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Long noncoding RNA associated-competing endogenous RNAs in gastric cancer

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Some long noncoding RNAs (lncRNAs) play important roles in the regulation of gene expression by acting as competing endogenous RNAs (ceRNAs). However, the roles of lncRNA associated-ceRNAs in oncogenesis are not fully understood. Here, based on lncRNA microarray data of gastric cancer, bioinformatic algorithm miRcode and microRNA (miRNA) targets database TarBase, we first constructed an lncRNA-miRNA-mRNA network. Then, we confirmed it by data of six types of other cancer including head and neck squamous cell carcinoma, prostate cancer, papillary thyroid carcinoma, pituitary gonadotrope tumors, ovarian cancer, and chronic lymphocytic leukemia. The results showed a clear cancer-associated ceRNA network. Eight lncRNAs (AC009499.1, GACAT1, GACAT3, H19, LINC00152, AP000288.2, FER1L4, and RP4-620F22.3) and nine miRNAs (miR-18a-5p, miR-18b-5p, miR-19a-3p, miR-20b-5p, miR-106a-5p, miR-106b-5p, miR-31-5p, miR-139-5p, and miR-195-5p) were involved. For instance, through its miRNA response elements (MREs) to compete for miR-106a-5p, lncRNA-FER1L4 regulates the expression of PTEN, RB1, RUNX1, VEGFA, CDKN1A, E2F1, HIPK3, IL-10, and PAK7. Furthermore, cellular experimental results indicated that FER1L4-small interfering RNA (siRNA) simultaneously suppressed FER1L4 and RB1 mRNA level. These results suggest that lncRNAs harbor MREs and play important roles in post-transcriptional regulation in cancer.

MicroRNAs (miRNAs) play important roles in gene expression regulation[1]. Each miRNAs may repress up to hundreds of transcripts, while each transcript may be targeted by multiple miRNAs[2]. Their regulatory networks participate in a variety of biological processes, including development, oncogenesis and tumor metastasis[3–5].

Salmena and colleagues previously proposed a competing endogenous RNA (ceRNA) hypothesis[6], which was supported by numerous evidences[7–11]. The hypothesis described a complex post-transcriptional regulatory network mediated by miRNAs: by sharing one or more miRNA response elements (MREs), protein-coding and noncoding RNAs compete for binding to miRNAs and then regulate each other's expression (Fig. 1). Later, more studies provided convincing evidences for this hypothesis. Pandolfi group focused on phosphatase and tensin homologue (PTEN) and its ceRNAs[12,13]. They demonstrated the existence of ceRNA interaction among mRNAs *in vitro* and *in vivo*. Sumazin *et al.* analysed gene expression data in glioblastoma, and found more than 7,000 transcripts acting as ceRNAs[14]. Further study showed that ceRNAs are not limited to mRNAs. Linc-MD1, a long noncoding RNA (lncRNA), regulates myoblast differentiation by competing for binding to miR-133 and miR-135[15].

To annotate the associations between noncoding RNAs and diseases, several disease-associated bioinformatic methods and databases including LncRNADisease[16], HMDD[17] and dbDEMC[18] have been developed. Otherwise, computational tools for miRNA-target interaction prediction have been developed[19].

The above studies showed a new layer of post-transcriptional regulation. However, to understand the roles of ceRNA networks in pathological conditions, more work is required. It is found that active translation of mRNA impedes interaction of miRNAs and their targets[20]. Contrast to mRNAs, noncoding RNAs are more effective ceRNAs without any interference from translation[6]. Considering these findings and the fact that gastric cancer is one of the most frequent causes of mortality in the world[21], in the current study, we first focused on gastric cancer associated-lncRNAs and constructed a ceRNA network *in silico*. Then, to confirm this network, we performed a regression analysis using several sets of gene expression data of other types of cancer including head

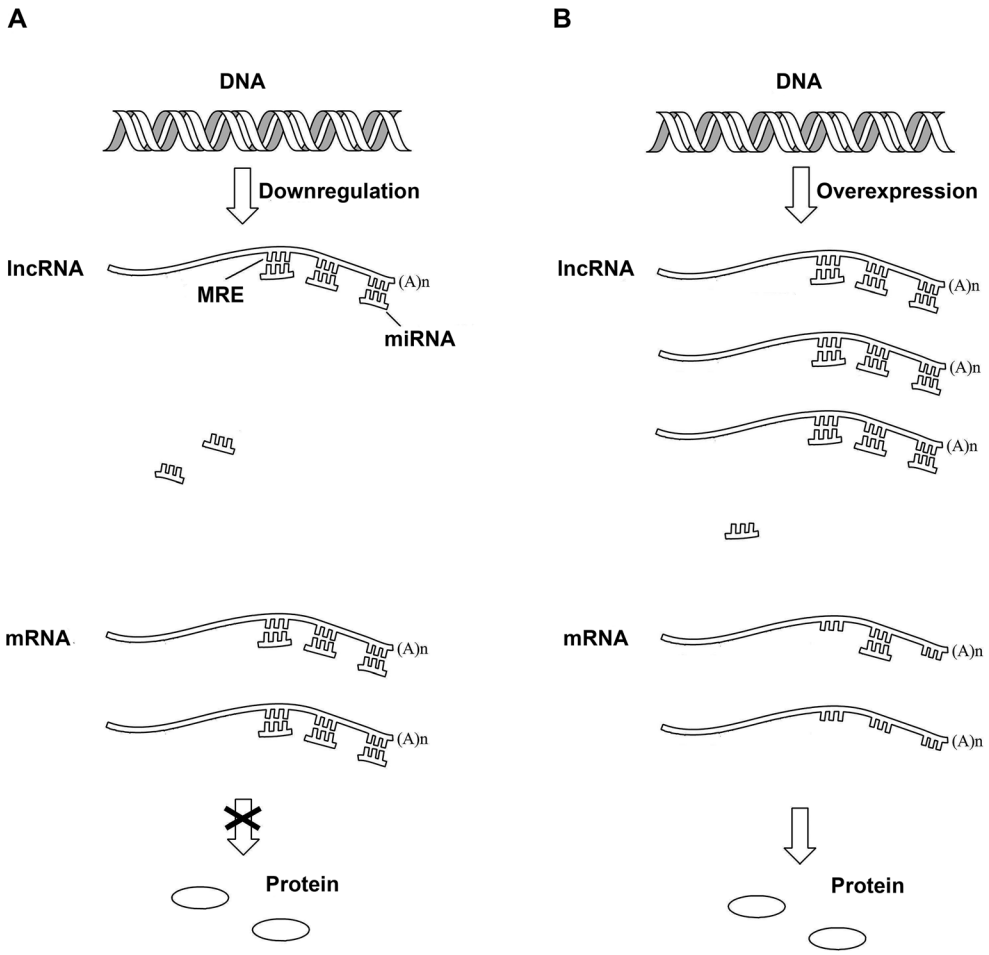

Figure 1 | Competing endogenous RNAs (ceRNAs) function as microRNA (miRNA) sponges sequester miRNAs to regulate expression level of other transcripts sharing common miRNA response elements (MREs). (A) Downregulation of long noncoding RNAs (lncRNA) leads more miRNA molecules free to bind to mRNA that contain the same MREs, thus its protein expression level decreases. (B) Overexpression of lncRNA leads fewer miRNA molecules to bind to mRNA, thus its protein expression level increases.

and neck squamous cell carcinoma, prostate cancer, papillary thyroid carcinoma, pituitary gonadotrope tumors, chronic lymphocytic leukaemia, and ovarian cancer. Finally, we used several experimental data to prove the ceRNA network. This new approach of predicting cancer associated-ceRNA network might help us easily searching for candidate cancer-associated ceRNAs.

Table 1 | A collection of differentially expressed lncRNAs between gastric cancer tissues and paracancerous tissues

lncRNA	Gene ID	Expression change	Fold change	*P*-value
AC009499.1	ENSG00000203386	Up-regulation	5.4	0.040
GACAT1	ENSG00000232991	Up-regulation	4.1	0.008
GACAT3	ENSG00000236289	Up-regulation	3.3	0.007
H19	ENSG00000130600	Up-regulation	5.9	0.025
LINC00152	ENSG00000222041	Up-regulation	3.4	0.004
RMRP	ENSG00000199916	Up-regulation	3.8	0.043
RP11-179G5.4	ENSG00000235082	Up-regulation	3.2	0.022
RP11-187O7.3	ENSG00000259124	Up-regulation	3.1	0.031
RPPH1	ENSG00000259001	Up-regulation	3.0	0.001
ABHD11-AS1	ENSG00000225969	Down-regulation	3.0	0.027
AC073871.2	ENSG00000182648	Down-regulation	3.0	0.002
AKR7A2P1	ENSG00000229020	Down-regulation	3.1	0.001
AP000288.2	ENSG00000227757	Down-regulation	3.8	0.027
FER1L4	ENSG00000088340	Down-regulation	9.2	0.047
RP1-15D23.2	ENSG00000224228	Down-regulation	5.2	0.048
RP4-620F22.3	ENSG00000238081	Down-regulation	3.8	0.021
RP4-740C4.4	ENSG00000229813	Down-regulation	3.0	0.039

Table 2 | Putative miRNAs targeting lncRNA

lncRNA	miRNAs
AC009499.1	miR-18a-5p, miR-18b-5p
GACAT1	miR-106a-5p
GACAT3	miR-195-5p, miR-497-5p
H19	miR-17-5p, miR-18a-5p, miR-18b-5p, miR-19a-3p, miR-20a-5p, miR-20b-5p, miR-106a-5p, miR-106b-5p
LINC00152	miR-18a-5p, miR-18b-5p, miR-31-5p, miR-139-5p, miR-195-5p, miR-497-5p
ABHD11-AS1	miR-133b
AP000288.2	miR-19a-3p
FER1L4	miR-18a-5p, miR-18b-5p, miR-106a-5p, miR-133b, miR-139-5p, miR-195-5p, miR-497-5p
RP4-620F22.3	miR-195-5p, miR-497-5p

Results

Aberrantly expressed lncRNAs in gastric cancer. From the lncRNAs expression profiles (data accessible at NCBI GEO database, accession GSE47850, Guo, 2013), we selected 53 lncRNAs differentially expressed between gastric cancer tissues and paracancerous tissues (fold change ≥ 3.0[22], P-value < 0.05). To enhance the data reliability, we only retained lncRNAs included in Encyclopedia of DNA Elements (ENCODE)[23]. Finally, 17 lncRNAs (9, up-regulated; 8 down-regulated) were selected (Table 1).

Gastric cancer associated-lncRNAs' MREs predicted by miRcode. Our previous study has found several gastric cancer-associated miRNAs that include miR-17-5p, miR-18a-5p, miR-18b-5p, miR-19a-3p, miR-20a-5p, miR-20b-5p, miR-21-5p, miR-31-5p, miR-106a-5p, miR-106b-5p, miR-133b, miR-139-5p, miR-195-5p, miR-340-3p, miR-378a-3p, miR-421, miR-497-5p and miR-658[21]. Here, we focused on whether these miRNAs are targeted on above lncRNAs. Since miRNAs are interacting with lncRNAs through their MREs within ceRNA network, the first thing we should do was to search for the potential MREs in lncRNAs. MREs predicted by miRcode showed that 13 miRNAs may interact with nine of seventeen lncRNAs (Table 2).

mRNAs targeted by miRNAs. To establish lncRNA-miRNA-mRNA network (ceRNA network), the next step we should do was to search for miRNAs' mRNA targets. Based on those miRNAs that might interact with lncRNAs (Table 2), we searched miRNAs' mRNA targets with experimental support using TarBase[24]. The results showed that nine miRNAs including miR-18a-5p, miR-18b-5p, miR-19a-3p, miR-20b-5p, miR-106a-5p, miR-106b-5p, miR-31-5p, miR-139-5p, and miR-195-5p may be included (Table 3). Most of their targets are cancer-associated genes such as PTEN, TNF-α, STAT3, VEGFA, E2F1, RB1, p21, MMP16, BCL2, CCND1, CDK6, CyclinD1, etc. Their functions are involved in cell proliferation, cell cycle, apoptosis, invasion and metastasis.

ceRNA network. Based on the above data from gastric cancer (Table 2 and Table 3), we constructed an lncRNA-miRNA-mRNA ceRNA network. To get more robust results, we assigned a P-value to each relationship by re-sampling analysis (see Methods). As shown in Figure 2, in this ceRNA network, eight lncRNAs and nine miRNAs were involved.

Positive correlation between ceRNAs' expression levels. Our constructed ceRNA network shows that lncRNAs could interact with mRNA in gastric cancer (Fig. 2). To confirm this finding, we performed a regression analysis using several sets of gene expression data of other types of cancer including head and neck squamous cell carcinoma, prostate cancer, papillary thyroid carcinoma, pituitary gonadotrope tumors, chronic lymphocytic leukemia, and ovarian cancer. The results reveal a very good or perfect positive correlation between ceRNAs' expression levels (Fig. 3). For example, FER1L4 interacted with RUNX1 mediated by miR-106a-5p (Fig. 3A and B); LINC00152 interacted with THBS1 mediated by miR-18a-5p (Fig. 3C and D); while H19 interacted with MYCN mediated by miR-19a-3p (Fig. 3E and F).

FER1L4-RB1 is one pair of ceRNAs associated by miR-106a-5p. RB1 is one of miR-106a-5p's targets, and has been validated by dual luciferase reporter assay[25]. In this study, the interaction between FER1L4 and miR-106a-5p was first predicted by miRcode (Table 2). Then dual luciferase reporter assay showed that the luciferase activity of the mutant FER1L4 plasmid was about 56% higher than that of the wild-type plasmid (Fig. 4). This indicated that the mutations introduced in the seed matches impair the ability of miR-106a-5p to bind to FER1L4. Take together, both FER1L4 and RB1 are targets of miR-106a-5p. This is the basic condition of ceRNAs[6].

Next, we observed whether FER1L4 and RB1 expression levels are associated. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis revealed that transfection of small interfering RNA (siRNA) against FER1L4 not only reduced FER1L4 levels in GES-1, AGS, MGC-803 and SGC-7901, but also reduced RB1 levels in all cells tested (Fig. 5). Since FER1L4 is the target of miR-106a-5p (Table 2 and Fig. 4), FER1L4 knockdown led to more miR-106a-5p free to bind to other targets; and RB1 mRNA is one of them.

Discussion

Studies revealed that lncRNAs play important regulatory roles in gene expression regulation and contribute to oncogenesis and tumor

Table 3 | Validated mRNAs' targets from TarBase

miRNA	mRNAs targeted by miRNAs
miR-18a-5p	ATM, BCL2L11, CA12, CA13, CCNL1, CKAP5, CREBL2, CTDSPL, CTGF, DICER1, ERα, ESR1, HIF1A, HOXA9, MID1, PTEN, RAB23, RAB5A, SERTAD3, Smad4, TGFBR2, THBS1, TNFSF11, VIL2
miR-18b-5p	ERα, ESR1
miR-19a-3p	BCL2L11, Bim, CCND1, CTGF, DPYSL2, ERBB4, ESR1, HOXA5, MECP2, MYCN, NR4A2, PRMT5, PTEN, RAB14, Smad4, SOCS1, TGFBR2, THBS1, TNF-α, VPS4B
miR-20b-5p	ARID4B, BAMBI, CDKN1A, ESR1, HIF1A, HIPK3, MUC17, PPARG, STAT3, VEGFA
miR-31-5p	CASR, CXCL12, ETS1, FOXP3, FZD3, HOXC13, ITGA5, KLF13, LATS2, MMP16, MPRIP, NFAT5, NUMB, PPP2R2A, RET, RHOA, SELE, TIAM1, YY1
miR-106a-5p	CDKN1A, E2F1, HIPK3, IL-10, PAK7, PTEN, RB1, RUNX1, VEGFA
miR-106b-5p	AFP, AKAP11, BRMS1L, CABP2, CASP7, CD34, CDC37L1, CDK5R2, CDKN1A, CLOCK, DNAJB6, E2F1, EIF5A2, ELK3, HMGB3, IFNAR2, JAK1, KDR, KIF23, LIMK1, MXI1, p21, PCAF, PKD2, PTEN, RB1, RBL2, RUNX1T1, Smad2/3, Smad9, SSX2, TβRII, TLR2, VEGFA
miR-139-5p	HOXA10
miR-195-5p	BCL2, BCL2L11, CCND1, CDK6, CyclinD1, E2F3, KRT7, MECP2, SKI, VEGFA, WEE1

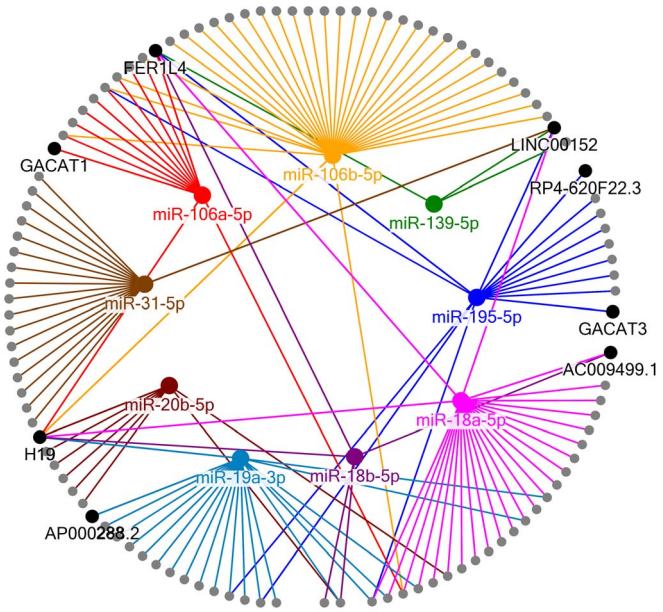

Figure 2 | ceRNA network in gastric cancer. Coloured nodes represent miRNAs; black nodes represent lncRNAs; gray nodes represent mRNAs; coloured edges indicate miRNA-target interactions. mRNAs' names are not shown.

metastasis[26,27]. LncRNAs can function as endogenous miRNA sponges as a part of ceRNA network[15,28,29]. In current study, based on lncRNA microarray and miRNA microarray data, we constructed the ceRNA network jointed by lncRNAs, miRNAs and mRNAs. Previous works have proposed several different strategies, such as RNA22 and miRanda[12,30], to predict miRNA-target interactions. However, these algorithms were mainly used in the prediction of miRNA-mRNA interaction. Since that lncRNAs have recently emerged as major players in governing fundamental biological processes and disease occurrence; and that ~18% of the protein-coding genes that produce lncRNAs are associated with cancers, whereas only 9% of all human protein-coding genes are associated with cancers[31], here, we focused on searching for lncRNA-miRNA interactions. We used miRcode, an lncRNA-miRNA interaction prediction algorithm, to predict MREs in lncRNAs. In addition, to enhance the data reliability, validated mRNA targets were acquired from TarBase only with effective experimental supporting data. These defined rules are beneficial to screening for candidate ceRNAs.

Our analysis suggested lncRNAs harbor potential MREs and participate in a complex ceRNA network (Fig. 2). The network brings to light an unknown miRNA regulatory network in gastric cancer. It also suggests that lncRNAs may play crucial roles in gastric cancer occurrences and development (Table 1). As Sumazin *et al.* proved, ceRNA network is a scale-free network in which miRNAs mediated interactions exhibit symmetric behavior[14]. We found that many oncogenes and tumor suppressors participate in the ceRNA network in gastric cancer (Table 3). For instance, mediated by miR-106a-5p, PTEN, RB1, RUNX1, VEGFA, CDKN1A, E2F1, HIPK3, IL-10, or PAK7 may interact with lncRNA-FER1L4, GACAT1 and H19. Previous study has showed that miR-106a-5p, the core element in this network, is one of the typical onco-miRNAs[32]. FER1L4 and RB1 are targeted by miR-106a-5p. siRNA silencing of FER1L4 led to RB1 mRNA level decrease (Fig. 5).

In addition, we analysed ceRNAs' expression in several sets of data from cancers including solid cancers and leukemia. We found that there were excellent positive correlations between their expression levels (Fig. 3). This coexpression character corresponds with ceRNA hypothesis.

Recently, two groups have identified a circular RNA (circRNA) that contains ~70 MREs with miR-7[33,34]. The circRNA sequesters miR-7 away from its targets and suppresses its function. Thus circular RNAs are a new class of ceRNAs[35,36]. On the other hand, to predict ceRNAs' effect on transcriptional network, Ala and colleagues developed a computational model of ceRNA[37]. Further investigations of ceRNA have implications in many fields. For instant, to block the functions of miRNAs, Tang *et al.* constructed an artificial ceRNA named short tandem target mimic (STTM)[38]. STTM is similar to miRNA sponge, but contains different kinds of MREs that bind various miRNAs. Based on the ceRNA hypothesis, Liu *et al.* further presented a long intergenic noncoding RNA (lincRNA) database named Linc2GO[39]. Li *et al.* also developed a database named starBase v2.0 for predicting miRNA-ceRNA interactions[40]. Moreover, ceRNA might be a new therapeutic approach to diseases[41].

In conclusion, we proposed a new approach to construct ceRNA network. It might help us to easily searching for ceRNAs involved in cancer-associated lncRNAs. Our findings suggest that lncRNAs may harbor MREs and participate in a complex ceRNA network.

Methods

The methods were carried out in "accordance" with the approved guidelines.

Tissue samples. Biopsy samples of gastric cancer tissues and paracancerous tissues were obtained from the Affiliated Hospital of Ningbo University School of Medicine. The paracancerous tissues were 5 cm from the edge of tumor; and there were no obvious tumor cells, as evaluated by an experienced pathologist. There was no radiotherapy or chemotherapy prior to the endoscopy examination. The Human Research Ethics Committee from Ningbo University approved all aspects. Written informed consent was taken from all subjects.

RNA extraction. Total RNA was extracted by using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quantity was measured by a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). RNA purity was evaluated by the A_{260}/A_{280} ratio.

lncRNA microarray and computational analysis. For lncRNA expression profiling, the Human LncRNA Array (Arraystar, Rockville, MD, USA) which covered 18,534 lncRNAs was used. After hybridization and washing, the arrays were scanned by an Axon GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA). Raw data were extracted and normalized by NimbleScan v2.5 software package (Roche NimbleGen, Madison, WI, USA).

Construction of the ceRNA network. The construction of ceRNA network included three steps (Fig. 6): (i) lncRNA screening: lncRNAs that were up- or down-regulated fold change ≥ 3.0 and P-value < 0.05 were first retained; then to enhance the data reliability, lncRNA whose sequences have not been recorded in ENCODE were removed; (ii) lncRNA-miRNA interactions were predicted by miRcode (http://www.mircode.org/); (iii) mRNAs that were targeted by miRNAs with experimental support were from TarBase (http://www.microrna.gr/tarbase).

Re-sampling analysis of ceRNA network. To get more robust results of ceRNA network, we assigned a P-value to each relationship by re-sampling analysis[42]. First, we re-sampled the set of above miRNAs from the pool of all human miRNAs in miRBase database and re-inferred the ceRNA relationships. Then we observed whether the previously predicted ceRNA relationships occurred in the newly inferred dataset. We repeated the re-sampling 10,000 times and counted the number of times that each ceRNA relationship occurred. The P-value was defined as the number of occurred times over 10,000. Finally, we obtained the ceRNA relationships using the cutoff of 0.01.

Regression analysis of ceRNAs' expression level. Several sets of gene expression data in cancers were analysed (data accessible at NCBI GEO database, accession GDS1667, Slebos *et al*, 2005; accession GDS4159, Vitari *et al*, 2011; accession GDS1665, He *et al*, 2005; accession GDS4275, Michaelis *et al*, 2011; accession GDS4168, Gutierrez *et al*, 2010; accession GDS4066, Spillman *et al*, 2011). Regression analysis and correlation coefficient were generated using SPSS Statistics v20.0 software package (IBM, Armonk, NY, USA).

Cells and culture conditions. Human gastric epithelial cell line, GES-1, was obtained from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). Human gastric cancer cell lines, AGS, MGC-803 and SGC-7901, were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Human embryonic kidney cell line, HEK 293T, was obtained from GeneChem Co., Ltd. (Shanghai, China). GES-1, AGS, MGC-803 and SGC-7901 were grown in RPMI Medium 1640 (Life Technologies) plus 10% fetal bovine serum (FBS). HEK 293T was grown in DMEM (Life Technologies) plus 10%

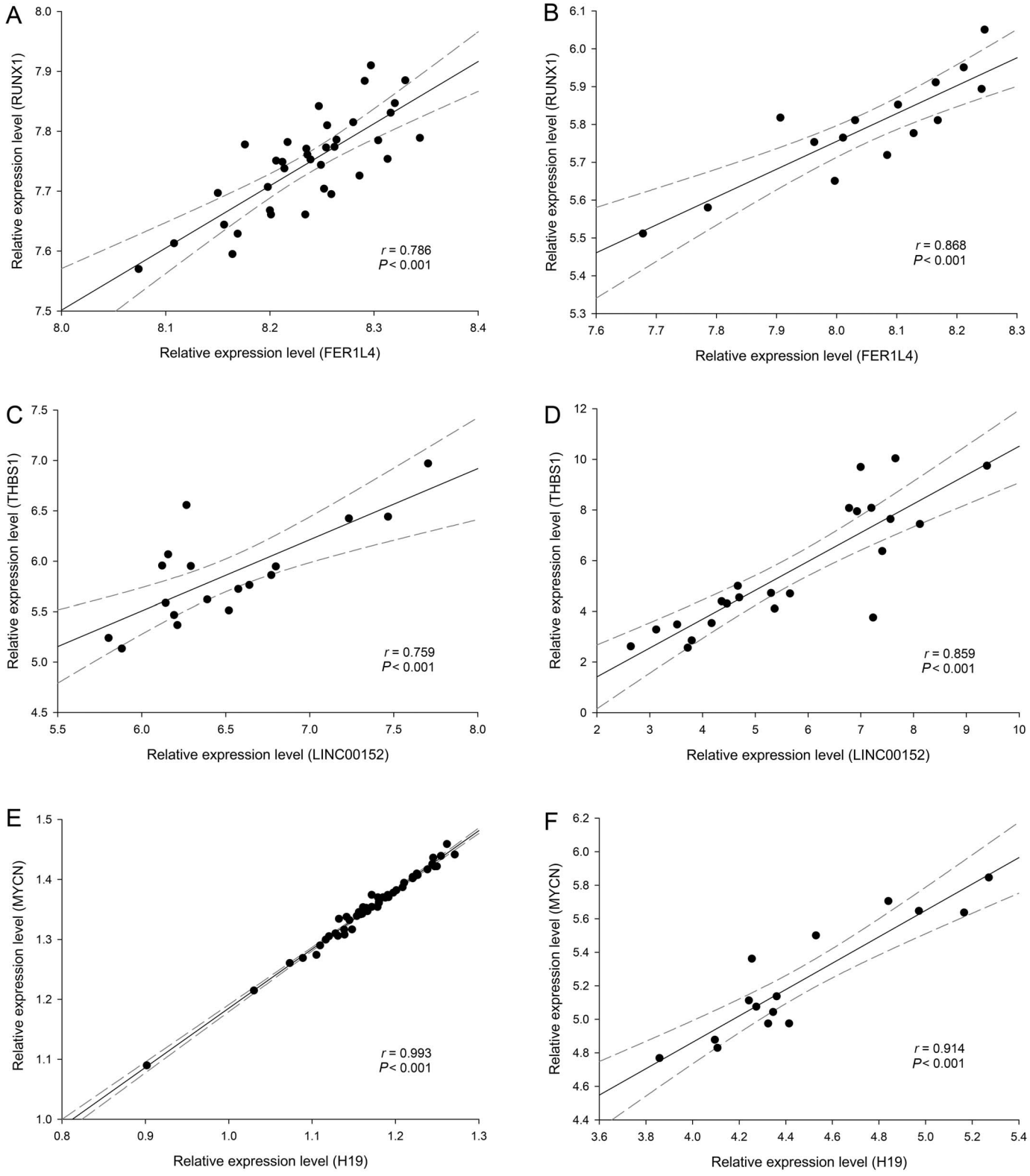

Figure 3 | Linear regression of ceRNAs' expression level. Dashed lines represent 95% confidence interval. (A) FER1L4 *vs* RUNX1 (head and neck squamous cell carcinoma, $n = 36$). (B) FER1L4 *vs* RUNX1 (prostate cancer, $n = 15$). (C) LINC00152 *vs* THBS1 (papillary thyroid carcinoma, $n = 18$). (D) LINC00152 *vs* THBS1 (pituitary gonadotrope tumors, $n = 23$). (E) H19 *vs* MYCN (chronic lymphocytic leukemia, $n = 52$). (F) H19 *vs* MYCN (ovarian cancer, $n = 15$).

FBS. All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2. Cells were counted using a TC10 Automated Cell Counter (Bio-Rad).

Dual luciferase reporter assay. miR-106a-5p expression plasmid (GV268) was purchased from GeneChem Co., Ltd. (Shanghai, China). The wild-type and mutant DNA sequences of FER1L4 were custom synthesized by GeneChem Co., Ltd. and cloned into GV272 Firefly luciferase plasmid (GeneChem). The miR-106a-5p MRE of wild-type FER1L4 was 5′-GCACUU-3′, while the mutant sequence was 5′-UACAGG-3′. HEK 293T of 80% confluence in 24-well plates was transfected using Lipofectamine 2000 Reagent (Life Technologies) according to the manufacturer's

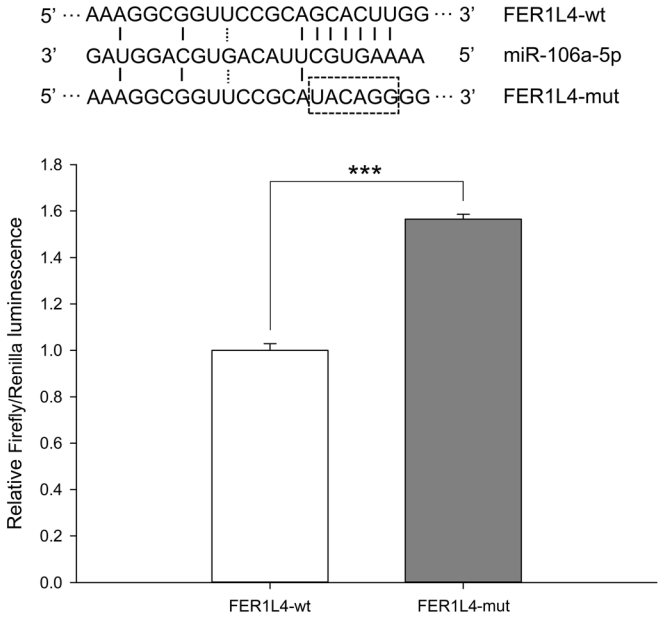

Figure 4 | Relative Firefly/Renilla luminescence (mean ± SD, $n = 3$) mediated by luciferase plasmid harboring the wild-type or mutant FER1L4 sequence upon transfeciton with miR-106a-5p expression plasmid. It indicates the direct interaction between FER1L4 and miR-106a-5p. $***P < 0.001$.

protocol. Firefly luciferase plasmid and miR-106a-5p expression plasmid were co-transfected with pRL-TK Renilla luciferase vector (Promega, Madison, WI, USA) for normalization. 48 hours after transfection, luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega). All experiments were performed in triplicate.

Transient transfection. For the transfection of siRNA, GES-1, AGS, MGC-803 and SGC-7901 (2×10^5) were seeded in 6-well plates. The following day they were transfected with 120 nM siRNAs using Lipofectamine 2000 Reagent (Life Technologies). FER1L4 siRNA was: 5′-CAGGACAGCUUCGAGUUAATT-3′ (sense) and 5′-UUAACUCGAAGCUGUCCUGTT-3′ (antisense). Negative control siRNA was: 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense).

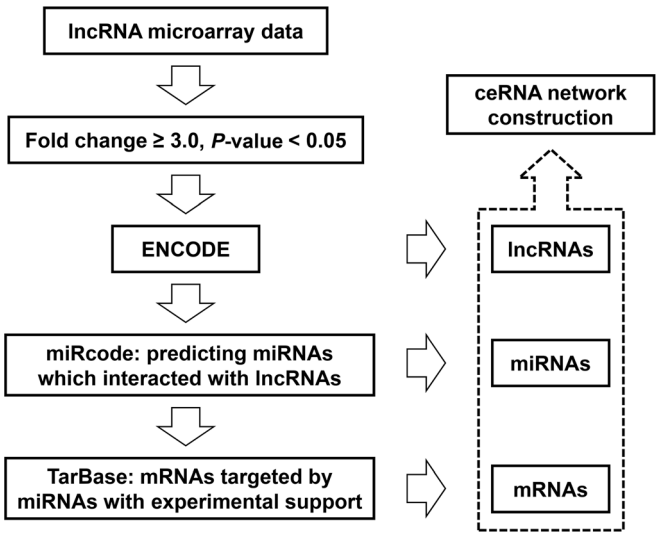

Figure 6 | A flowchart of ceRNA network construction. (i) lncRNAs that are fold change ≥ 3.0 and P-value < 0.05 were retained; (ii) lncRNAs that have not been recorded in ENCODE were removed; (iii) miRNA-lncRNA interactions were predicted by miRcode; (iv) mRNAs that targeted by miRNAs were captured from TarBase; (v) ceRNA network construction.

qRT-PCR analysis. qRT-PCR was performed using GoTaq 2-Step RT-qPCR System (Promega) in a Mx3005P QPCR System (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Relative quantification of gene expression was performed with $2^{-\Delta\Delta Ct}$ method[43]. FER1L4 primers: forward 5′-CCGTGTTGAGGTGCTGTTC-3′ and reverse 5′-GGCAAGTCCACTGTCAGATG-3′. RB1 primers: forward 5′-TGGCG-TGCGCTCTTGAGGTT-3′ and reverse 5′-AGAGCCATGCAAGGGATTCC-ATGA-3′. GAPDH primers: forward 5′-AAGGTGAAGGTCGGAGTCAA-3′ and reverse 5′-AATGAAGGGGTCATTGATGG-3′. All experiments were performed in triplicate.

Statistical analysis. Data were presented as mean ± SD. Differences among groups were evaluated by two-tailed Student's t-test by using SPSS Statistics v20.0 software package (IBM). $P < 0.05$ was considered to be statistically significant.

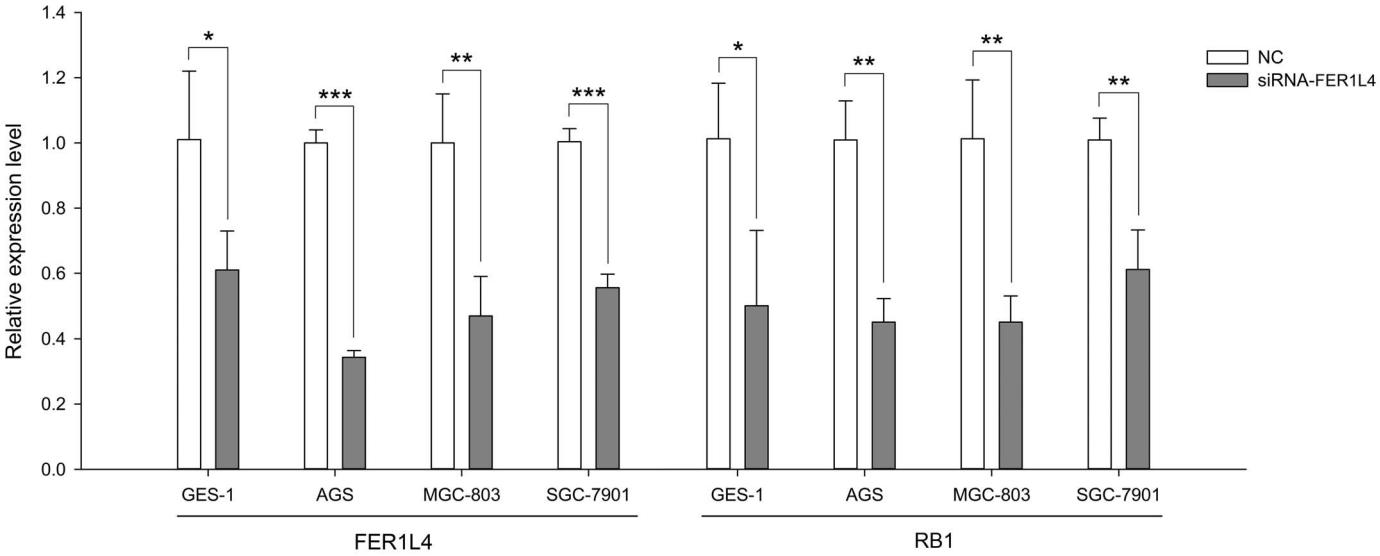

Figure 5 | qRT-PCR analysis of expression levels (mean ± SD, $n = 3$) of FER1L4 and RB1 in GES-1, AGS, MGC-803 and SGC-7901 treated with siRNA against FER1L4. NC, negative control. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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Author contributions

T.X. and J.G. conceived and designed the experiments. T.X. Q.L. X.J. Y.S. B.X. performed the experiments. T.X. Q.L. X.J. B.X. Y.X. J.G. analyzed the data. T.X. J.G. wrote the paper.

Additional information

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