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Long non-coding RNAs as potential biomarkers or therapeutic targets in gastric cancer

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ABSTRACT

Aim: This study aimed to find lncRNAs and mRNAs that were expressed differently by combining microarray datasets from different studies. This was done to find important target genes in gastric cancer for anti-cancer therapy.

Background: Gastric cancer (GC) is the fourth most frequent and second-most deadly malignancy worldwide. Thus, genetic diagnosis and treatment should focus on genetic and epigenetic variables. Based on several studies, disordered expression of non-coding RNAs (ncRNAs), such as lncRNAs, regulate gastric cancer invasion and metastasis. Besides, lncRNAs cooperatively regulate gene expression and GC progression.

Methods: We obtained differentially expressed mRNAs (DEmRNAs) and lncRNAs (DElncRNAs) from three GC tissue microarray datasets by meta-analysis and screened genes using the "Limma" package. Then, using the RNAInter database, we allocated DEmRNAs to each DElncRNA. ClusterProfiler and GOplot programs were used to analyze function enrichment pathways and gene ontologies for final DEmRNAs.

Results: A total of 9 differentially expressed lncRNAs (DElncRNAs) (5 up-regulated and 4 down-regulated), and 856 DEmRNAs (451 up-regulated and 405 down-regulated) between tumor and adjacent normal samples were found. Finally, 117 differentially expressed mRNAs were predicted as interactors of six DElncRNAs (H19, WT1-AS, EMX2OS, HOTAIR, ZEB1-AS1, and LINC00261).

Conclusion: In order to promote cancer therapeutics and give knowledge on the process of carcinogenesis, our study projected a network of drug-gene interactions for discovered genes and presented relevant prospective biomarkers for the prognosis of patients with stomach cancer.

Keywords: Biomarker, Gastric cancer, Messenger RNA (mRNA), Long non-coding RNAs (lncRNAs), Microarray analysis.

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Introduction

Gastric cancer (GC) is a type of malignant epithelial neoplasm, especially in developing countries. Although its incidence and mortality rates have declined over the last few decades, gastric cancer is one of the most

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frequent cancers worldwide (1). Considering the lack of specific diagnostic biomarkers, most gastric cancer patients are diagnosed at the advanced stages, which leads to a low survival rate (2). Late-stage gastric carcinoma is associated with perivascular invasion, peritoneal dissemination, and lymph node metastasis (LNM). In order to find important biomarkers or genetic signatures for early GC diagnosis and treatment targets, it is crucial to investigate the molecular pathways driving gastric carcinogenesis (3, 4).

Gastric cancer initiation, and progression result from cooperation between genetic alterations and epigenetic factors in a multi-step process (5). Several studies found that aberrant expression of non-coding RNAs (ncRNAs) as epigenetic elements significantly contributes to GC's pathogenesis by regulating invasion and metastasis via diverse signaling pathways. These recently discovered RNA molecules are capable of distinguishing between early-stage GC and healthy cases. In addition, non-coding RNAs have greater sensitivity and diagnostic efficacy than clinical screening indicators such as CA19-9, CEA, and others. Therefore, they can serve as novel potential targets for cancer treatment (6, 7). NcRNAs usually encode no protein which are generally divided into short ncRNAs, and long ncRNAs (lncRNAs) based on their transcript sizes (8). LncRNAs are long transcripts (>200 nucleotides) with compound mechanisms. Functionally, these molecules have vital roles in several biological processes and can regulate the expression of oncogenes and tumor suppressor genes at different levels (9). Numerous studies have demonstrated that the up- and down-regulation of lncRNAs affects several cancer types, including gastric cancer, by altering the cell cycle, apoptosis, migration, invasion, metastasis, cell proliferation, and tumorigenicity (10, 11). LncRNAs modify gastric cancer cells via several tumor signaling pathways, such as Notch, mTOR, NF- κb, and Wnt (3). Many studies were carried out to understand underlying molecular mechanisms of lncRNAs in gastric tumorigenesis and progression, although the complex mechanism of lncRNAs has not yet been fully elucidated. Investigations have found that lncRNAs act as key gene regulators at transcriptional and post-transcriptional levels (12). In order to predominantly control gene expression both indirectly and directly in the nucleus, lncRNAs cause chromatin remodeling at the transcriptional level and bind to the target gene's promoter. In the cytoplasm, lncRNAs directly interact with the target gene's mRNA to impact its stability and expression (4, 13). At the post-transcriptional level, an interaction between cytoplasmic lncRNAs and miRNA enables them to regulate each other and modulate gene expression via multiple mechanisms (12).

Numerous studies have proposed that using the presence of GC-related lncRNAs tumor initiation might help diagnose and monitor gastric cancer since lncRNAs exhibit a high degree of cell type- and disease-specificity (14). Additionally, lncRNAs collaborate to control GC development and gene expression (15). Over the last decade, gene expression microarrays have helped to recognize essential dysfunctional genes in cancer. We carried out microarray analysis of the expression profiling of lncRNAs and mRNAs from gastric cancer tissues. We matched adjacent noncancerous tissues to identify differentially expressed lncRNAs and mRNAs. Studying these data will provide valuable information on the mechanism of carcinogenesis and enable researchers to detect the critical target genes for anti-cancer therapy.

Methods

Data collection

The mRNA and lncRNA expression datasets of gastric cancer were searched using these keywords: “gastric cancer”, “lncRNA”, “mRNA”, “Homo sapiens” [porgn: txid9606]', and “Expression profiling by array” in Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). Three GSE profiles (GSE109476, GSE158662, and GSE65801) were selected and analyzed after a systematic review. All datasets were related to lncRNA and mRNA expression and were based on GPL24530 (Agilent-033010; custom-annotation; probe name version), GPL22755 (Agilent-076500 Human lncRNA+mRNA array (Probe name version)), and GPL14550 (Agilent-028004 SurePrint G3 Human GE 8x60K Microarray (Probe Name Version)), respectively.

Microarray data processing and integrative meta-analysis

The statistical programming language R was used to process the data and carry out integration processes. The platforms (Agilent) used by the datasets listed are identical. The batch effect (non-biological differences) was eliminated using the Surrogate Variable Analysis (SVA) software tool (16). PCA and boxplot were checked after batch effect removal. The meta-analysis

outcome is a unit expression matrix (the combination of three datasets of this study).

Identification of differentially expressed mRNAs and lncRNAs

Firstly, all differentially expressed genes (DEGs) (List O) were identified by limma R package in Bioconductor based on the difference in their expression values between tumor and adjacent normal samples. Significant differential expression was identified with a log fold change ≥ |1| and an adjusted p-value threshold of 0.05 (17). Then, differentially expressed lncRNAs (DElncRNAs) (List A), and differentially expressed mRNAs (DEmRNAs) (List B) were separated by Venny 2.1 free online tool (https://bioinfogp.cnb.csic.es/tools/venny/).

Determination of mRNAs for each lncRNA

A database called RNAInter (RNA Interactome Database) supports the development of the interactome and offers details on the biological purposes and molecular mechanisms of RNA, including certain interactions like RNA-RNA, RNA-protein, and RNA-DNA (18). We selected the items as follows, categories: lncRNA, species: Homo Sapience, interaction type: RNA-Protein interaction, detection method: computational prediction, and the interval of confidence score: between 0.1 and 1. We obtained all mRNAs as interactors with DElncRNAs based on mentioned setting (list C). Some DElncRNAs did not have any interactions, so they were not considered. Then, Venny 2.1 was used to deduce commonalities of mRNA (List D) across the B and C data lists. Finally, we built a lncRNA-mRNA network and visualized the network using Cytoscape 3.9.1.

Gene Ontology (GO) and pathway enrichment analyses

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) were used to recognize biological mechanisms. ClusterProfiler package in R (19) was run to attitude?? GO and KEGG pathway enrichment analyses of list D.

Drug-gene networks

To generate drug-gene network, we focused on up-

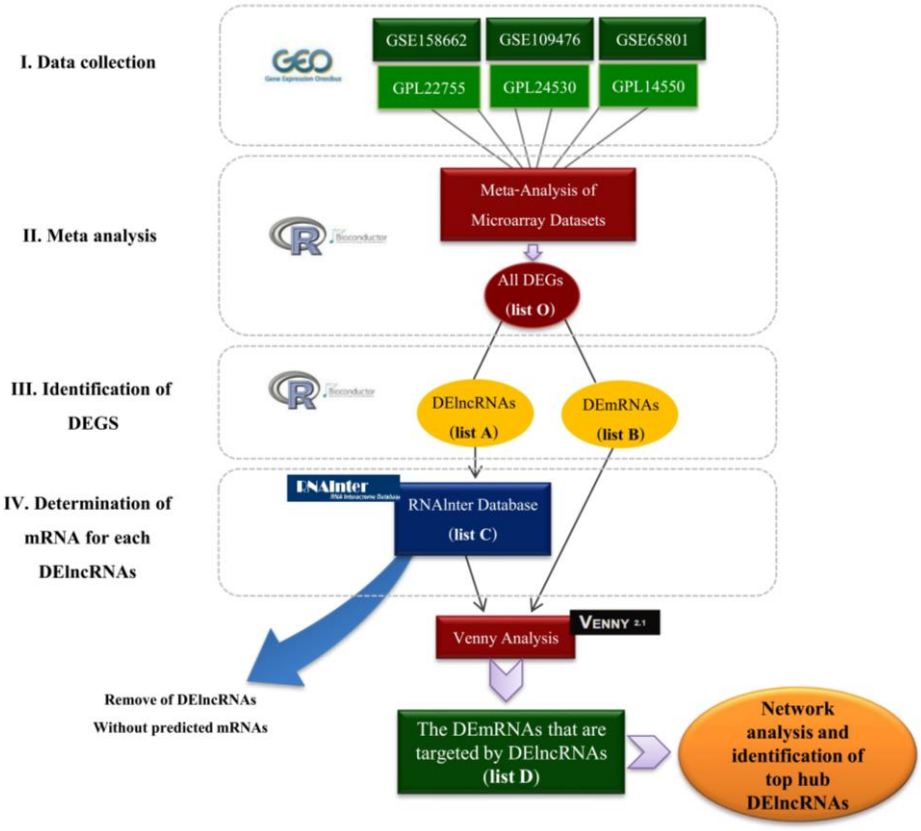

Figure 1. Informatics pipeline for the identification of lncRNAs and interactors in gastric cancer

regulated DEmRNAs, which were common targets of hub DElncRNAs (DElncRNAs with the most targets). Alluvial diagrams created with the ggalluvial package demonstrated the unique interplay between hub DElncRNAs and typical up-regulated DEmRNAs. DGIdb database was used to construct the drug-gene networks. The network is used to represent interactions between drugs and genes from different resources. DGIdb 3.0 (20) provides a user-friendly interface to search and filter to access the desired data.

Results

Identification of DElncRNAs and DEmRNAs in gastric cancer

We summarized the analysis pipeline in Figure 1. Three microarray gene expression datasets, GSE109476 (GPL24530), GSE158662 (GPL22755), and GSE65801 (GPL14550), were used in this study. Each dataset contains 10 (5 adjacent normal & 5 tumor), 6 (3 adjacent normal & 3 tumor) and 64 (32 adjacent normal & 32 tumor) samples, respectively. After integrating all datasets and batch effect removal, all DEGs (865 DEGs containing DElncRNAs and DEmRNAs) were obtained by limma package between tumor (n=40) and adjacent normal samples (n=40), among all DEGs (List O (Supplementary File 1)). We identified 9 DElncRNAs (list A): 5 up-regulated and 4 down-regulated (Table 1) and 856 DEmRNAs (list B): 451 up-regulated and 405 down-regulated, respectively (Supplementary File 2).

Finding mRNAs for each DElncRNAs

We determined mRNAs that are targeted by DElncRNAs via RNAInter. Among 9 DElncRNAs (5 up and 4 down-regulated), only 6 DElncRNAs (5 up-regulated (H19, WT1-AS, EMX2OS, HOTAIR, and ZEB1-AS1), and one down-regulated (LINC00261)) target some mRNAs based on computational prediction in RNAInter. For H19, WT1-AS, EMX2OS, HOTAIR, ZEB1-AS1, and LINC00261, respectively, 1635, 1538, 88, 168, 3095, and 79 mRNA were predicted as interactors by RNAInter analysis (list C) (Supplementary File 3). No mRNAs were predicted for other DElncRNAs (C11orf40, HSDL2-AS1 (C9orf147), and LINC00319) that are shown in dark grey in Table 1. VENN diagram was used to predict DE-mRNAs (list B) and 117 target mRNAs as interactors (list C), overlapping DElnRNAs (list D) (Table 2). We visualized the interaction between final DElncRNAs and list D by Cytoscape (Figure 2). Moreover, ABCC9, MRC2, and TOP2A genes were common between all up-regulated DEmRNAs in hub DElncRNAs, including H19, WT1-AS, HOTAIR, and ZEB1-AS1 (Figure 3). But, RBMS3 was targeted by EMX2OS. On the other hand, ABCC8 and CUX2 genes

Table 1. List of DElncRNAs

	Symbol	Official name & Accession numbers	Function
Up DElncRNAs	H19	H19 imprinted maternally expressed transcript (NR_002196)	A tumor suppressor, prevents uncontrolled cell growth.
	WT1-AS	WT1 antisense RNA (NR_023920)	The antisense transcript of the Wilm's tumor gene, encodes a zinc finger transcription domain possessing tumor suppressor or oncogenic properties.
	EMX2OS	EMX2 opposite strand/antisense RNA (NR_002791)	Induces proliferation, invasion, and sphere formation in ovarian cancer. Inhibits Wilms' tumour cell stemness, migration, invasion and epithelial-mesenchymal transition (EMT).
	HOTAIR	HOX transcript antisense RNA (NR_003716)	A key component of many processes in cancer, such as proliferation, survival, migration, drug resistance, and genomic stability.
	ZEB1-AS1	ZEB1 antisense RNA 1 (NR_024284)	Modifieshistons via epigenetically activating ZEB1. Induces tumor progression.
Down DElncRNAs	LINC00261	long intergenic non-protein coding RNA 261 (NR_001558)	Promotes differentiation and apoptosis by acting as a negative regulator of cell growth
	C11orf40	chromosome 11 putative open reading frame 40	unknown
	HSDL2-AS1	HSDL2 antisense RNA 1 (Also known as C9orf147)	unknown
	LINC00319	long intergenic non-protein coding RNA 319	Promotes osteosarcoma progression. Sponges miR-335-5p to accelerate the development of tumor growth and metastasis of gastric cancer.

Table 2. The DEmRNAs (interactor) for DElncRNAs (list D). The kinase and TF elements represented as green and yellow, respectively.

	LncRNAs	Interactors	
Up DElncRNAs	H19	31 Up DEmRNAs	COL1A1,SDS,COL3A1,COL4A1,COL5A2,COL18A1,C3,ONECUT3,PDE3A, COL5A3,ADAMTS12,ABCC9,ANXA1,TOP2A,GRAP,ANTXR1,PLEKHG2, MRC2,MEIS3,HMOX1,CCDC8,TRPM2,TM4SF1,MEGF6,NRXN2,HOXA10, COL15A1,CEP170,PRR5L,MCAM,FANCA
		19 Down DEmRNAs	PIK3C2G,GC,ANKRD24,ABCC8,DUOX2,NEUROD1,PROM2,ZBTB7C,B4GAL NT3,SUSD4,FER1L4,TFCP2L1,SUCLG2,ELL2,GGT6,RASEF,NIP7,C5,CUX2
	WT1-AS	36 Up DEmRNAs	OLFM4,SULF1,VIL1,COL3A1,COL4A1,COL5A2,ITGBL1,COL18A1,C3,ONEC UT3,COL5A3,ADAMTS12,ABCC9,ANXA1,HTRA3,TOP2A,FSTL1,PPP1R1B,C HRNA5,GRAP,PLEKHG2,DPEP1,MRC2,HMOX1,TRPM2,CLEC11A,PODN,TM 4SF1,MEGF6,NRXN2,HOXA10,COL15A1,CEP170,HHIPL1,PRR5L,FANCA
		23 Down DEmRNAs	PIK3C2G,GC,ANKRD24,DUOX1,ABCC8,DUOX2,NEUROD1,FER1L6,EGF R,LDHD,PROM2,ZBTB7C,APLP1,SUSD4,FER1L4,TFCP2L1,ELL2,ADARB2 ,GGT6,NIP7,KRT26,C5,CUX2
	EMX2OS	Just one Up DEmRNAs	RBMS3
	HOTAIR	3 Up DEmRNAs	ABCC9,TOP2A,MRC2, RBMS3
		2 Down DEmRNAs	ABCC8,CUX2
	ZEB1-AS1	70 Up DEmRNAs	OLFM4,SULF1,COL1A1,CHRDL2,VIL1,SLC39A5,TIMP1,COL6A3,SDS,CO L3A1,GUCY1A2,TDO2,FSCN1,SNAI1,COL4A1,COL5A2,ITGBL1,COL18A1 ,C3,MSC,ONECUT3,HOXC6,PDE3A,COL5A3,ADAMTS12,ABCC9,SPC25,A NXA1,HTRA3,TOP2A,HOXD10,FSTL1,DGKH,BTBD19,GJA5,PPP1R1B,CH RNA5,GRAP,ANTXR1,AFAP1L1,CPVL,PLEKHG2,DPEP1,ITGB8,DLGAP5, KRT23,ADAMTS4,MRC2,SHCBP1,MEIS3,HMOX1,CCDC8,TRPM2,CLEC11 A,PODN,TM4SF1,MEGF6,NRXN2,GPRC5A,HOXA10,COL15A1,CEP170,H HIPL1,ADCY3,PRR5L,LAMC2,MCAM,RBMS3,LTBP2,FANCA
		49 Down DEmRNAs	CXCL17,PIK3C2G,GC,HOMER2,FGA,ANKRD24,DUOX1,IRX3,CYP2C18,AB CC8,DUOX2,NEUROD1,FER1L6,IRX5,RBPJL,TM4SF4,EGFR,LDHD,TRPV6, PROM2,HMGCS2,FGB,PALM3,CYP2C9,ROBO3,ZBTB7C,ALDH1A1,APLP1, B4GALNT3,SLC4A4,PXMP2,SUSD4,AKR1B1,FER1L4,DGKD,TFCP2L1,SUC LG2,SSC5D,ELL2,ADARB2,GGT6,NIP7,KRT26,C5,CUX2,SDR42E1
Down DElncRNAs	LINC00261	Just one Up DEmRNAs	RBMS3

were common in all down-regulated mRNAs including H19, WT1-AS, HOTAIR, and ZEB1-AS1. In addition, LINC00261 as a down-regulated lncRNA has one target in this study (RBMS3). We selected ABCC9, MRC2, and TOP2A genes for the drug-gene network analysis.

Gene Ontology (GO) and pathway enrichment analysis

The clusterProfiler and GOplot packages were used to find the enriched pathways and GO with P adjust <0.05 shown by dot and chord plots, respectively. The most common metabolisms in list D, according to KEGG pathway analysis, were protein digestion and absorption, mineral absorption, cytochrome P450-mediated xenobiotic metabolism, cytochrome P45-mediated drug metabolism, stomach acid secretion, and but anoate metabolism (Figure 4A). Besides, response to xenobiotic stimulus, extracellular matrix organization, extracellular structure organization, external encapsulating structure organization, digestion, detoxification of copper ion, stress response to copper ion, cellular response to cadmium ion, collagen fibril organization, and the detoxification of inorganic compound were significant in GO category (Figure 4B).

The drug-gene interaction network

DGIdb 3.0 database was used to study potential drugs for gastric cancer treatment and extract drug-gene interactions. The identified medicines associated with the database-detected upregulated and downregulated genes are shown in Figure 5. The relationships between the genes are shown by displaying the gene complexes independently in various groupings. The cyan and yellow nodes represent

drugs and genes, respectively. No interactions were found for MRC2 gene which is interacted with the extracellular matrix.

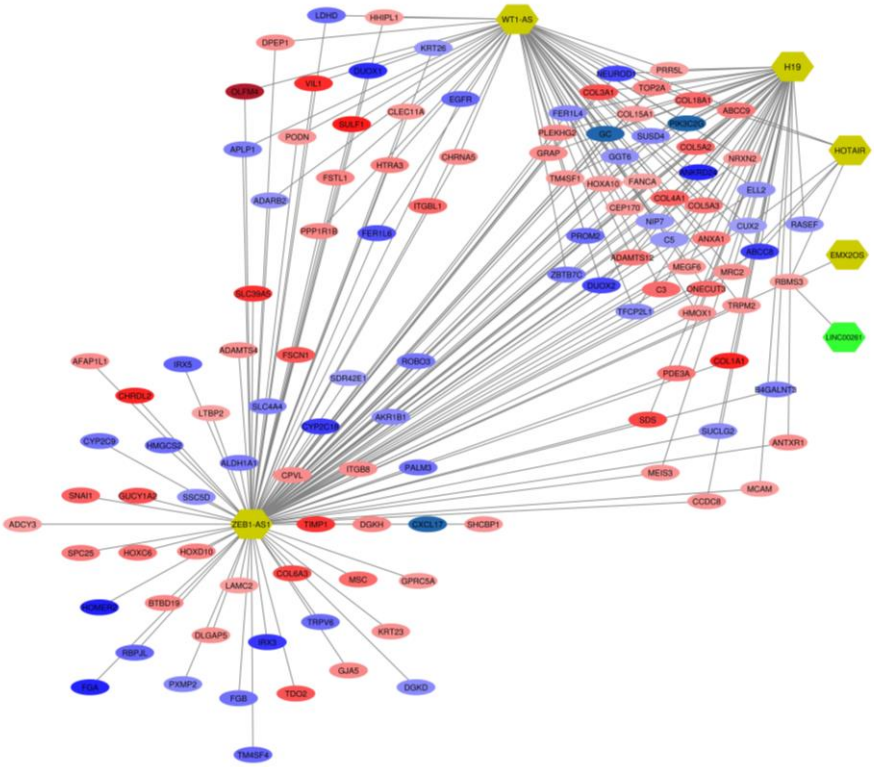

Figure 2. Networks were constructed from up & down-regulated mRNAs and DElncRNAs. Yellow and Green hexagonal represent up & down-regulated DElncRNAs and light red and blue circles represent up & down-regulated mRNAs.

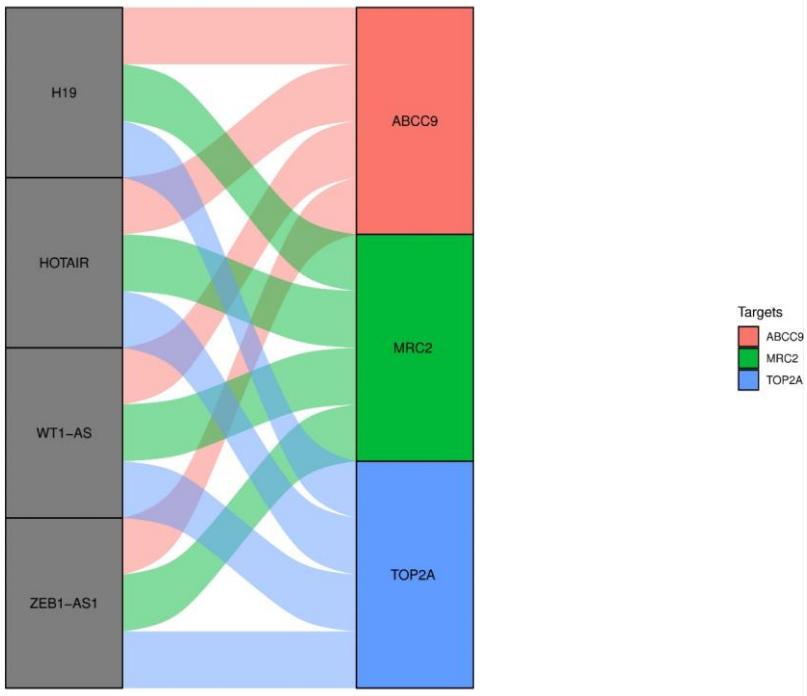

Figure 3. Alluvial diagrams show the interaction between ABCC9, MRC2, and TOP2A that are up-regulated and targeted by the most up-regulated DElncRNAs.

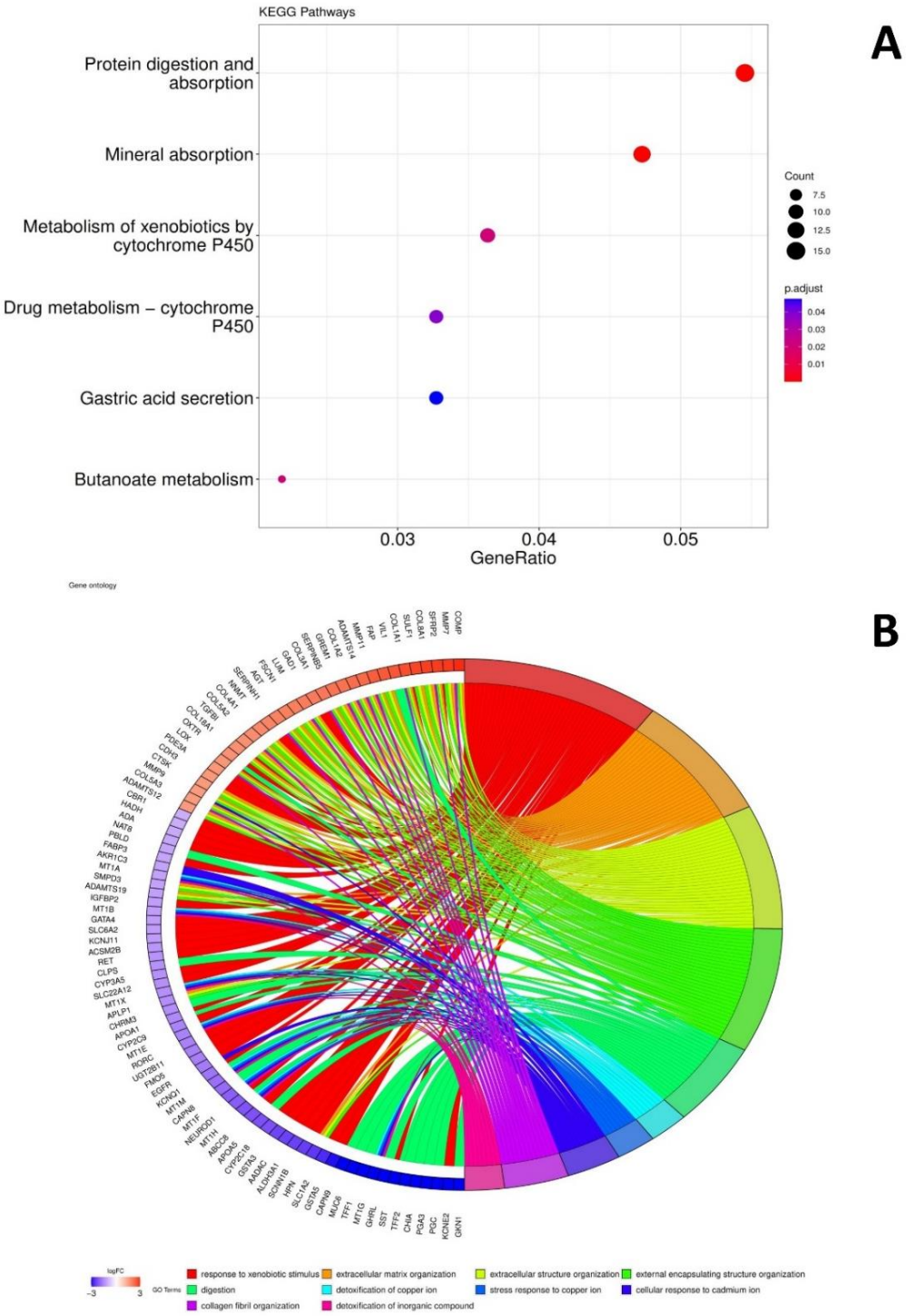

Figure 4. Functional enrichment analysis of list D. Top KEGG pathways (A); Top GO terms (B).

Discussion

One major class of non-coding RNAs is long non-coding RNAs (lncRNAs). The length of LncRNAs is 200-100,000 nucleotides (nt) (19). Recent studies showed survival benefits of chemotherapy and surgery in the patients with GC, but it is still one of the most common cancers, and the leading cause of cancer

deaths worldwide. Consequently, it is important to discover effective biomarkers. According to Zhu et al., lncRNAs have an impact on the migration, proliferation, and death of gastric cancer cells (21). The possibility of lncRNA-HMlincRNA717 serving as a biomarker for gastric cancer has been suggested by similar investigations (22, 23). Lu et al., 2016 reported that lncRNA BC032469 enhanced proliferation in gastric cancer (23). Li et al., 2017 indicated that MIAT competitively interacted with miR-29a-3p and changed gastric cancer's biological activities (24).

In another study, Liu et al., 2015 showed that LincHOTAIR epigenetically controlled miR34a and motivated epithelial-to-mesenchymal transition (EMT), enhancing metastasis in gastric cancer (25). However, there have not been enough extensive analyses of lncRNAs in gastric cancer. The present study aimed to find a specific mRNA-miRNA-lncRNA network in which lncRNAs play an important role as biomarkers in gastric cancer.

At first, lncRNAs and mRNAs showed significantly different expressions in gastric cancer. Moreover, the binding power of each lncRNA to mRNAs was measured, and the results showed that only 5 out of 14 lncRNAs were able to bind to mRNAs (five up-regulated (H19, WT1-AS, EMX2OS, HOTAIR, and ZEB1-AS1) and four down-regulated (LINC00261, C11orf40, HSDL2-AS1, and LINC00319)). However, three of down-regulated lncRNAs were incapable of binding with the mRNAs. The subsequent KEGG pathway analysis revealed that the pathways of protein metabolism and absorption and mineral absorption are the most important in gastric cancer. Besides, the metabolism of xenobiotics by cytochrome p450, drug metabolism-cytochrome p450, gastric acid secretion, and butanoate metabolism pathways were significant in BP category. Moreover, in MF, extracellular matrix structural constituent conferring tensile strength and platelet−derived growth factor binding was supplemented.

Current study found 9 DElnRNAs (list A): 5 up-regulated and 4 down-regulated (Table 1) and 856 DEmRNAs (list B): 451 up-regulated and 405 down-regulated.

Previous studies showed that H19 is associated with gastric cancer severity through the miR-22-3p/Snail1 axisaxis (26, 27). Moreover, Du et al., 2016 indicated that WT1-AS could affect the proliferation, migration, and invasion of gastric cancer cells (28). This work has demonstrated that when compared to nearby normal tissues, gastric cancer considerably upregulates the expression of H19 and WT1-AS. Additionally, the drug-gene interaction research revealed certain medications that already target these genes and are used to treat GC. In another study, Dong et al., 2019 showed that HOTAIR is up-regulated in gastric cancer, and it was correlated with the invasion of cancer cells (29). EMX2OS plays important role in many kinds of cancers, such as gastric cancer Moreover, age, grade, stage, and cancer status are all associated with EMX2OS (30). Our data indicated that HOTAIR, EMX2OS, and ZEB1-AS1 are up-regulated in GC. On the other hand, we found that LINC00261 is down-regulated in gastric cancer.

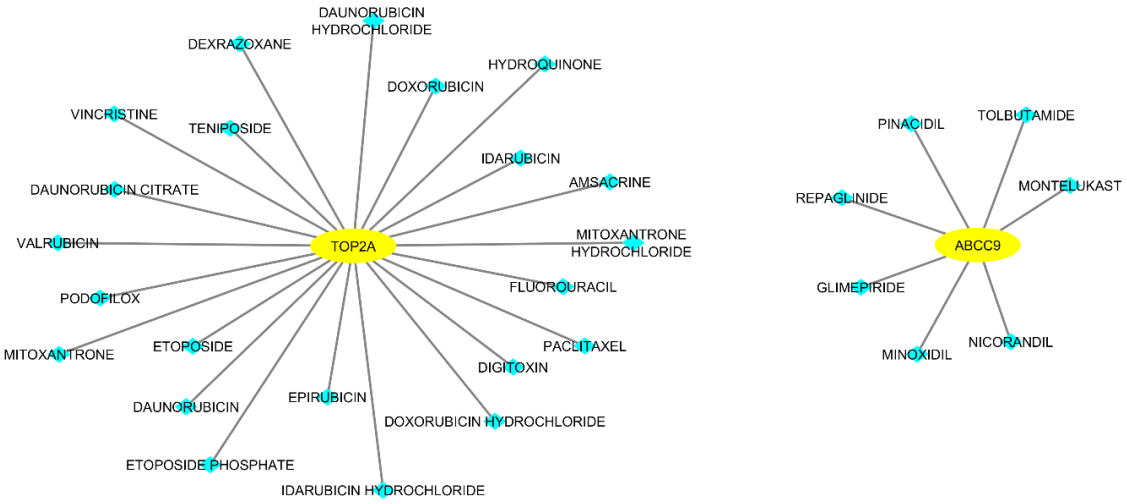

Figure 5. The drug-gene interaction network for up-regulated detected genes produced by DGIdb 3.0

The results of this investigation confirm other studies that indicated LINC00261 was downregulated in gastric cancer. A low prognosis for gastric cancer is indicated by the down-regulation of LINC00261, which also prevents metastasis (31). Furthermore, the interaction analysis of all five up-regulated lncRNAs showed that TOP2A, MRC2, RBMS3, and ABCC9 interacted with lncRNAs (Fig. 2). Significant changes in the expression of TOP2A, MRC2, RBMS3, and ABCC9 mRNAs in gastric cancer were shown in previous studies (32-34).

Different projects should be designed to enable us to deliver drugs in a predictable time frame for gastric cancer (35). In this matter, bioinformatics analysis is crucial. Four lncRNAs modified different pathways in gastric cancer tissue via the interactions with various mRNAs. However, more investigations are needed to confirm these results.

LncRNAs generally show their regulatory functions by affecting gene expression. The present study measured the potential target mRNAs for all lncRNAs by analyzing their expression pattern in gastric cancer. The cytoskeleton and invasion of cancer cells may be related, according to further study of lncRNA activity. These lncRNAs may remain in stomach cancer as markers. By altering the activity of regulatory elements and chromosomal conformation, LncRNAs can have an impact on gene expression (36). GO analysis indicated that the detected DEGs were significantly enriched in biological processes (BP). Furthermore, DEGs enriched in following pathways: response to xenobiotic stimulus, digestion, and collagen fibril organization.

Conclusion

In conclusion, we showed a novel mRNA-miRNA-lncRNA network using bioinformatics analysis. This network can be helpful in the prognosis of patients with gastric cancer. Thus, it may establish new insights into the molecular mechanism of gastric cancer. Besides, the analysis of drug-gene interactions revealed that potential FDA-approved drugs interact with the identified target genes. We have identified pathway networks, candidate genes, and drugs for better management of GC.

Conflict of interests

The authors declare no conflict of interest, financial or otherwise.

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