

Identification of lncRNA-associated competing endogenous RNA networks for occurrence and prognosis of gastric carcinoma

Lianmin Ye

Wenzhou Medical University First Affiliated Hospital

Wumin Jin (✉ jydigestion@163.com)

Wenzhou Medical University First Affiliated Hospital

Research

Keywords: Gastric cancer; Competing endogenous RNA; Bioinformatics; Prognosis

Posted Date: July 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-45596/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published at Journal of Clinical Laboratory Analysis on October 26th, 2021. See the published version at <https://doi.org/10.1002/jcla.24028>.

Abstract

Background: Gastric cancer (GC) is one of the common digestive malignancies worldwide and causes a severe public health issue. So far, the underlying mechanisms of GC are largely unclear. Thus, our aim is to identify the long non-coding RNA (lncRNA) associated competing endogenous RNA (ceRNA) specialized for occurrence and progression in GC.

Methods: The comprehensive online dataset, TCGA, was downloaded and used for identification of differentially expressed (DE) lncRNA, miRNA and mRNA screen with the value of $\log_{2}FC = 1$ and $FDR < 0.05$, respectively. The interactions between lncRNA and miRNA as well mRNA and miRNA were predicted via multiple online databases, such as miRcode and Targetscan. Then the ceRNA network was constructed accompanied with gene set enrichment analysis and survival analysis. In addition, RT-qPCR and in vitro assay was carried out to validate the effect of the hub lncRNAs.

Results: We identified 1485 lncRNAs, 312 miRNAs and 4260 mRNAs were differentially expressed between GC and normal tissues, respectively. Then, we generated a ceRNA network with 909 edges and 253 nodes including 76 lncRNA, 18 miRNA and 159 mRNA. This ceRNA network was involved in MET activates PTK2 signalling, MET promotes cell motility and non-integrin membrane-ECM interactions. Next, by univariate and multivariate analysis, there were 9 hub lncRNAs emerged and were associated subnetwork involved in actin filament binding and MAPK signaling pathway. The in vitro assay indicated lncRNA INHBA-AS1 and CCDC144NL-AS1 may positively related to the GC aggressive features, including proliferation, invasion and migration.

Conclusion: In summary, we constructed a ceRNA network involved in the GC development. Moreover, we also identified 9 hub lncRNA-associated network related to prognosis of GC and validated two out of them as promising oncogenes. This may provide potential biomarkers or therapeutic target for GC in future.

Background

Gastric cancer (GC) is one of the frequent digestive system cancer, which accounts for the second cause of cancer mortality worldwide by 2018 (1). The cases in China account for more than 40% total number of GC worldwide due to a high incidence rate and a large population (2). In addition, the increasing GC cases may be explained by that GC patients were more likely be advanced stage when diagnosed because of non-early specific symptoms. The late diagnosis can significantly effect on the 5-year survival rate. In the past decade, due to the advancement of therapeutic treatment and medicine, the prognosis has improved, but it is still not satisfied for the disease-free survival time (3). Thus, it is challenging and necessary to understand the underlying mechanisms of GC occurrence and progression and develop novel biomarker or treatment target.

In the other hand, long non-coding RNA (lncRNA) is a well-known member of the non-coding RNA family with the length over 200 nt (4, 5). In the past decade, the accumulating knowledges indicate the importance role of the aberrant expression of lncRNAs in GC (6, 7). LncRNA can display specific regulatory effect in cancer research field. Specifically, it can function as sequence-specific recruitment of proteins, competing endogenous RNA (ceRNA) regulation and molecular scaffolding of protein complexes, in which the ceRNA regulation is widely investigated in the recent years. It is hypothesised that lncRNA can modulate miRNA-regulated mRNA expression by competitively binding miRNAs through endogenous molecular sponges (8). This regulatory mechanism provides a potential explanation to interpret the roles of lncRNA in various cancers (9). The previous studies have already identified the lncRNA was involved in GC via ceRNA (10–12). Those indicated the potential significance to investigate the lncRNA-associated ceRNA network and promote us to elucidate the underlying mechanisms of specific lncRNA-miRNA-mRNA axis.

Therefore, we employed the online open dataset to construct the ceRNA network. Furthermore, the hub lncRNAs with sub ceRNA network related to prognosis were further identified. To confirm the reliability and validity of the results of network, the potential effect of two hub lncRNAs were validated in vitro. Overall, the present study showed the occurrence and progression related ceRNA network and determined 11 hub lncRNA for further investigation in future.

Methods

Data resources and Differential expression analysis

We download the RNA sequence data with $\log_2(\text{fpm} + 1)$ transformed (lncRNA and mRNA, level 3; Illumina HiSeq RNA-Seq platform), miRNA sequence data (Illumina HiSeq miRNA-Seq platform), and clinical information from the Xena dataset ([https://xenabrowser.net/datapages/?dataset=TCGA-STAD.htseq_fpm-
uq.tsv&host=https%3A%2F%2Fgdc.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443](https://xenabrowser.net/datapages/?dataset=TCGA-STAD.htseq_fpm-
uq.tsv&host=https%3A%2F%2Fgdc.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443)). Annotation information for RNA-seq data was provided by the Ensemble database derived from “biomaRt” package (13). The expression value was analysed by the “limma” package (14) to screen differentially expressed (DE) lncRNAs, miRNAs and mRNAs between 32 normal samples and 372 cancer samples. We set the $|\log_2(\text{Fold change})| > 1$ and p value < 0.05 as the thresholds to identify DE genes. DE lncRNA, miRNA and mRNA were presented as a volcano plot.

Cerna Network Construction

To construct a ceRNA network, we predicted the interactions between lncRNA and miRNA as well as mRNA and miRNA. For miRNA interacted with mRNA, we used “miRNAatp” package (<https://bioconductor.org/packages/release/bioc/html/miRNAatp.html>), which consisted of commonly used five reliable and online datasets, including Pictar (<https://pictar.mdc-berlin.de/>), DIANA(15), Targetscan (16), miranda (17), mirdb (18). Only when the interaction was identified at least three datasets, we considered it as potential interactions. The lncRNA-miRNA interactions were predicted utilising the miRcode (19).

After obtaining the potential interactions, we extracted the candidate axis lncRNA-miRNA-mRNA and submitted to ceRNA construction. Given the potential regulatory mechanisms between lncRNA to mRNA, we used the “GDCRNATools” package (<http://bioconductor.org/packages/devel/bioc/vignettes/GDCRNATools/inst/doc/GDCRNATools.html>), which can identify the interaction based on those two criterions, 1) expression of lncRNA and mRNA must be positively correlated and 2) those common miRNAs should play similar roles in regulating the expression of lncRNA and mRNA. Those two respects were indicated via the Pearson correlation and regulation similarity (20).

Prognosis Related Lncrna Identification

To further identify the potential prognosis related lncRNA, we grouped the samples into high- and low-expression subgroups based on the mean expression. Then, univariate Cox regression analysis was used to find the prognosis-related lncRNA. Then, the lncRNA achieving statistical significance ($P < 0.05$) were submitted into multivariate Cox analysis adjusting with age, gender, TNM stage and histological grade. In addition, the KM plot with log-rank test was carried out to further validate the prognosis related result.

Gene Sets Enrichment Analysis

To reveal the function of lncRNA-associated ceRNA network, the DE mRNA derived from ceRNA were subjected to gene sets enrichment analysis based on gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and REACTOME annotation with the R package clusterProfiler (21). GO is a structured standard biological model established by the GO consortium, including biological processes (BP), molecular functions (MF), and cellular components (CC) (22). KEGG is widely used as a reference for integrating large-scale molecular datasets generated by sequencing and high-throughput experimental technologies (23). RACTOME is an open-source, open access, manually curated and peer-reviewed pathway database (<https://reactome.org/>). Gene sets with a p value < 0.05 were considered as significant.

Cell Line Culture, Rna Extraction And Real-time Pcr

GC cell line, MKN45, was purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ThermoFisher Scientific, Waltham, MA, USA) combined with 10% fetal bovine serum (FBS).

The primers were designed and purchased from GenePharma (Shanghai, China, Table 1). Total RNA was extracted using the ReliaPrep RNA Cell Miniprep System (Promega). Total RNA was first reversely transcribed into complementary DNA through the iScript Reverse Transcription Kit (Bio-Rad). Then, SYBR Green Supermix (Bio-Rad) was employed to perform quantitative-PCR (qPCR). which was ran in Bio-Rad CFX 96 PCR instrument (Bio-Rad). The 2- $\Delta\Delta$ CT method was used to evaluate the relative gene expression. The GAPDH and U6 were used as the internal control for lncRNA/mRNA and miRNA, respectively.

Table 1
Forward and reverse primer sequences used for quantitative PCR.

Gene	Sequences (5'↯3')
INHBA-AS1	F: CCTTTCCAGTCAGGTGGGTC
	R: CCCAGGGGAAGCATCACTTT
CCDC144NL-AS1	F: CCCTCCTCACGGCATAGAAC
	R: TTAGTGGTTCCTTGGGAGTCG
COL5A2	F: CAAACTGGGCGGAAGCAAG
	R: TTCACCATATCCTTCATCCTCG
MATN3	F: GACAGAACAGGGTCCCATCA
	R: GCACACTTGTCACGGACTGA
GAPDH	F: CTGGGCTACACTGAGCACC
	R: AAGTGGTCGTTGAGGGCAATG
hsa-miR-98	RT: CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTatgccca
	F: AACAAGCTGCACATGCTGGG
	R: AACAATCTGCACATGCTGGGG
hsa-miR-128-1	RT: CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTgaagca
	F: AACAAGTGAGCTGTTGGATTCCG
	R: AACAATTGAGCTGTTGGATTCCGG
U6	F: CTCGCTTCGGCAGCAC
	R: AACGCTTCACGAA TTTGCGT
F, forward; R, reverse.	

SiRNA Knockdown In Cell Line

siRNA designed and chemically synthesized by GenePharma (Shanghai, China). The sequences of siRNA against INHBA-AS1 was: 5'-GUCUCAUGACCACAGCUAAtt-3' (Sense) and 5'-UUAGCUGUGGUCAUGAGACct-3' (Anti-Sense) and while siRNA against CCDC144NL-AS1 was 5'-UAGGUAGAUGGUGGAAUGAtt-3' (Sense) and 5'-UCAUUCACCAUCUACCUAtg-3' (Anti-Sense). Before the transfection, MKN45 cells were cultured with antibiotics-free DMEM for 24 hours in advance. Transfections of the siRNAs were manipulated via the lipofectamine RNAiMAX (Invitrogen, USA) and the same protocols were carried out following the manufacturer's instruction in all those two lncRNA.

Proliferation Assay

To evaluate the proliferation speed, 3×10^3 cells were seeded into a 96-well plate per well (Greiner bio-one), and then a time-series assay each 2 days were carried out in triplicate. We used the MTS CellTiter 96 One Solution Cell

Proliferation Assay (Promega) to measure the proliferation. With 1, 3, 5, and 7 days, the absorbance at 490 nm was measured using the microtiter plate spectrophotometer (Benchmark Plus, Bio-Rad) according to the manufacturer's protocol. Subsequently, proliferation was normalised based on the absorbance of the first day and calculated by the changes between readings.

Transwell Migration And Invasion Assay

For cell migration and invasion assay, 24-well transwell inserts with a pore size of 0.8 mm (Corning) were used. After siRNA transfection, 1×10^5 cells were seeded in the upper chamber, and 650 μ l of complete medium was added to the lower chamber as a chemo-attractant. The 100 μ l Matrigel (Corning) with concentration of 20 mg/ml was pre-coated in the upside of insert for invasion assay. The cells were allowed to migrate or invade toward the chamber for 12 hour and 16 hour, respectively. The migrated and invaded cells on the underside of the membrane were fixed with 4% paraformaldehyde, stained with DAPI (Beyotime Biotechnology, Shanghai, China), and quantified from microscopic fields.

Statistical analysis

The Student's t-test was used to compared continues variables. Chi-squared test was employed to categorical variables. P values have 2 tails and only when it is less than 0.05 was considered significant. All the figures and statistical analysis were carried out via R software.

Results

Identification of differently expressed lncRNA, miRNA and mRNA

To better understand the DE genes associated with GC tumorigenesis, we initially identified the DE lncRNA, miRNA and mRNA between normals ($n = 32$) and cancers ($n = 321$), respectively. Then, as shown in Fig. 1, with the cut-off value of $|\log_{2}FC| \geq 1$ and $FDR < 0.05$, there were 1485 DE lncRNA (1260 up-regulation and 225 down-regulation), 312 DE miRNA (290 up-regulation 12 down-regulation) and 4260 DE mRNA (2347 up-regulation and 1913 down-regulation). The details of those DE lncRNA, miRNA and mRNA were showed in supplemental Table 1 (see Additional file 1).

Cerna Network Construction And Function Analysis

To construct the ceRNA, we first generated the interaction between DE miRNA and lncRNA, and miRNA and mRNA. There were 6082 pairs of lncRNA-miRNAs interactions predicted via miRcode database. Then, 938 interactions between miRNA and mRNA were found via the above-mentioned criterion. After that, we identified 24847 potential lncRNA-miRNA-mRNA axes consisting of 892 lncRNA, 18 miRNA and 278 mRNA.

The ceRNA network was constructed via "GDCRNATools" package and "gdcCEAnalysis" function, which can provide the pearson correlation and regulation pattern to further determine the promising ceRNA. Finally, a ceRNA network with 909 edges and 253 nodes including 76 lncRNA, 18 miRNA and 159 mRNA was generated with Pearson correlation coefficient ≥ 0.5 , Pearson correlation p value > 0.05 and regulation similarity $\neq 0$ (Fig. 2 and supplemental Table 2 see Additional file 1).

Next, we performed gene sets enrichment analysis to better understand the potential biological processes and pathways of those 159 DE mRNA derived from the ceRNA network. We first divided the DE mRNA into two groups, including up- (n = 33) and down-regulated (n = 126) genes. Then, we employed GO, KEGG and REACTOME dataset and identified 20 significant GO terms, 1 KEGG and 22 REACTOME for up-regulated genes (supplemental Table 3 see Additional file 1). In meantime, there were 220 significant GO terms and 5 REACTOME for down-regulated genes (supplemental Table 4 see Additional file 1). Then, we displayed the top 20 significant gene sets including GO, KEGG and REACTOME in the Fig. 3.

Survival-associated Lncrna And Mrna Identification

To investigate the potential prognosis related lncRNA, we utilised univariate Cox analysis to filter those 76 lncRNA derived from the above ceRNA network. Then, the lncRNA and mRNA with p value achieve 0.05 were further subjected into multivariable Cox analysis with adjustment of age, gender, histological grade and TNM stage. Then, there were 11 lncRNA associated with overall survival (OS) (Table 2). Intriguingly, they were all negatively related to OS. The mean expression of lncRNA was utilised as cut-off value to determine the high- and low-expression groups. Then, the log-rank test was applied to validate the relationship between the OS and those lncRNA (Fig. 4).

Reconstruction And Function Analysis Of Hub Lncrna-associated Cerna Network

We assumed that those 11 lncRNAs may play critical roles in the GC-related ceRNA network. Accordingly, we extracted the corresponding 59 mRNA derived from the ceRNA network. Then, the univariate and multivariate Cox analysis were employed to filter the prognosis-related mRNA with the same procedures. There were 13 out of 59 mRNA showing the independent relationship with OS (Table 2). Also, we performed the KM plot with log-rank test to validate the relationship between mRNA and OS and all those 13 mRNA remained with p value < 0.05 (Fig. 5).

Table 2
Univariate and multivariate Cox analysis for lncRNA and mRNA for overall survival of GC.

RNA	Symbol	Univariable Cox analysis				Multivariable Cox analysis			
		HR	95% CI lower	95% CI upper	P value	HR	95% CI lower	95% CI upper	P value
lncRNA	LINC02731	1.527	1.109	2.103	0.036	1.574	1.139	2.175	0.007
lncRNA	MIR99AHG	1.447	1.052	1.992	0.008	1.433	1.036	1.984	0.005
lncRNA	INHBA-AS1	1.419	1.034	1.949	0.024	1.420	1.033	1.953	0.007
lncRNA	LINC02613	1.480	1.078	2.032	0.023	1.550	1.126	2.133	0.003
lncRNA	CCDC144NL-AS1	1.439	1.046	1.981	0.018	1.445	1.047	1.994	0.006
lncRNA	VLDLR-AS1	1.379	1.004	1.896	0.030	1.502	1.090	2.069	0.004
lncRNA	LINC01497	1.596	1.159	2.198	0.045	1.509	1.086	2.097	0.005
lncRNA	LIFR-AS1	1.466	1.066	2.016	0.005	1.446	1.046	1.998	0.006
lncRNA	A2M-AS1	1.472	1.070	2.024	0.044	1.437	1.042	1.980	0.004
lncRNA	LINC01537	1.502	1.093	2.064	0.002	1.580	1.142	2.185	0.003
lncRNA	LINC00702	1.457	1.057	2.007	0.025	1.410	1.019	1.950	0.006
mRNA	NOVA1	1.789	1.294	2.473	0.010	1.856	1.340	2.569	0.006
mRNA	NPAS3	1.442	1.050	1.981	0.023	1.579	1.145	2.178	0.005
mRNA	CACNB2	1.412	1.023	1.949	0.030	1.377	0.996	1.902	0.008
mRNA	PDE7B	1.543	1.118	2.129	0.015	1.474	1.064	2.042	0.007
mRNA	CACNA2D3	1.467	1.051	2.047	0.025	1.368	0.980	1.911	0.006
mRNA	COL21A1	1.488	1.057	2.096	0.047	1.536	1.076	2.192	0.004
mRNA	SLC35F1	1.376	1.002	1.891	0.004	1.410	1.025	1.939	0.008
mRNA	PKIA	1.436	1.036	1.991	0.019	1.401	1.009	1.945	0.007
mRNA	KLF9	1.385	1.008	1.904	0.017	1.327	0.957	1.839	0.006
mRNA	PKNOX2	1.616	1.153	2.265	0.012	1.788	1.267	2.522	0.003
mRNA	COL5A2	1.387	1.009	1.906	0.021	1.490	1.072	2.071	0.014
mRNA	MATN3	1.651	1.197	2.277	0.000	1.695	1.222	2.351	0.009
mRNA	SNAP25	1.436	1.046	1.971	0.024	1.359	0.986	1.874	0.008

Next, a sub ceRNA network consisting 24 edges and 27 nodes, including 9 lncRNA, 5 miRNA and 13 mRNA, was reconstructed (Fig. 6A). Thus, those 9 lncRNA were considered as hub lncRNA, including LINC02731, MIR99AHG, INHBA-AS1, CCDC144NL-AS1, VLDLR-AS1, LIFR-AS1, A2M-AS1, LINC01537 and LINC00702. To reveal the potential biological function of this sub network, the functional enrichment analysis was carried out for those 13 mRNA and

obtained 8 significant GO terms, 8 KEGG and 30 REACTOME sets, such as MAPK signalling pathway (Fig. 6B and supplemental Table 5 see Additional file 1).

Inhba-as1 And Ccdc144nl-as1 Are Potential Oncogene In Gc

First, all those 9 hub lncRNA displayed inverse association with OS, and then we only selected the lncRNA that higher expressed in cancer tissues compared to normal. In this way, two lncRNA, INHBA-AS1 and CCDC144NL-AS1, were kept. Subsequently, to validate the findings, siRNA-mediated silencing of lncRNAs were measured by RT-qPCR, and the knockdown efficiencies of INHBA-AS1 and CCDC144NL-AS1 were achieved significant in MKN45 cell line (Fig. 7A).

Next, we investigated the potential effect of INHBA-AS1 and CCDC144NL-AS1 in MKN45 cell line. Knockdown of those 2 lncRNA both suppressed cell proliferation as determined by MTS assays (Fig. 7B). The results of migration and invasion assay indicated that MKN45 cell line with INHBA-AS1 and CCDC144NL-AS1 knockdown significantly less migrated and invaded than their counterpart (Fig. 7C and D). Based on the ceRNA axis, we selected hsa-mir-98 and hsa-mir-128-1 to verify the relationships between lncRNA and miRNA. Using RT-qPCR, we observed that knockdown of INHBA-AS1 and CCDC144NL-AS1 significantly increased the hsa-miR-98 and hsa-miR-128-1 expression level, respectively (Fig. 7E). Accordingly, the expression of COL5A2 and MATN3 showed a significant decrease compared to controls (Fig. 7F). These results indicated that INHBA-AS1 and CCDC144NL-AS1 might have oncogenic function and act as ceRNA to sponge miRNAs in GC.

Discussion

So far, the GC is the one of the top-ranking digestive cancer and has become a worldwide public concern. Thus, it is worthwhile to investigate the potential biomarkers and promising tumour promoting or suppressor genes in GC. In our study, we identified lncRNA-associated ceRNA network involving in GC tumorigenesis, which was based on analysis of gene expression data obtained from TCGA databases. Then, we identified 9 hub lncRNA accompanied with the sub ceRNA network related to OS. Among those 9 lncRNA, we partially validated the effect of INHBA-AS1 and CCDC144NL-AS1 in vitro and found they were promising oncogene in GC.

As mentioned above, lncRNA can influence the expression of mRNA via competitively binding to shared miRNAs, which is defined as ceRNA and may play critical role in regulation of the cancer development and progression (8). This lncRNA-miRNA-mRNA regulatory mechanism has been identified in GC. For instance, lncRNA LINC01133 can inhibit GC progression by sponging hsa-mir-106a-3p and then effect on APC expression (10). In addition, lncRNA PTENP1 can regulate PTEN expression via binding to hsa-mir-106b and hsa-mir-93 in GC (11). Except those individual cases, some other studies carried out integrated analysis for ceRNA. Arun et al. employed 13 GCs and paired adjacent normal tissues and found the crucial ceRNA network for GC (12). Similarly, Liu et al. integrative analysis of lncRNA-associated ceRNA network and validated their findings in vitro in GC (24). However, one of the cell lines they used, BGC823, is considered as HeLa derivative contamination (25). Our study first investigated the differential expressed lncRNA, miRNA and mRNA between normal and GC, respectively, and then constructed a GC development related ceRNA network. This network is involving in up-regulation of MET activates PTK2 signalling, MET promotes cell motility and non-integrin membrane-ECM interactions. Based on REACTOME annotation, the MET activates PTK2 signalling is related to MET receptor activates the focal adhesion kinase FAK1, which plays crucial roles in focal adhesions (FAs). Specifically, FAs are large macromolecular complexes of integrins that mediate cell-ECMs interactions and facilitate the metastatic process (26, 27). Metastasis is a well-known aggressive feature for cancer. Previous studies identified that FAs is strongly associated with metastasis and lower survival rates (26, 28–30). Moreover, FAs can impact on

various tumour behaviours, such as migration, invasion and proliferation (31). This may partially explain the influence of ceRNA network on GC. In addition, MET promotes cell motility may also contribute GC development (32). Non-integrin membrane-ECM interactions, such as dystroglycan and 37/67 laminin receptor, is found to be related to variety of epithelial cancers (33).

Subsequently, we further filtered 9 hub lncRNA, including LINC02731, MIR99AHG, INHBA-AS1, CCDC144NL-AS1, VLDLR-AS1, LIFR-AS1, A2M-AS1, LINC01537 and LINC00702. The hub lncRNA associated ceRNA subnetwork involved in actin filament binding and MAPK signalling pathway. Filament is a form of dense meshwork generated by lamellipodia, at the leading edge of a migrating or extending cell, which facilitate cellular movement and play essential roles in tumour cell metastasis (34). MAPK signaling pathway is involved various promoting-cancer mechanisms, such as anti-drug, inflammation and immune evasion(35–38). In term of individual lncRNA, most of them have already found to be related to development and progression of various cancers. For instance, MIR100HG has been validated as an oncogene in the development of myeloid leukemia in vitro (39). In addition, it was also positively related to poorer prognosis in GC based on dataset other than TCGA (40). lncRNA INHBA-AS1 can promote multiple invasion features, including cell growth, migration, and invasion in oral squamous cell carcinoma, which targeting on hsa-mir-143-3p (41). The INHBA-AS1 in GC plasma was found higher expressed compared to the one in heavy controls (42). Knockdown of lncRNA CCDC144NL-AS1 attenuates migration and invasion phenotypes in endometrial stromal cells (43). The expression of VLDLR-AS1 was independently related to prognosis in thymoma (44). The LIFR-AS1/hsa-mir-29a/TNFAIP3 axis played effect on resistance of photodynamic therapy in colorectal cancer (45). High expression of lncRNA LIFR-AS1 was correlated with poor survival in GC (46). Up-regulated lncRNA, A2M-AS1, was associated with invasion and migration in breast cancer (47). Except that, LINC00702 enhanced the progression of ovarian cancer through increasing EZH2 expression (48). Then, LINC00702/hsa-mir-4652-3p/ZEB1 axis is promising to promote the progression of malignant meningioma through activating Wnt/ β -catenin pathway (49). Taken together, most of those 9 hub lncRNAs were promising tumour-promoting genes in diverse cancer, and were worthwhile for further investigation in GC.

Then, given the regulatory direction of those 9 lncRNA, INHBA-AS1 and CCDC144NL-AS1 and related axis were further verified in vitro and showed the potential promoting influence on proliferation, migration and invasion. This indicated those two lncRNA were promising to play oncogenic roles in GC. In term of their targeted mRNA, INHBA-AS1-regulated COL5A2 and CCDC144NL-AS1-regulated MATN3 have been found to play critical role in GC prognosis (50, 51). MATN3 is a member of the matrilin protein family, a noncollagenous extracellular matrix. It has been widely investigated in bone and cartilage related fields and broadened to malignant tumours in recent years (52–54). Specifically, it can induce the expression of MMP1, MMP3, MMP13, pro-inflammatory cytokines, iNOS, and COX2, indicating MATN3 can regulate extracellular matrix degradation (53). The gene COL5A2, collagen type V alpha 2 chain, encodes an alpha chain for one of the low abundance fibrillar collagens. It has been reported to play critical role in the pathological process in multiple cancers including the colorectal cancer, ovarian cancer and bladder cancer (55, 56). Moreover, COL5A2 was highly correlated with cell extracellular matrix organization, vascularization and EMTs process function, and those function were known to be involved in cancer invasion and metastasis (55).

There are several limitations in our study. Firstly, we only employed TCGA dataset that is comprehensive cancer database. Then, although we combined well-designed bioinformatics study and partially in vitro validation, there was no in-depth in vitro evidence, such as dual-luciferase reporter assay and mice model. Thirdly, the current study was of a retrospective nature, as it was based on data from TCGA dataset without validating it in a prospective clinical trial. Therefore, some vital experiments such as luciferase reporter systems and co-immunoprecipitation assays as well as mice model will be helpful to further validate the correlation among lncRNA-associated ceRNA network. Besides, a large clinical sample size needed to be collected in our future study to verify the ceRNA network.

Conclusion

So far, the molecular mechanisms of ceRNA networks effect on GC occurrence and progression are not far from understood. In the present study, we established a comprehensive lncRNA-miRNA-mRNA triple ceRNA network related to development of GC. Moreover, we identified 9 hub lncRNA with ceRNA subnetwork that are promising diagnostic and prognostic values for GC patients. Then, we used experimentally validation to emerge 2 out of the 9 hub lncRNA, INHBA-AS1 and CCDC144NL-AS1, accompanied their corresponding miRNA and mRNA, as potential oncogenic roles in GC. However, these findings need to be further confirmed by more basic experiments and larger-scale clinical trials in the future.

List Of Abbreviations

GC Gastric cancer

TCGA The Cancer Genome Atlas

DE differentially expressed

qPCR quantitative-PCR

lncRNA long non-coding RNA

ceRNA competing endogenous RNA

GO gene ontology

KEGG Kyoto Encyclopedia of Genes and Genomes

BP biological processes

MF molecular functions

CC cellular components

FBS fetal bovine serum

Declarations

Availability of data and materials

The datasets supporting the conclusion of this article are included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that there is no conflict of interest.

Funding

Not applicable.

Authors' contributions

The conception and design of the study: Lianmin Ye and Wumin Jin. Acquisition of data, analysis and interpretation of data: Lianmin Ye and Wumin Jin. Drafting the article and revising it critically for important intellectual content: Lianmin Ye and Wumin Jin. Final approval of the version to be submitted: Lianmin Ye and Wumin Jin. The author(s) read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394–424.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 2015;136(5):E359-86.
3. Zeichner SB, Goldstein DA, Kohn C, Flowers CR. Cost-effectiveness of precision medicine in gastrointestinal stromal tumor and gastric adenocarcinoma. *J Gastrointest Oncol.* 2017;8(3):513–23.
4. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell.* 2009;136(4):629–41.
5. de Oliveira JC, Oliveira LC, Mathias C, Pedrosa GA, Lemos DS, Salviano-Silva A, et al. Long non-coding RNAs in cancer: Another layer of complexity. *J Gene Med.* 2019;21(1):e3065.
6. Sakai S, Ohhata T, Kitagawa K, Uchida C, Aoshima T, Niida H, et al. Long Noncoding RNA ELIT-1 Acts as a Smad3 Cofactor to Facilitate TGFbeta/Smad Signaling and Promote Epithelial-Mesenchymal Transition. *Cancer Res.* 2019;79(11):2821–38.
7. Xu TP, Wang WY, Ma P, Shuai Y, Zhao K, Wang YF, et al. Upregulation of the long noncoding RNA FOXD2-AS1 promotes carcinogenesis by epigenetically silencing EphB3 through EZH2 and LSD1, and predicts poor prognosis in gastric cancer. *Oncogene.* 2018;37(36):5020–36.
8. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell.* 2011;146(3):353–8.
9. Ala U. Competing Endogenous RNAs, Non-Coding RNAs and Diseases: An Intertwined Story. *Cells.* 2020;9(7).
10. Yang XZ, Cheng TT, He QJ, Lei ZY, Chi J, Tang Z, et al. LINC01133 as ceRNA inhibits gastric cancer progression by sponging miR-106a-3p to regulate APC expression and the Wnt/beta-catenin pathway. *Mol Cancer.* 2018;17(1):126.
11. Zhang R, Guo Y, Ma Z, Ma G, Xue Q, Li F, et al. Long non-coding RNA PTENP1 functions as a ceRNA to modulate PTEN level by decoying miR-106b and miR-93 in gastric cancer. *Oncotarget.* 2017;8(16):26079–89.
12. Arun K, Arunkumar G, Bennet D, Chandramohan SM, Murugan AK, Munirajan AK. Comprehensive analysis of aberrantly expressed lncRNAs and construction of ceRNA network in gastric cancer. *Oncotarget.*

2018;9(26):18386–99.

13. Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, et al. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics*. 2005;21(16):3439–40.
14. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
15. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res*. 2015;43(Database issue):D153-9.
16. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *Elife*. 2015;4.
17. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res*. 2008;36(Database issue):D149-53.
18. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res*. 2015;43(Database issue):D146-52.
19. Jeggari A, Marks DS, Larsson E. miRcode: a map of putative microRNA target sites in the long non-coding transcriptome. *Bioinformatics*. 2012;28(15):2062–3.
20. Paci P, Colombo T, Farina L. Computational analysis identifies a sponge interaction network between long non-coding RNAs and messenger RNAs in human breast cancer. *BMC Syst Biol*. 2014;8:83.
21. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. 2012;16(5):284–7.
22. The Gene Ontology C. Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res*. 2017;45(D1):D331-D8.
23. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res*. 2017;45(D1):D353-D61.
24. Liu H, Zhang Z, Wu N, Guo H, Zhang H, Fan D, et al. Integrative Analysis of Dysregulated lncRNA-Associated ceRNA Network Reveals Functional lncRNAs in Gastric Cancer. *Genes (Basel)*. 2018;9(6).
25. Ye F, Chen C, Qin J, Liu J, Zheng C. Genetic profiling reveals an alarming rate of cross-contamination among human cell lines used in China. *FASEB J*. 2015;29(10):4268–72.
26. Nagano M, Hoshino D, Koshikawa N, Akizawa T, Seiki M. Turnover of focal adhesions and cancer cell migration. *Int J Cell Biol*. 2012;2012:310616.
27. The Gene Ontology C. The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Res*. 2019;47(D1):D330-D8.
28. Di K, Wong YC, Wang X. Id-1 promotes TGF-beta1-induced cell motility through HSP27 activation and disassembly of adherens junction in prostate epithelial cells. *Exp Cell Res*. 2007;313(19):3983–99.
29. Cai HX, Yang LC, Song XH, Liu ZR, Chen YB, Dong GK. Expression of paxillin and FAK mRNA and the related clinical significance in esophageal carcinoma. *Mol Med Rep*. 2012;5(2):469–72.
30. Chen D, Zhang B, Kang J, Ma X, Lu Y, Gong L. Expression and clinical significance of FAK, ILK, and PTEN in salivary adenoid cystic carcinoma. *Acta Otolaryngol*. 2013;133(2):203–8.
31. Wozniak MA, Modzelewska K, Kwong L, Keely PJ. Focal adhesion regulation of cell behavior. *Biochim Biophys Acta*. 2004;1692(2–3):103–19.

32. Muharram G, Sahgal P, Korpela T, De Franceschi N, Kaukonen R, Clark K, et al. Tensin-4-Dependent MET Stabilization Is Essential for Survival and Proliferation in Carcinoma Cells. *Dev Cell*. 2014;29(5):629–30.
33. Cloutier G, Sallenbach-Morrisette A, Beaulieu JF. Non-integrin laminin receptors in epithelia. *Tissue Cell*. 2019;56:71–8.
34. Machesky LM. Lamellipodia and filopodia in metastasis and invasion. *FEBS Lett*. 2008;582(14):2102–11.
35. Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med*. 2006;203(7):1651–6.
36. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta*. 2010;1802(4):396–405.
37. Huang P, Han J, Hui L. MAPK signaling in inflammation-associated cancer development. *Protein Cell*. 2010;1(3):218–26.
38. Corcoran RB, Ebi H, Turke AB, Coffee EM, Nishino M, Cogdill AP, et al. EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov*. 2012;2(3):227–35.
39. Emmrich S, Streltsov A, Schmidt F, Thangapandi VR, Reinhardt D, Klusmann JH. LincRNAs MONC and MIR100HG act as oncogenes in acute megakaryoblastic leukemia. *Mol Cancer*. 2014;13:171.
40. Zhu X, Tian X, Yu C, Shen C, Yan T, Hong J, et al. A long non-coding RNA signature to improve prognosis prediction of gastric cancer. *Mol Cancer*. 2016;15(1):60.
41. Ma WQ, Chen J, Fang W, Yang XQ, Zhu A, Zhang D, et al. LncRNA INHBA-AS1 promotes cell growth, migration, and invasion of oral squamous cell carcinoma by sponging miR-143-3p. *Eur Rev Med Pharmacol Sci*. 2020;24(4):1821–8.
42. Ke D, Li H, Zhang Y, An Y, Fu H, Fang X, et al. The combination of circulating long noncoding RNAs AK001058, INHBA-AS1, MIR4435-2HG, and CEBPA-AS1 fragments in plasma serve as diagnostic markers for gastric cancer. *Oncotarget*. 2017;8(13):21516–25.
43. Zhang C, Wu W, Zhu H, Yu X, Zhang Y, Ye X, et al. Knockdown of long noncoding RNA CCDC144NL-AS1 attenuates migration and invasion phenotypes in endometrial stromal cells from endometriosis. *Biol Reprod*. 2019;100(4):939–49.
44. Gong J, Jin S, Pan X, Wang G, Ye L, Tao H, et al. Identification of Long Non-Coding RNAs for Predicting Prognosis Among Patients with Thymoma. *Clin Lab*. 2018;64(7):1193–8.
45. Liu K, Yao H, Wen Y, Zhao H, Zhou N, Lei S, et al. Functional role of a long non-coding RNA LIFR-AS1/miR-29a/TNFAIP3 axis in colorectal cancer resistance to photodynamic therapy. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864(9 Pt B):2871–80.
46. Wang HF, Lv JQ, Li HH, Wang W, Lin FQ. High long non-coding LIFR-AS1 expression correlates with poor survival in gastric carcinoma. *Eur Rev Med Pharmacol Sci*. 2020;24(10):5378–84.
47. Fang K, Caixia H, Xiufen Z, Zijian G, Li L. Screening of a Novel Upregulated lncRNA, A2M-AS1, That Promotes Invasion and Migration and Signifies Poor Prognosis in Breast Cancer. *Biomed Res Int*. 2020;2020:9747826.
48. Wang L, Ye TY, Wu H, Chen SY, Weng JR, Xi XW. LINC00702 accelerates the progression of ovarian cancer through interacting with EZH2 to inhibit the transcription of KLF2. *Eur Rev Med Pharmacol Sci*. 2019;23(3 Suppl):201–8.
49. Li T, Ren J, Ma J, Wu J, Zhang R, Yuan H, et al. LINC00702/miR-4652-3p/ZEB1 axis promotes the progression of malignant meningioma through activating Wnt/beta-catenin pathway. *Biomed Pharmacother*. 2019;113:108718.
50. Wu PL, He YF, Yao HH, Hu B. Martrilin-3 (MATN3) Overexpression in Gastric Adenocarcinoma and its Prognostic Significance. *Med Sci Monit*. 2018;24:348–55.

51. Hongyu Shen LW, Chen Q, Xu J, Zhang J, Fang L, Wang J, Weifei Fan. The prognostic value of COL3A1/FBN1/COL5A2/SPARC-mir-29a-3p-H19 associated ceRNA network in Gastric Cancer through bioinformatic exploration. *J Cancer Metastasis Treat.* 2020;11(17):4933–46.
52. Klatt AR, Becker AK, Neacsu CD, Paulsson M, Wagener R. The matrilins: modulators of extracellular matrix assembly. *Int J Biochem Cell Biol.* 2011;43(3):320–30.
53. Klatt AR, Klinger G, Paul-Klausch B, Kuhn G, Renno JH, Wagener R, et al. Matrilin-3 activates the expression of osteoarthritis-associated genes in primary human chondrocytes. *FEBS Lett.* 2009;583(22):3611–7.
54. Wagener R, Ehlen HW, Ko YP, Kobbe B, Mann HH, Sengle G, et al. The matrilins—adaptor proteins in the extracellular matrix. *FEBS Lett.* 2005;579(15):3323–9.
55. Januchowski R, Zawierucha P, Rucinski M, Zabel M. Microarray-based detection and expression analysis of extracellular matrix proteins in drugresistant ovarian cancer cell lines. *Oncol Rep.* 2014;32(5):1981–90.
56. Fischer H, Stenling R, Rubio C, Lindblom A. Colorectal carcinogenesis is associated with stromal expression of COL11A1 and COL5A2. *Carcinogenesis.* 2001;22(6):875–8.

Figures

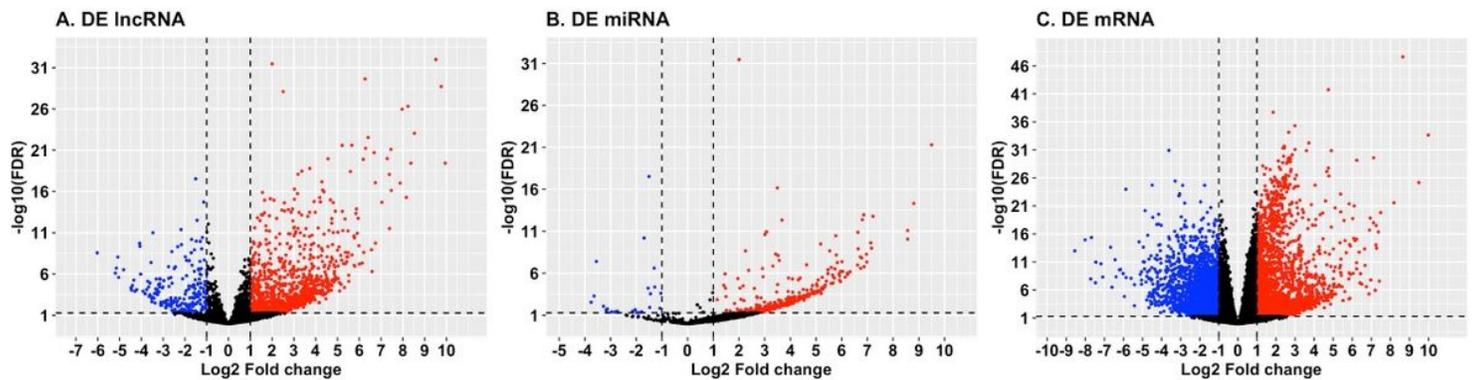


Figure 1

Volcano plots showing up- and down-regulated (A) lncRNAs, (B) miRNAs and (C) mRNA. The red dots represent high expression lncRNA/miRNA/mRNA with $\text{LogFC} \geq 1$ and $\text{FDR} < 0.05$, and the blue dots represent low expression of lncRNA/miRNA/mRNA with $\text{LogFC} \leq -1$ and $\text{FDR} < 0.05$.

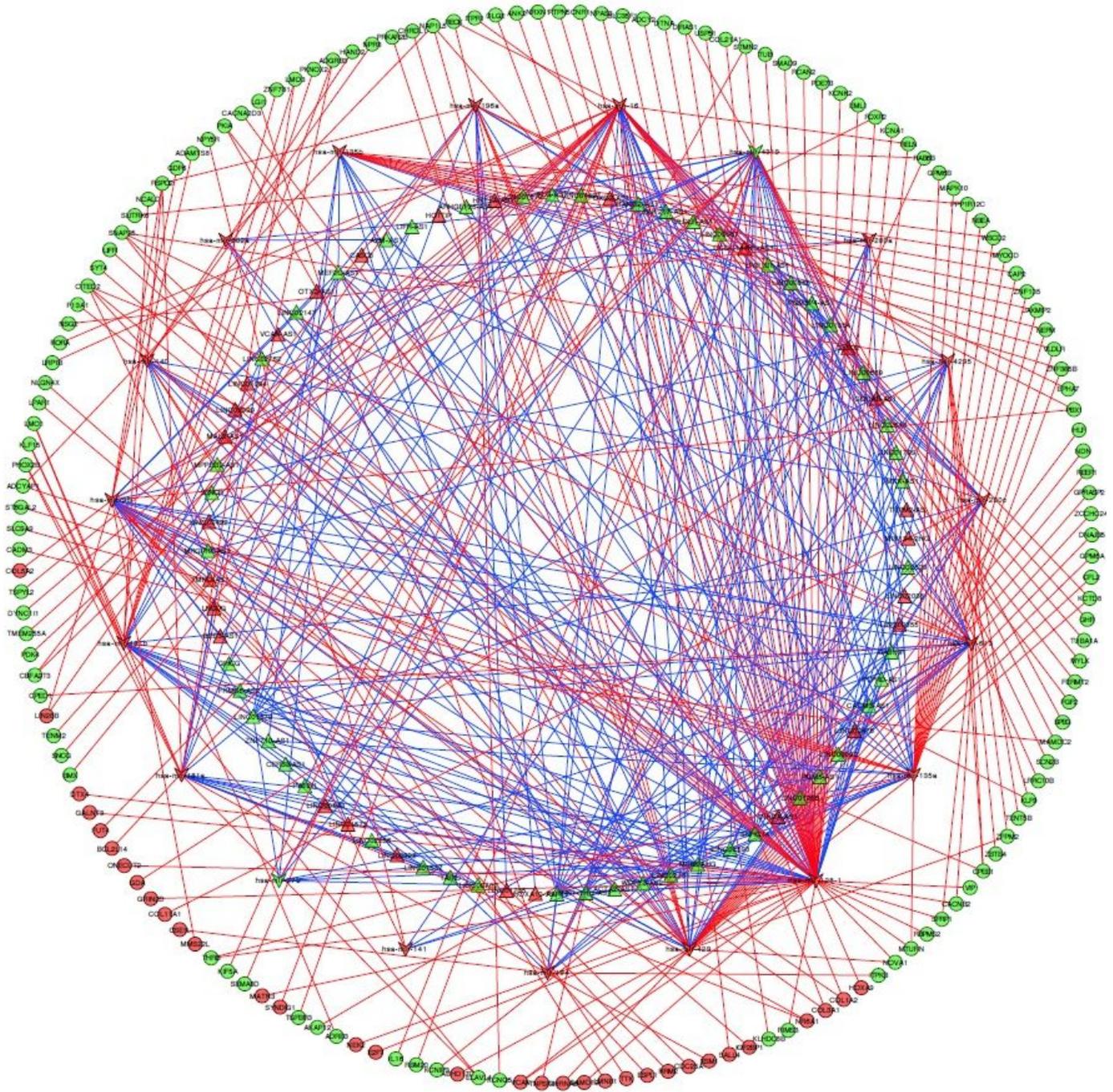
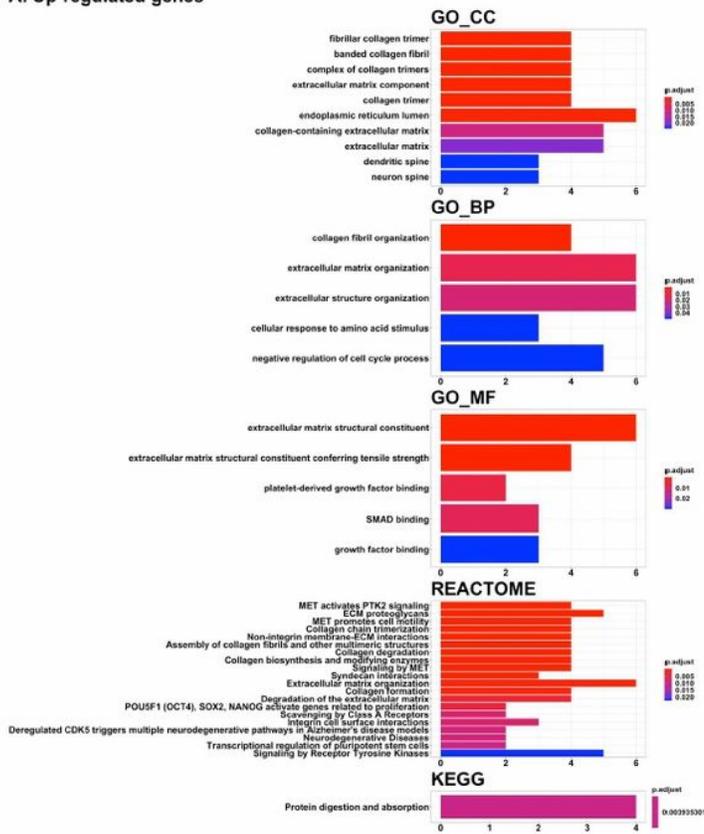


Figure 2

The ceRNA network including lncRNA, miRNA and mRNA. Red and green represent up- and down-regulated direction between normal and cancer tissues, respectively. Ellipses, rectangles, and rhomboids represent miRNA, mRNA and lncRNA, respectively. The node of V shape: miRNA; the node of triangle shape: lncRNA; the node of cycle: protein coding gene; connecting line of red: lncRNA-miRNA; connecting line of blue: miRNA-mRNA.

A. Up-regulated genes



B. Down-regulated genes

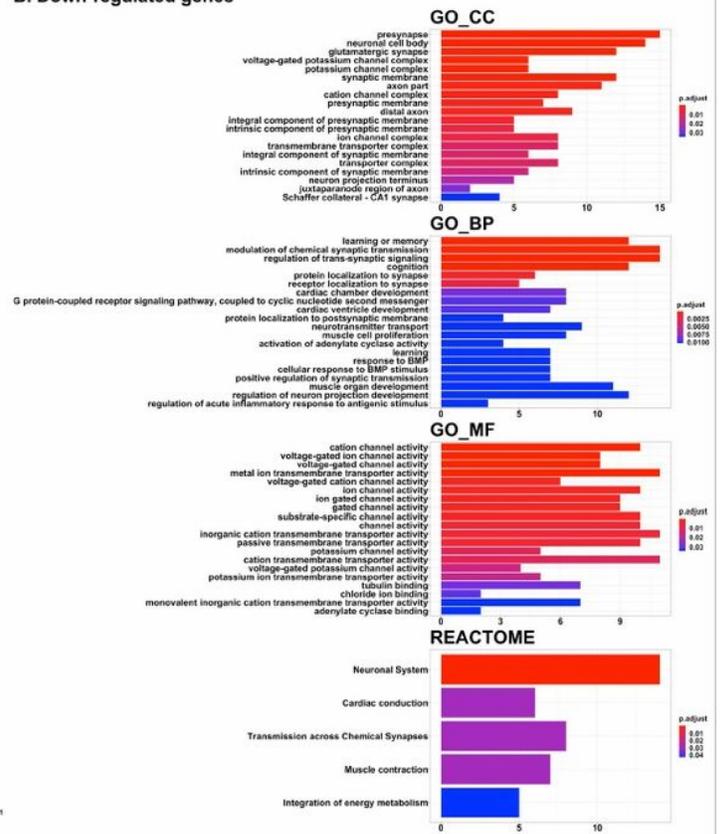


Figure 3

GO terms, REACTOME and KEGG interpretation for functions of (A) up- and (B) down-regulated mRNAs derived from ceRNA network in GC. CC: cellular component; BP: biological pathway; MF: molecular function.

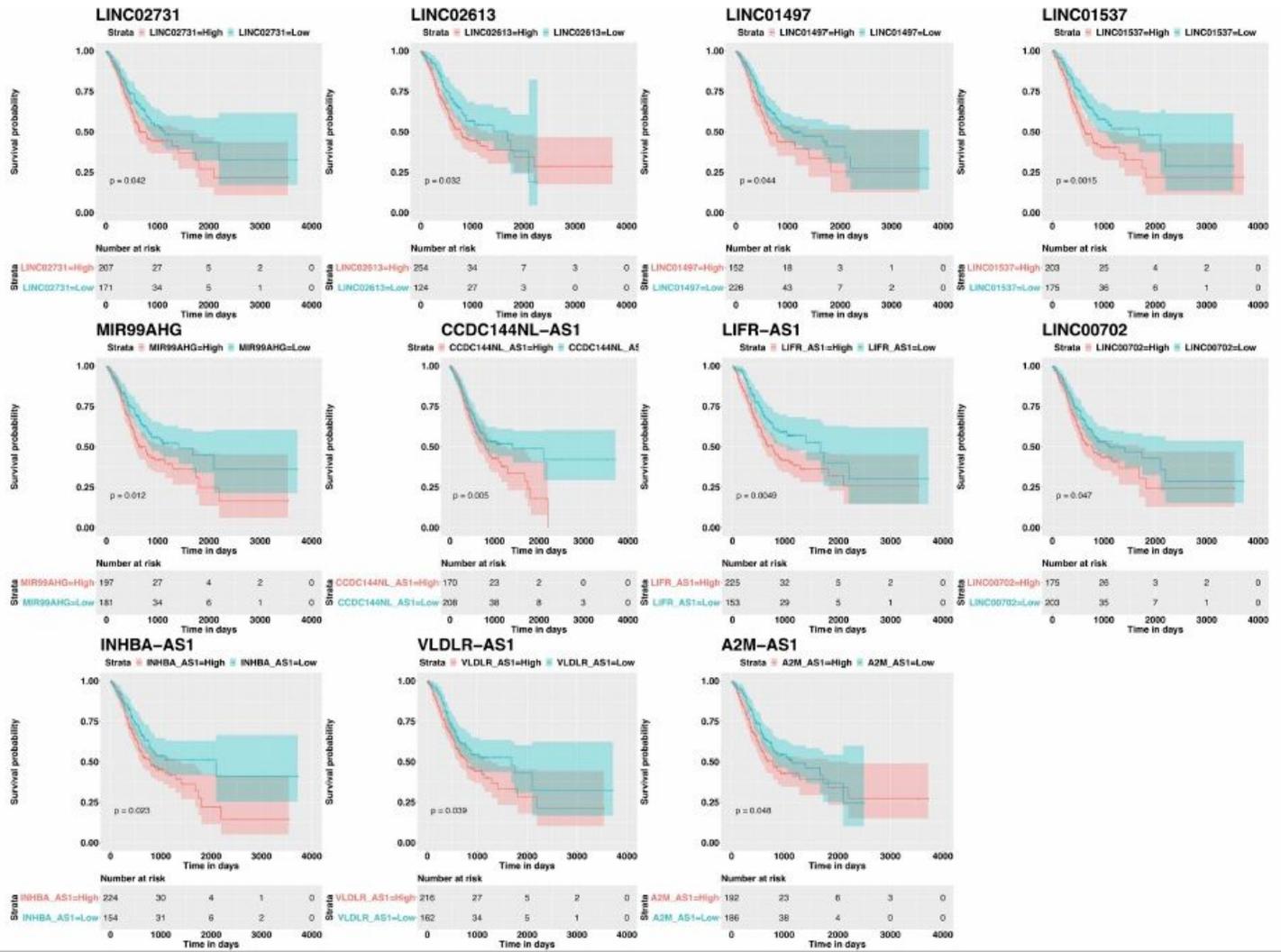


Figure 4

Kaplan-Meier survival analysis for the correlation of differential expression lncRNA with overall survival of the patients with GC. Patients with expression \geq mean expression were considered as high expression and otherwise as low expression.

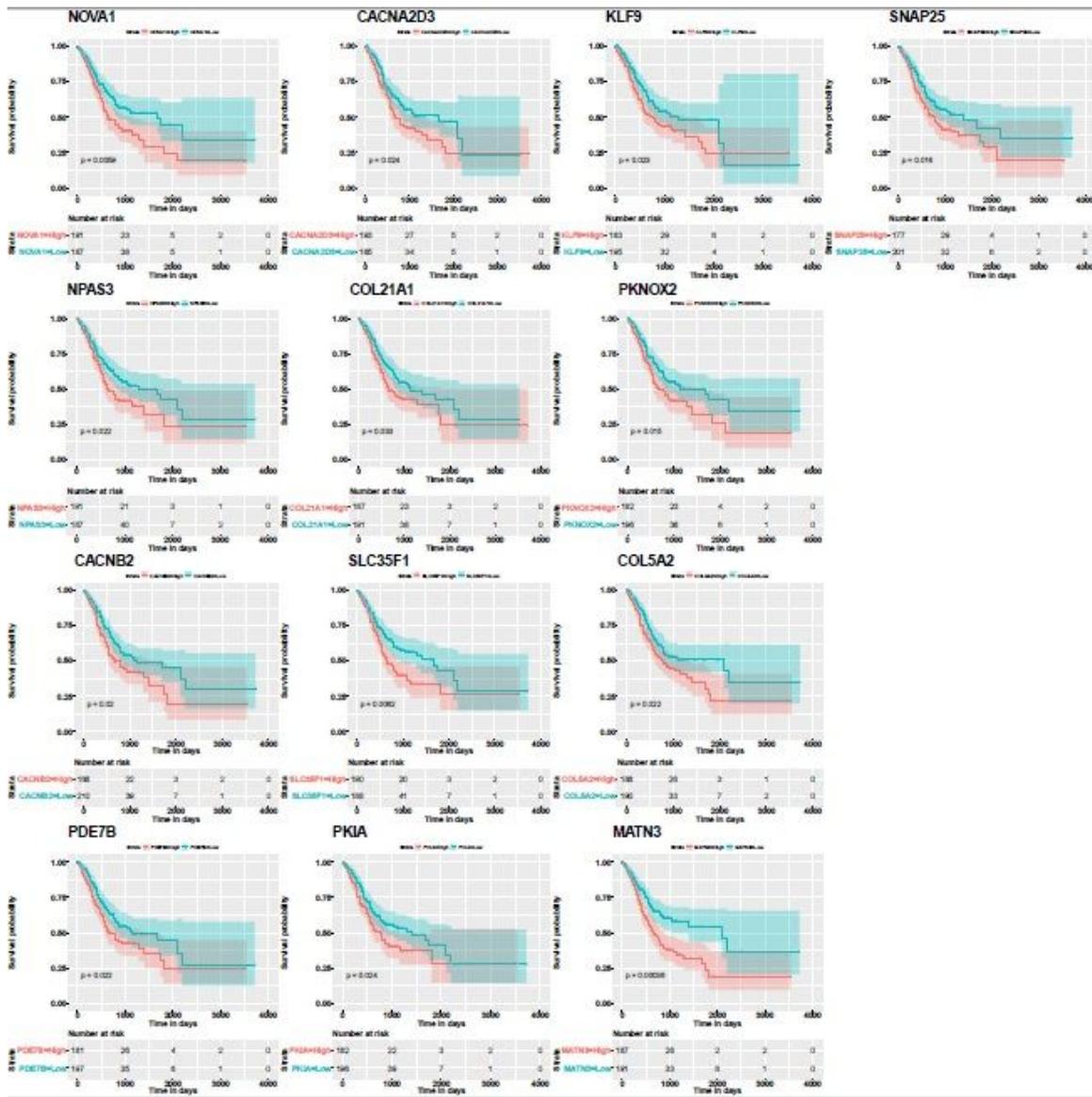


Figure 5

Kaplan-Meier survival analysis for the correlation of differential expression mRNA with overall survival of the patients with GC. Patients with expression \geq mean expression were considered as high expression and otherwise as low expression.

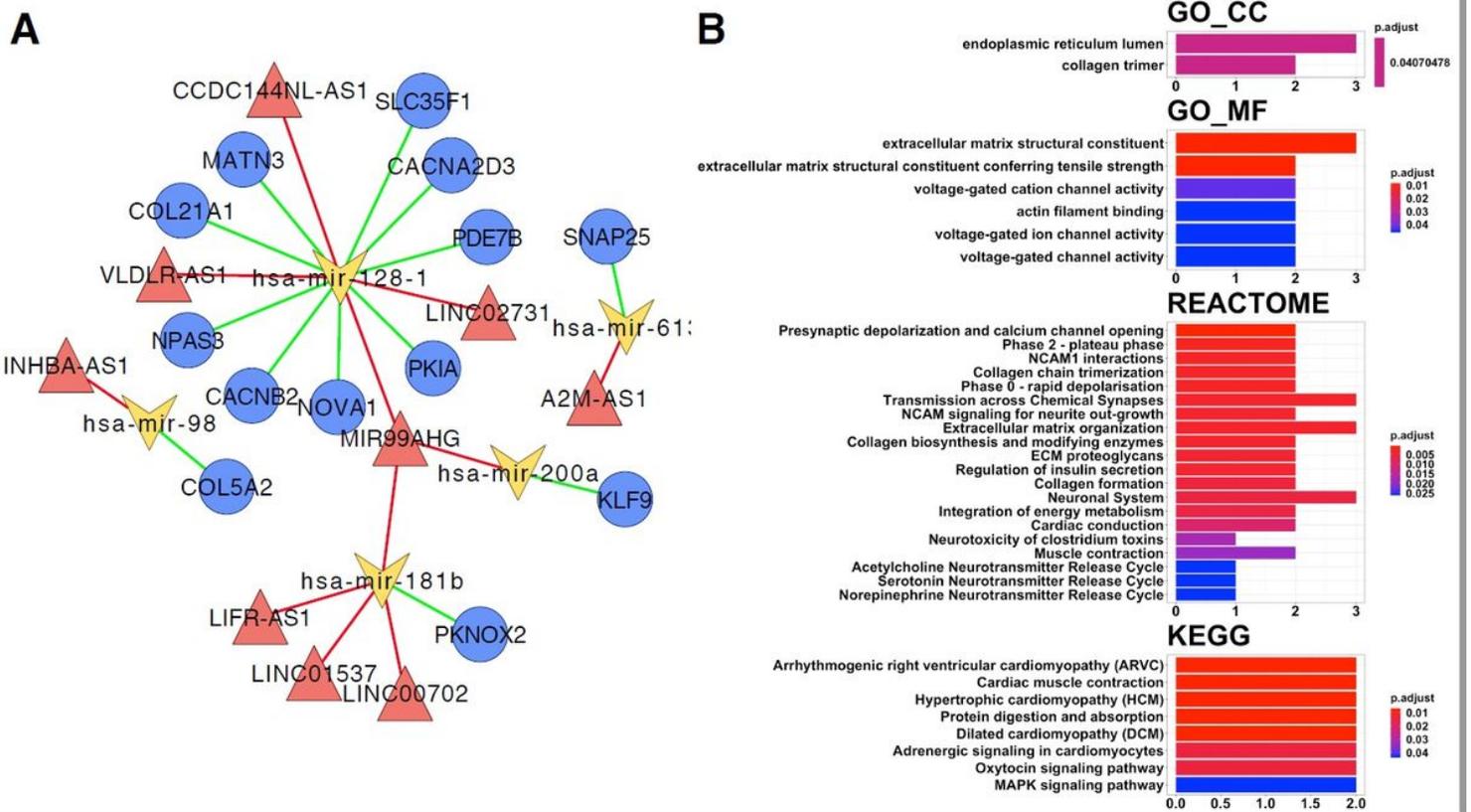


Figure 6

The hub lncRNA associated subnetwork with functional interpretation. (A) The subnetwork including lncRNA, miRNA and mRNA. Blue ellipses, red rectangles, and yellow rhomboids represent miRNA, mRNAs and lncRNAs, respectively. (B) Functional interpretation of GO terms, REACTOME and KEGG.

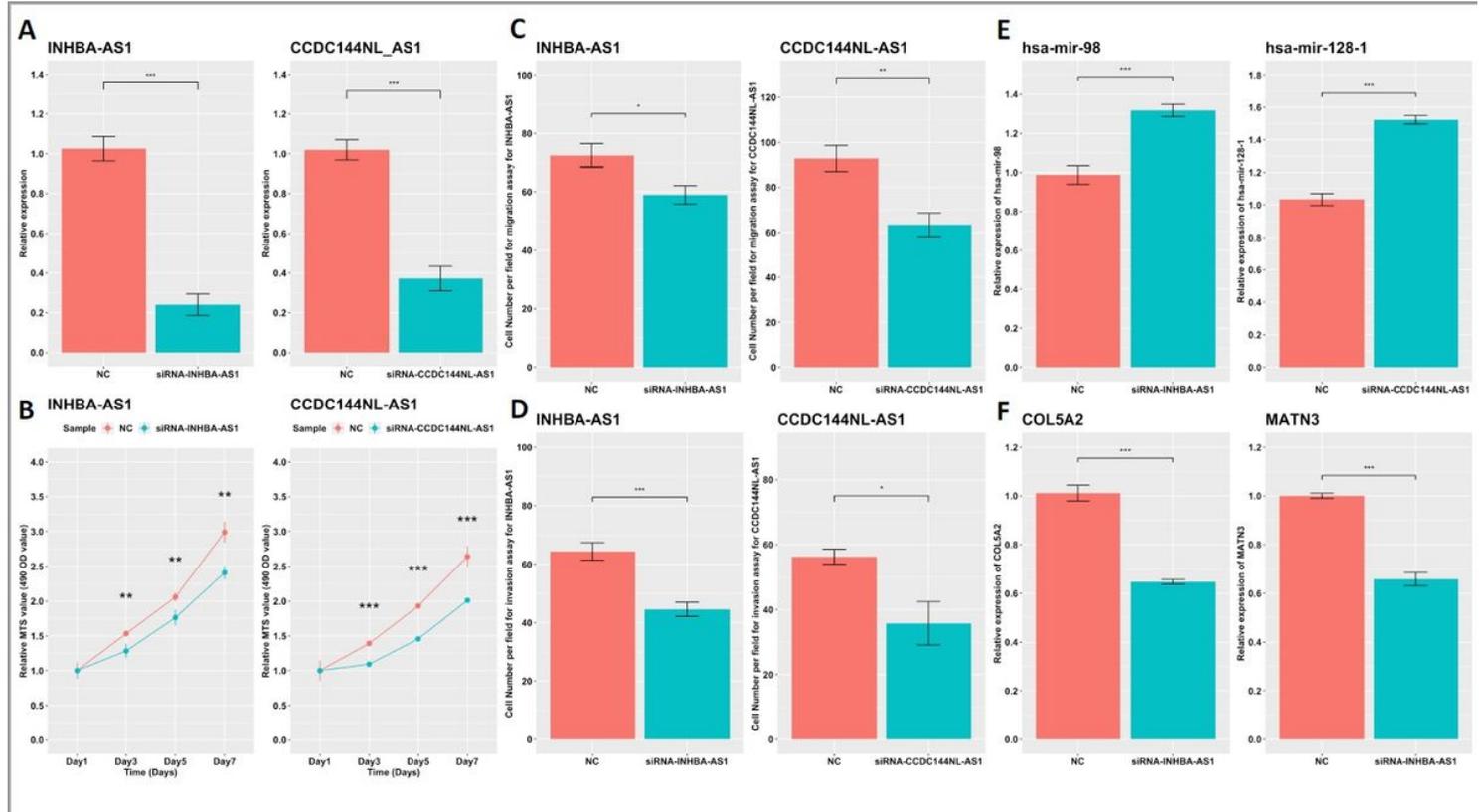


Figure 7

The expression of INHBA-AS1/hsa-mir-98/COL5A2 and CCDC144NL-AS1/hsa-mir-128-1/MATN3 axis and in vitro function assay. (A) The expression of INHBA-AS1 and CCDC144NL-AS1 with siRNA knockdown. (B) Proliferation assay with INHBA-AS1 and CCDC144NL-AS1 knockout, respectively. (C) Migration assay with INHBA-AS1 and CCDC144NL-AS1 knockout, respectively. (D) Invasion assay with INHBA-AS1 and CCDC144NL-AS1 knockout, respectively. (E) The expression of hsa-mir-98 and hsa-mir-128-1 with with INHBA-AS1 and CCDC144NL-AS1 knockout, respectively. (F) The expression of COL5A2 and MATN3 with with INHBA-AS1 and CCDC144NL-AS1 knockout, respectively. *** Indicated $p < 0.001$; ** indicated $p < 0.01$; * indicated $p < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.xlsx](#)