[b]			0.233[b]
Low	29	1					21	9		22	8	
High	9	5					12	2		13	1	
CERS5			0.630[b]			0.456[b]						<0.001[b]
Low	29	4		21	12					31	2	
High	9	2		9	2					4	7	
CERS6			1.000[b]			0.233[b]			<0.001[b]			
Low	30	5		22	13		31	4				
High	8	1		8	1		2	7				

[a]Pearson's χ^2 test; [b]Fisher's exact test; [c]Linear by linear association. CERS, ceramide synthase; BMI, body mass index; CEA, carcinoembryonic antigen.

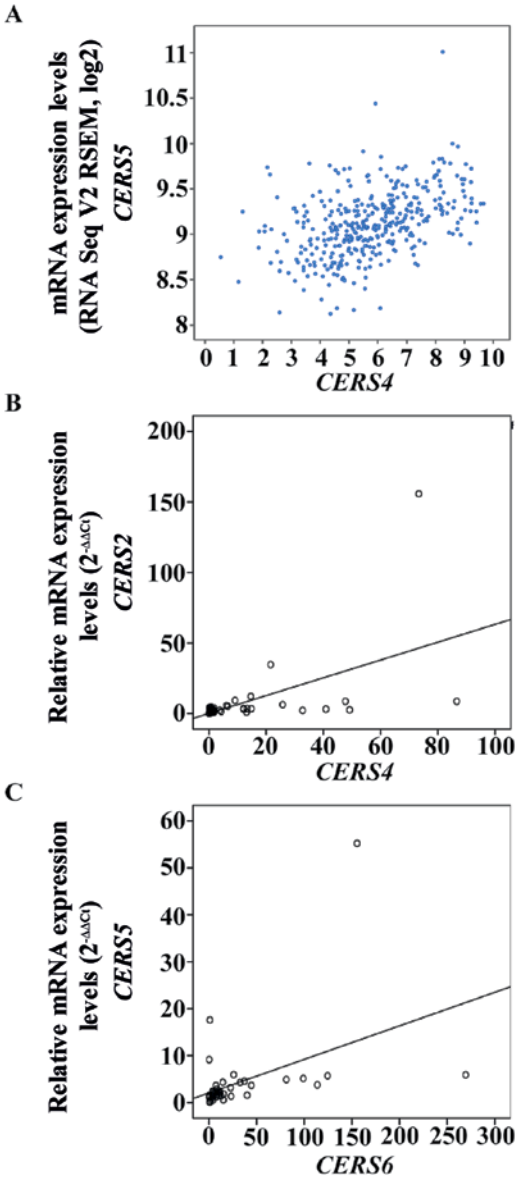

Figure 6. Correlation between mRNA expression levels of inter-individual CerS in the TCGA-COADREAD cohort and in the cohort of Korean patients with CRC. (A) *CERS4* and *CERS5* in the TCGA-COADREAD cohort. (B) *CERS2* and *CERS4* in the cohort of Korean patients with CRC. (C) *CERS5* and *CERS6* in the cohort of Korean patients with CRC. CERS, ceramide synthase; TCGA-COADREAD, The Cancer Genome Atlas Colon and Rectal Cancer; CRC, colorectal cancer.

Discussion

Sphingolipid metabolism serves a critical role in mammalian cell growth arrest and survival (33). Accumulating evidence have demonstrated that CerS, a major component in sphingolipid metabolism (7), regulates various biological phenomenon, including apoptosis (34), cancer (17,35), ER stress (36), hepatopathy (37), hypoxia/re-oxygenation injury (38), lipid metabolism (39), neurodegeneration (40), and sensitivity to chemotherapeutic drugs and radiation (30). Although aberrant CerS expression is correlated with cell death and proliferation (10-14) in various types of cancer, much uncertainty remains regarding the dysregulated mRNA levels of CerS gene in CRC and the clinical implications of this.

The aims of the present study were to investigate the mRNA expression levels and functions of CerS genes, which are primarily expressed in the intestine (30,41), and analyze their clinicopathological implications in patients with CRC. To begin with, significantly dysregulated sphingolipid metabolism-related genes were identified in the heat-maps of 6 independent CRC cohorts (Fig. 2). The hierarchical clustering results demonstrated considerable dysregulation of sphingolipid metabolism-related genes in CRC tissues compared with corresponding NST of independent CRC cohorts. Among the considerably altered genes, certain genes were overlapping over 6 independent cohorts. *SPHK1* and *UGGT2* were significantly upregulated in CRC tissues. This result is in accordance with those of recent studies that indicated that SPHK1 is overexpressed and serves an important role in tumorigenesis, proliferation, invasiveness and metastasis in CRC (42,43). On the other hand, *HPGD*, *LPAR1*, *NAAA*, *SMPD1* and *SMPDL3A* were all significantly downregulated in CRC tissues. HPGD is a cytoplasmic enzyme responsible for degrading PGE2 in colorectal tissue (44), and functions as a tumor suppressor gene in various types of cancer (45-48). The present study observed that *HPGD* was downregulated in 6 independent CRC cohorts (Fig. 2). However, little is known regarding the cellular functions and clinicopathological implications of *LPAR1*, *NAAA*, *SMPD1*, *SMPDL3A* and *UGGT2* in CRC. Therefore, further studies investigating the functional role of these genes in CRC are required as these transcripts may be diagnostic markers or promising therapeutic candidates.

Additionally, the differential mRNA levels of *CERS2*, *CERS4*, *CERS5* and *CERS6* in CRC and NST were analyzed in 1,001 patients with CRC from 6 independent publicly-available CRC cohorts and a cohort of Korean patients with CRC. The results of the present study should be interpreted with caution as qPCR, RNA-Seq and microarray are different experimental platforms with different sensitivities, principles and dynamic ranges. Nonetheless, the results revealed that *CERS2* was significantly upregulated in the majority of cohorts (Fig. 3; Table III) and in the cohort of Korean patients with CRC (Fig. 4; Table IV). It was recently demonstrated that *CERS2*-overexpression had no effect on the viability of HCT116 cells, whereas overexpressing *CERS2* plus the addition of very-long chain acyl-CoAs significantly enhanced colony formation in HCT116 cells (18). Unlike these previous results, the present study revealed that *CERS2*-overexpression reduced the viability of various human CRC cells, including HCT116 cells (Fig. 5). The primary technical differences between these two experiments are the culture time following transfection and the use of different expression plasmids. Additionally, knockdown experiments were performed using shRNAs against *CERS2* mRNA and *CERS6* mRNA, knockdown of CerS2 or CerS6 did not affect the proliferation of CRC SW403 and SW480 cells (data not shown). Although the exact mechanism that underlies the effect on cell viability was not elucidated in the present study, it is possible that sustained cell culture time following transfection may affect the synthesis of ceramides of various chain lengths.

It has been reported that increased expression of CerS6 and C16:0-Ceramide resulted in a sensitization of SW620 cells to TRAIL-induced apoptosis (49), and *CERS6*-overexpression significantly inhibited the colony formation capacity and increased the apoptosis of HCT116 cells (18). In accordance

with these previous results, the present study demonstrated that *CERS6* is significantly upregulated in CRC tissues, compared with NST (Figs. 3 and 4; Table IV) and *CERS6*-overexpression led to inhibition of cell viability in various human CRC cells (Fig. 5). Notably, controversial results have demonstrated that CerS6 and C16:0-Ceramide protected cells against ER-stress in human head and neck squamous cell carcinomas (36). Although oncogenes are usually upregulated in cancer tissues compared with non-neoplastic tissues, previous studies and the results presented in the present study indicated that the roles of CerSs and ceramides of specific chain lengths are complicated and cell type-dependent. Notably, it was demonstrated that *CERS4* was significantly upregulated, but only in the cohort of Korean patients with CRC (Fig. 4), while it was downregulated in the TCGA-COADREAD cohort (Fig. 3). Future studies specifically focused on *CERS4* in different CRC populations are required in order to understand this phenomena.

Additionally, the present study evaluated correlations between inter-individual mRNA expression levels of CerS genes and their clinicopathological implications in patients with CRC. A recent study revealed that inter-individual differences in the mRNA expression levels of CerS genes are significantly correlated with each other in cancer tissues (17). Furthermore, Combinational patterns of CerS expression are involved in sphingolipid metabolism (31,32). The results of analyzing correlations between inter-individual CerS genes mRNA expression levels revealed a correlation between *CERS4* and *CERS5* in TCGA-COADREAD, between *CERS2* and *CERS4*, and between *CERS5* and *CERS6* in the cohort of Korean patients with CRC. However, combinational patterns of CerS expression may be associated with sphingolipid metabolism. Therefore, it will be important to determine which components serve critical roles in sphingolipid metabolism in different disease and tissue settings. To the best of our knowledge, the present study was the first to investigate the clinicopathological implications of dysregulated CerS genes mRNA expression in CRC. However, no correlation was observed between mRNA expression levels of specific CerS genes and the investigated clinicopathological parameters.

In conclusion, the present study revealed that the mRNA expression levels of *CERS2*, *CERS4*, *CERS5* and *CERS6* were significantly upregulated or downregulated in various independent CRC cohorts, suggesting that dysregulated CerS gene expression may serve a role in CRC development.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request. The primary and processed data used to generate the analyses presented here can be downloaded by registered users from The Cancer Genome Atlas at http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp.

Authors' contributions

SWJ, WJP and SK contributed to the conception and design of the study, analysis of the data, interpretation of results and the writing of the manuscript. SWJ, WJP, HM and SK contributed to the acquisition of data. SWJ, WJP, HM, SKB, IH and SK performed the experiments. TKK contributed to the conception and design of the study. JWP and IH contributed to the conception and design of the study and provided guidance regarding the clinical implications of the study. WJP and SK reviewed and edited the manuscript. All authors read and approved the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The experimental study was approved by the Institutional Review Board of Keimyung University Dongsan Medical Center (approval no. 2015-11-059-001). Written informed consent was obtained from each study participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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