Detection of lncRNAs Involved in Gastric Cancer Development and Carcinogenic Mechanism

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The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Hyo joo Byun

December 2020

This certifies that the Doctoral Dissertation

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December 2020

ACKNOWLEDGEMENTS

3 년반의 박사학위과정을 마무리하며 감사한 분들께 짧은 글로 감사의 마음을 전합니다. 어릴 적 초등학교 장래희망에 적어두었던 과학자라는 꿈을 한 계단 계단 밟아가는 과정에 도움을 주셨던 모든 분들께 감사의 말씀을 전합니다.

먼저, 정말 큰 꿈이었던 박사학위를 성취하게끔 이끌어 주신 지도교수님 이상길 교수님께 감사드립니다. 교수님의 자상한 지도와 가르침으로 학위과정을 뜻깊게 마치게 되었습니다. 부족한 저에게 연구와 배움의 기회를 주신 것에 대해 큰 감사를 드립니다.

아울러 진료 및 연구활동으로 바쁘신 와중에도 매 심사과정중에 깊은 관심과 가르침으로 학위논문을 지도해주신 심사위원 교수님들, 남기택 교수님, 김현실 교수님, 김지현 교수님, 김현기 교수님께 감사 드립니다.

학위과정 동안 응원하여 주셨던 분들에게도 감사의 인사를 이글을 통해 전합니다. 박사과정이라는 시작과 끝, 매 순간에 많은 배움과 가르침을 주셨던 윤정호 선생님, 진심으로 감사드립니다. 또한 함께 실험에 대한 고민을 하고 박사과정을 진행했던 서승인 선생님, 많은 디스커션을 해주시는 정다현 선생님, 황보람선생님, 이소담 선생님, 유병민 선생님께도 감사의 말씀 전합니다.

힘들 때마다 힘이 되어주던 자랑스러운 고등학교친구들 아란이, 주혜, 지혜, 은비, 소영이, 그 누구보다도 뒤에서 진심으로 응원과 기도해주던 선아 그리고 실험적으로나 내적으로 항상 전폭적인 지지를 해주던 현지, 지현이, 가연언니, 혜린이, 실험실 선배로써 많이 부족하지만 잘 따라와주고 있는 착한 서연이에게도 고마움을 전합니다.

영원한 제 편이시고 이세상 누구보다 저를 사랑하시는 아버지, 어머니께 항상 존경하고 감사하고 사랑한다는 말을 이 글을 통해 전합니다. 감사한 마음을 글로써 다 표현하지 못하지만 앞으로도 항상 자랑스러운 딸이자 아버지, 어머니와 같은 훌륭한 사람이 되기 위해 노력하겠습니다.

물심양면으로 많은 도움을 주시고 넘치는 사랑을 주시는 아버님, 어머님, 지윤언니와 슬기아주버님께도 진심으로 감사의 말씀을 드립니다.

인생에 중요한 순간들 마다 큰 조언을 주는 석주오빠에게도 쑥스러워 한번도 꺼내지 못한 고맙다는 말을 전합니다. 또한 부모님만큼 큰 기대와 지지를 해주셨던 큰고모님, 미국 큰아버지, 한국 큰아버지, 작은고모님, 하늘나라에 계신 그리운 외할머님께도 감사의 말씀 올립니다.

마지막으로 박사학위 시작과 함께 모든 순간을 함께하였던 사랑하는 남편이자 가장 친한 친구이자 오빠인 박상호, 연애할 때에 장난으로 졸업식 때 꼭 함께하겠다고 했던 약속을 이제 부부로써 함께 할 수 있어 더욱 뜻깊고 앞으로도 지금처럼 항상 사랑하고 아끼고 배려하겠다고 다시 한번 되새기며 감사의 말을 전합니다.

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ABSTRACT

Detection of lncRNAs Involved in Gastric Cancer Development and Carcinogenic Mechanism

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(Directed by Professor Sang Kil Lee)

Gastric cancer is one of the most common tumors worldwide. Endoscopic screening has led to an increase in the rate of diagnosis of early gastric cancer, however when patients experience symptoms of gastric cancer and had endoscopy, there is a high probability of advanced gastric cancer, and the accuracy of serum markers in gastric cancer is significantly lower than that of other carcinomas. So that implication of therapeutic targets and diagnostic markers for gastric cancer is needed. Recently, Long non-coding RNAs

(lncRNAs) have been suggested the regulation of gene transcription in various cancers.

To understand the biological process including tumorigenesis, progression, and diversity related to lncRNAs in gastric cancer, we performed microarray analysis on 3 human gastric normal tissues (Group 1), 3 gastric normal tissues with single cancer (Group 2) and paired adjacent normal (Group 3), gastric cancer tissues (Group 4) with multiple cancer. These results were divided into 4 groups to select candidate lncRNAs for each stage of gastric cancer development, and comparative analysis was conducted.

In first comparison, lncRNAs, which is up-regulated in Group 3 verse Group 2 was selected to reveal the methylation status of early gastric cancer. In second comparison, we sorted the intersection lncRNAs which is upregulated in Group 4 verse Group 1 and Group 4 verse Group 3 that contributes to the progression of cancer development. After grouping organization, reverse transcription-quantitative polymerase chain reaction was used to validation in cohorts and further functional studies.

Thus, the first comparison results showed that lncRNA, CACS9 and LINC01186 resulted in methylation of tumor suppressor genes (GAS1, PCDH10, RUNX3) related to early gastric cancer.

In second comparison, LINC00853 were upregulated in gastric cancer tissues compared to paired adjacent normal tissues and promotes the epithelial to mesenchymal transition (EMT) process through MAP17- PDZK1-AKT pathway.

In particular, this study showed the expression of lncRNAs in different types of human gastric cancer that the analyzing of molecular mechanism including tumorigenesis, oncogenic function and also stated that the implication for improving lncRNA-based diagnosis biomarkers and therapeutics approach in gastric cancer.

Key words: Long non-coding RNA, LINC00853, LINC01186, CASC9, Microarray, Gastric cancer

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I. INTRODUCTION

Gastric cancer is one of the most aggressive gastrointestinal malignancy in worldwide, despite of decline in incidence and mortality[1,2]. With the popularization of national early cancer screening programs, the prognosis for patients with early gastric carcinoma has a 5 years survival rate greater than 90%. However, majority of gastric cancer patients have advanced with malignant proliferation and metastatic diseases at diagnosis[3,4]. Tumor markers, such as CEA, CA 19-9 and CA 72-4 are not preferred for gastric cancer screening

due to their low sensitivity and false positives[5,6]. Therefore, there is a need for a biomarker capable of specifically detecting early stages and explaining new mechanisms associated with gastric cancer metastasis in advanced stages in gastric cancer.

Long noncoding RNA (lncRNA) is transcripts that contains longer than 200 nucleotides that do not code the proteins. It have been exerted the function of biological process including cancer development by which modulates the gene expression through transcription regulation, post-transcription regulation, chromatin modification[7-10]. Additionally, lots of research have found that abnormal lncRNA expression contributes to the mechanism of tumorigenesis and metastasis[11-13]. However, there are relatively few papers on the discovery of lncRNA for detection in early gastric cancer and fewer papers have been expanded to advanced gastric cancer.

The progression of adenoma-carcinoma in gastric tumorigenesis is expected to present in a subset of series and resulted in multiple events of genetic modifications[14,15]. Therefore, a molecular research approach to all stages of gastric cancer progression is needed. Dozens of results showed that epigenetic activation of tumor suppressor genes increases in precancerous lesion and may result in early events contributing to gastric tumorigenesis[16-19]. In the studies of advanced gastric cancer, plenteous lncRNAs were proposed as biomarker through systematic assessment of metastasis and clinical analysis for high-risk gastric cancer patients[20], blocking the EMT processes[21], regulating histone-modifying complexes[22], or identifying molecular mechanisms involved in the gastric cancer

progression[23].

Microarray provides a comprehensive approach, which means non-coding lncRNA and protein-coding mRNA expression datasets on the same panel to allow their co-expressional and correlational studies to profile gene expression[24]. In this reason, it is widely used for lncRNA detection in various carcinomas, such as breast[25], heart[26], bladder[27], liver[28], and prostate[29] etc. as validation for accurate lncRNAs. However, the identification of lncRNAs in a series of development processes from early gastric cancer to advanced gastric cancer and its cancer related mechanisms have not been discovered yet.

In this study, Firstly, we performed lncRNA microarray analysis using different types of total 12 gastric samples. Next, we divided into 4 groups to assume the expression of lncRNAs at various stages in gastric cancer. (Group 1 indicates healthy normal tissue, Group 2 means adjacent normal tissue from single cancer, Group 3 reveals adjacent normal tissue from multiple cancer and Group 4 shows cancer tissue from multiple cancer) Finally, we sorted 2 lncRNAs (LINC01186, CASC9), which is upregulated in adjacent normal tissue from multiple cancer compared to adjacent normal tissue from single cancer, associated with early gastric cancer progression and lncRNA, LINC00853, which is upregulated in paired gastric cancer tissues compared to adjacent normal tissues, associated with advanced gastric cancer with metastasis.

We showed that LINC01186 and CASC9 regulated the methylation of RUNX3, PCDH10, GAS1, tumor suppressor genes to illustrate the effect on early stage of gastric tumorigenesis.

we also determined that a lncRNA, LINC00853 elevated cancer progression by modulating PDZK1/AKT signaling pathways via directly cis-acting with MAP17. Thus, LINC01186, CASC9 and LINC00853 has the potential to become a new biomarker for diagnosis and therapy in diverse gastric cancer developmental stages.

II. MATERIALS AND METHODS

1. Gastric cancer Patients and tissue sampling

One hundred fresh gastric cancer tissue and paired adjacent gastric normal tissue samples were collected from 100 patients who underwent surgical resection for gastric cancer at Severance Hospital, Yonsei University College of Medicine. All samples were frozen in liquid nitrogen immediately after resection and stored at −80°C until use. All tissue samples were obtained after receiving written informed consent from patients according to the Declaration of Helsinki, and this study was approved by Institutional Review Board (IRB) of the Yonsei University College of Medicine (#4-2011-0753).

2. Cell lines and cell culture

Gastric cancer cell lines and normal cell line were purchased from the Korean Cell Line Bank (KCLB, SNU, Seoul, Korea) and the American Type Culture Collection (ATCC,

Rockville, MD, USA). The cells were cultured in RPMI-1640 medium or DMEM medium (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Scientific). All cells were incubated in a humidified atmosphere of 5% CO2 at 37℃.

3. Small interfering RNA (siRNA) transfection

For transfection, AGS, MKN28 and MKN74 cells (3×10^5) were seeded in 6-well plates and incubated in a 37°C incubator. After 24 hours, the cells were treated with each targeted lncRNAs siRNA 50 uM and RNAi negative control (50 uM, siCT; Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. siRNAs used for loss of function are represented in Table 1.

Table 1. siRNAs sequences for targeting lncRNAs

Name	**Direction**	**Sequence (5' to 3')**
LINC01186_1	Forward	5'- CCACUGGGUGAAACUGCAA -3'
	Reverse	5'- UUGCAGUUUCACCCAGUGG -3'
LINC01186_2	Forward	5'- GGUGAAACUGCAAGUCGAG -3'
	Reverse	5'- CUCGACUUGCAGUUUCACC -3'
CASC9_1	Forward	5'- GCCCAGAAGACAGUGGAAU -3'
	Reverse	5'- AUUCCACUGUCUUCUGGGC -3'
CASC9_2	Forward	5'- GACAGUGGAAUGAGAUCUU -3'
	Reverse	5'- AAGAUCUCAUUCCACUGUC -3'
LINC00853_1	Forward	5'- GGAGGGCUCCCAGGACC -3'
	Reverse	5'- GGUCCUGGGAGCCCUCC -3'
LINC00853_2	Forward	5'- CCUGAUCUAAUGGAUCC -3'
	Reverse	5'- GGAUCCAUUAGAUCAGG -3'
MAP17	Forward	5'- GGAACAGAUGGAAGGUACU -3'
	Reverse	5'- AGUACCUUCCAUCUGUUCC -3'

4. Construction of LINC00853 overexpression plasmid

THE LINC00853 was amplified by a PCR system (Roche Applied Science). To insert the cDNA into the pcDNA3.1 (+) expression vector,

LINC00853_NheI_F(acccaagctggctagcGCCGCGCCTGAAGCTCAACT),
LINC00853_Xbal_R (aaacgggccctctagaTGACACATGCAGAAATACTAT), were used as cloning primers. The pcDNA3.1 (+) expression vector was purchased from Addgene. AGS cells were transfected with 1 μg of pcDNA3.1-LINC00853 for 24 hours via Lipofectamine 2000 (Invitrogen).

5. Mutagenesis Assay

Mutagenesis of the MAP17 binding site in LINC00853 was conducted using aQ5 Site-Directed Mutagenesis kit.
LINC00853_NheI_F acccaagctggctagcGCCGCGCCTGAAGCTCAACT,
LINC00853_Xbal_R aaacgggccctctagaTGACACATGCAGAAATACTAT were used as the cloning primers to insert the wild-type (WT) LINC00853 cDNA into pcDNA3.1.

LINC00853-Mut_AS: CTGCTCTGAGCAAAATGGAAGACGATGGC,
LINC00853-Mut_S: GCCATCGTCTTCCATTTTGCTCAGAGCAG
primers were used to create LINC00853-Mut. LINC00853-Wt and LINC00853-Mut sequences were confirmed by Sanger sequencing.

6. Treatment of AGS and MKN74 cell with 5-aza-CdR

For 5-Aza-2′-CdR (Sigma-Aldrich, St. Louis, Missouri, USA) treatment, a 10 mM stock solution was prepared by dissolving the compound in DMSO, which was then diluted in cell culture medium to the indicated concentrations. AGS and MKN74 cells (2.5 × 105) were seeded into a 6-well culture plate on day 0 and exposed to 0, 10 μM 5-aza-CdR (Sigma-Aldrich) for 3 days. The cells treated with 5-aza-CdR were harvested and used for detection of cell proliferation.

7. Total RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from gastric cancer tissues and cell lines using TRIzol reagent (Invitrogen). RNA was quantified using an Nanodrop (ND-100; NanodropTechnologies lnc., Wilmington, DE, USA), and purity was determined rely on the 260/280 nm ratio and products were loaded on 1% agarose gel. For cDNA synthesis, 2.0 μg of total RNA was used reverse transcribed with SuperscriptTMII (Invitrogen) following the manufacturer's protocol. The relative level of LINC001186, CASC9 and LINC00853 were measured by real-time PCR using iQ SYBR Green Supermix (Applied Biosystems Inc, Carlsbad, CA, USA). The Ct value of the sample was normalized to the U6 or GAPDH expression, and the $2^{-\Delta\Delta}$Ct value was calculated. Primers used for qRT-PCR are represented in Table 2.

Table 2. Primer sequences for qRT-PCR

Gene	Direction	Sequence (5' to 3')
LINC00704	Forward	5'- CACATACGCACACATCCACA-3'
	Reverse	5'- TCCTGGGAAGCTTGCTTTTA -3'
RP11-273G15.2	Forward	5'- GCCATTGGGAACTTTCTTCA-3'
	Reverse	5'- AGGACGCTTCGGTCTCTGTA-3'
RP11-73M7.1	Forward	5'- GCACAAGTTGGTCTGCTTGA-3'
	Reverse	5'- TGGACAAGCCAGAGAGTCCT-3'
LINC01186	Forward	5'- CCCCTTCTAAAACGGGAAAG-3'
	Reverse	5'- AAGTCACATCTGGCCCAAAC-3'
CASC9	Forward	5'- GCTGTAGACCGGAGCTGTTC-3'
	Reverse	5'- AACACCATGAATGTGGCTGA-3'
GAS1	Forward	5'-CGGAGCTTGACTTCTTGGAC-3'
	Reverse	5'-CGTCCTGAACACTGCAGCTA-3'
RUNX3	Forward	5'-TAGGAAGCACGAGGAAAGGA-3'
	Reverse	5'-CAGATGAGTGCAGCAGGTGT-3'
PCDH10	Forward	5'-CCCAGTCAGCTGGTATGGAT-3'
	Reverse	5'-GCAGATTGCTGCGATAATCA-3'
PTEN	Forward	5'-CATAACGATGGCTGTGGTTG-3'
	Reverse	5'-CCCCCACTTTAGTGCACAGT-3'
LINC00853	Forward	5'-CAGAAAAGCTCCCGAAACTG-3'
	Reverse	5'-TTCCTTTGCCGGTAAAATTG-3'
LINC00634	Forward	5'-CTTGGAACTGGTGAGGGTGT-3'
	Reverse	5'-CATCTCATCTCCCCATGCTT-3'
LINC01535	Forward	5'-TGAATGCAGCTTTCTTGGTG-3'
	Reverse	5'-CAGGCTGATGGGGTATCTGT-3'
GAPLINC	Forward	5'-GTTTCCTGGAAGGGCATTTT-3'
	Reverse	5'-GTGCCTGAGTCCAGCTTCTC-3'
RP11-57A1.1	Forward	5'-GAGGCAACACTGCAGATGAA-3'

	Reverse	5'-AGAGGTTTCTGGCTGACTCG-3'
RP11-326C3.15	Forward	5'- GTGCTCCAGACCTTTTCCTG-3'
	Reverse	5'-TGCACCCATCAACACAGACT-3'
RP11-561O23.8	Forward	5'-GTGCTCCAGACCTTTTCCTG-3'
	Reverse	5'-TGCACCCATCAACACAGACT-3'
CYP4A22	Forward	5'-TTCAGCACGTCTCCTTGATG-3'
	Reverse	5'-ATGGCCTGGATGTAGGACTG-3'
MAP17	Forward	5'-TCAGGTCCAGTGAGCATGAG-3'
	Reverse	5'-ACTGGACATCCATCCCATGT-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCAGGAATTTGCGT-3'
GAPDH	Forward	5'-CCGGGAAACTGTGGCGTGATGG-3'
	Reverse	5'- AGGTGGAGGAGTGGGTGTCGCTGTT-3'

8. Cell proliferation analysis

Gastric cancer cells were transfected with 50uM siLINC01186, siCASC9, siLINC00853, siMAP17 and siCT from 0 to 72 hours. Cell proliferation was performed by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI, USA) in 96-well culture plates at time dependent condition. The plate was allowed to react with MTS reagent for 1 hour in the dark and products of the reaction were measured with a spectrophotometric plate reader set at 490 nm (Multiskan™ Microplate Photometer, Thermo Scientific). For rescue experiments, cells were transfected with pcDNA-LINC00853 at 48 hours after siRNA transfection, and cell proliferation was measured.

9. Apoptosis analysis

AGS and MKN74 cells were transfected with LINC00853 siRNA or siCT, and the cell pellet was isolated after 48 hours. The cell pellet was resuspended in 1 × binding buffer (BD Bioscience, San Jose, CA, USA) and phosphate-buffered saline (PBS). Cells were stained with propidium iodide and fluorescein isothiocyanate (FITC) annexin V using a FITC-Annexin V kit (BD Bioscience). For analysis by flow cytometry using a FACS verse instrument (BD Biosciences), stained cells were incubated at for 15 minutes. Data were analyzed using Flow Jo software (Treestar, Ashland, OR, USA). Three experiments were conducted for each assay. For apoptosis analysis, 2 gastric cancer cell lines were washed with PBS and resuspeded in 1× binding buffer (BD biosciences). Fluorescein

isothiocyanate (FITC) Annexin V and propidium iodide staining was conducted by the FITC Annexin V detection kit (BD biosciences) according to the manufacturer's protocol. The ratio of percentage apoptosis was measured by flow cytometry (BD biosciences).

10. Cell cycle analysis

Transfected AGS cells and MKN74 cells were washed with PBS and fixed with 75% ethanol at −20°C overnight. Cells were resuspended in PBS and treated with RNase for 30 minutes at room temperature. The nuclei of the fixed cells were stained with 50 mg/ml PI (Sigma-Aldrich) in the dark for 20 minutes and then determined via flow cytometry (BD Biosciences).

11. Scratch wound healing assay and Invasion assay

AGS cells and MKN74 cells transfected with siLINC00853s and siMAP17 or siCT were re-seeded in 6 well cultured plates. When cells organizied an approximately 60%~80% confluency, scratch the bottom of well using a P-20 tip equally. The width of scratched cells was measured at 0, 24 hours or 48 hours by microscopy. For invasion assay, using the same cell line and condition above and replated on BD BioCoat trans-wells (BD Biosciences) followed to the manufacturer's protocol. After 24 hours or 48 hours, non-invading cells within the insert chamber were removed and the upper layer of the trans-well was wiped

with a cotton swab briefly. Fixing the membrane of the bottom part of the upper chamber with 5% acetaldehyde buffer and stained with Crystal violet solution. The invading cells on the membrane were counted under a bright-field microscope.

12. Soft agar colony formation assay

To analyze tumorigenicity in vitro, base and top agarose were coated in 96well cultured plates using CytoSelect™ 96-Well Cell Transformation Assay (CELL BIOLABS, INC, San Diego, CA, USA). 1.5ml of 2X DMEM containing 1% Agarose was poured into each well as a base layer. After 1h solidification, the transfected AGS cells were resuspended in 2X DMEM containing 0.7% Agarose as a top layer and maintained in 37°C incubator for 2-3 weeks. After daily observation, colonies were captured under bright field microscopy.

13. Western blot

Transfected cells with siLINC00853 and siMAP17 or siCT were lysed in 1X RIPA buffer (Cell Signaling Technology, MA, USA) containing protease inhibitor. Isolated proteins were loaded by 8–15% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked for 1 hour at room temperature in tris–phosphate buffer containing

0.1% Tween 20 with 5% BSA (BD biosciences), and incubated with primary antibodies at 4°C cold room for overnight.

The following primary antibodies were used for Western blot analysis: epithelial marker E-cadherin (1:1000, BD biosciences); mesenchymal marker N-cadherin (1:1000, BD biosciences), Vimentin (1:200, Santa Cruz Biotechnology, Texas, DA, USA, sc-373717), Snail (1:1000, Cell Signaling Technology, Danvers, MA USA, # 3879S), PARP (1:1000, Cell Signaling Technology, #9542), Bcl-xl (1:1000, Cell Signaling Technology, #2764), Bax (1:1000, Santa Cruz Biotechnology, sc-493), Caspase-9 (1:1000, Cell Signaling Technology; 9504), MAP17 antibody (1:1000, abcam, ab156014), Phospho-Akt (Ser473) antibody (1:1000, Cell Signaling Technology, #12694), Anti-PDZK1 (1:1000, abcam, Cambridge, UK, ab121248) and LaminB (1:4000, Santa Cruz Biotechnology, sc-374015) and β-actin (1:5000, Bioworld Technology, Louis Park, MN, USA, AP0060). The signal was developed in ECL solution (GenDEPOT, Barker, TX, USA) and exposed to an Image Quant LAS 4000 bio-molecular image for 2 minutes.

14. RNA immunoprecipitation (RIP)

Cells were lysed with IP buffer (Thermo Fisher Scientific) and resuspended in RIP buffer (Abcam) with mixtures contained RNase inhibitor (GenDEPOT) and protease inhibitor (GenDEPOT). For shearing of chromatin, 20 cycles (for each cycle 170–190 W) of shearing under cooling conditions with sonicator. The antibodies were applied to the

supernatant and then incubated overnight at 4°C with a rotator. After incubation, 20 μL of MagnaChip protein magnetic beads (Millipore) was added and reacted on a rotator at 4°C for 1 hour. After washing twice with RIP buffer, samples were dissolved with TRIzol reagent or RIPA buffer for further studies.

15. Methylation-specific PCR (MS-PCR)

Genomic DNA was extracted AGS and MKN74 cells using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). An EZ DNA methylation-gold kit (ZymoResearch, Irvine, CA, USA) was performed for DNA bisulfate transformation. Primers were designed for specific methylated and unmethylated sites. Primers used for MS-PCR are represented in Table 3.

Table 3. Primer sequences for MS-PCR

Gene	Direction	Sequence (5' to 3')
GAS1	Forward	5'-AGTGAGTTTTTTCGTTATTATCGG-3'
Methylation	Reverse	5'-AAATATCTTCATAAATCCATACGCG-3'
GAS1	Forward	5'-AGTGAGTTTTTTTTGTTATTATTGG-3'
Unmethylation	Reverse	5'-AAATATCTTCATAAATCCATACACACC-3'
RUNX3	Forward	5'-ATAGATGGGGTAGGATTTAGTACGT-3'
Methylation	Reverse	5'-AAAACCTACCCTATAAACCTAAACG-3'
RUNX3	Forward	5'-ATAGATGGGGTAGGATTTAGTATGT-3'
Unmethylation	Reverse	5'-AAAAAAACCAACAATTAAAAACAA-3'
PCDH10	Forward	5'-GATCGAGAGTAGTAAGCGGTGTATC-3'
Methylation	Reverse	5'-TATAAACGAATTAATCGAAAACGAA-3'
PCDH10	Forward	5'-TGGATTGAGAGTAGTAAGTGGTGTATT-3'
Unmethylation	Reverse	5'-TATAAACAAATTAATCAAAAACAAA-3'
PTEN	Forward	5'-GGGAGTAATTATTTTTAGTTAGAGGC-3'
Methylation	Reverse	5'-ATACATAAACTTATCTTCCCGTCGTA -3'
PTEN	Forward	5'-GAGTAATTATTTTTAGTTAGAGGTGT-3'
Unmethylation	Reverse	5'-ATACATAAACTTATCTTCCCATCATA-3'

16. EMSA (Electrophoretic Mobility-Shift Assay)

MAP17 and LINC00853 binding activity was detected using EMSA (Promega Corp., Madison, WI, USA). The electrophoretic mobility shift assay (EMSA) kit, oligonucleotide probes (MAP17), and reporter lysis buffer were from Promega. AGS cells were grown to ~90% confluence with nuclear protein extracts prepared using the Nuclear Extract kit (Affymetrix, CA, USA). EMSA was performed using the EMSA gel shift kit (Panomics, Fremont, CA, USA), according to the manufacturer's protocol. Briefly, nuclear protein was subject to hybridization to a double stranded, biotin-labeled oligonucleotide probe, containing the consensus-binding site for MAP17 (sense strand, 5′-CGGAAACAAGGCAGATGGAGTCC-3′). The protein-DNA complexes were resolved on a 6% non-denaturing PAGE gel and then transferred to a Pall Biodyne B nylon membrane (Pall Life Sciences, Pensacola, FL, USA). Signal detection was with streptavidin-HRP and a chemiluminescent substrate.

17. Microarray analysis

Total RNA was extracted from total twelve gastric tissues using TRIZOL reagent (Invitrogen). RNA quality was verified using an Multiskan™ Microplate Photometer (Thermo Scientific). LncRNA expression profiling was conducted using ArrayStar Human

LncRNA array 2.0 (ArrayStar, Rockville, MD, USA). Target LncRNAs were selected for further experiment if their fold change exceeds 2.0, and their P values were less than 0.05.

18. Statistical analysis

All analyzing data for continuous variables are presented as the mean ± standard error. Categorical variables were demonstrated as the number with proportion. Statistical tests included the t-test, $\chi 2$ test, Fisher's exact test and one-way ANOVA test. All statistical processes were accomplished using the statistical software SPSS for Windows (version 18.0; SPSS Inc., Chicago, IL, USA).

III. RESULTS

1. Overview of profiles of gastric cancer related lncRNAs expression from microarray data

To identify the expression of lncRNAs in different stage of gastric cancers, microarray was performed using different types of 12 gastric normal, adjacent normal and cancer tissues. In total, 39,317 LncRNAs and 21,174 mRNAs were identified. Based on these lncRNAs, a comparison on each group combination was analyzed (Fig. 1a). To discover the lncRNAs which associated with early gastric cancer development, the first comparison group was selected as adjacent normal tissues of patients with single and multiple cancers, displaying greater than 2-fold differences in expression with a p-value ≤ 0.05. The total of 36 lncRNAs were significantly upregulated in adjacent normal tissue in multiple cancer compared to single cancer patients (Fig. 1b). Then, to identify the lncRNA related to gastric tumorigenesis and its progression, we sorted that a total of 16453 lncRNAs were upregulated in cancer tissue with multiple cancer patient compared to healthy normal tissue and total 16572 lncRNAs were upregulated in cancer tissue with multiple cancer compared to its adjacent normal tissue (fold c>2, p-value<0.05). After selection of Top 20 lncRNAs in each comparison set and combining intersection lncRNAs for further validation (Fig. 1c).

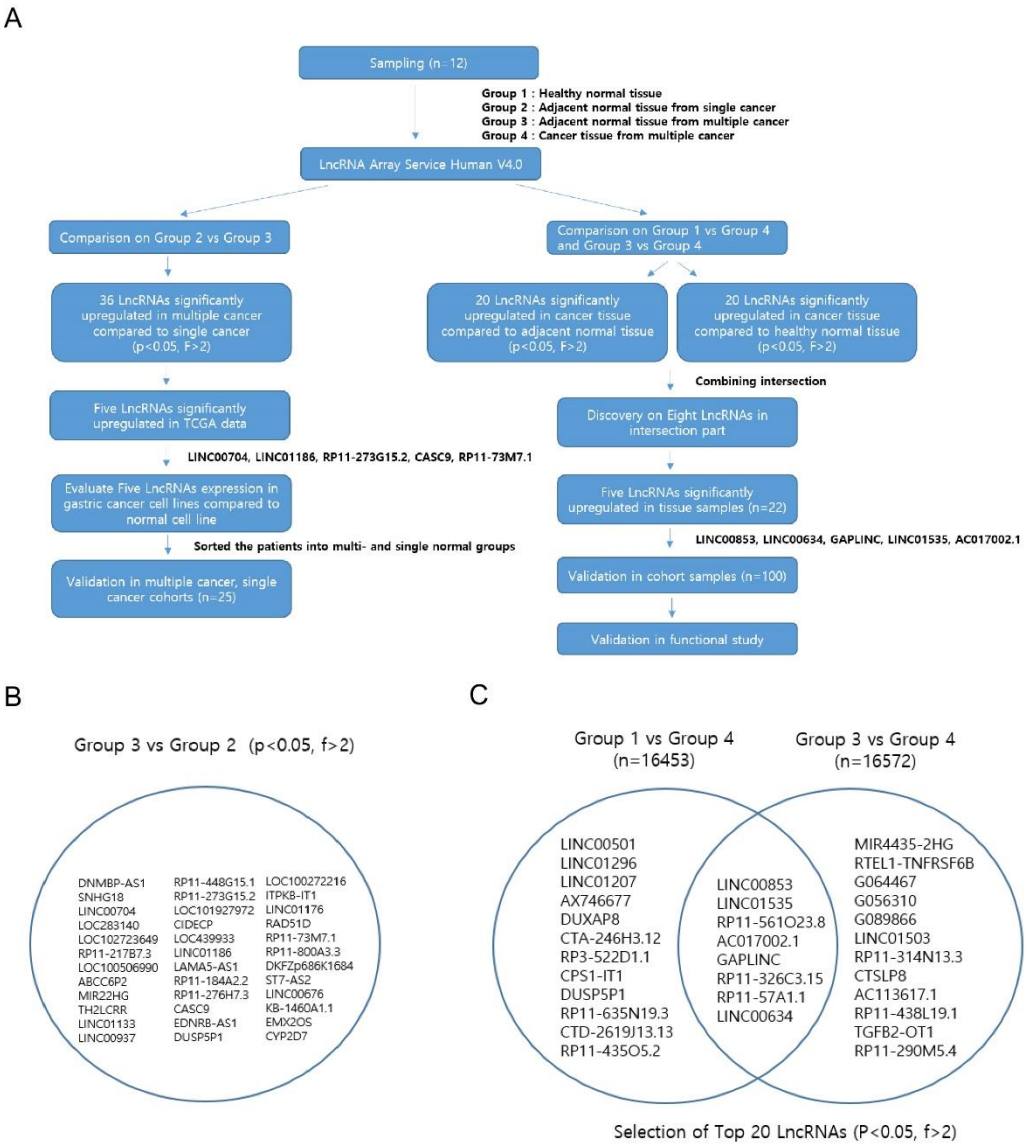

Figure 1. Summary of identification and sorting of lncRNAs in gastric cancer. (Group 1 : Healthy normal tissue, Group 2 : Adjacent normal tissue from single cancer, Group 3 : Adjacent normal tissue from multiple cancer, Group 4 : Cancer tissue from multiple cancer.)

(A) Schematic representation of lncRNAs derived from microarray workflow. (fold change > 2, $p < 0.05$) (B) Venn diagram showed a selection of candidate lncRNAs upregulated in Group 3 compared to Group 2. (C) Venn diagram analysis showed intersection of lncRNAs which is highly expressed in comparison between Group 1 vs Group 4 and Group 3 vs Group 4.

2. LINC01186, CASC9 presented upregulating in adjacent normal tissue with multiple cancer patients

To find relevant lncRNAs that are associated with early gastric cancer, 36 lncRNAs were conducted with STAD-TCGA datasets with median value (http://gepia.cancer-pku.cn/). As a result, 5 lncRNAs were significantly upregulated in tumor compared to normal tissues (Fig. 2a). Among the 5 lncRNAs, LINC00704, RP11-273G15.2, RP11-73M7.1, LINC01186 and CASC9 (Table 4), were validated in adjacent normal tissues with single cancer and multiple cancer patients (Fig. 2b). And we further analyzed the expression of 5 lncRNAs in gastric cancer cell lines compared to normal cell line (Fig. 2c). Based on the results, LINC01186 and CASC9 were used for further molecular experiments to verify the methylation status associated with early gastric cancer.

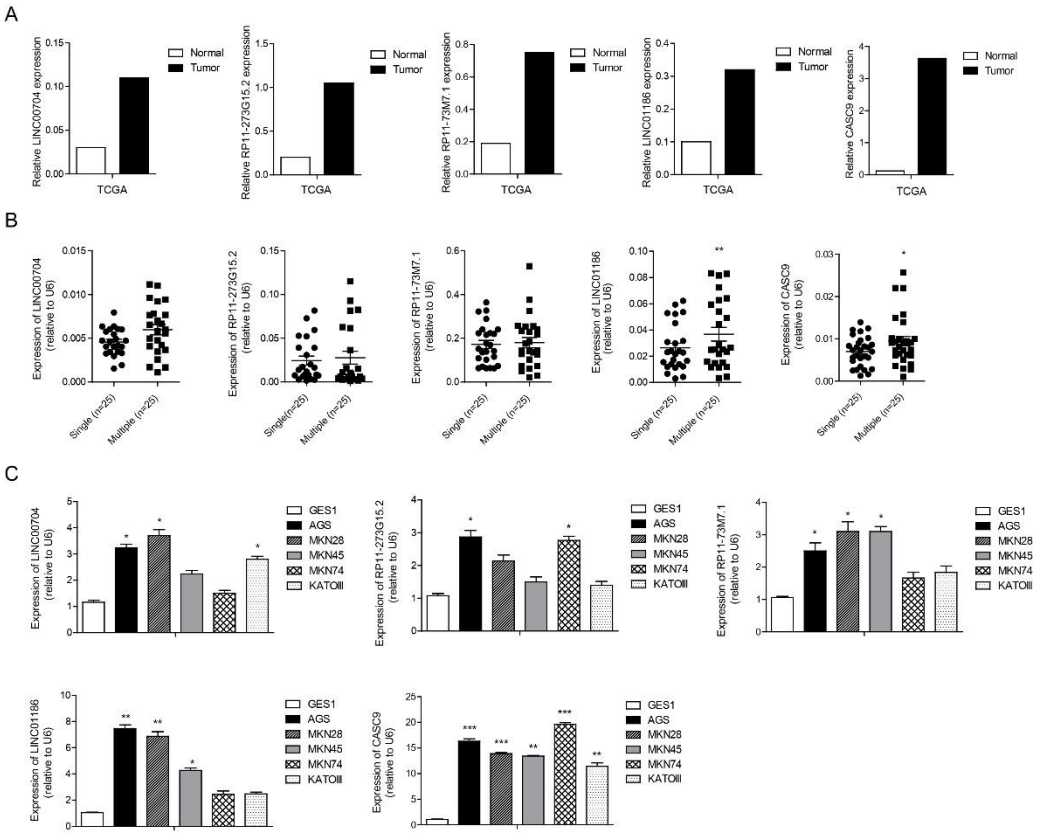

Figure 2. Validation of the aberrantly upregulated lncRNAs in Group 2 verse Group 3. (A) Bar plot showed the 5 lncRNAs expression level in tumor and normal tissues using TCGA data and (B) in adjacent normal tissue from single and multiple cancer (n=25). (C) Highly expressed up-5 lncRNAs were evaluated using qRT-PCR and calculated by $2^{-\Delta\Delta}$Ct method in gastric normal and cancer cell line. Error bars represent the mean ± SD from 3 independent experiments (*P ≤ 0.05,**P ≤ 0.01, ***P ≤ 0.001).

Table 4. Highly expressed lncRNAs in Group 2 vs Group 3

gene_id	locus	fold Change	p-value	strand
LINC01186 (lncRNA)	chrX:46,325,924-46,327,645	2.4989363	0.005017469	-
CASC9 (lncRNA)	chr8:75,233,404-75,278,461	39.3329976	0.0169997956	-
LINC00704 (lncRNA)	Chr10:4692376-4720262	6.2632034	0.0240714794	-
RP11-73M7.1 (lncRNA)	Chr1:144063465-144099798	0.020870475	2.1977822	-
RP11-273G15.2 (lncRNA)	Chr8:144063465-144099798	2.5331256	0.0011664124	-

3. Inhibition of LINC01186 modulated cell proliferation and methylation status of tumor suppressor genes

The expression of LINC01186 was significantly higher in cancer tissues compared with adjacent normal tissues of patients with gastric cancer ($p \leq 0.05$) (Fig. 3a). qRT-PCR data from fractionated RNA represented that LINC01186 in AGS cell were dominantly localized in the nucleus (Fig. 3b). To investigate whether LINC01186 is involved in methylation status of early gastric cancer development, Firstly, loss of function in LINC01186 was performed with two different siRNAs and showed about 50% decline its function in AGS cell ($p \leq 0.05$, $p \leq 0.001$) (Fig. 3c). Next, the MTT assay indicated that 5-aza-CdR significantly decreased the proliferation ability of AGS cell compared with control cells (with 10μmol/L, $p \leq 0.05$), and in the 5-aza-CdR (10 μmol/L) + si-LINC01186 groups was significantly decreased the proliferation more than that in the 5-aza-CdR (10 μmol/L) + siCT group both in 48 hours, 72 hours ($p \leq 0.05$, $p \leq 0.001$) (Fig. 3d). And we selected that the tumor suppressor genes (RUNX3, PCDH10, GAS1, PTEN), which is frequently inactivated in gastric cancer, to analyze the potential of these genes in early gastric cancer detection marker. The mRNA levels of these genes were clearly upregulated by siLINC01186 compare to the siCT (Fig. 3e) and MS-PCR was performed to check the methylation status after the transfection of siRNAs. Inhibition of LINC01186 reduced the methylation level of RUNX3, PCDH10 and GAS1 but not in PTEN (Fig. 3f).

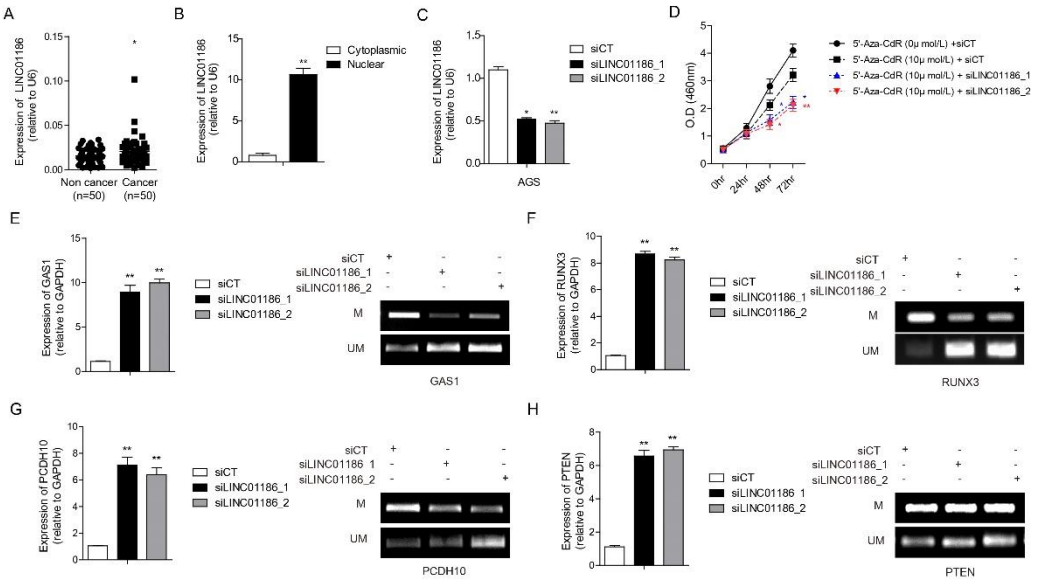

Figure 3. Knock-down of LINC01186 expression using siRNAs and epigenetically regulates the genes associate with early gastric cancer progression. (A) Expression of LINC01186 was upregulated in gastric cancer tissues compared to adjacent normal tissue (n=50). (B) Localization of LINC01186 was identified using qRT-PCR after nuclear and cytosolic separation. (C) Expression of LINC01186 was detected in AGS cell transfected with siRNAs. (D) Knock-down of LINC01186 could further increase the inhibitory effect of 5-aza-CdR on proliferation in AGS cell. Expression level of (E) GAS1, (F) RUNX3, (G) PCDH10 and (H) PTEN were measured by qRT-PCR and DNA methylation status were confirmed by MS-PCR in AGS cell line with siCT or siLINC01186. Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01).

4. Knock-down of CASC9 regulated cell proliferation and methylation status of tumor suppressor genes

The expression of CASC9 was significantly upregulated in cancer tissues compared with adjacent normal tissues of patients with gastric cancer ($p \leq 0.01$) (Fig. 4a). And then, we isolated fractionated RNA from MKN74 cell to detect its cellular localization, CASC9 was located in evenly nucleus and cytosol (Fig. 4b). To identify whether CASC9 is involved in methylation status of early gastric cancer development, Firstly, generation of siRNAs for CASC9 were done and showed about 50% decline its function in MKN74 cell ($p \leq 0.05$) (Fig. 4c). Next, the MTT assay showed that 5-aza-CdR decreased the proliferation ability of MKN74 cell compared with control cells (with 10μmol/L, $p \leq 0.05$), and in the 5-aza-CdR (10 μmol/L) + si-CASC9 groups were significantly decreased the proliferation more than that in the 5-aza-CdR (10 μmol/L) + siCT group in 72 hours ($p \leq 0.05$) (Fig. 4d). And same as the previous approach, we analyze the mRNA levels of RUNX3, PCDH10, GAS1 and PTEN were clearly upregulated by siCASC9 compare to the siCT (Fig. 4e~h) and MS-PCR was performed to check the methylation status after the transfection of siRNAs. Inhibition of CASC9 reduced the methylation level of RUNX3, PCDH10 and GAS1 but not in PTEN (Fig. 4e~h).

Taken together, these results showed that LINC01186 and CASC9 regulates epigenetic inactivation of tumor suppressor genes, related with an event during early gastric cancer development.

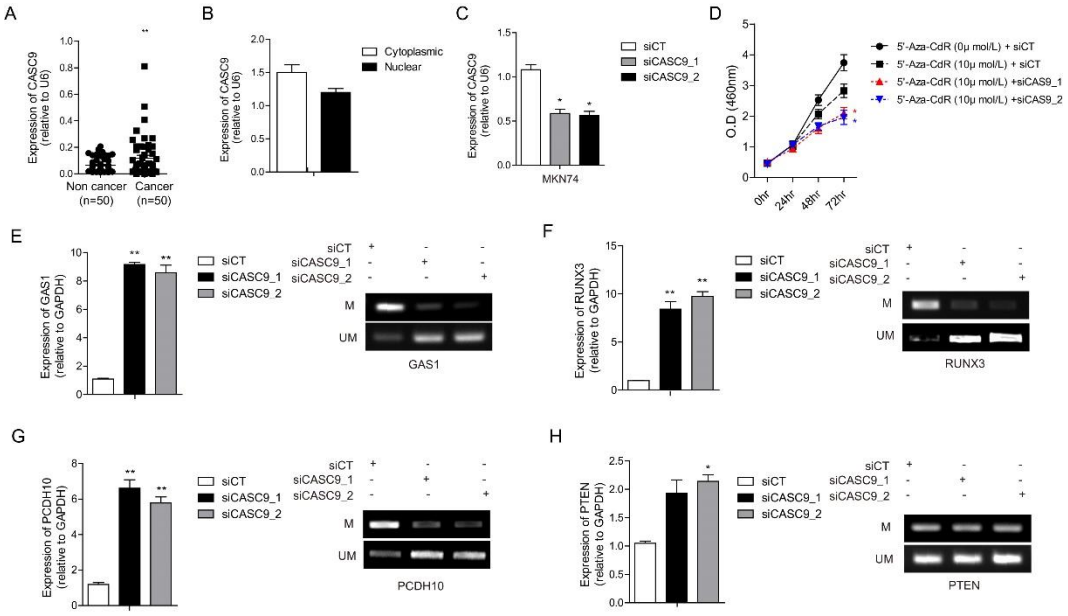

Figure 4. Production of Loss and gain function in lncRNA, CASC9 and its epigenetically regulates the genes associate with early gastric cancer progression. (A) Expression of CASC9 was upregulated in gastric cancer tissues compared to adjacent normal tissue (n=50). (B) Localization of CASC9 was identified using qRT-PCR after nuclear and cytosolic separation. (C) Expression of CASC9 was detected in MKN74 cells transfected with two different siRNAs. (D) Knock-down of CASC9 could further increase the inhibitory effect of 5-aza-CdR on proliferation in MKN74 cell. Expression level of (E) GAS1, (F) RUNX3, (G) PCDH10 and (H) PTEN were measured by qRT-PCR and DNA methylation status were confirmed by MS-PCR in MKN74 cell line under the condition of siCT or siCASC9. Error bars represent the mean ± SD from 3 independent experiments.

The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01).

5. Validation of candidate lncRNA in advanced gastric cancer by qRT-PCR analysis

To investigate the lncRNAs associated with gastric cancer tumorigenesis, we sorted the lncRNA by comparision on grouping organizations. The candidate lncRNAs were chosen by multiple standard: (1) LncRNAs which is upregualted in Group 4 compared to Group 1 with fold change > 2, p-value ≤ 0.05 ; (2) LncRNAs which is upwarded in Group 4 compared to Group 3 with fold change > 2, p-value ≤ 0.05 ; (3) Top 20 lncRNAs among the ones satisfying the each conditions of No. 1 and No. 2 ; (4) overlapping the Top 20 lncRNAs in each comparision (Fig. 5a). After sorting the 8 candidate lncRNAs (Table 5), we further evaluated the expression levels of lncRNAs that were most differentially expressed between cancer tissues and adjacent normal tissues from 22 patients with gastric cancer. The expression level of LINC00853, LINC00634, LINC01535, GAPLINC and AC017002.1 in gastric cancer tissues were indeed clearly up-regulated compared with those in adjacent normal tissues (p ≤ 0.05, all of them), on the other hand, RP11-57A1.1, RP11-326C3.15 and RP11-561O23.8 had no difference in expression of each lncRNAs (Fig. 5b). Except for lncRNAs, which has already been studied, LINC00853, which is expected to be involved in the gastric cancer process, was used as a further study.

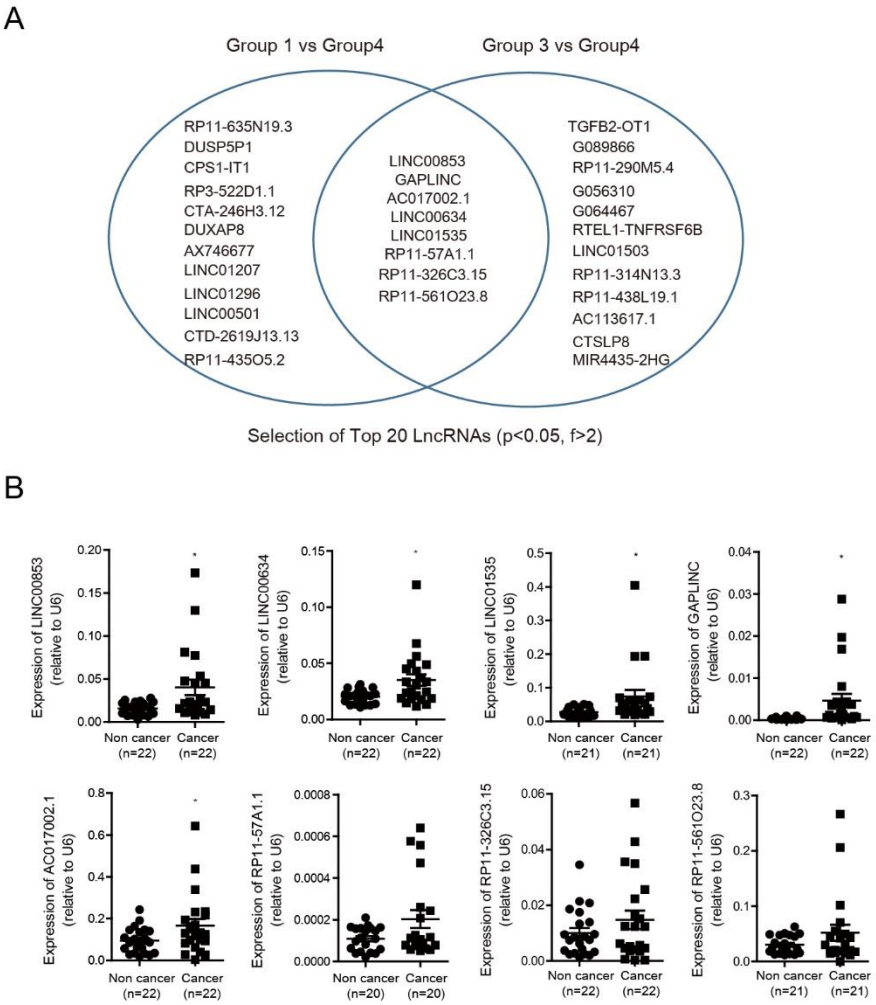

Figure 5. Sorting and validation of lncRNAs in gastric cancer tissues. (A) Venn diagram of differentially expressed lncRNAs in microarrays. Comparison on Group 1 verse Group 4 were indicated in left side while comparison on Group 3 verse Group 4 were indicated in right side (fold change > 2, $p \leq 0.05$). (B) Expression of LINC00853 was higher than that

of LINC00634, LINC01535, GAPLINC, AC017002.1, RP11-57A1.1, RP11-326C3.15 and RP11-561O23.8 in gastric cancer tissues than in the adjacent normal tissue (n=22).

Table 5. Intersection of lncRNAs in four Groups

gene_id	locus	fold Change	*p*-value	strand	class
LINC00853 (lncRNA)	chr1:47,179,250-47,180,339	13.99233	0.00922	+	intergenic
LINC00634 (lncRNA)	chr22:41,952,187-41,958,942	2.20438	0.04606	+	intergenic
LINC01535 (lncRNA)	chr19:37,251,885-37,265,535	1.83725	0.02407	+	intergenic
RP11-561O23.8 (lncRNA)	chr9:68,228,651-68,229,312	7.02695	0.01787	+	intergenic
RP11-57A1.1 (lncRNA)	chr17:72,425,939-72,428,852	65.46701	0.0018	+	intergenic
RP11-326C3.15 (lncRNA)	chr11:333,192-333,688	2.87825	0.03443	+	intergenic
GAPLINC (lncRNA)	chr18:3,466,250-3,478,978	4.61183	0.01621	+	intergenic
AC017002.1 (lncRNA)	chr2:111,491,273-111,510,990	12.77413	0.00142	+	intergenic

6. LINC00853 is highly overexpressed in gastric cancer cell lines and cancer tissues and adjusted proliferation

The expression of LINC00853 in gastric cancer cell lines and tissues were performed using qRT-PCR. LINC00853 was significantly upregulated in gastric cancer in the genotype-tissue expression (GTEx) and The Cancer Genome Atlas (TCGA) datasets ($p \leq 0.001$) (Fig. 6a) and also verified in our 100 cohort samples ($p \leq 0.001$) (Fig. 6a).

LINC00853 was clearly elevated in five gastric cancer cell lines compared with normal cell line (GES-1) (Fig. 6b). We selected AGS and MKN74 cells, which express LINC00853 at relatively high levels, for further experiments. And then, to determine the cellular localization of LINC00853, we extracted the cytosolic and nuclear fractionation from AGS cell. LINC00853 was mostly located in nucleus (Fig. 6c).

To evaluate the effects of LINC00853 on proliferation of gastric cancer cell lines by MTS assay, gastric cancer cells were incubated with siRNAs for LINC00853 and pcDNA-LINC00853. After treatment of AGS and MKN74 cells with 50 μM siRNAs, there was an about 50% decline in LINC00853 expression compared to cells transfected with control siRNA (siCT) (Fig. 6e and 6f). pcDNA-LINC00853 induced overexpression of LINC00853 by about 30-fold compared with the pcDNA vector alone (Fig. 6d). Following transfection of siRNAs, transfection with both siLINC00853_1 and siLINC00853_2 decreased AGS and MKN74 cells viability from 48 hours to 72 hours compared to siCT. These changes were reversed by overexpression of LINC00853 (Fig. 6g and 6h).

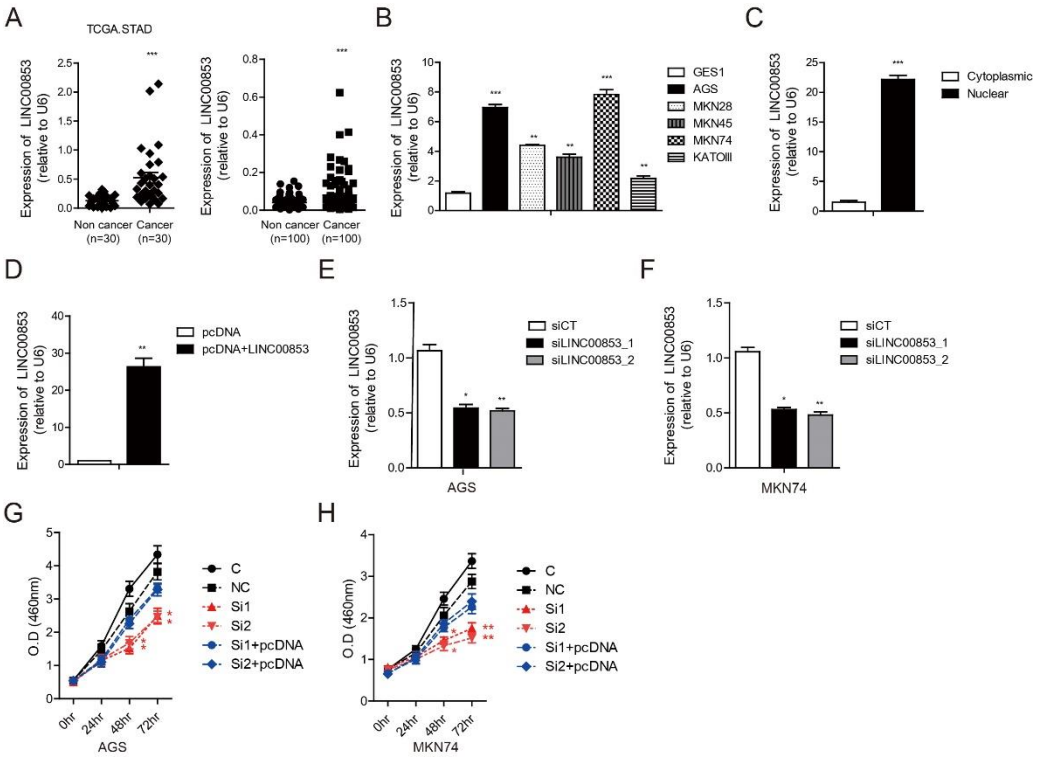

Figure 6. Expression of LINC00853 was upregulated in gastric cancer cell lines and tissues and construction of loss and gain function in gastric cancer cells. (A) LINC00853 expression was first analyzed in STAD-TCGA data (n=30) and estimated in gastric tissues and adjacent normal tissues (n=100) using quantitative real-time PCR and calculated by $2^{-\Delta\Delta}$Ct method. (B) LINC00853 expression was clearly upregulated in gastric cancer cells AGS, MKN28, MKN45, MKN74 and KATOIII compared to gastric normal cell, GES-1. (C) Expression of LINC00853 in the nuclear and cytoplasmic fractions in AGS cells as measured by qRT-PCR. LINC00853 expression was measured by qRT-PCR in AGS cells and MKN74 cells transfected with (D) overexpression of

LINC00853 (pcDNA-LINC00853) and two different siRNAs in (E) AGS and (F) MKN74 cells. Cell proliferation was detected by MTS assay in (G) AGS and (H) MKN74 cell lines. Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

7. Suppression of LINC00853 expression induces cell cycle arrest and apoptosis

To follow up on previous cell proliferation results on LINC00853, we evaluated the role of LINC00853 in cell cycle progression and apoptosis. When AGS and MKN74 cells were treated with two different siLINC00853s, the induction of apoptosis was confirmed by flow cytometry using PI/Annexin V staining (Fig. 7a). Both early and late apoptosis was increased by siLINC00853s with 2.5 to 3 times in the siLINC00853s-treated cells compared to the control group (Fig. 7c). To determine the quantitative relationship, caspase 3/7 protein was measured and it was found that siLINC00853s significantly increased caspase 3/7 activity by 1.5 and 2 times, respectively (Fig. 7e).

Furthermore, Treatment with siLINC00853s promotes cell cycle arrest in the G2/M phase and no changes in the number of AGS and MKN74 cells in subG1/G1 (Fig. 7b). Bar graph shows that only the number of cells in G2/M phase increased by siLINC00853s

with 3 to 3.5 times compared to siCT (Fig. 7d). Western blot analysis showed that siLINC00853 clearly increased the expression of parp, caspase 9, bax and decreased in the expression of bcl-xl in both cell lines (Fig. 7f). And also these changes were reversed by the transfection of pcDNA-LINC00853. Based on these results, we conclude that knock down of LINC00853 expression induced cell cycle arrest in G2/M phase which is correlated with inducing late apoptosis and decreased cell proliferation in gastric cancer cell lines.

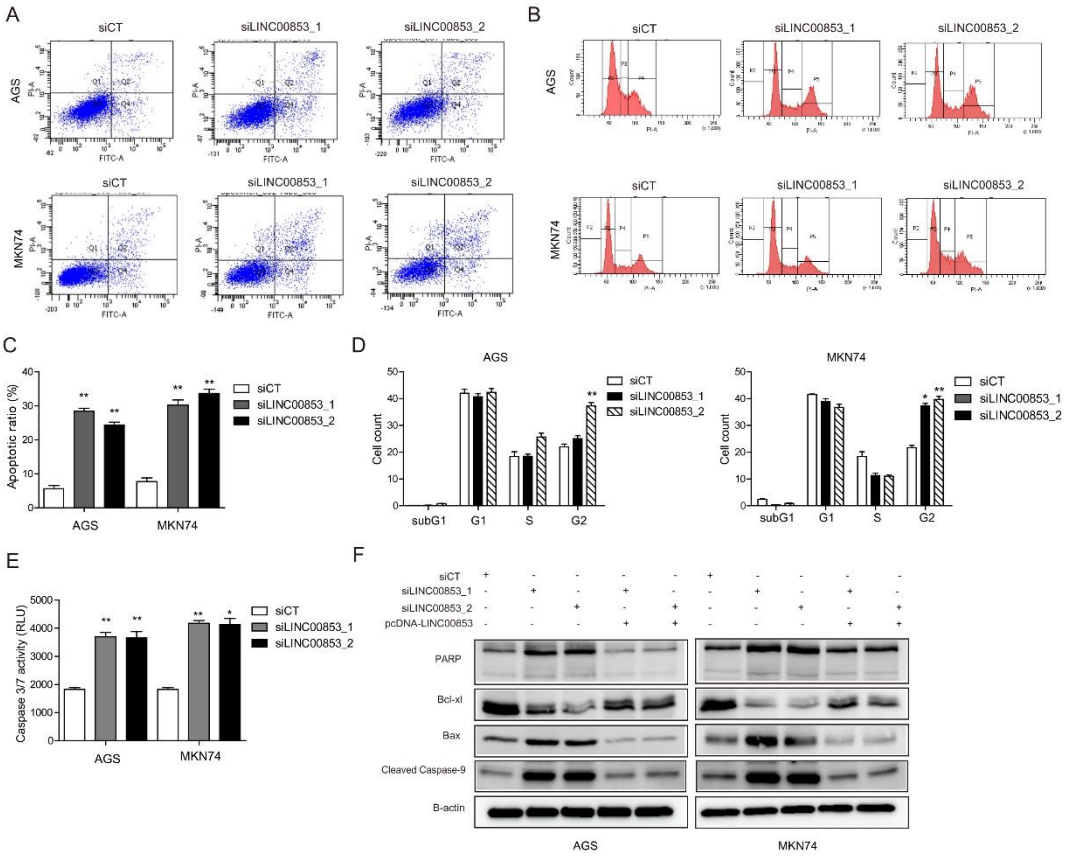

Figure 7. Inhibition of LINC00853 expression induce apoptosis and cell cycle arrest. (A) Apoptosis was performed by flow cytometry using PI/Annexin V staning. (B) Cell cycle assay were carried out with flow cytomertry in AGS and MKN74 cells. (C) The bar graph showed the percentage of apoptotic cells in each population stage. (D) The bar graph displayed the percentage of cells in subG1, G1, S, and G2 phases during knock-down of LINC00853 in AGS and MKN74 cell lines. (E) Caspase 3 activity assays were performed on AGS, MKN74 cells using a luminescence microplate reader. (F) Apoptosis markers were

assessed by western blot in AGS and MKN74 cells transfected with siCT or siLINC00853 and/or pcDNA-LINC00853. Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01).

8. siLINC00853 inhibits invasion and migration in gastric cancer cells

To investigate the role of LINC00853 in metastasis, we tested whether transfection of cells with siLINC00853s affected the epithelial-to-mesenchymal transition (EMT) process using the wound healing assay and Matrigel invasion assay. Transfection of cells with two different siLINC00853s significantly reduced migration ability in AGS and MKN74 cells (Fig. 8a). In an invasion assay, siLINC00853s repressed the invasion ability compared with siCT, consistent with the migration assay results (Fig. 8b). Next, we proceed a soft agar colony formation assay to measure in vitro cellular anchorage-independent growth. Number and size of forming colonies declined notably in cells transfected with siLINC00853_1 compared to cells transfected with siCT (Fig. 8c). Moreover, we evaluated the differential expression of EMT markers using western blot. Expression of the mesenchymal markers Snail, N-cadherin, and Vimentin was reduced, whereas that of E-cadherin was increased after transfection of cells with siLINC00853s compared to siCTs. Though, these expressions were reversed by transfection of pcDNA-LINC00853 (Fig. 8d).

Taken together, these show that LINC00853, which is a typical phenotype of cancer, is involved in invasion, migration and colony forming abilities in gastric cancer.

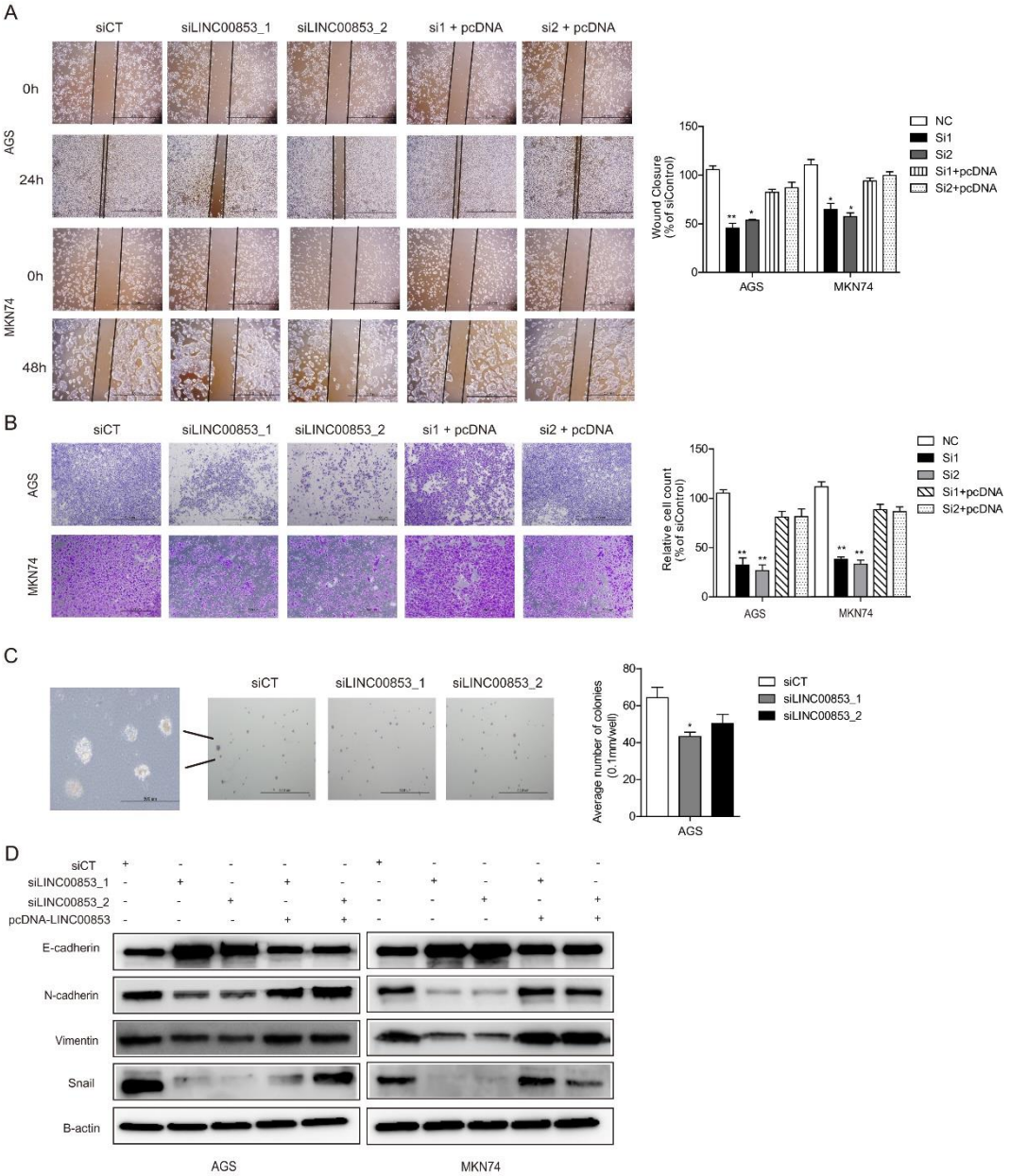

Figure 8. LINC00853 regulates cell migration, invasion and colony formation.

(A) Wound healing assay (B), invasion assay (C) and colony formation were conducted by transfected with siControl, siLINC00853 or siLINC00853 followed by pcDNA-LINC00853 in AGS and MKN74 cells. (D) Along the same way, EMT markers were determined by western blot. Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01).

9. Identification of candidate target gene for LINC00853

To elucidate the means by which LINC00853 regulates the biological functions in gastric cancer, we analyzed candidate genes that may regulate by *cis*-regulatory mechanisms of LINC00853. Genomic location of LINC00853 and its neighbor genes, CYP4A22 and MAP17 were displayed using UCSC Genome Browser (https://genome.ucsc.edu/) (Fig. 9a) Each candidate genes moderately upregulated in gastric cancer cell lines compared to normal cell (Fig. 9b and c).

And then, to examine the molecular relationship between LINC00853 and candidate genes, only MAP17 was down-regulated in the condition of siLINC00853s (Fig. 9d and Fig. 9e). Microarray data also provided that MAP17 was described as down-stream target of LINC00853 with positive regulation (Table 6).

Table 6. Down-stream target of LINC00853

gene_id	Genome realtionship	Nearby gene symbol	Fold change	*p*-value	Regulation
LINC00853 (lncRNA)	Downstream	PDZK1IP1 (MAP17)	5.3876968	0.0362763	Up

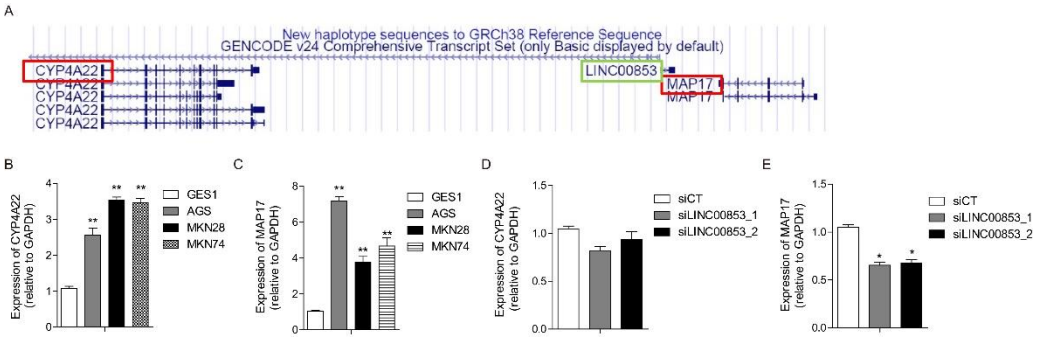

Figure 9. LINC00853 influences the expression of neighboring genes. (A) The LINC00853 genomic locus with its neighbor genes, CYP4A22 and MAP17. Both expression level of (B) CYP4A22 (C) and MAP17 were upregulated in gastric cancer cell lines compared to normal cell. (D, E) Expression levels of CYP4A22 and MAP17 were measured by qRT-PCR using knock down of LINC00853 into AGS cell. Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01).

10. LINC00853 interacts with MAP17 and its downstream pathways

We above showed that LINC00853 is located nearby MAP17 and regulates transcription level of MAP17 in gastric cancer cell line. To show the interaction between LINC00853 and MAP17, RNA immunoprecipitation (RIP) and MAP17 IP assays were performed in AGS cell line (Fig. 10a). In order to validate the interaction between LINC00853 and MAP17, we predicted the binding site for LINC00853 and MAP17 using web program (Fig. 10b)(http://rtools.cbrc.jp/cgi-bin//RNARNA/index.pl) and produced the plasmid vector that lack of binding site region. Cells transfected with LINC00853 overexpression displayed significantly increased MAP17 at both the RNA and protein level, whereas LINC00853-Mut overexpression (pcDNA-LINC00853) reduced the expression of MAP17 (Fig. 10c and d). LINC00853 located mostly in nucleus and binds to MAP17 gene promoters in order to exert transcriptional control so that we assayed levels of MAP17 in nuclear extracts prepared from pcDNA, pcDNA-LINC00853 and LINC00853-Mut in AGS cell.

As shown in Fig. 10e, there was a reduction in the level of MAP17 in nuclear extracts with LINC00853-Mut group. Furthermore, we performed Electrophoretic mobility shift assay (EMSA) within pcDNA, pcDNA-LINC00853 and LINC00853-Mut groups and showed bond to MAP17 probe in the condition of overexpression of LINC00853 group more than mutated LINC00853 group (Fig. 10f). To further determine LINC00853-mediated molecular mechanism, PDZK1/AKT signaling pathway, which is activated by

MAP17, was performed by western blot and found that the protein levels of MAP17, PDZK1 and p-AKT were significantly decreased after siLINC00853s, while overexpression of LINC00853 had the opposite effect (Fig. 10g).

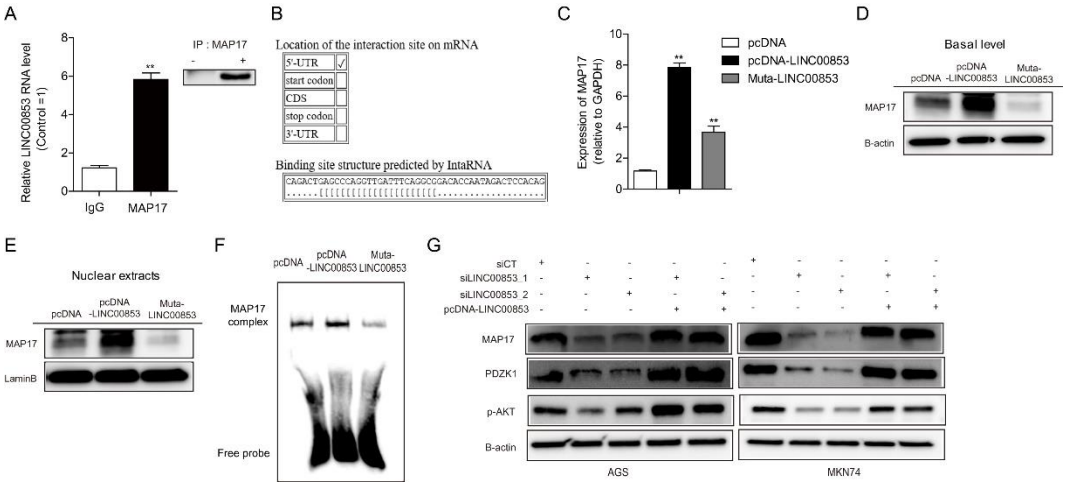

Figure 10. Cis-regulatory function of LINC00853 regulates the expression of MAP17/PDZK1/AKT pathway. (A) RIP assays were performed with anti-MAP17 and anti-IgG in lysates of AGS cells. The bar graph shows the relative RNA level of LINC00853 after anti-MAP17 IP using lysates. (B) The potential binding sites between LINC00853 and MAP17 were predicted using http://rtools.cbrc.jp/cgi-bin/RNARNA system. MAP17 (C) RNA expression and (D) protein basal levels were measured in AGS cells transfected with pcDNA, pcDNA-LINC00853, or LINC00853-Mutagenesis by qRT-PCR and western blot, respectively. (E) AGS cells were exposed to pcDNA, pcDNA-LINC00853, or LINC00853-

Mut and nuclear proteins were isolated and analyzed by western blotting. LaminB was used as a loading control. (F) The DNA binding activity of MAP17 was analyzed by gel shift assay, followed by transfected with pcDNA, pcDNA-LINC00853 and LINC00853-Mut. (G) Western blots were performed the target gene, MAP17 and it downstream PDZK/AKT signaling pathways in AGS and MKN74 cells. Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*P ≤ 0.05, **P ≤ 0.01).

11. MAP17 has oncogenic activity in gastric cancer

Lots of paper informed that MAP17 has an oncogenic function in various carcinomas and its molecular mechanism[30-32]. To figure out the MAP17's function in gastric cancer, firstly we validated the MAP17 expression in gastric cancer cell lines compared to normal cell line and confirmed in 50 paired gastric cohort samples. MAP17 was significantly upregulated in gastric cancer cell lines, but also in cancer tissues (Fig. 11a and 11b). Also the MAP17 expression in STAD-TCGA data on box plot presented upregulation in gastric cancer tissue compared to normal tissue (http://gepia.cancer-pku.cn/detail.php?gene=MAP17#iframe) (Fig. 11c). For further experiment analysis, we generate siRNA for loss of function on MAP17 and clearly showed about 50% diminution in MAP17 expression in AGS cell (Fig. 11d). Moreover, single knockdown of MAP17 and

double knockdown of MAP17 and LINC00853 appeared that the expression of MAP17 was more decreased in double knockdown group than single knockdown group (Fig. 11e).

In order to explain the role of MAP17 in gastric tumorigenesis, the MTT assay was performed on the condition that transfection with siCT, siMAP17 and double knockout of MAP17 and LINC00853. These results revealed that knockdown with MAP17 decreased cell proliferation compared to the siCT and in the double knockdown group showed the most decreased the proliferation ability (Fig. 11f).

Finally, to confirm the relationship between the LINC00853 and the downstream signal by the control of MAP17, we conducted the western blot analysis and noticed that the down-stream signaling pathway targets were down-regulated in both the single knockdown of MAP17 group and the double knockout group (Fig. 11g).

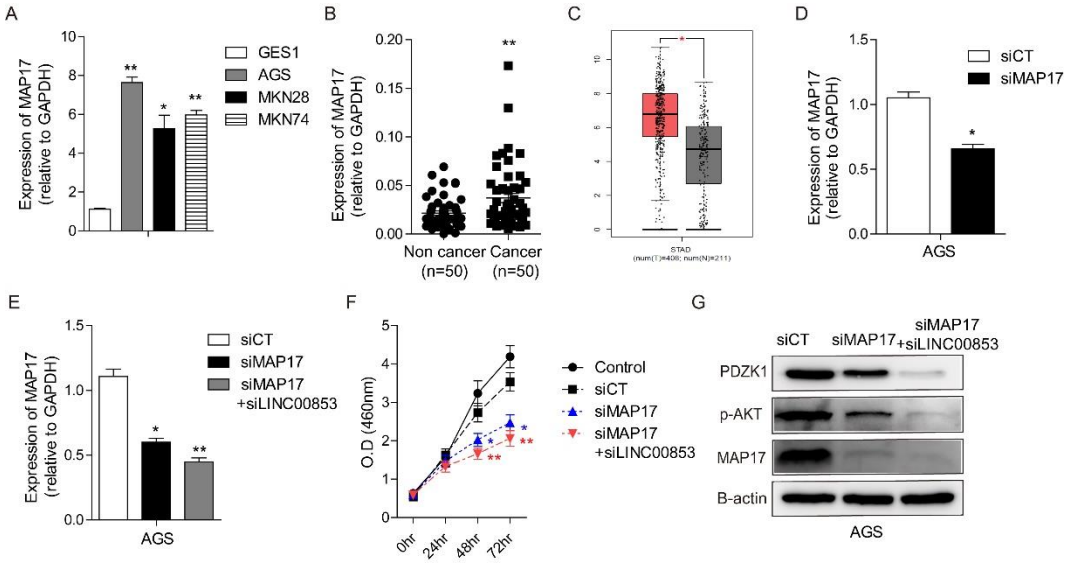

Figure 11. The effect of MAP17 on gastric tumorigenesis. (A) MAP17 expression was clearly upregulated in gastric cancer cells compared to normal gastric cell, GES-1. (B) Expression of MAP17 estimated in gastric tissues and adjacent normal tissues (n=50) using qRT-PCR and calculated by $2^{-\Delta\Delta}$Ct method. (C) Expression of MAP17 measured in STAD-TCGA data. (D) Loss of function on MAP17 expression was used siRNA by qRT-PCR. (E) Expression of MAP17 were analyzed using AGS cells transfected with siCT, siMAP17 and double knock-down between MAP17 and LINC00853. (F) Cell viability was assumed using MTS assay. (G) Western blot analysis was performed in the products of the LINC00853 target gene (MAP17) and its downstream proteins (PDZK1 and AKT). Error bars represent the mean ± SD from 3 independent experiments. The asterisk showed a statistically significant difference compared with scrambled control (*$P \leq 0.05$, **$P \leq 0.01$).

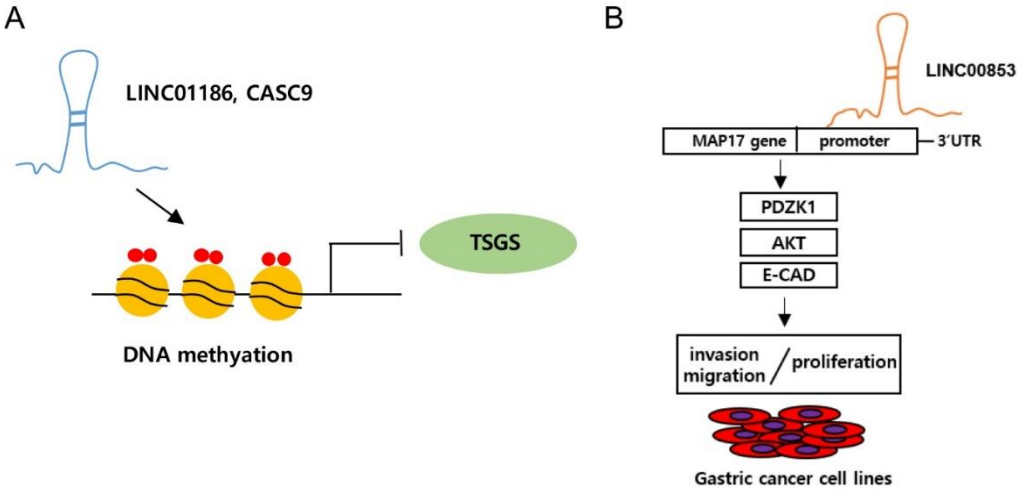

Figure 12. A graphic illustration of LINC01186, CASC9 and LINC00853. (A) LINC01186 and CASC9 regulates the methylation events of gastric tumor suppressor. (B) LINC00853 controls the gastric tumorigenesis through cis-acting with MAP17/PDZK1/AKT pathway.

IV. DISCUSSION

Gastric cancer is a type of malignant cancer with high incidence and mortality worldwide[33]. Also the most prevalent cancer in Korea and various factors such as genetic predisposition, eating habits, and Helicobacter pylori infection affect gastric cancer development[34]. Particularly in the case of early gastric cancer, endoscopic submucosal dissection (ESD), which replaces surgical gastrectomy as a method of treatment for early gastric cancer, is increasing as the remarkable development of endoscopic treatment techniques is accompanied[35]. However, Lots of patients with early-stage gastric cancer are seem to be asymptomatic and because of these symptoms, only after progressing to terminal cancer, the patient becomes aware of the pre-symptoms of cancer and progresses the endoscope[36]. In advanced gastric cancer, the accuracy of serum tumor marker test is poor and the rate of positivity is considerably lower than that of other carcinomas[37,38]. Also the molecular approach in gastric cancer has been relatively less experimentally verified. Therefore, it necessary that it is important to discover the biomarkers for each stage of gastric cancer development.

Recently, advances in experimental and computational technologies such as RNA-seq[39,40] and microarray[26,28,41] over the past decades have revealed the correlation between various types of human cancer and abnormal expression of lncRNA in cancer etiology. It

is already known that lncRNA contributes to the regulation of transcription, post-transcription, and epigenetic changes[9].

Interestingly, lncRNAs, which is differentially expressed in various carcinomas function as oncogenes or tumor suppressors, are shown to regulate the abilities of proliferation, growth suppression, motility, immortality, angiogenesis, and metastasis[8]. In particular, studies on lncRNAs in breast cancer[42], colorectal cancer[43], liver cancer[44] have been conducted with many mechanisms involved in the tumorigenesis. However, this is not the case in early gastric cancer screening and advanced gastric cancer prevention. Based on these mechanisms, the abnormally expressed lncRNA is considered to be an attractive potential therapeutic target and a biomarker for gastric cancer. It is not that lncRNAs associated with gastric cancer has not been published at all. However, studies dealing with early gastric cancer often ended at the screening stage[45], and few studies on advanced gastric cancer development have been explored with EMT process[46].

Here, we used microarray technique to validate the 3 lncRNAs expressed in totally 12 tissues at various types of gastric cancer. For the selection of lncRNA involved in early gastric cancer stage, lncRNAs with higher expression level in normal tissues with multiple cancer against were chosen by comparing to normal tissues with single cancer. Two lncRNA, LINC01186, CASC9 were expected to be a biomarker for gastric cancer progression and were validated using an additional 25 patient cohorts with early gastric cancer.

The factors that greatly influence epigenetic genetics are histone proteins and DNA methylation, of which DNA methylation induces transcriptional inhibitors and recruitment chromosomal changes. These changes are involved in the development and progression of gastric cancer, many of which genes are silenced by abnormal methylation of CpG islands. Aberrant DNA methylation of certain genes reveals frequently expressed in early stage of gastric cancer[36,47,48]. In our study, we sorted candidate tumor suppressors genes[49-52],which are expected to be useful in detecting their epigenetic methylation status and confirmed that it was demethylatyed by siLINC01186 and siCASC9.

To replenish the evidence for methylation is regulated by LINC01186 and CASC9 in early gastric cancer, the cell proliferation assay was confirmed by introducing 5'-AZA, a drug that inhibits methylation[53], into two gastric cancer cell lines with knockdown of LINC01186 and CASC9. To be sum up, LINC01186 and CASC9 are likely to be used as biomarkers in early gastric cancer stage.

Many studies on lncRNAs in advanced gastric cancer mainly focused on the tumorigenesis process through infiltration and invasion, that is, regulation of the EMT process[54,55]. Therefore, we focused on LINC00853, a high-expression lncRNA in gastric cancer tissue of patients with multiple cancers among various combinations. It was confirmed that LINC00853 promotes the abilities of proliferation, migration, invasion, apoptosis in gastric cancer cell lines.

As mentioned above, lncRNA regulates transcription by cis-acting that regulates the transcription of surrounding genes or trans-acting, which is involved in many roles in cells outside of the surrounding gene or chromatin, affects the structure in the cell nucleus, proteins and RNAs[56]. During transcription regulation by cis acting, functional gene regulatory elements, such as enhancers located in lncRNA, often regulate the expression of surrounding neighbor genes[57]

MAP17 is a neighbor gene of LINC00853 and also representative oncogene that is largely expressed in various carcinomas[30,31]. Therefore, LINC00853 regulated the expression of the peripheral gene, MAP17 and suggested the function of cis-acting by binding it to the promoter region. In addition, MAP17's downstream signaling pathway is PDZK1/AKT/ECAD [58],which is one of the pathways involved in the gastric cancer signal pathway. According to MAP17 gene regulate by cis-acting of LINC00853, PDZK1/AKT/ECAD signaling pathway also down-regulated during knock-down of LINC00853.

The initial experiment plan was to validate the lncRNAs in early gastric cancer and advanced cancer, since the number of experimental groups is too small and the diversity and accuracy of each stages in gastric tissues are slightly poor, there is a point that is insufficient as a diagnostic marker for early gastric cancer and advanced cancer. Therefore, using lncRNAs discovered in our results, it is expected that the initial purpose of the study

will be achieved only when further studies are performed on the tissues of each stage of gastric cancer. In addition, it is considered necessary to study the mechanism of LINC00853 promoting metastasis more deeply through in vivo follow-up study in gastric cancer.

However, our results clearly suggested that LINC01186, CASC9 can regulated methylation status in tumor suppressor genes and LINC00853 modulated EMT process through MAP17/PDZK1/AKT pathways in gastric cancer (Fig. 12a and b).

In this regards, the analysis of lncRNAs in gastric cancer has potential as a new therapeutic approach as a biomarker.

V. CONCLUSION

In order to generate biomarkers for each stages during gastric cancer development, microarray analysis was performed in different types of gastric tissues.

LINC01186 and CASC9, which is capable of detecting methylation status related in early gastric cancer, were found through analysis on the normal tissues from multiple cancer patients and single cancer patients. It has been shown that the LINC01186 and CASC9 regulates methylation, which is important for the early gastric cancer development process.

In addition, LINC00853 was selected as a lncRNA capable of detecting advanced gastric cancer and showed significantly upregulated in gastric cancer tissues compared with that in adjacent normal tissue of patients with gastric cancer. Furthermore, it has been found that LINC00853 accelerates the gastric cancer tumorigenesis by regulating the MAP17 and its downstream pathway.

To be sum up, we identified LINC01186, CASC9 and LINC00853 as a potential target for biomarkers for gastric cancer.

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ABSTRACT (IN KOREAN)

위암발생과정과 발암 기전에 연관된 lncRNA의 발견

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변 효 주

위암은 전세계적으로 가장 흔한 암 종에 속하는 암의 하나이다. 내시경의발달로 조기위암의 발견이 조금은 앞당겨졌지만, 환자들이 증상 느끼고 내시경을 시행하였을 때는 이미 진행성, 말기 위암일 확률이 높고, 위암에서의 혈청표지자의 정확도는 다른 암종에 비해 심히 낮은 편이다. 따라서 위암에서의 치료적측면의 마커와 진단가능한 마커의 도입이 필요함을 시사한다. 최근 연구들은 Long non-coding RNAs (lncRNAs)가 다양한 암종에서 유전자의 전사적조절을 함을 밝혔다.

위암에서의 발암과정, 전이 등에 연관된 lncRNA을 밝히기 위해서, 우리는 총 12개의 위암조직으로 (그룹1은 3개의 정상인의 정상조직, 그룹2는 3개의 단일 위암을 가진 환자의 인접한 정상조직, 그룹3은 3개의 다중위암을 가진 환자의 인접한 정상조직, 그룹4는 3개의 다중위암을 가진 환자의 위암조직 microarray 분석을 진행하였다. 이 결과는 위암의 발달 진행 과정의 단계별 lncRNA를 선별하기 위해 총 4개의 그룹으로 나뉘어 분석을 진행하였다.

첫번째로는, 조기위암의 발달과 연관된 메틸화를 조절할 수 있는 lncRNA를 선별하기 위해 그룹2와 그룹3중의 비교에서 그룹3에서 높은 것을 선별하였다. 두번째로는, 진행성 위암과 연관된 전이를 조절 할 수 있는 lncRNA를 선별하기 위해 그룹1과 그룹4의 비교에서 그룹4에서 높은 것과 그룹3과 그룹4의 비교에서 그룹4에서 높은 것의 교집합을 찾았다. 이러한 그룹화를 통한 비교를 한 후, 코호트에서의 검증과 추가적인 기능적 실험 탐구를 위해 실시간중합효소연쇄반응을 진행하였다.

따라서 첫번째 비교결과는 CASC9과 LINC01186는 종양억제유전자 (PTEN, PCDH10, RUNX3) 들의 메틸화를 촉진시켰다. 두번째 비교결과는 LINC00853이 위암조직에서 가장 높게 발현됨을 확인하였고, 위암과 연관된 PDZK1-AKT-MAP17 신호 경로를 조절함으로써 위암에서의 침윤과 전이에 관련된 기전을 촉진한다.

결론적으로, 이 연구는 위암에서 lncRNA의 전이 과정과 분자적 기능과 그의 발현을 보여주고, 위암에서의 치료적 약물 및 바이오 마커로써의

가능성을 시사한다.

핵심되는말: 긴 비번역 RNA, LINC00853, LINC01186, CASC9, 마이크로어레이분석법, 위암