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Identification of a ceRNA Network Driven by Copy Number Variations in Esophageal Cancer

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Background: Copy number variation (CNV) is associated with progression of esophageal cancer (EC), a common gastrointestinal neoplasm.

Methods: Using sequencing data, CNV data, and clinical data of EC transcriptome samples obtained from public databases, we performed differential expression analysis on sequencing data. Differentially expressed CNV-driven lncRNAs were screened using the chi-square test, and CNV-driven lncRNA-associated miRNAs and mRNAs were predicted. Cytoscape software was then used to construct ceRNA networks. Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses were performed to investigate biological functions of mRNAs in the ceRNA network. Survival curves were plotted to explore correlations between lncRNAs in the ceRNA network and overall survival of CNV patients. Multiple databases were used to predict lncRNAs-related drugs.

Results: A dysregulated lncRNA-associated ceRNA network driven by CNV in EC, including 11 lncRNAs, 11 miRNAs and 159 mRNAs, was constructed. Downstream enrichment of mRNAs was related to biological processes such as extracellular matrix organization, indicating that these mRNAs mainly participate in intercellular exchange between tumor cells. Additionally, expression of all lncRNAs in the ceRNA network, except LINC00950, LINC01270 and MIR181A1HG, was correlated with patients' CNV. In addition, none of the 11 lncRNAs was significantly correlated with overall survival of CNV patients. CH5424802 and PD-033299CNV mainly affected the RTK signaling pathway and the cell cycle of tumor cells via RP11-180N14.1 and RP11-273 G15.2 in the ceRNA network.

Conclusions: This study identified 11 CNV-driven lncRNAs that might affect EC development, 2 of which have promising effects if applied to drug treatment. These findings might assist in identifying novel treatments for EC. (J Nippon Med Sch 2023; 90: 426–438)

Key words: esophageal cancer, lncRNA, ceRNA, CNV-driven lncRNA, bioinformatics

Introduction

The incidence rate for esophageal cancer (EC), one of the most common malignant tumor diseases, is increasing[1,2]. EC can be classified into 2 major categories by histological features: esophageal squamous cell carcinoma (ESCC) is more prevalent in Asia, Africa, and South America and esophageal adenocarcinoma (EAC) is more prevalent in North America, Europe, and Oceania[1,3]. EC is mainly treated by surgical resection, which can achieve desirable effects and prolong survival if completed at an early stage. However, even though patients with advanced EC have a much-improved prognosis because of chemotherapy, radiotherapy, or both before and after surgery, the 5-year survival rate remains 15-25%[4,5]. Hence, for better effects and prognosis, new therapeutic targets and corresponding mechanisms targeting the underlying pathogenesis of EC deserve more attention.

In contrast to insertion, deletion, substitution, and other gene variants, DNA copy number variation (CNV) refers to an increase or decrease in gene copy numbers[6].

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Because of the link between DNA copy number and DNA-coding RNA expression, the medical community tends to agree that CNV is associated with tumor incidence, because a gain or loss in tumor-related gene copy number often triggers oncogene expression or inactivates tumor suppressor genes[6]. Gains in the copy number of IGHG3 gene locus on human chromosome 14 were correlated with its overexpression and hence increased prostate cancer incidence and mortality[7]. Other CNVs were found to be tumor biomarkers as well. After a comprehensive assessment of colorectal carcinoma tissues, including mRNA expression level, DNA methylation level, and CNV, Liu et al.[8] found that 10 CNV-related lncRNAs could identify patient prognosis for 5 colorectal carcinoma subtypes. In bladder cancer, analyzing tumor and urine samples[9] identified CNVs of 3 genes with high abbreviation frequencies that were capable of predicting disease-free survival. Similarly, an analysis of CNV in EC may help explain its pathogenesis.

Competing endogenous RNAs (ceRNAs) are tumor-specific regulatory pathways that affect tumor specificity at the protein level[10]. As lncRNAs share miRNA response elements with mRNA, ceRNA could regulate gene expression by competing for shared miRNAs[11]. Studies proved that various RNAs, including mRNA, lncRNA, circular RNA, and miRNA, possess ceRNA activity to regulate tumor cells, microenvironment, and tumor biological functions[12]. New insights into ceRNA both unveil the mechanism towards its mutual regulation with RNA and add to knowledge of lncRNA functions[13]. Accumulating evidence from studies shows that lncRNA could be a prognostic biomarker. Hu et al.[14] discovered that high expression of lncRNA PVT1 not only boosts proliferation, migration, and invasion of tumor cells but also is related to postoperative metastasis and poor prognosis. Ferroptosis-related lncRNAs in EC were analyzed by Liu et al.[15] to predict the prognosis of EC patients. However, the relationship between ceRNA and therapeutic targets is not well understood.

This comprehensive analysis of CNV, lncRNA, miRNA, and mRNA used datasets from public databases. A LncRNA-associated ceRNA network driven by CNVs in EC was built. Functional enrichment analysis was performed to reveal and identify the functional roles and underlying mechanisms of ceRNA networks in EC. Subsequently, we performed CNV differential expression analysis between lncRNA-coding genes and lncRNAs for 11 lncRNAs in the ceRNA network. Finally, we identified lncRNAs associated with tumor drug efficacy in EC to inspire development of new clinical treatments for EC.

Materials and Methods

1.1 Differential Expression Analysis of Genes

The TCGA-ESCA dataset was downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) and included lncRNA and mRNA expression (normal: 11, tumor: 160), mature miRNA expression (normal: 13, tumor: 185), CNV (normal: 188, tumor: 185), and clinical data (patients' general condition, TNM stage, pathology, and survival situation are provided in Supplementary Table 1: https://doi.org/10.1272/jnms.JNMS.2023_90-611). Differential expression analysis was conducted on lncRNAs, miRNAs, and mRNAs by using the edge R package ($|\log FC| > 1.5$, $FDR < 0.05$) and returned DElncRNAs, DEmiRNAs, and DEmRNAs.

1.2 Identification of CNV-Driven Dysregulated lncRNAs

Genes with copy numbers that differed in normal and tumor tissues were compared by the chi-square test and corrected by Bonferroni adjustment (adjusted p-value <0.05). We calculated the intersection of lncRNAs with gains in copy number and upregulated DElncRNAs and identified a pool of CNV-driven upregulated lncRNAS. Similarly, CNV-driven downregulated DElncRNAs were obtained by calculating the intersection of lncRNAs with losses in copy number and downregulated DElncRNAs.

Tumor tissue samples with available data on CNV and expression were chosen and submitted to the Kruskal-Wallis test. Combined with data at hand and test results, the distribution and correlation analysis of CNV and expression were generated ($p<0.05$). A strong correlation between lncRNA CNV and expression identified candidates as CNV-driven lncRNAs.

1.3 Identification of ceRNA Network Regulated by CNV-Driven lncRNAs

lncBase and starBase databases predicted miRNAs that could interact with CNV-driven lncRNAs. Based on negative regulation between lncRNAs and miRNAs in the ceRNA network, differentially expressed miRNAs (DEmiRNAs) that intersected with the prediction results were selected for correlation analysis (cor<-0.2), which eventually identified target miRNAs downstream regulated by CNV-driven lncRNAs. Similarly, possible target mRNAs downstream of miRNA were plotted by the starBase, miRDB, mirDIP, and targetScan databases. Based on the negative regulation between mRNAs and miRNAs in the ceRNA network, we obtained a pool of candidate mRNAs after calculating the intersection of differentially

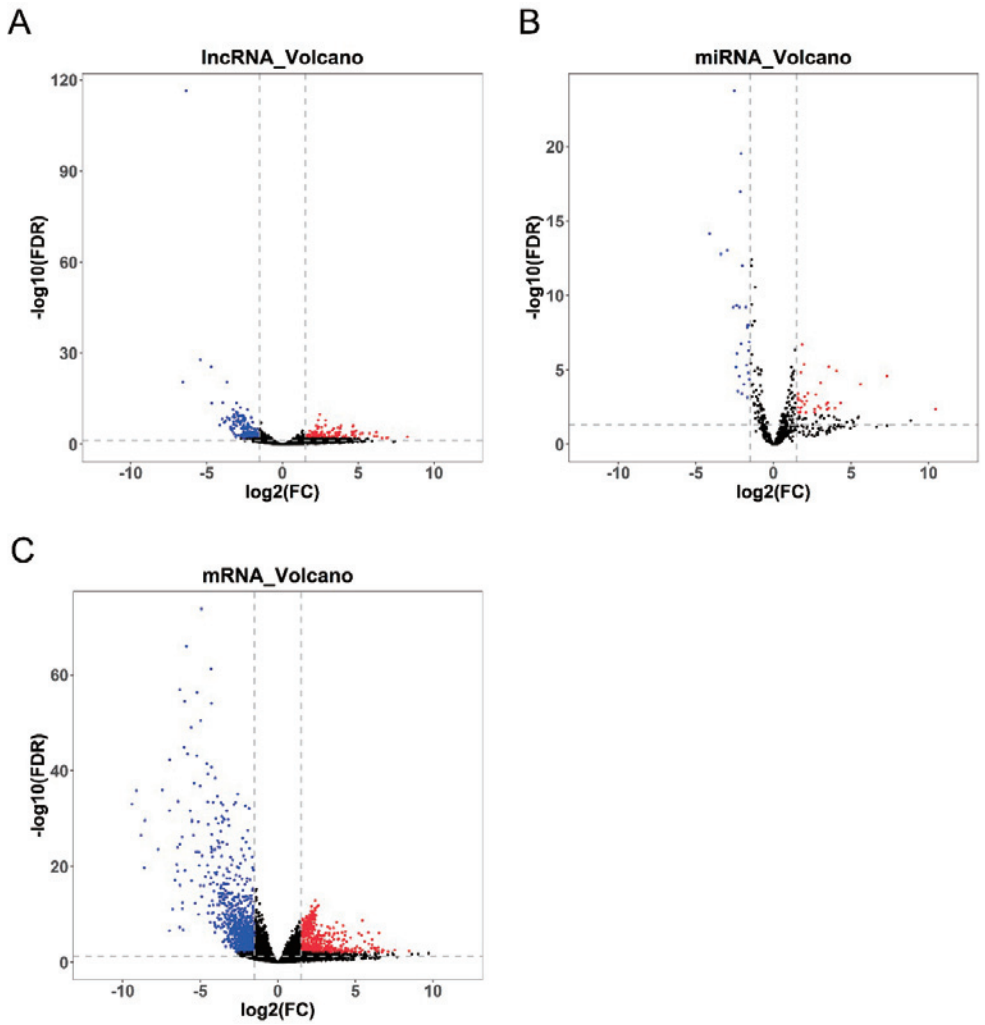

Fig. 1 Differential expression analysis of genes

(A) Volcano plot of DElncRNAs in the normal and tumor groups; (B) Volcano plot of DEmiRNAs in the normal and tumor groups; (C) Volcano plot of DEmRNAs in the normal and tumor groups. Red represents significantly upregulated RNAs and blue represents significantly downregulated RNAs.

expressed mRNAs (DEmRNAs) and the prediction results. The target mRNA downstream of the miRNA was specified via correlation analysis (cor<-0.2).

LncRNAs, miRNAs, and mRNAs in the lncRNA-miRNA-mRNA network were retrieved and imported to Cytoscape software to establish a ceRNA network. Regions of lncRNA copy number amplification (or deletion) were mapped using the GISTIC2 package.

1.4 Gene Set Enrichment Analysis

To investigate the role of dysregulated mRNAs in EC, we uploaded the selected potential target mRNAs to the Metascape (http://metascape.org) database for functional analysis.

1.5 Correlation between lncRNAs in the Dysregulated ceRNA Network and Prognosis

To investigate the effect of lncRNAs CNV in dysregulated ceRNA networks on patient prognosis, we grouped lncRNAs by CNV status in ceRNA networks and plotted survival curves using the "survival" package to assess their prognostic effect.

1.6 Prediction of Drugs Associated with lncRNAs in the Dysregulated ceRNA Network

The response of 1,001 tumor cell lines to 265 drugs was obtained in the GDSC database. Wang et al.[16] conducted bootstrapping procedures on 265 drugs by using Elastic Net regression to identify key genes that might be associated with cancer drug response.

Results

2.1 Differentially Expressed Genes in EC

Differential analysis of the transcriptome data from the TCGA-ESCA dataset, using the edgeR package, acquired

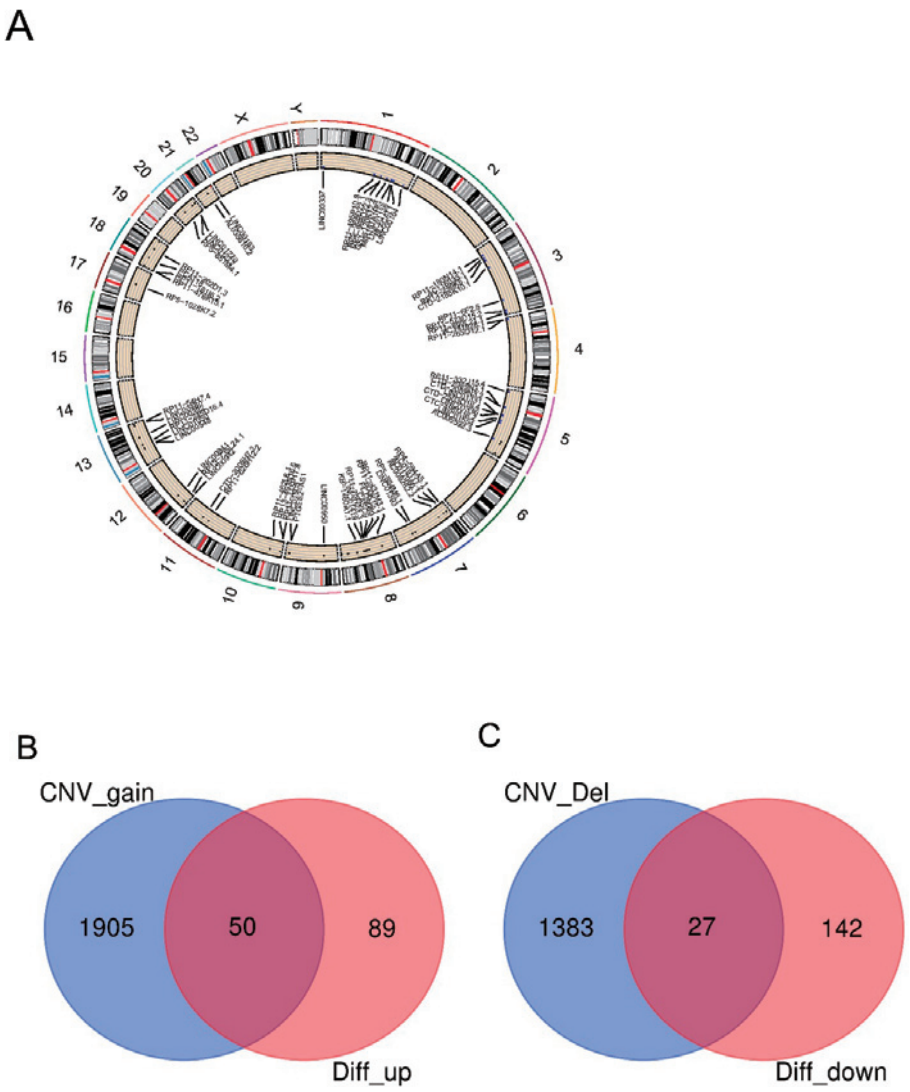

Fig. 2 Identification of CNV-driven lncRNAs

(A) Circle plot of EC lncRNA CNV, where the black scatter in the inner circle indicates gains in corresponding gene copy number and the blue scatter indicates losses; (B) Venn diagram displaying lncRNA with copy number gains and upregulated genes; (C) Venn diagram displaying lncRNA with copy number losses and downregulated genes.

308 DElncRNAs, among which 139 were upregulated and 169 downregulated (**Fig. 1A**); 1,606 DEmiRNAs, among which 590 were upregulated and 1,016 downregulated (**Fig. 1B**); and 62 DEmRNAs, among which 35 were upregulated and 27 downregulated (**Fig. 1C**).

2.2 Identification of lncRNAs Driven by CNV

To identify lncRNAs with CNV in EC tissues, we used the chi-square test to analyze lncRNA sequencing data from normal and tumor tissues; 3,365 lncRNAs appeared with significant CNV in the EC tumor groups (**Fig. 2A**). Fifty overexpressed CNV-driven lncRNAs were obtained by overlapping upregulated lncRNAs with lncRNAs with gains in copy number (**Fig. 2B**). Similarly, 27 downregulated CNV-driven lncRNAs were obtained from the intersection between downregulated lncRNAs and lncRNAs with copy number losses (**Fig. 2C**). Thirty lncRNAs with a strong correlation between CNV and expression in correlation analysis with the Kruskal-Wallis test were confirmed as CNV-driven lncRNAs.

2.3 The Dysregulated lncRNA-Associated ceRNA Network Driven by CNV

With CNV-driven lncRNAs specified in the previous section, multiple databases were used to predict the interaction of lncRNA-miRNA and miRNA-mRNA to obtain a dysregulated lncRNA-associated ceRNA network driven by CNV in EC, which included 11 lncRNAs, 11 miRNAs, and 159 mRNAs (**Fig. 3A～C**; **Table 1**). The ceRNA network was then visualized on Cytoscape for straightforward observation of the interaction network among genes (**Fig. 4A**). We mapped the chromosomes for

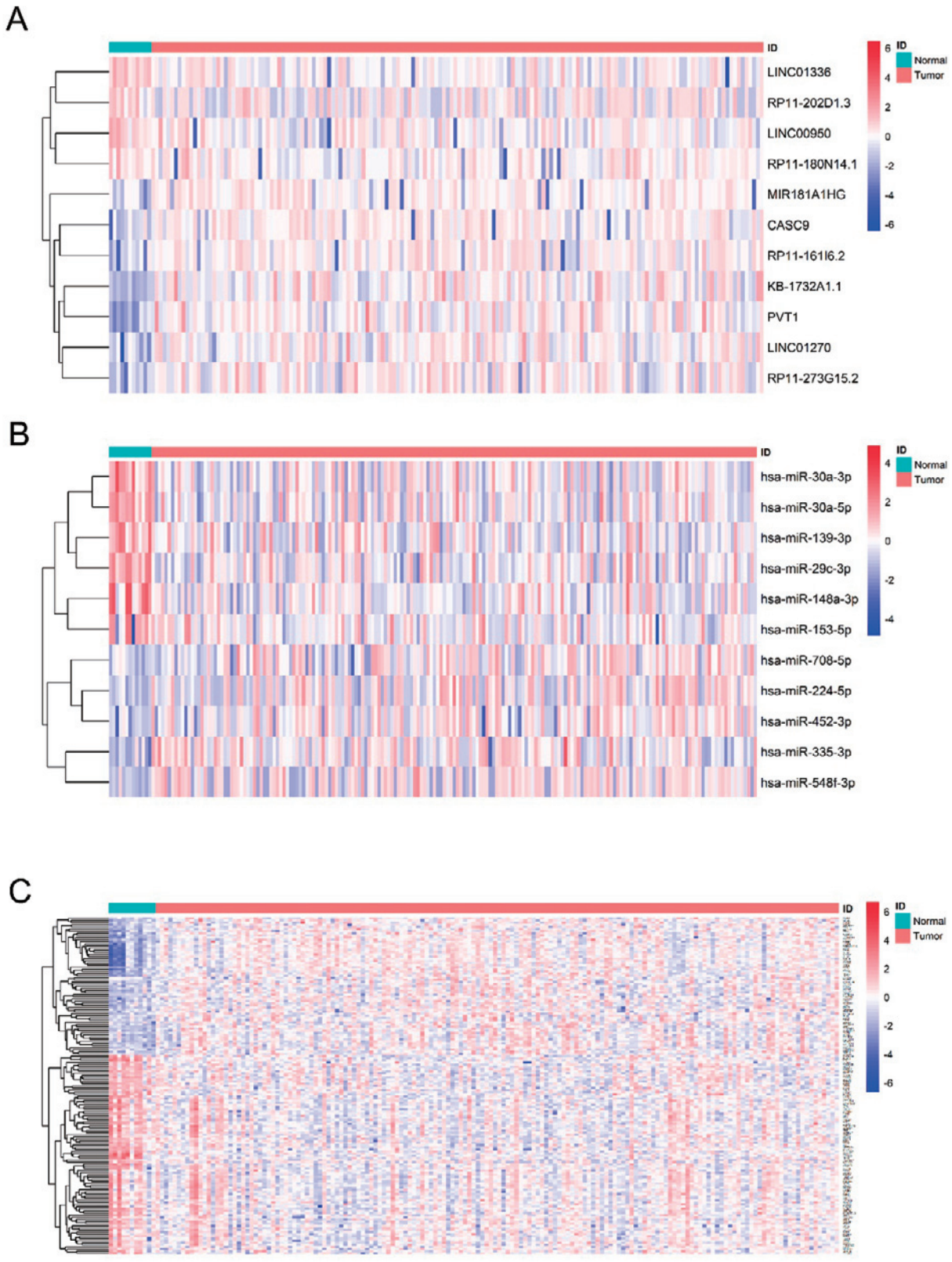

Fig. 3 Heatmap of RNA expression in the dysregulated ceRNA network

(A) Heatmap of lncRNA expression in the dysregulated ceRNA network; (B) Heatmap of miRNA expression in the dysregulated ceRNA network; (C) Heatmap of mRNA expression in the dysregulated ceRNA network.

which the CNV indicated where part of the lncRNAs with copy number gain (or deletion) had been marked in the amplification (or deletion) region map at their corresponding positions (**Fig. 4B and C**).

Table 1 Chromosomes location of lncRNAs in the CNV-driven ceRNA network

Gene	Chr	Start	End	Band
LINC01336	chr5	75047719	75052843	q13.3
CASC9	chr8	75223404	75324741	q21.13
KB-1732A1.1	chr8	102805517	102809971	q22.3
PVT1	chr8	127794533	128101253	q24.21
RP11-273G15.2	chr8	142981738	143018437	q24.3
LINC00950	chr9	35858738	35865518	p13.3
MIR181A1HG	chr1	198807493	198937429	q32.1
RP11-161I6.2	chr18	1883524	2240847	p11.32
RP11-202D1.3	chr18	53568447	53597851	q21.2
LINC01270	chr20	50292720	50314922	q13.13
RP11-180N14.1	chr3	21412222	21413944	p24.3

2.4 GO Functional and KEGG Pathway Enrichment Analyses

To investigate how dysregulated ceRNA affects biological function, GO functional and KEGG pathway enrichment analyses were conducted on 159 ceRNA network-related mRNAs. The results suggest that these genetic changes could lead to extracellular matrix organization, regulation of systemic processes, multicellular organismal homeostasis, appendage development, muscle system processes, sensory organ development, cellular response to hormone stimulus, p53 signaling pathway, protein kinase A signaling, response to epinephrine, cellular response to organic cyclic compound, transport across blood-brain barrier, and cell-cell adhesion via plasma-membrane adhesion molecules, among other processes (**Fig. 5A～C**).

2.5 CNV of lncRNAs Involved in the Dysregulated ceRNA Network May Affect the Prognosis of EC Patients

To explore the association between lncRNA-coding gene CNV and differential expression of lncRNA, we focused on 11 lncRNAs in EC tumor tissue sample and classified them as Amplification and Deletion groups by CNV, for the purpose of comparison with samples with normal copy numbers. Except for LINC00950 and MIR 181A1HG, gene expression level was associated with CNV (**Fig. 6A～K**).

Subsequently, the link between CNV and survival condition was plotted by drawing survival curves for the 11 lncRNAs, using the survival package. There was no obvious correlation between CNV of 11 lncRNAs and overall patient survival (**Fig. 7**).

2.6 Drug Response-Associated lncRNAs in the Dysregulated ceRNA Network

After investigating the relationship between lncRNAs in the ceRNA network and cancer drug response, we found a link between RP11-180N14.1 and CH5424802, RP 11-273 G15.2, and PD-0332991 (Predictive Score ≥0.25; **Table 2**).

Discussion

Cancer is strongly linked to genomic mutation, including small size mutations and CNV, i.e., copy number deletions, duplications, and amplifications[17]. CNV is a hallmark of cancer. Since it is correlated with the expression of the corresponding RNA, CNV is thought to contribute to altered expression of coding RNAs. This study focused on CNV changes in the tumor tissue of EC patients. In this study, the TCGA database was used to obtain DElncRNAs, DEmiRNAs, and DEmRNAs, and then 30 CNV-driven lncRNAs in EC were obtained by calculating the intersections of DElncRNAs and CNV-related lncRNAs. A lncRNA-associated ceRNA network driven by CNV was then built with bioinformatics tools through many databases, including 11 lncRNAs, 11 miRNAs, and 159 mRNAs. Among these 11 lncRNAs, expression levels of LINC00950 and MIR181A1HG were correlated with CNV patients, but the relationship between the 11 lncRNAs and CNV patients' prognosis was weak. Based on databases, RP11-180N14.1 and RP11-273 G15.2-related drugs were predicted. The association between CNV and EC has been reported previously. Dong et al.[18], in a comprehensive analysis of CNV in EC and transcriptional expression profiles, identified FAM60A as the prognostic factor of EC. The tumor suppressor FAM60A, when downregulated by CNV deletion, regulated the cell cycle,

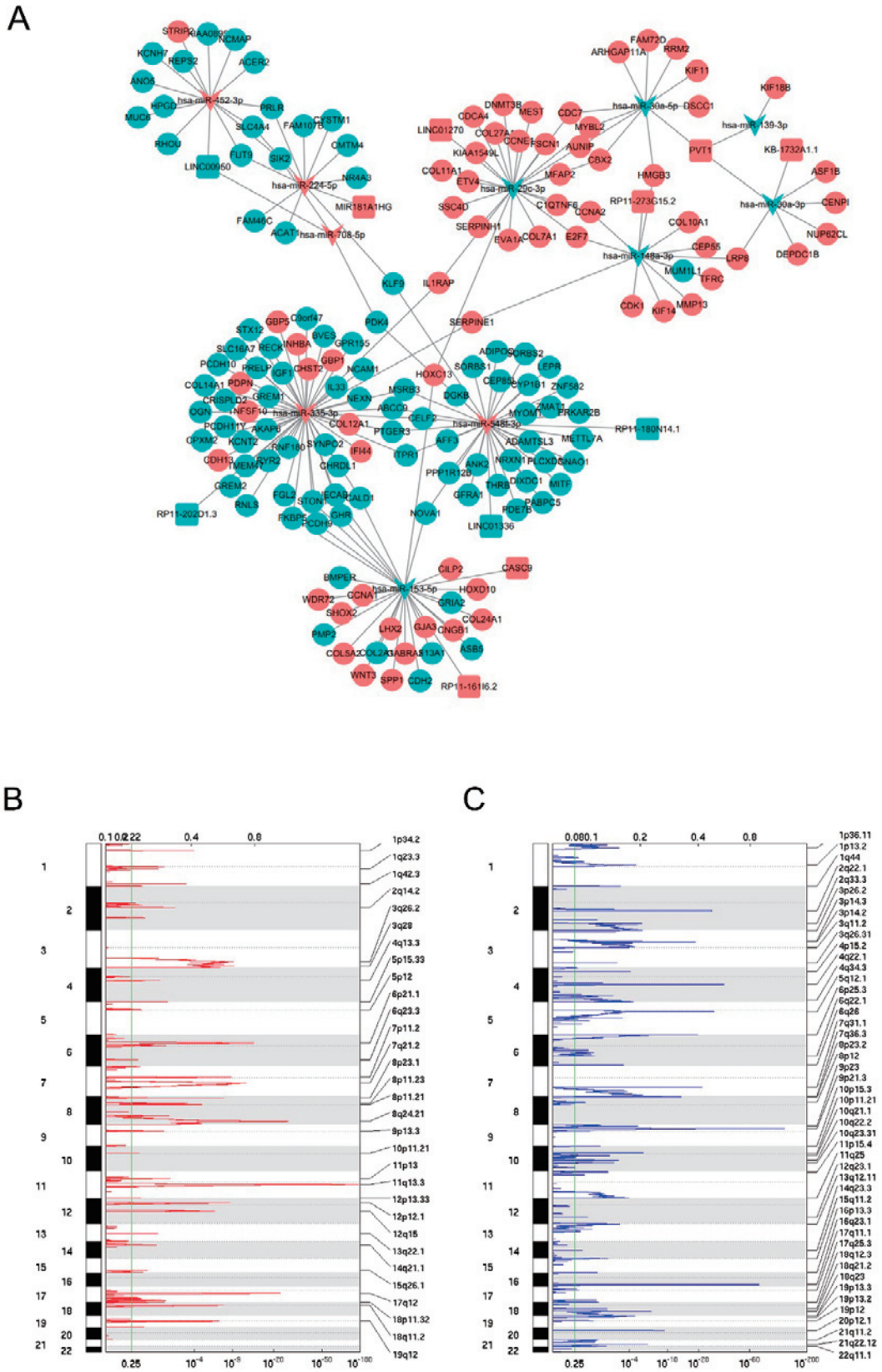

Fig. 4 Building a lncRNA-associated dysregulated ceRNA network driven by CNV

(A) A dysregulated lncRNA-associated ceRNA network driven by CNV. The squares represent lncRNA, the arrowheads represent miRNA, and the circles represent mRNA. Red indicates upregulated genes, and blue indicates downregulated genes; (B) LncRNA copy number amplification in EC; (C) LncRNA copy number deletion in EC.

Note: Some lncRNAs in the ceRNA network were marked in the right correspondingly.

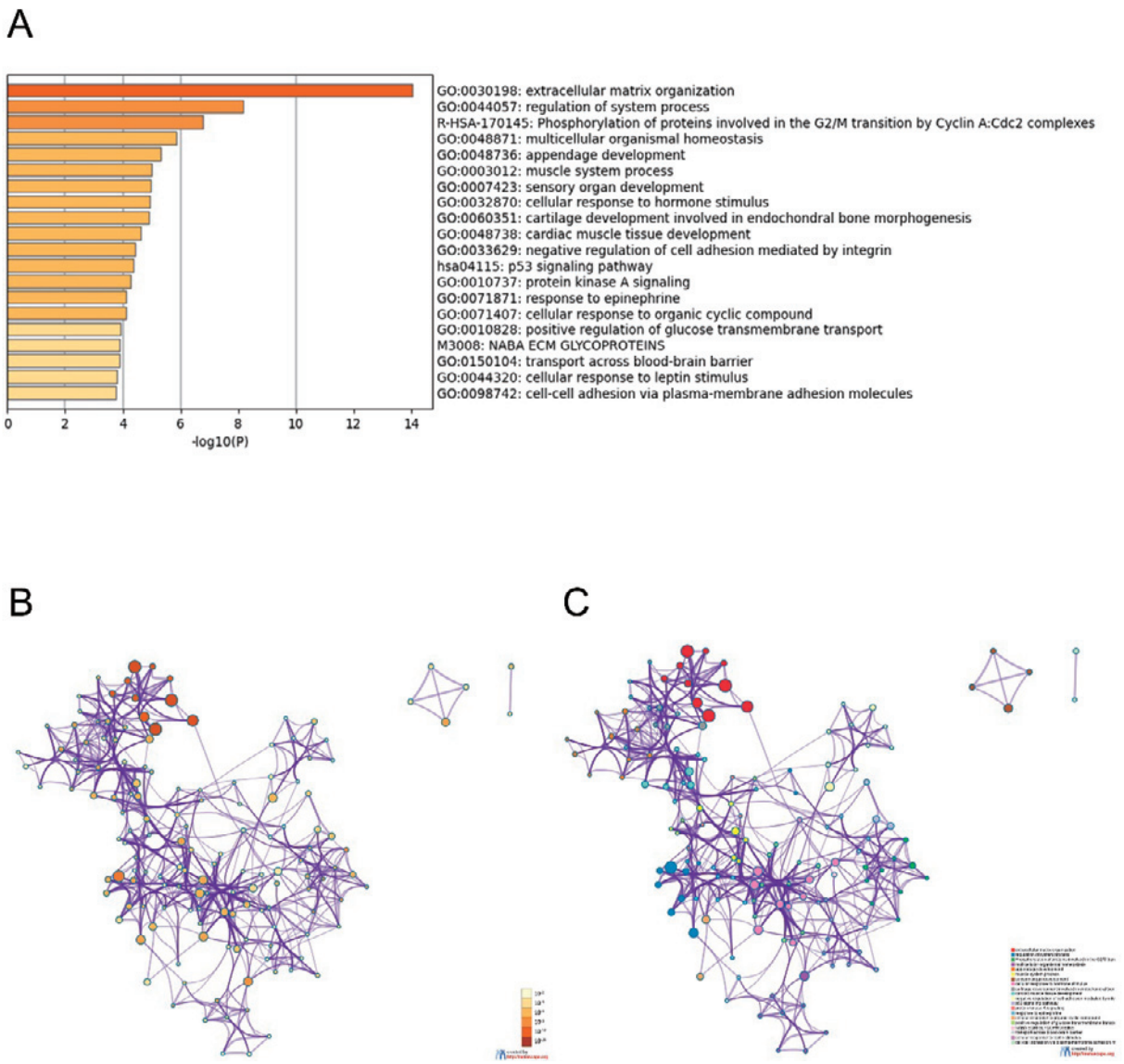

Fig. 5 Enrichment analysis of the dysregulated ceRNA network

(A) Heatmap of enriched GO and KEGG pathway in descending order of p-values; (B) Network of GO and KEGG enrichment analyses. The colors represent p-values; (C) Network of GO and KEGG enrichment analyses. The colors represent enrichment items.

thereby affecting the biological functions of EC cells. Hence, a deep understanding of how CNV affects EC genes is crucial to clarifying the EC mechanism and improving cancer treatment.

After the ceRNA mechanism was first described, researchers have increasingly focused on its role in biological functions. Several studies have demonstrated that interactions between mRNAs, miRNAs, and lncRNAs are closely related to tumor progression. In this study, DElncRNA analysis identified 11 lncRNAs (CASC9, KB-1732A1.1, PVT1, LINC00950, LINC01270, LINC01336, RP 11-273 G15.2, MIR181A1HG, RP11-161I6.2, RP11-202D1.3, and RP11-180N14.1) that were driven by CNV. Hence, a ceRNA network of EC was built. LncRNA CASC9 is overexpressed in a variety of tumors and promotes tumor cell proliferation, differentiation, and invasion[19–21]. CASC9 overexpression also promotes malignant tumor progression in EC. Gao et al.[22] found that CASC9 both promotes tumor development, by regulating epithelial to mesenchymal transition, and indicates a poor prognosis in patients with ESCC when highly expressed. KB-1732A 1.1 (lincK) was found to promote breast cancer progression by promoting proliferation and EMT[23]. High expression of lncRNA PVT1 in tumor tissues is associated with a poor prognosis for EC patients, as PVT1 can promote malignant progression of EC by regulating the the miR-128/ZEB1 signaling axis[14,24]. Liu et al.[25] found that LINC 00950 (NGX6) overexpression in osteosarcoma cells could

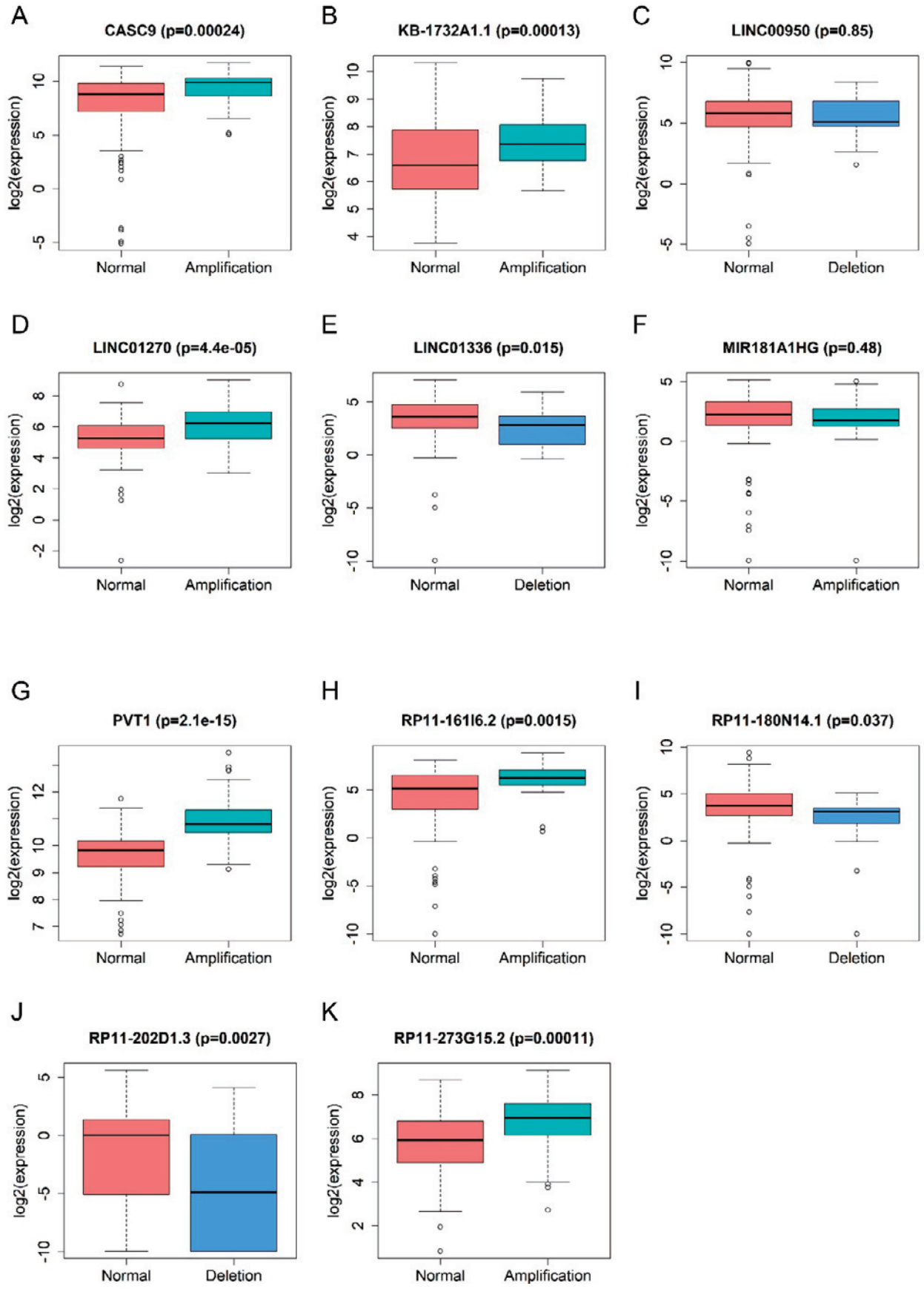

Fig. 6 Boxplots for expression of lncRNAs in the dysregulated ceRNA network
Pink represents the normal group; blue represents the deletion group; green represents the amplification group.

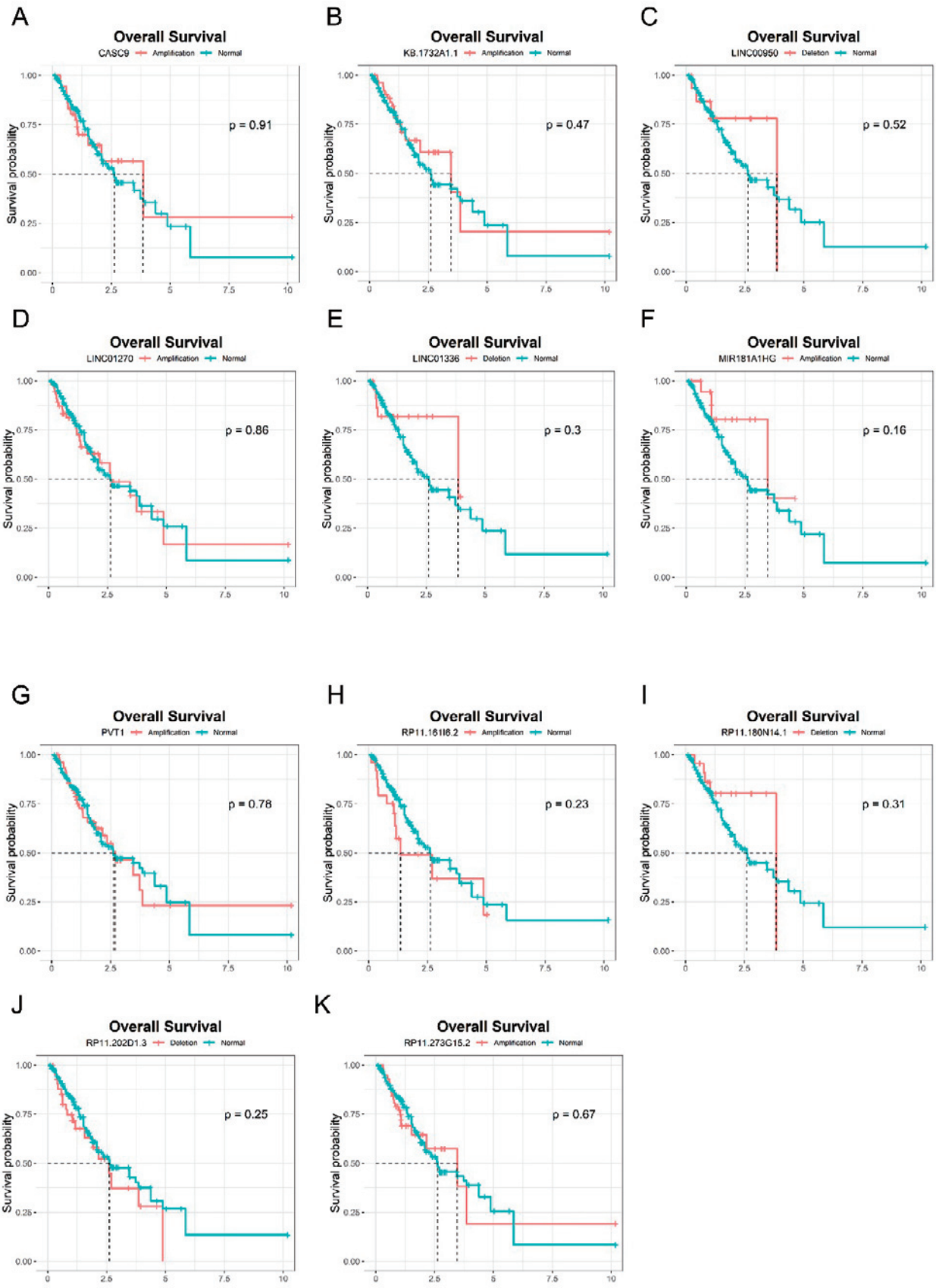

Fig. 7 Overall survival analysis of lncRNA involved in the dysregulated ceRNA network

lead to less active, migratory, and invasive cells, as it could also induce osteosarcoma cell apoptosis by inhibiting the W/β-catenin signaling pathway. Li et al.[26] proved that, by silencing the highly expressed lncRNA LINC

Table 2 Prediction of drugs associated with lncRNAs in the dysregulated ceRNA network

Gene ID	Ensembl ID	Drug	Predictive Score	Category
RP11-180N14.1	ENSG00000272511	CH5424802	0.255	RTK signaling
RP11-273G15.2	ENSG00000247317	PD-0332991	0.265	cell cycle

01270 in EC, EC cell migration and invasion were inhibited, and EC cells were more sensitive to 5-FU. The above studies showed that 5 lncRNAs among the 11 obtained CNV-driven lncRNAs were closely related to cancer progression. Knockdown of MIR181A1HG was also found to affect DNA damage in multipotent mesenchymal stromal cells and impact cell cycle progression[27]. This suggests that MIR181A1HG has a similar effect on the cell cycle by affecting cancer cell DNA in EC. These hypotheses need to be confirmed in future studies. Other lncRNAs identified in this study, namely LINC01336, RP 11-273 G15.2, RP11-161I6.2, RP11-202D1.3, and RP11-180N 14.1, are currently poorly understood, and studies of the effects of these lncRNAs on EC are expected. Past and present evidence suggests that some lncRNAs selected to construct the EC dysregulated ceRNA network in this study affect the pathogenesis and development of tumors if dysregulated. Thus, the ceRNA network constructed by CNV-driven lncRNAs is credible.

Subsequently, we performed GO functional and KEGG pathway enrichment analyses of the pathways affected by the ceRNA network on EC and found that these genetic changes may cause changes in cell-cell communication and transmembrane trafficking, as well as other biological changes, in tumor cells. The tumor microenvironment is pivotal in tumor progression. Defined as the acellular component of tissues, extracellular matrix (ECM) organization provides biochemical and basic structural support for cellular components. Specific arrangements and orientation of ECM components form tissue-specific microenvironments that have critical roles in tumor progression[28–30]. Alterations in ECM density and composition have been shown to promote tumor growth and progression. For example, ECM degradation in matrix metalloproteinase-related pathways promoted tumor cell invasion and thus malignant tumor progression in TME[31]. In breast cancer, ECM and ECM-related functions may serve as new therapeutic targets[32]. As this study proved that ceRNA may affect the ECM pathway in EC, we suggest that this ceRNA network might affect EC progression through ECM. Thus, knowledge of the changes in biological function caused by ceRNA networks could drive breakthroughs in EC treatment.

In sum, this study used CNV, lncRNA, miRNA, and mRNA data in EC from public databases to construct a dysregulated ceRNA network regulated by CNV-driven lncRNAs. The network was shown to be linked with biological processes such as communication between tumor cells and transmembrane trafficking. Further analysis of the results found that CNV-driven lncRNA was associated with drug efficacy.

This study is a pure bioinformatics analysis and thus limitations. All data used in this study were obtained from the TCGA database. Samples in this database were obtained from counties around the world, including the United States, and encompass different ethnic groups and cancer types. Although this study aimed to identify an applicable set of CNV-driven lncRNAs, differences between samples may limit the usefulness of the results. To make the experimental results more reliable, we plan to collect more samples and establish our own database in the future. In addition, cell studies are needed to support the findings.

Conflict of Interest: None declared.

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