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Supplemental information

Metformin inhibits gastric cancer cell proliferation by regulation of a novel Loc100506691-CHAC1 axis

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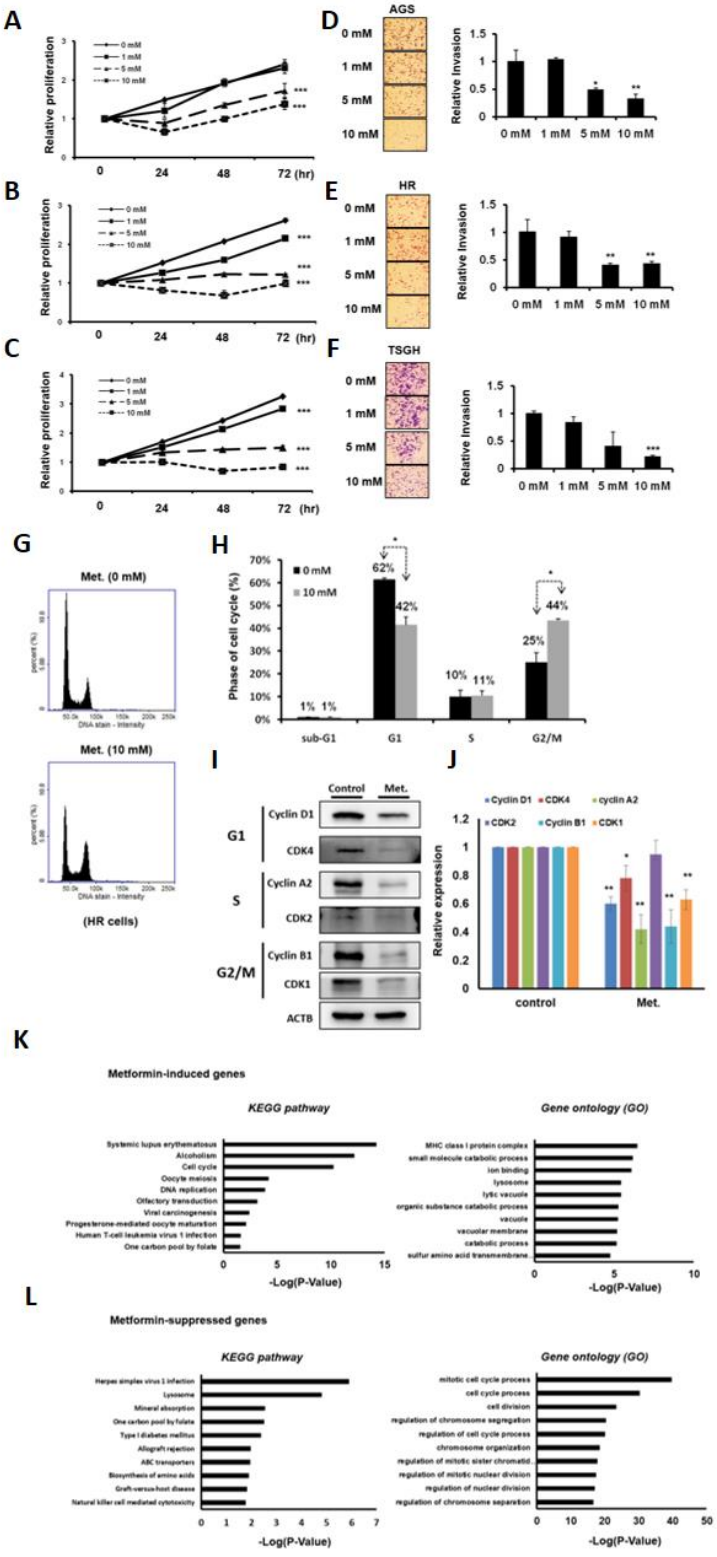
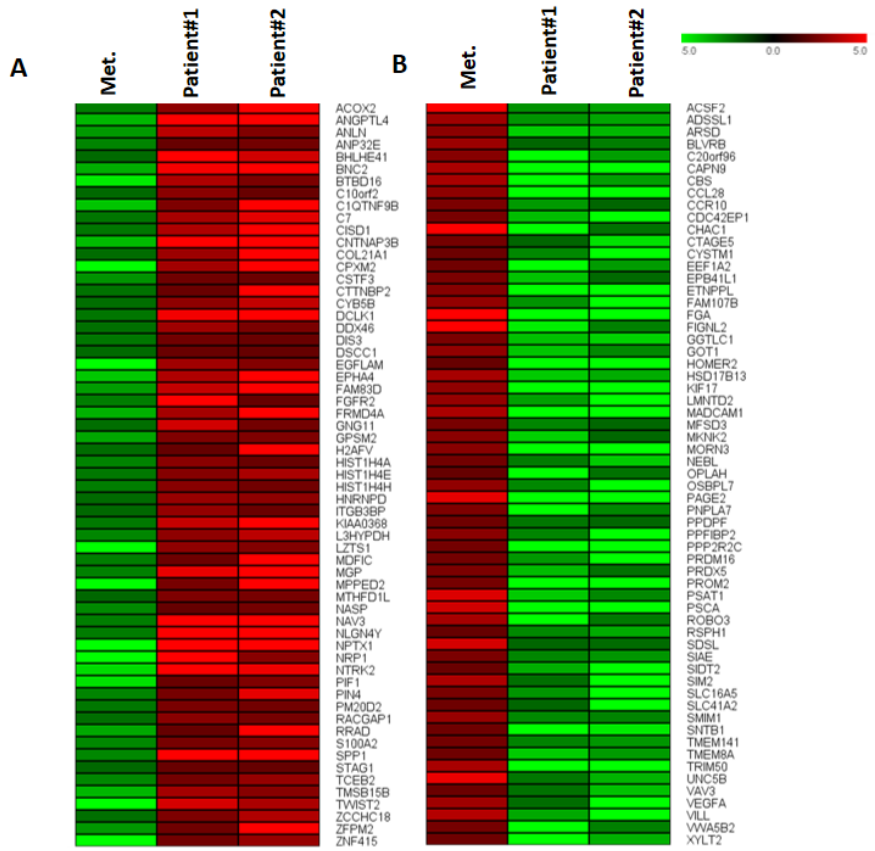


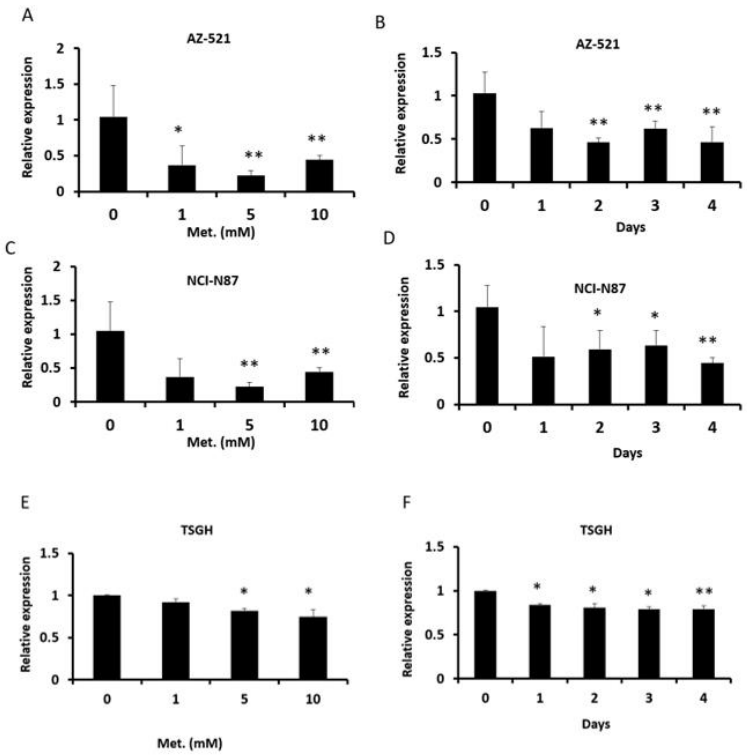
Supplementary Figure 1. Metformin could inhibit gastric cancer cell proliferation and motility ability. (A)–(C) AGS, HR, and TSGH were treated with various doses of metformin (0, 1, 5, and 10 mM). Next, cell proliferation was measured after 0, 24, 48, and 72 h. (D)–(F) Cell invasion ability was examined in

AGS, HR, and TSGH with various doses of metformin (left panel), and the relative invasion ability was quantified (right panels). (G) and (H) Cell cycle distributions of HR cells with and without metformin (10 mM) were analyzed and quantified through image flow cytometry. (I) and (J) Cell cycle-related genes were examined and quantified in HR with and without metformin through western blotting. (*$p < .05$, **$p < .01$, ***$p < .001$). (K) and (L) Metformin-induced and -suppressed genes were respectively subjected to Kyoto encyclopedia of genes and genomes (KEGG) and gene ontology (GO) pathway enrichment analysis.

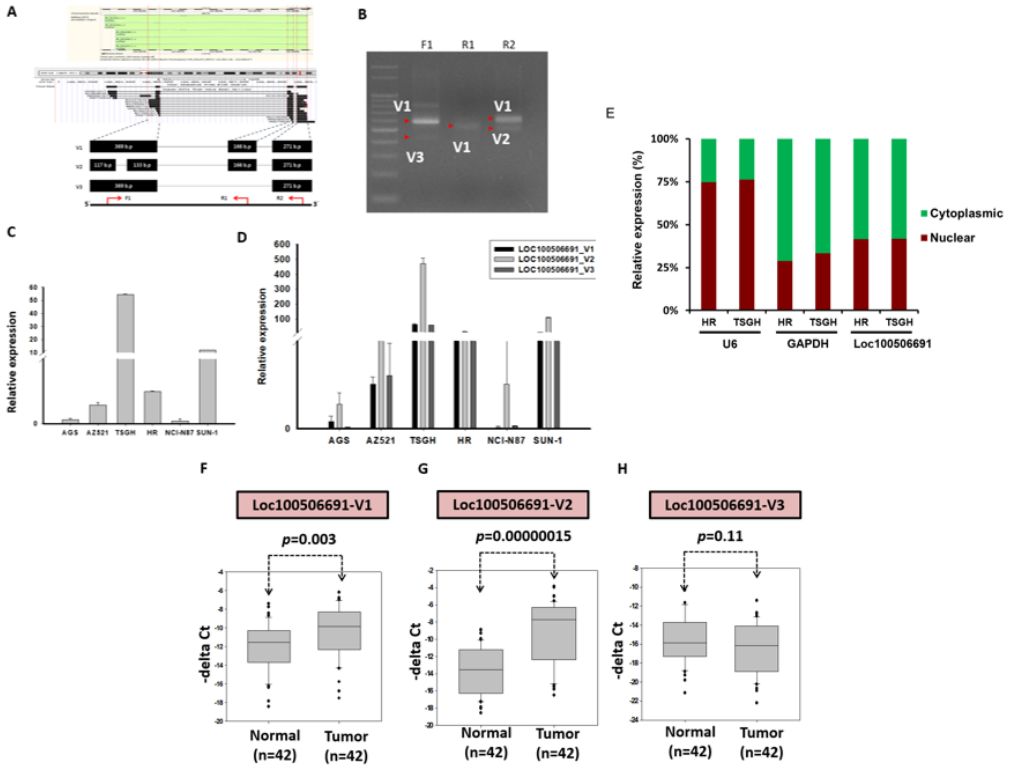


Supplementary Figure 2. Metformin-regulated protein-coding genes in gastric cancer identified using the microarray approach

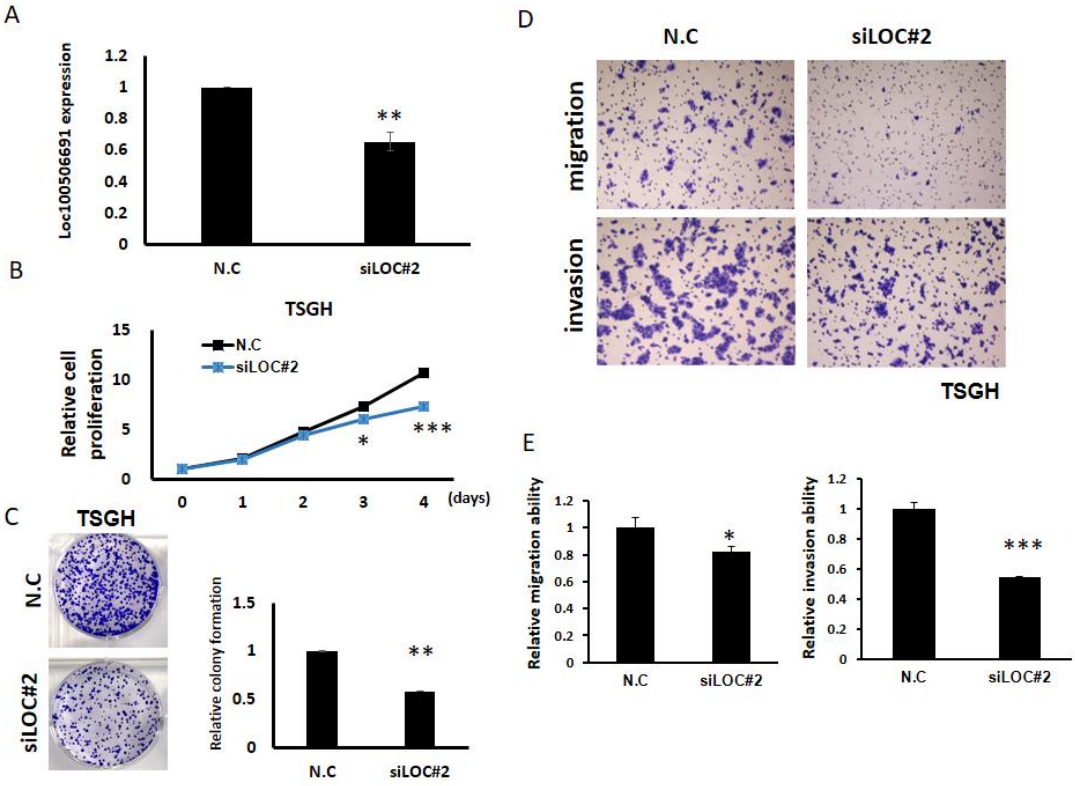
By combining the transcriptome profiles of gastric cancer and the corresponding adjacent normal tissue form two patients with gastric cancer, we precisely identified metformin-associated protein-coding gene candidates in gastric cancer. (A) and (B) Putative oncogenic and tumor-suppressive protein-coding gene candidates for regulating expression in HR with metformin treatment and two gastric cancers.



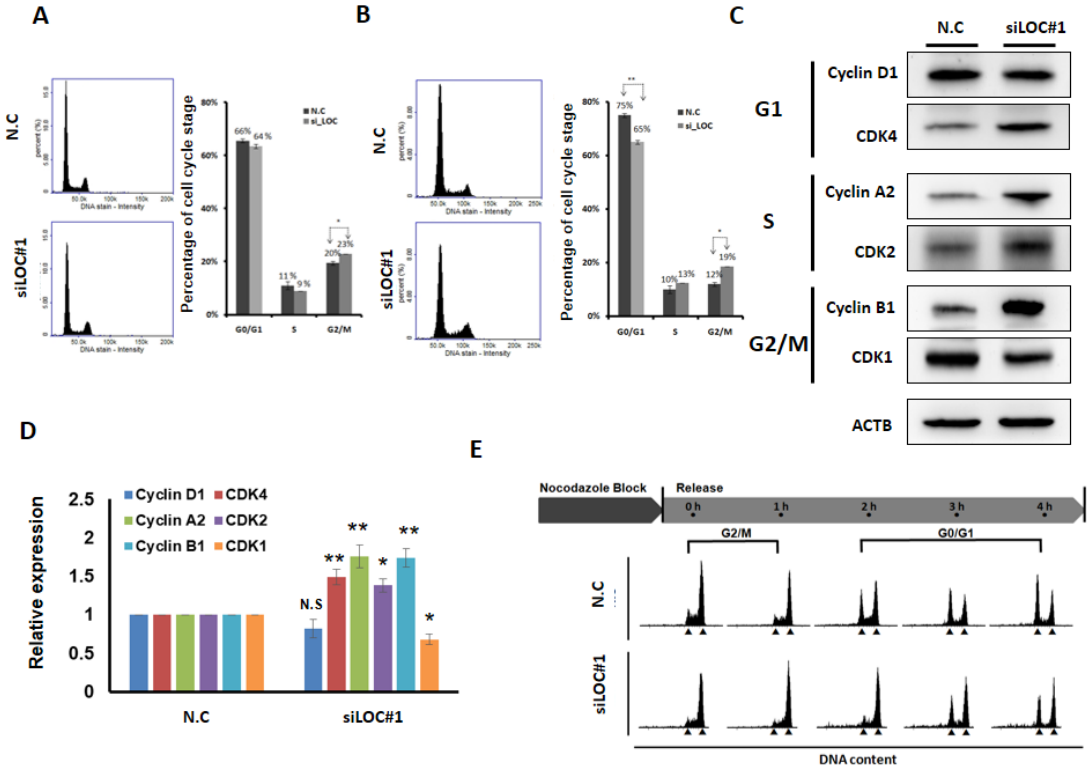
Supplementary Figure 3. Loc100506691 expression was suppressed by metformin treatment in gastric cancer cells. (A)–(C) Expression levels of Loc100506691 were examined using real-time PCR in AZ-521, NCI-N87, and TSGH cells following metformin treatment at various doses (0, 1, 5, and 10 mM) for 4 days. (D)–(E) Expression levels of Loc100506691 were examined using real-time PCR in AZ-521, NCI-N87, and TSGH cells following metformin treatment (10 mM) at different durations (0, 1, 2, 3, and 4 days). (*$p < .05$, **$p < .01$, ***$p < .001$).



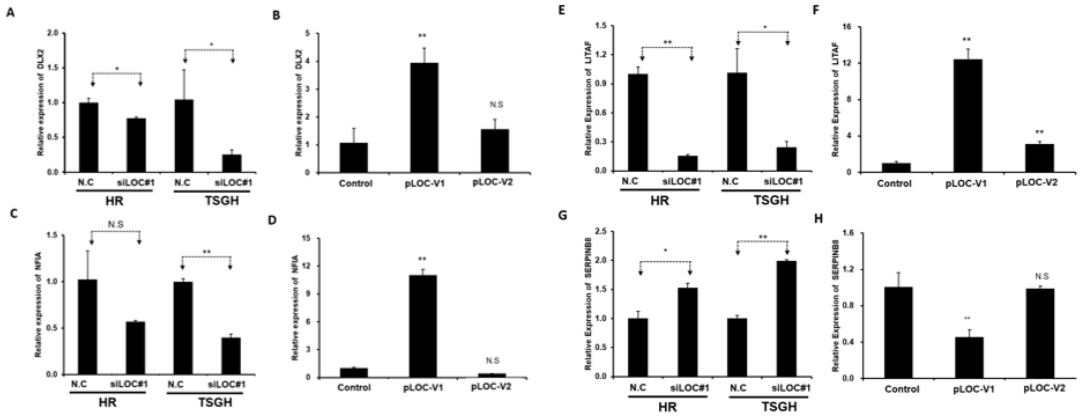
Supplementary Figure 4. Expression levels of Loc100506691 isoforms in human gastric cancer cells and gastric cancer tissues. (A) Locations of the Loc100506691 genes were determined by the UCSC Genome Browser of the human genome (upper panels). Expression sequence transcripts are presented in the lower panel. (B) Identification of the full length of Loc100506691 was performed by 5'- and 3'-RACE. (C) Expression levels of Loc100506691 were examined by employing the real-time PCR approach in human gastric cancer cells, namely AGS, AZ-521, HR, TSGH, NCI-N87, and SUN-1. (D) Expression levels of three isoforms were examined by performing real-time PCR in human gastric cancer cells. (E) The localization of Loc100506691 was evaluated in TSGH and HR cells. (F)–(H) Expression levels of three isoforms of Loc100506691 were examined in gastric cancer and compared with those of the corresponding adjacent normal tissue through real-time PCR.



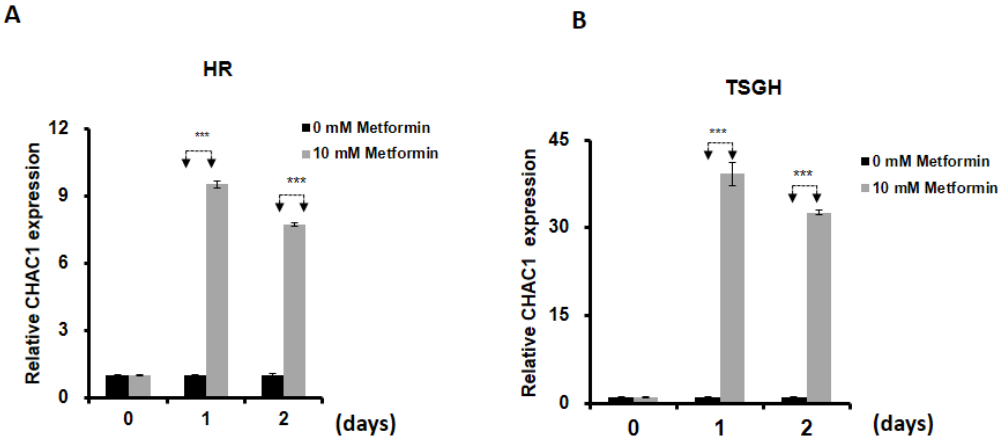
Supplementary Figure 5. Loc100506691 knockdown suppressed gastric cancer cell growth and motility. (A) and (B) Expression levels of Loc100506691 were examined in HR and TSGH after siLOC#2 transfection. (C)-(E) Cell proliferation, colony formation ability, migration and invasion ability were assessed in TSGH with siLOC#2 transfection. (*$p < .05$, **$p < .01$, ***$p < .001$).



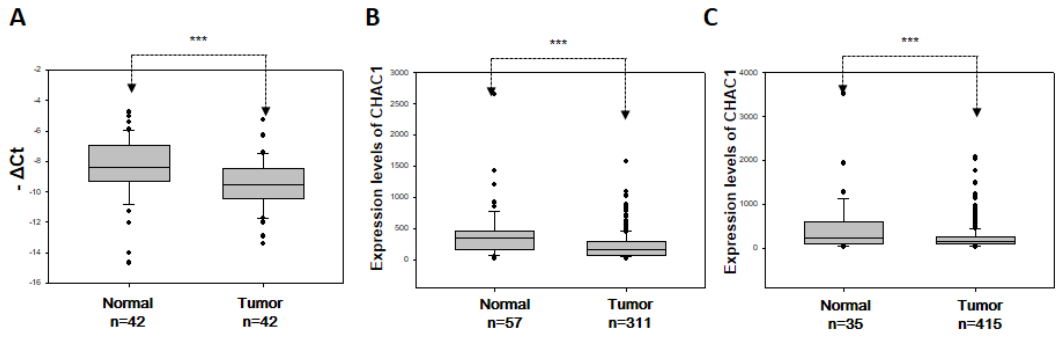
Supplementary Figure 6. Knockdown of Loc100506691 impaired gastric cancer cell growth by inducing cell cycle arrest in the G2/M phase (A) and (B) The cell cycle of HR and TSGH was examined following Loc100506691 knockdown for 48 h. (C) and (D) Expression levels of cell cycle-related genes were examined and quantified using Western blotting. (E) The cell cycle of TSGH was synchronized using a nocodazole block for 14 h, and the cells were subsequently released and analyzed at 1, 2, 3, and 4 h. (*$p < .05$, **$p < .01$, ***$p < .001$).



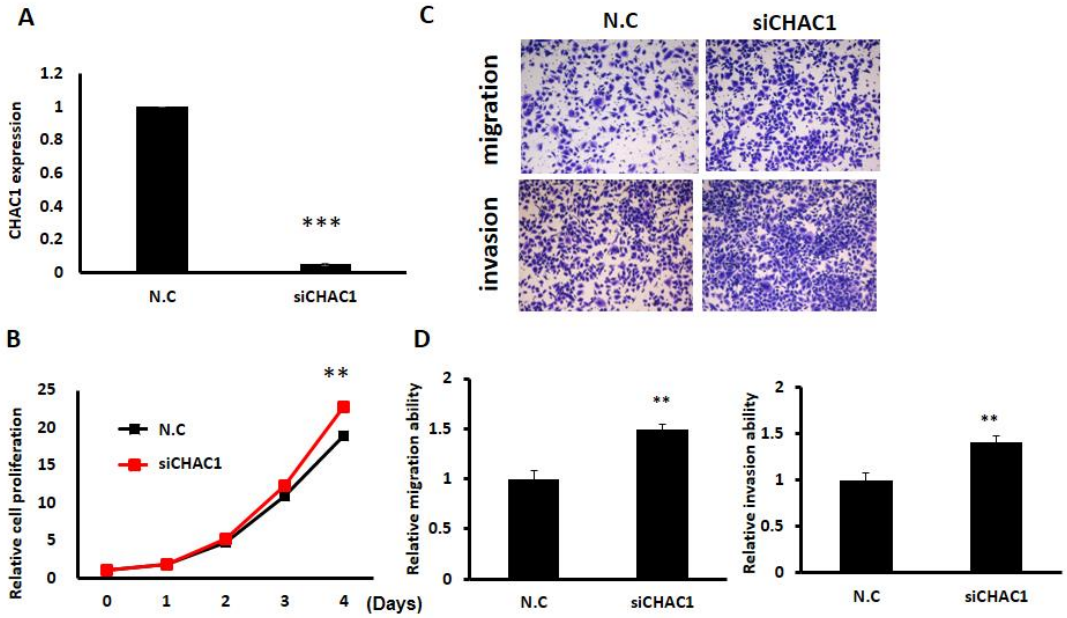
Supplementary Figure 7. Identification of putative downstream genes of Loc100506691 in gastric cancer by adopting a microarray approach. Expression levels of DLX2 (A), NF1A (C), LITAF (E), and SERPINB8 (G) were examined through real-time PCR in TSGH and HR cells with Loc100506691 knockdown. Expression levels of DLX2 (B), NF1A (D), LITAF (F), and SERPINB8 (H) were assessed in HR cells with pLoc100506691-V1 or-V2 overexpression by adopting the real-time PCR approach. (*p < .05, **p < .01, ***p < .001).



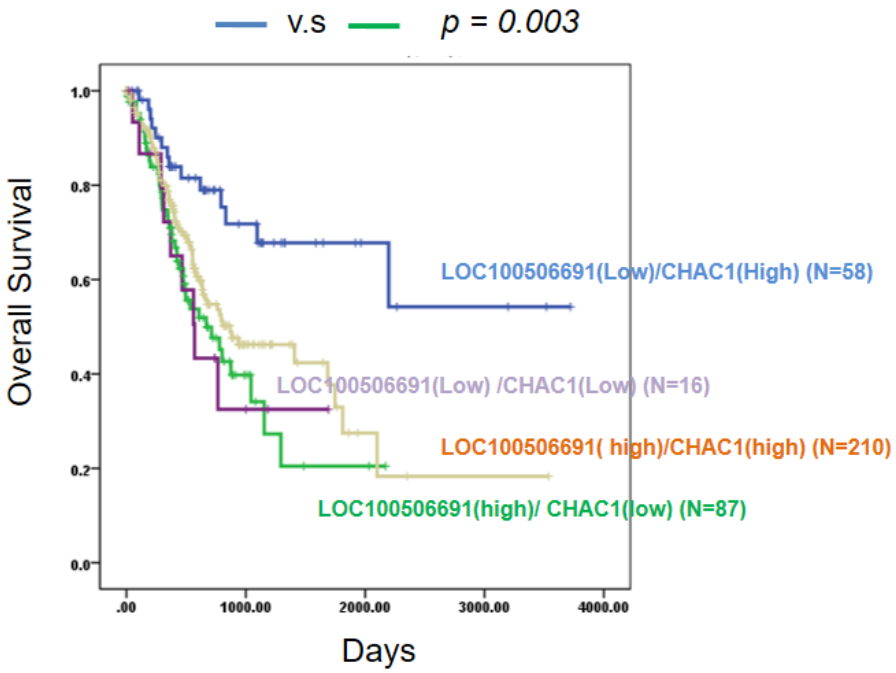
Supplementary Figure 8. Expression levels of CHAC1 were upregulated in gastric cancer cells following metformin treatment (A) and (B) CHAC1 expression levels were assessed using real-time PCR in HR and TSGH following metformin treatment. (*p < .05, **p < .01, ***p < .001).



Supplementary Figure 9. Expression levels of CHAC1 assessed in gastric cancer. (A) Expression levels of CHAC1 were examined through real-time PCR in gastric cancer and compared with those of the corresponding adjacent healthy tissue. (B) and (C) Expression levels of CHAC1 obtained from the TCGA and GENT databases were analyzed.



Supplementary Figure 10. CHAC1 knockdown suppressed HR cell growth and motility. (A) CHAC1 expression levels were examined in HR after siCHAC1 transfection. (B)-(D) Cell proliferation, migration and invasion ability were assessed in HR with siCHAC1 transfection. (*$p < .05$, **$p < .01$, ***$p < .001$).



Supplementary Figure 11. The combination of high Loc100506691 and low CHAC1 expression levels was highly correlated with the poor survival curve of patients with gastric cancer. Patients with gastric cancer were separated into two groups representing high and low Loc100506691 or CHAC1 expression on the basis of best cutoff value (Loc100506691: 0.184 and CHAC1: 2.06).

Supplementary Table 1. Correlation of Loc100506691 expression with clinicopathological characteristics of 360 patients with gastric cancer.

Variables	Loc100506691 (n=360)			
	No. (%)	Mean±SD	Median	p-value
Pathology stage				
I	54 (15.0)	0.82±1.14	0.36	0.893[a]
II	116 (32.2)	0.92±1.26	0.46	
III	151 (41.9)	0.86±0.99	0.55	
IV	39 (10.8)	0.79±0.78	0.54	
pT stage				
T1	19 (5.3)	0.77±0.93	0.45	0.274[a]
T2	77 (21.4)	0.76±1.07	0.35	
T3	165 (45.8)	0.82±0.99	0.52	
T4	99 (27.5)	1.04±1.25	0.72	
pN stage (n=357)				
N0	114 (31.9)	1.03±1.39	0.53	0.658[b]
N1	94 (26.3)	0.83±0.99	0.45	
N2	75 (21.0)	0.83±0.93	0.52	
N3	74 (20.7)	0.70±0.75	0.51	
pM stage				
M0	335 (93.1)	0.86±1.10	0.49	0.800[c]
M1	25 (6.9)	0.92±0.87	0.55	

[a]*p-value were estimated by one-way ANOVA test.*
[b]*p-values were estimated by Kruskal-Wallis 1-way ANOVA test.*
[c]*p-value were estimated by student's T test.*

Supplementary Table 2. Univariate and multivariate Cox's regression analyses of Loc100506691 expression for overall survival of patients with gastric cancer.

Characteristic	No. (%)	OS			
		CHR (95% CI)	P-value	AHR (95% CI)	P-value
Loc100506691	(n=)				
Low	71 (19.9)	1.00		1.00	
High	285 (80.1)	1.82 (1.15-2.88)	0.011	1.86 (1.17-2.95)	0.009

Abbreviation: OS, Overall survival; CHR, crude hazard ratio; AHR, adjusted hazard ratio

AHR were adjusted for AJCC pathological stage (II,III and IV VS. I).

Supplementary Table 3. The sequence of three isoforms of Loc100506691was examined by Sanger sequencing.

Isoforms	Sequence
V1 (806bp)	5- AAAGAATTCTGCTGGAGCATCTGGAGTTGCTCAAATGGAGTGGCTGTGGCCATCTTTCCTTACACGTGGGGAGGAATCAGCCTGAGAATGAAACCATCAC AGGAAAGGGCTGAAAGACGGTGAGTGTGTGCTTTCTAAGGGCAGCATTTGGGCCTTGGCCCCAGCCGGCCCTGAAGCCAGCACAACCCCCGGAACGTC TCAATTTGTGAGCCAATAAATGCCCTTGATGGTTTAGGCAAGTTTTCGCTGGGTCTTACCCGACGTGAGCCCCCACTCCTCCATATGGACCTGTTTTGGACC AATGAGGCATCCCCTTCTGTAGTCCTCAACACGCGGAGCTCCACCACTCCTGAGCAGTGTGACCTCAGGTGTGACCTCAGGTGCTTGCTGCAGAGGCATC GGGGGTCTCTGGCCAGAGGTGACATCTGAAGCAATCGGGATCCTGTTTGGTTTTGCCACATCCGACCTGCCGCCCAGCTGGGGCAAGACAGCCACGCGC GGCGGATGCACCGGCCCTGAACTCTTTCTCGAGTAGTCCCCAGAGATTTCAGGAAGAGAGAGAAGAGGTAAAGAGACCAGAAACAAAGTTTTTTGCAA GCAAAGAACTGCAACAGGCCCCAGCTGTTAGGTTTGAGGGGCGTATGGGGCAGGCGACTGAACTGCAGAGTTGGAGCCAGAGGTACCATTGCTGACCC CTGAGCCCAGGAGCCTTGAGAATACTGACAGTGTTAATAATAACTGCAATCATCCAACATATGATGCTAGAATAACTGGACATCTGTGTGCAAAAATATTAAA CCTATACCTTGCACCTT-3
V2 (685bp)	AAGAATTCTGCTGGAGCATCTGGAGTTGCTCAAATGGAGTGGCTGTGGCCATCTTTCCTTACACGTGGGGAGGAATCAGCCTGAGAATGAAACCATCACA GGAAAGGGCTGAAGACGGCAAGTTTTCACTGGGTCTTACCCGACGTGAGCCCCCACTCCTCCATATGGACCTGTTTTGGACCAATGAGGCATCCTCTTCT GTAGTCCTCAACACGCGGAGCTCCACCACTCCTGAGCAGTGTGACCTCAGGTGCTTGCTGCAGAGGCATCGGGGGTCTCTGGCCAGAGGTGACATCTGA AGCAATCGGGATCCTGTTTGGTTTTGCCACATCCGACCTGCCGCCCAGCTGGGGCAAGACAGCCACGCGCGGCGGATGCACCGGCCCTGAACTCTTTCTC GAGTAGTCCCCAGAGATTTCAGGAAGAGAGAGAAGAGGTAAAGAGACCAGAAACAAAGTCTTCTGCAAGCAAAGAACTGCAACAGGCCCCAGCTGTTA GGTTTGAGGGGCGTATGGGGCAGGCGACTGAACTGCAGAGTTGGAGCCAGAGGTACCATTGCTGACCCCTGAGCCCAGGAGCCTTGAGAATACTGACA GTGTTAATAATAACTGCAATCATCCAACATATGATGCTAGAATAACTGGACATCTGTGTGCAAAAATATTAAACCTATACCTTGCA CCTT
V3 (640bp)	AAGAATTCTGCTGGAGCATCTGGAGTTGCTCAAATGGAGTGGCTGTGGCCATCTTTCCTTACACGTGGGGAGGAATCAGCCTGAGAATGAAACCATCACA GGAAAGGGCTGAAAGACGGTGAGTGTGTGCTTTCTAAGGGCAGCATTTGGGCCTTGGCCCCAGCCGGCCCTGAAGCCAGCACAACCCCCGGAACGTCT CAATTTGTGAGCCAATAAATGCCCTTGATGGTTTAGGCAAGTTTTCACTGGGTCTTACCCGACGTGAGCCCCCACTCCTCCATATGGACCTGTTTTGGACCA ATGAGGCATCCTCTTCTGTAGTCCTCAACACGCGGAGCTCCACCACTCCTGAGCAGTGTGACCTCAGATTTCAGGAAGAGAGAGAAGAGGTAAAGAGAC CAGAAgCAAAGTCTTCTGCAAGCAAAGAACTGCAACAGGCCCCAGCTGTTAGGTTTGAGGGGCGTATGGGGCAGGCGACTGAACTGCAGAGTTGGAGC CAGAGGTACCATTGCTGACCCCTGAGCCCAGGAGCCTTGAGAATACTGACAGTGTTAATAATAACTGCAATCATCCAACATATGATGCTAGAATAACTGGAC ATCTGTGTGCAAAAATATTAAACCTATACCTTGCACCTT

Supplementary Table 4. Univariate and multivariate Cox's regression analyses of Loc100506691and CHAC1 expression for overall survival of patients with gastric cancer.

Characteristic	No. (%)	OS			
		CHR (95% CI)	P-value	AHR (95% CI)	P-value
(n=371)					
LOC100506691 low , CHAC1 high	58 (15.6)	1.00		1.00	
LOC100506691 high, CHAC1 low	87 (23.5)	1.48 (1.04-2.11)	0.031	1.49 (1.04-2.13)	0.029
LOC100506691 high, CHAC1 high	210 (56.6)	1.08 (0.78-1.49)	0.648	1.10 (0.79-1.52)	0.586
LOC100506691 low , CHAC1 low	16 (4.3)	1.44 (0.73-2.83)	0.288	1.33 (0.67-2.61)	0.417

Abbreviation: OS, Overall survival; CHR, crude hazard ratio; AHR, adjusted hazard ratio

AHR were adjusted for AJCC pathological stage (II,III and IV VS. I).

Supplementary Table 5. The Primers used in this study of qPCR

Primer	**5'~3'**
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG
Loc100506691-F1	GTCCCCAGAGATTTCAGGAAGAG
Loc100506691-F2	TGAGCCAATAAATGCCCTTG
Loc100506691-F3	GAAAGGGCTGAAAGACGGCA
Loc100506691-R1	CCATACGCCCCTCAAACCTAA
Loc100506691-R2	ACAGGATCCCGATTGCTTCA
Loc100506691-R3	TCTCTCTTCCTGAAATCTGAG
H19-F	ATC GGT GCC TCA GCG TTC GG
H19-R	CTG TCC TCG CCG TCA CAC CG
RBMS3-AS3-F	GAGCAAACAACTGCATATGGCT
RBMS3-AS3-R	TGGCATTGGAGACTATTGTGTT
CHAC1-F	GTGTGGTGACGCTCCTTGAA
CHAC1-R	TGGTATCGTAGCCACCAAGC
DLX2-F	CCTTACTCCGCCAAGAGCAG
DLX2-R	TCCTCCTTCTCAGGCTCGTT
NF1A-F	ACAGGACCCAGAGCAAGTCC
NF1A-R	CAACATTGGGGTGGGAAGGA
LITAF-F	CCATCCGCACCTCCATCCTA
LITAF-R	TGGATAGGGCGGTCCAAAAA
SERPINB8-F	TATTCACCGAGGTTTCCAGTCA
SERPINB8-R	TCCAGCTCTGCCTGATAGAAC
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
miR-26a-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCCTATC
miR-330-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCCTAAGA

miR-26a-5p-GSF	CGGCGGTTCAAGTAATCCAGGA
miR-330-5p-GSF	CGGCGGTCTCTGGGCCTGTGTC
Universal-R	CTGGTGTCGTGGAGTCGGCAATTC