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RESEARCH ARTICLE

Integrated Bioinformatics Analysis Reveals ceRNA Triplet Related to Apoptosis in Gastric Cancer

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Abstract: Apoptosis is a form of programmed cell death and evading the apoptosis is a milestone in gastric cancer (GC) tumorigenesis. Various RNAs such as miRNA (microRNA), mRNA (messenger RNA), lncRNA (long non-coding RNA), circRNA (circular RNA), play crucial roles in the apoptosis mechanism. In this present study, we aimed to understand the role played by these RNAs in apoptosis in GC. We constructed a ceRNA (competitive endogenous RNA) network using the expression profiles obtained from SRA (Sequence Read Archive) and GEO (Gene Expression Omnibus) datasets. After network construction, the differentially expressed mRNAs were used for gene prioritization. The prioritized genes and the RNAs interacting with them were analyzed to study their role in GC apoptosis. Then, we performed functional and pathway analysis to understand the role played by these genes in gastric cancer. This resulted in 37 miRNA-mRNA interactions, one miRNA-lncRNA interaction, and 17 miRNA-circRNA interactions. The binding site analysis resulted in 10 miRNAs that share common MRE (miRNA Response Elements) among mRNA, lncRNAs, circRNA. Besides, we found 33 miRNA-mRNA-circRNA and two miRNA-mRNA-lncRNA valid interactions. Integration of multiple omics datasets revealed dysregulated genes, including IGF1, MME, CCND2, CDH2, and COL1A1, implicated in GC apoptosis. Functional enrichment analysis highlighted pathways related to apoptosis, with CCND2 emerging as a key player. The integrative methodology has revealed a new potential diagnostic biomarker for the regulation of gastric cancer (GC) apoptosis: the CCND2-miR-141-3p-hsa_circ_0008035 ceRNA triplet. This discovery offers fresh perspectives into the intricate regulatory pathways governing gene expression in GC, promising significant insights into its pathology.

Keywords: gastric cancer, ceRNA network, apoptosis, gene prioritization

1. Introduction

Gastric cancer (GC), claiming over 10.8 million cases globally (5.6 % of the total number of cases), in 2020 according to the International Agency for Research on Cancer, remains a prevalent malignancy and a significant public health threat worldwide (https://gco.iarc.fr/today/). GC progresses in the gastric mucosa, and it is asymptomatic, and the risk factors include Helicobacter pylori infection, smoking, excess salt intake, genetics, etc. Despite the development of new drugs and biomarkers, an appropriate remedy for early diagnosis of GC is still an afar[1].

Apoptosis is the mechanism by which cells commit suicide at the event when they become damaged. It is essential for the normal operation of the cells. Defects in apoptotic pathways contribute to various diseases such as cancer[2].
It's considered one of the defining characteristics of cancer[3]. The cancer cells tweak this self-destructing ability of cells by which the mechanism is halted. Thus the loss of apoptosis can result in tumor initiation, progression, and metastasis[4].

A vast network where coding and non-coding RNAs interact by vying for the same miRNA through miRNA-binding sites, thus controlling miRNA activity, is termed a ceRNA (competitive endogenous RNA) network[5]. RNA molecule that possesses

even a single MRE and if it can bind to miRNA can be considered as a ceRNA[6]. Also, these ncRNAs act as ceRNAs to tuning mRNA expression and controlling protein levels, which provides to the occurrence and development of tumors[7].

Gene prioritization is the process by which genes are ranked by their relevance of generating a disease phenotype. For prioritizing genes in a disease, a set of features such as gene expression and function, mutation, pathways involved, etc. are considered. In this study, we have analyzed the role of mRNAs and non-coding RNAs such as miRNA, lncRNA, and circRNA in GC apoptosis by constructing a ceRNA network. Figure 1 shows the workflow of the study.

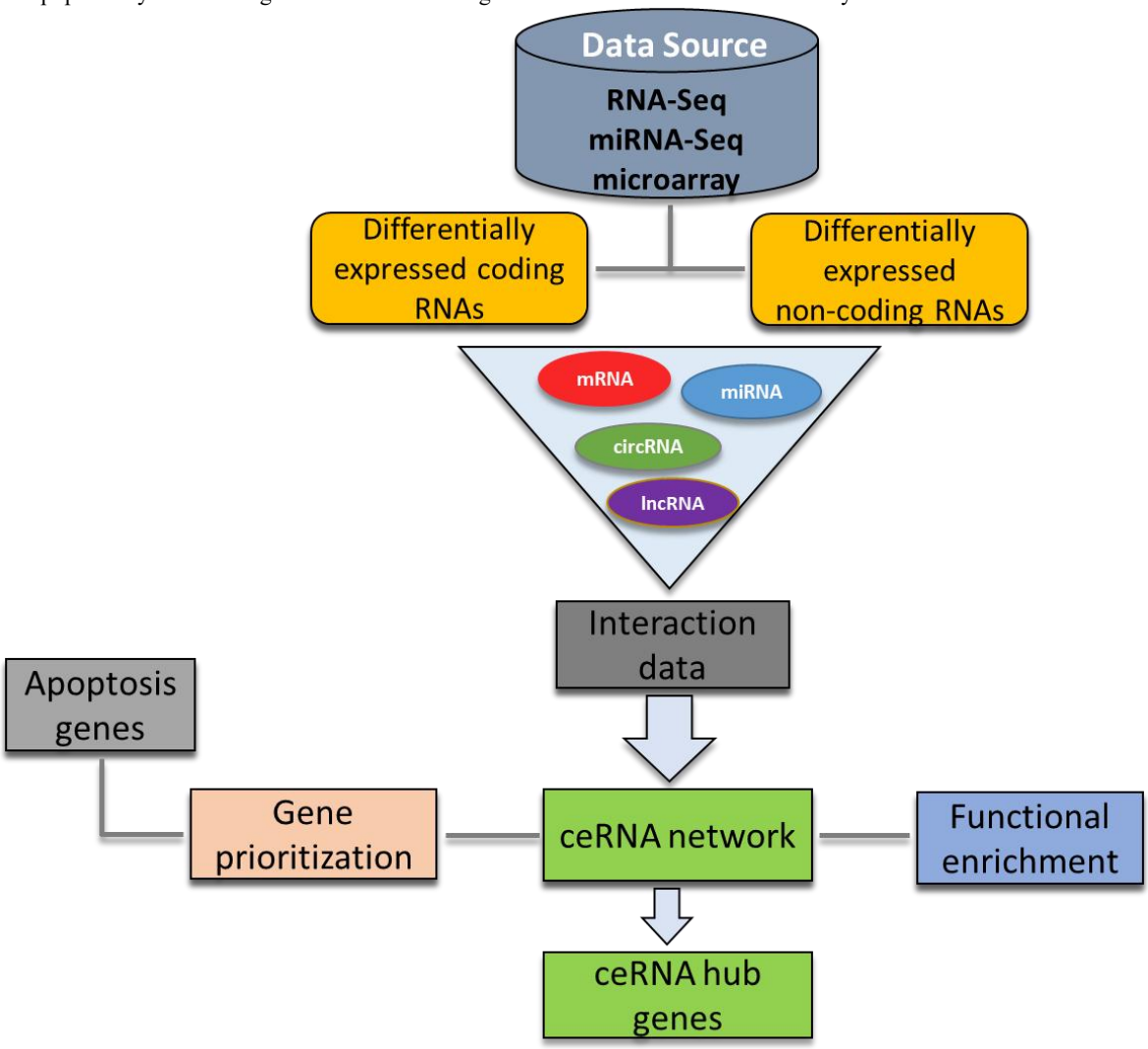

Figure 1. Workflow of the study

2. Materials and Methods

2.1. Datasets

The datasets for miRNA, mRNA, and lncRNA expression profiles were obtained from the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra). Additionally, circRNA expression data was retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) (Table 1). The miRNA-seq dataset, comprising 25 samples from both normal and tumor groups, was utilized for miRNA analysis. The mRNA-seq dataset, encompassing 44 samples, provided data for both mRNA and lncRNA analyses. Furthermore, the circRNA-seq dataset, consisting of 12 samples, was utilized for circRNA analysis.

Table 1. Details of the database and number of samples used in the analysis

Sl. No.	Dataset	Bio project No.	# Tumor samples	# Normal samples
1	miRNA	PRJEB27213	12	13
2	mRNA, lncRNA	PRJNA318782	38	6
3	circRNA	GSE83521	6	6

2.2. Identification of dysregulated genes

The raw data from miRNA-seq, mRNA-seq, and circRNA-seq expression profiles were filtered and quality was assessed using FASTQC tool[8]. The preprocessed data were then used to find the differentially expressed miRNAs, mRNAs, lncRNA, and circRNA. Differentially expressed genes were identified via DeSeq2 tool[9]. Genes exhibiting a log fold change of 1.5 and an adjusted p-value of 0.05 were selected as differentially expressed.

2.3. Interaction data

The interaction data of the DE miRNAs, mRNAs, lncRNAs, and circRNAs (miRNA- mRNA, miRNA- lncRNA, and miRNA- circRNA) were obtained from the ENCORI (The Encyclopedia of RNA Interactomes) database of starBase[10]. ENCORI provides miRNA-mRNA interaction data from seven different databases such as microT[11], miRanda[12], miRmap[13], PicTar[14], PITA[15], RNA22[16] and TargetScan[17]. To enhance result reliability, interactions between miRNA and mRNA were selected if they were consistent across more than two databases. The interactions between miRNA- lncRNA, and miRNA- circRNA were chosen if they were supported by at least one CLIP-Seq data.

2.4. ceRNA network construction

For constructing the ceRNA network, we leveraged the concept that coding and non-coding genes tend to compete through miRNA Response Elements (MREs) when they share common miRNAs. This competitive interaction was explored within the ceRNA network, where two RNA pairs (mRNA-lncRNA and mRNA-circRNA) were considered potential ceRNA pairs if they could bind to common miRNAs. The following steps were undertaken:

1) **Identification of Potential ceRNA Pairs:** We selected RNA pairs (mRNA-lncRNA and mRNA-circRNA) based on their ability to bind to common miRNAs. This selection was carried out using the hypergeometric test to assess the statistical significance of shared miRNA binding.

2) **Binding Site Evaluation:** The binding sites of the paired RNAs were evaluated using the ENCORI database, which provides miRNA-mRNA binding site predictions supported by any of the two databases among microT, miRanda, miRmap, PicTar, PITA, RNA22, and TargetScan. For miRNA-lncRNA and miRNA-circRNA interactions, binding site predictions were supported by the miRanda program.

3) **ceRNA Network Construction:** Following the acquisition and validation of regulatory data, the ceRNA network was built. This network, illustrating the competitive interplay among coding and non-coding RNAs via shared miRNAs, was visualized using the Cytoscape tool[18].

This provides a comprehensive understanding of the ceRNA network, emphasizing the competitive relationships between coding and non-coding genes mediated by shared miRNAs. This approach allows for a nuanced exploration of the intricate regulatory mechanisms within the cellular environment.

2.5. Gene prioritization

Gene prioritization ranks the genes based on the likelihood of their relationship with the disease. For gene prioritization, the well-known prioritization method ToppGene[19] was used. A list of apoptosis genes[20] were used as the training set and differentially expressed mRNAs in the ceRNA network were used as candidate genes. In the training parameters section “All feature” parameter was selected. Based on the value of the “Rank” output parameter the ranking of genes was done.

2.6. Network analysis: functional and pathway enrichment

Association between the genes and disease can be unraveled using functional and pathway enrichment. Metascape tool[21] was used in this study to enrich the GO (Gene Ontology) terms and the KEGG pathways. Attributing the gene set obtained from gene prioritization, pathway and enrichment analyses were carried out with KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, and CORUM[22]. Default value "all genes" was used as background genes. Terms satisfying the conditions of $p < 0.01$, a minimum count of 3, and an enrichment factor > 1.5 were gathered and categorized into clusters.

3. Results and Discussion

3.1. Differential expression analysis

The differential expression analysis reveals 50 dysregulated miRNAs (37 upregulated and 13 downregulated), 29 differentially expressed mRNAs (18 upregulated and 11 downregulated), five differentially expressed lncRNAs (4 upregulated and 1 downregulated). 43 differentially expressed circRNA (8 upregulated and 35 downregulated) were found. Supplementary Table 1 shows the differentially expressed miRNAs, mRNAs, lncRNAs, and circRNA.

3.2. ceRNA network

To understand the interaction of miRNAs with mRNAs, lncRNAs, and circRNAs we used the ENCORI database. Our analysis revealed:

- **Direct interactions**:
  - 37 miRNA-mRNA interactions
  - 1 miRNA-lncRNA interaction
  - 17 miRNA-circRNA interactions
- **Shared binding sites**:
  - 10 miRNAs targeted all three RNA types (mRNA, lncRNA, and circRNA) through common binding sites.
- **ceRNA network**: We identified a total of 35 interactions suggesting competition between these RNAs:
  - 33 miRNA-mRNA-circRNA interactions
  - 2 miRNA-mRNA-lncRNA interactions

The regulatory data of the RNAs (miRNA-mRNA, miRNA-lncRNA, and miRNA-circRNA) were incorporated to produce the ceRNA network and visualized in the Cytoscape (Figure 2).

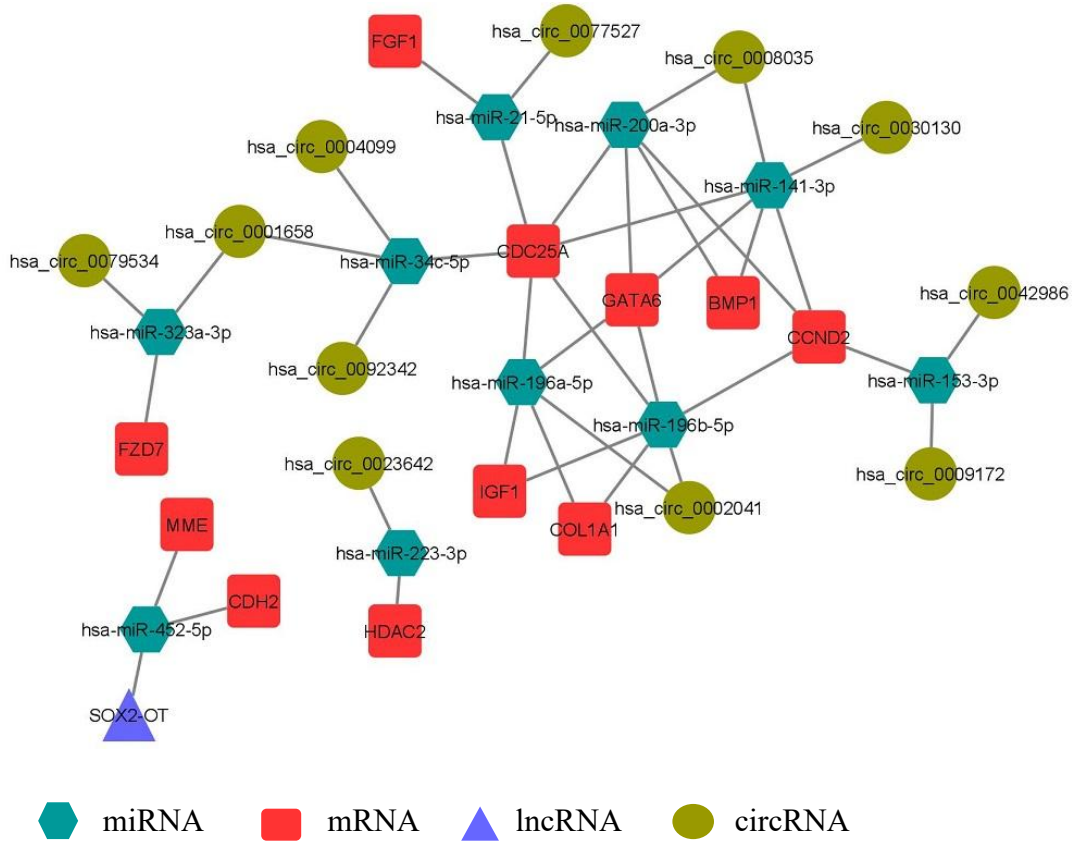

Figure 2. The reconstructed ceRNA network with mRNA, miRNA, lncRNA and circRNA interactions

3.3. Gene prioritization of DE mRNAs

The mRNAs which were differentially expressed and interact with other RNAs were chosen for gene prioritization. A list of 58 genes related to apoptosis from the study by Jourdan et al., was given as a training set[20]. The 13 differentially expressed mRNAs were given as the candidate set. The gene prioritization using ToppGene[19] gave a list with the rankings of the genes using the "All features" parameter. The following genes IGF1, MME, CCND2, CDH2, and COL1A1 which were in the top five positions were selected for further studies. The significant genes in the top five ranks is given in table 2.

Table 2. Genes in top five rank of gene prioritization

Rank	Gene	Description
1	IGF1	Insulin-like growth factor 1 (IGF-1) or Somatomedin C
2	MME	Membrane metallo-endo peptidase enzyme (CD10 or Cluster of differentiation 10)
3	CCND2	G1/S-specific cyclin-D2 protein (cyclin-dependent kinase)
4	CDH2	Cadherin-2 (CDH2) or neural cadherin (NCAD) protein
5	COL1A1	Collagen, type I, alpha 1 (alpha-1 type I collagen) protein

3.4. ceRNA network and apoptosis

Apoptosis is pivotal in carcinogenesis. This study delves into the involvement of the constructed ceRNA network in apoptosis. For that, the genes which were prioritized using gene prioritization were analyzed and their interactions with other genes in the network were studied.

The gene in the top ranking, IGF1 is the target gene of the miRNAs, miR-196a-5p, and miR-196b-5p. Biological processes like apoptosis, proliferation, metastasis, etc. are controlled in GC through IGF1 mediated signaling pathways[23; 24]. A study by Wang et al., suggests that targeting NEDD4 (Neural precursor cell-expressed developmentally downregulated 4), a protein that promotes IGF1/IGF1R signaling by affecting the PTEN-IRS1 axis, could be a promising strategy for treating GC driven by the IGF1 signaling pathway[25]. Another study by Basu and Kopchick builds on the challenges of IGF1R inhibitor failures and proposes an intriguing solution: targeting GH (Growth Hormone) and IGF1 action through GHR inhibition. This study suggests that GHR (Growth Hormone Receptor) inhibition holds promise as a strategy for overcoming cancer drug resistance due to its dual effect on GH and IGF1, its potential to improve insulin sensitivity, and its ability to target the multifaceted nature of cancer[26].

The second-highest ranked gene, MME, is the target gene of miRNA, miR-452-5p. MME/CD10 expression in stromal cells boosts the invasion and metastasis of differentiated gastric carcinoma[27]. Also, MME/CD10 increased expression is associated with increased apoptosis lymphomas[28]. But whether this gene plays a role in apoptosis in gastric cancer is not yet studied. A study by Zheng et al., highlights the regulatory role of miR-452-5p in gastric cancer. They demonstrate that circATP2B1, a circular RNA, acts as a tumor suppressor by reducing the activity of miR-452-5p, a miRNA potentially promoting cancer cell proliferation and invasion in GC[29]. There are studies which show the role of miR-452-5p in apoptosis in other cancers. A study by Li investigated the role of microRNAs (miRNAs) in breast cancer, specifically focusing on miR-452-5p. They found this miRNA to be upregulated in breast cancer tissues, suggesting it plays a role in tumor development[30]. Building on the understanding of miR-452-5p's anti-apoptotic role in colorectal cancer, this study by Lin et al. delves deeper into the complex mechanisms by which miRNAs regulate apoptosis. Their findings further emphasize the context-dependent nature of miRNA function in cancer progression[31].

The third mRNA in the list is CCND2. It is the mRNA with the highest number of miRNAs interacting with it (miR-141-3p, miR-153-3p, miR-196b-5p, and miR-200a-3p). Though the role of CCND2 in apoptosis in humans has already been published[32], no studies have implicated its role in apoptosis in gastric cancer. Another study by Demirci et al., suggests that CCND2, along with RHOA, LRP5, FZD8, and DVL2, may be a potential target for gastric cancer therapy due to their significant overexpression in tumor tissues and their potential role in early carcinogenesis[33]. The miRNA, miR-141 targets and inhibits YAP1 gene expression thereby inducing apoptosis in GC[34]. The study by Ju et al., highlights the importance of miR-141-3p as a potential tumor suppressor in gastric cancer. Their findings demonstrate that a long non-coding RNA, LINC00467, acts as an oncogene in GC by sponging miR-141-3p[35]. The miRNA, miR-153-3p has a role as a tumor suppressor in gastric cancer by directly targeting the KLF5 gene[36], and miR-153-3p accelerates cell apoptosis in gastric cancer cells[37]. miR-153-3p targets the circRNAs, hsa_circ_0042986 and hsa_circ_0009172 which were already found in the previous studies on GC[38; 39]. It has been found that hsa_circ_0009172 is downregulated in GC tissues, but the functions of both the circRNAs are unknown. miR-200a-3p is upregulated in RCC (Renal cell carcinoma) and induced apoptosis and cell cycle arrest in RCC cell lines[40]. miR-200a-3p was upregulated in the GC tissues[41]. The role of this miRNA in GC apoptosis is not yet studied. The circRNA, hsa_circ_0008035 is targeted by the miR-200a-3p and miR-141-3p and is a well-studied circRNA in GC. hsa_circ_0008035 promotes cell growth and repress apoptosis[42], and contributes to tumorigenesis[43].

The gene, CDH2, is the fourth mRNA in the list. It is the target gene of miRNA, miR-452-5p. In gastric cancer, expression of CDH2 is upregulated following demethylation[44], and miR-145 suppresses metastasis in GC by inhibiting CDH2 expression[45]. Also, CDH2 is targeted by miR-194 and suppresses apoptosis in osteosarcoma cells[46]. miR-452-5p has been predicted to be present in GC[47]. The study by Dai et al., 2023[48] sheds light on the complex role of CDH2 in gastric cancer, suggesting it might be stabilized by lncRNA SNHG14 and contribute to tumor progression. Their findings propose that lncRNA SNHG14 promotes cancer cell proliferation, invasion, and migration by stabilizing CDH2, which may activate a cellular process called EMT (Epithelial-to-Mesenchymal Transition). EMT is linked to increased migratory and invasive abilities of cancer cells.

However, the role of CDH2 in gastric cancer appears to be multifaceted, warranting further research to comprehensively elucidate its function and its interaction with lncRNA SNHG14.

The fifth gene in the ranking COL1A1 is the target gene of miRNAs, miR-196a-5p and miR-196b-5p. Knockdown of COL1A1 and upregulation of let-7i, both produced the same results i.e., anti-proliferative, reduced migration, and invasion in gastric cancer cells[49]. COL1A1 might contribute to GC metastasis and its expression might be regulated by RUNX2, also it could be a potential target for advancing novel therapeutic strategies for gastric cancer[50]. Also, by upregulation, COL1A1 plays an anti-apoptotic role in cervical cancer[51]. The study by Jin et.al. suggested that miR-196a-5p downregulation inhibits apoptosis in gastric adenocarcinoma[52]. Also, overexpression of miR-196b-5p GC cell growth and invasion in gastric cancer[53] and the miRNA plays a role in apoptosis in colorectal cancer[32].

All three genes, IGF1, CCND2, and COL1A1 are the target genes of miR-196b-5p. Also, in the whole network, it is the miRNA with five mRNAs (CCND2, CDC25A, COL1A1, GATA6, and IGF1) and one circRNA (hsa_circ_0002041) interacting with it. In colorectal cancer, miR-196b-5p regulates apoptosis by repressing FAS expression[54]. Even though studies of miR-196b-5p regulating metastasis[55], transcriptional regulation of miR-196b-5p[56], etc. in gastric cancer are available, this miRNA regulating apoptosis is not yet studied. mRNAs, MME, and CDH2 are the target genes of the miRNA, miR-452-5p. The

role of this miRNA in GC or apoptosis is not yet elucidated. In the ceRNA- network, this miRNA also targets the lncRNA, SOX2-OT. Even though the lncRNA, SOX2-OT is found in elevated levels in GC[57] the knowledge about the mechanism played by the lncRNA SOX2-OT is still limited.

3.5. Functional enrichment analysis

To identify the potential regulatory mechanism of the ceRNA network involved in gastric cancer a functional enrichment was conducted based on GO annotation using Metascape tool[21]. Functional enrichment analysis conducted using the Metascape tool offers a comprehensive approach to understanding the biological processes and pathways associated with a set of genes or proteins. Metascape integrates multiple databases and analysis tools to provide a holistic view of functional annotations, including gene ontology (GO) terms, KEGG pathways etc. In our study, 113 biological process terms and KEGG pathways were enriched in the ceRNA network (Figure 3 & 4).

The functional enrichment studies showed that CCND2, GATA6, HDAC2, and IGFI negatively regulate the apoptotic process. As stated above, both CCND2 and IGF1 are the target genes of miR-196b-5p. From this, we can presume that the miRNA, miR-196b-5p, and its target genes CCND2 and IGF1 have a vital role in gastric cancer apoptosis. We also found in the analysis that CCND2 is present in the GO terms related to cell cycle regulation which is a part of apoptosis.

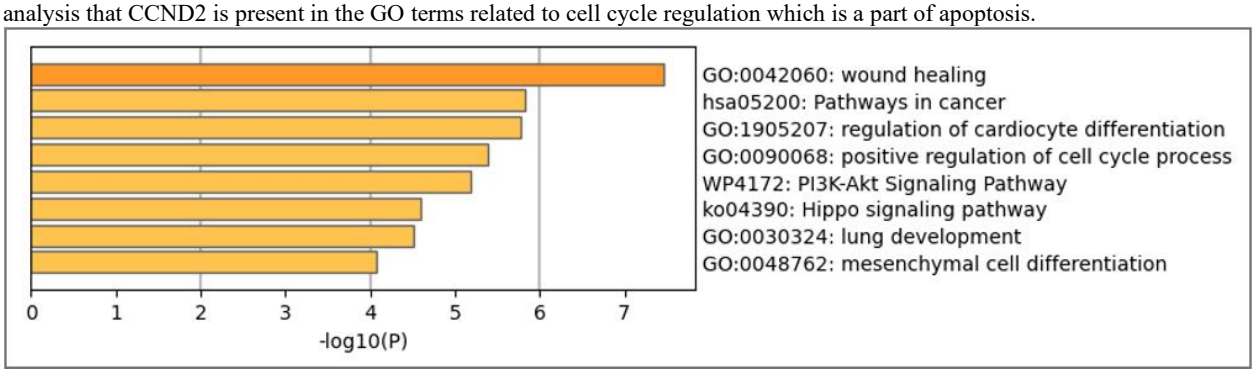

Figure 3. Functional enrichment analysis by Metascape

On analyzing the KEGG pathways obtained through the enrichment we got pathways related to apoptosis such as cell cycle[58], hippo signaling pathway[59], PI3K-Akt signaling pathway[60], mTOR signaling pathway[61], cellular senescence[62], wnt signaling pathway[63], MAPK pathway[64] and ras signaling pathway[65]. It is interesting to note that in most of the pathways, CCND2 and IGF1 were present. The role of IGF1 in GC apoptosis was substantiated earlier[23; 24]. But the role of CCND2 in GC apoptosis is not studied before. From this functional enrichment analysis, we can infer that CCND2 has a crucial role in GC apoptosis.

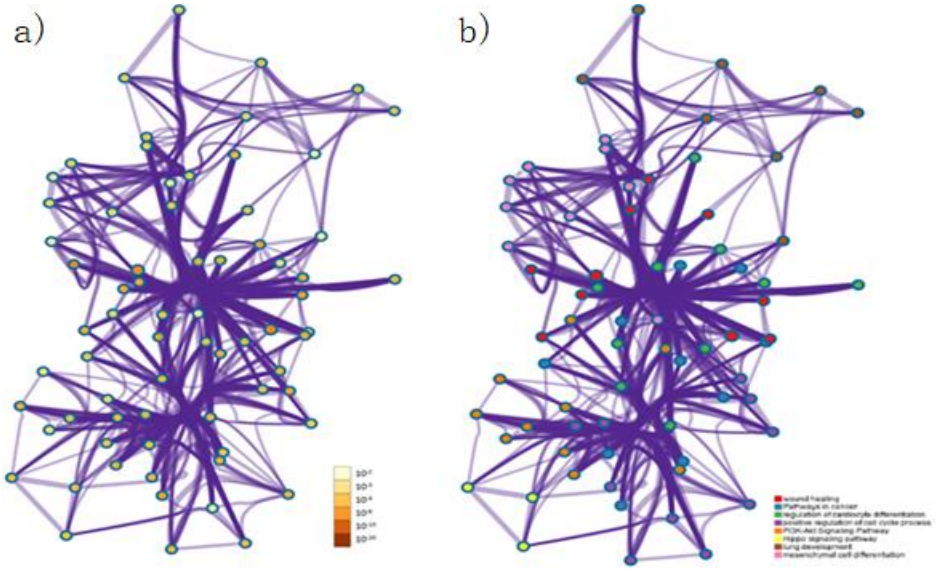

Figure 4. Enriched ontology clusters. a) Cluster colored by p-value; b) Cluster colored by cluster-ID. Every term is

depicted as a circular node, with its size corresponding to the number of input genes associated with it, while its color denotes its cluster affiliation (nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are connected by edges, with the thickness of each edge representing the similarity score.

The non-coding genes interacting with these prioritized genes in the ceRNA network were analyzed and we found some interesting results. CCND2, miR-141-3p, and hsa_circ_0008035 form a ceRNA triplet which may play an important role in regulating apoptosis (Figure 5). From literature surveys we understood that both miRNAs, miR-141-3p and circRNA, hsa_circ_0008035 have vital roles in GC apoptosis. Also, the functional enrichment of CCND2 also revealed its role in apoptosis pathways. The analysis of the ceRNA network gives us significant information to state that CCND2 may have a vital role in GC apoptosis. So, this ceRNA triplet can be a suitable diagnostic biomarker.

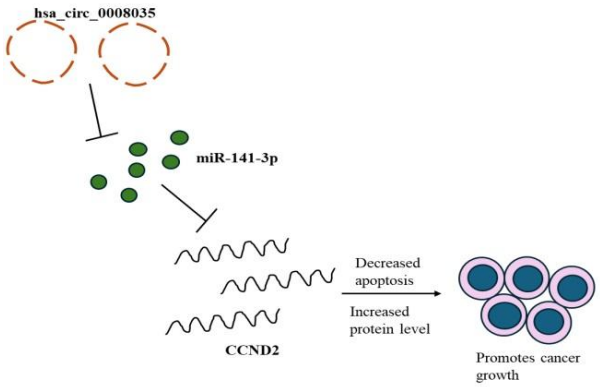

Figure 5. Schematic representation showing the possible role of ceRNA triplet, CCND2-miR-141-3p-hsa_circ_0008035 in GC.

The identification of this ceRNA triplet, CCND2-miR-141-3p-hsa_circ_0008035 presents an exciting opportunity for clinical translation. By understanding the regulatory networks involved in GC apoptosis, we can potentially develop new diagnostic biomarkers that leverage the dysregulated genes and ceRNA interactions uncovered in our study. Specifically, the CCND2-miR-141-3p-hsa_circ_0008035 ceRNA triplet holds promise as a diagnostic biomarker for GC. This novel biomarker could offer clinicians valuable insights into the apoptotic processes underlying GC progression, aiding in early detection, prognosis, and treatment stratification. Furthermore, the ceRNA-based biomarker approach provides a non-invasive and potentially cost-effective method for GC diagnosis, which could significantly impact patient care and outcomes. In summary, our research not only advances our understanding of the molecular mechanisms driving GC apoptosis but also offers promising avenues for the development of new diagnostic biomarkers with clinical relevance.

4. Conclusion

Our study provides valuable insights into the regulatory mechanisms underlying gastric cancer (GC) apoptosis through the construction and analysis of a competitive endogenous RNA (ceRNA) network. By integrating multiple omics datasets and employing bioinformatics tools, we identified dysregulated genes and elucidated their interactions within the ceRNA network. Through gene prioritization, we highlighted key genes implicated in GC apoptosis, including IGF1, MME, CCND2, CDH2, and COL1A1. Functional enrichment analysis revealed enrichment of pathways related to apoptosis, further emphasizing the importance of these genes in GC pathogenesis. Notably, our findings suggest a significant role for CCND2 in GC apoptosis, supported by its presence in both the ceRNA network and functional enrichment analyses. Additionally, the ceRNA triplet consisting of CCND2, miR-141-3p, and hsa_circ_0008035 emerges as a promising diagnostic biomarker for GC apoptosis regulation.

In addition to the insights gained from our study, future research could focus on validating the identified diagnostic biomarker, exploring additional ceRNA interactions in GC apoptosis, investigating the functional roles of other dysregulated genes within the ceRNA network, and elucidating the underlying mechanisms driving their interactions. Furthermore, prospective clinical studies are warranted to evaluate the diagnostic and prognostic significance of the CCND2-miR-141-3p-hsa_circ_0008035 ceRNA triplet in larger patient cohorts. Overall, our study contributes understanding of the molecular mechanisms governing GC apoptosis and identifies potential therapeutic targets and diagnostic biomarkers for further investigation.

Ethical Statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

Data Availability Statement

The data that support the findings of this study are openly available in NCBI database at https://www.ncbi.nlm.nih.gov/sra; in Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/; in ENCORI from https://academic.oup.com/nar/article/42/D1/D92/1063720?login=false.

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