LncRNA MAGI2-AS3 and ZEB2 Plays a Crucial Role in the Lung Squamous Cell Carcinoma:a Bioinformatics Study

Chenjing Hu

The Fifth People's Hospital of Suzhou Wujiang

JianLin Lu (✉ JianlinLu58@163.com)

The Fifth People's Hospital of Suzhou Wujiang

Zirui Wang

The Fifth People's Hospital of Suzhou Wujiang

Jianjun Lu

The Fifth People's Hospital of Suzhou Wujiang

Wenfei Qian

The Fifth People's Hospital of Suzhou Wujiang

Research

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Abstract

Purpose

Lung squamous cell carcinoma (LUSC) is a common type of non-small-cell lung cancer. Because of the limitations of targeted therapy and immunotherapy, LUSC treatments are ineffective. To better understand the underlying mechanisms of LUSC carcinogenesis, the present study aimed to identify novel factors and their signaling networks in LUSC, with a primary focus on the construction of a competing endogenous RNA (ceRNA) regulatory network.

Methods

We conducted a transcriptomic analysis of LUSC samples from The Cancer Genome Atlas database. Furthermore, we analyzed the data using bioinformatics methods.

Results

533 samples were selected for analysis, including 485 “PrimaryTumor” samples and 48 “SolidTissueNormal” samples. A total of 1853 DEgenes were identified. Bioinformatics analyses identified the most physiologically relevant and significantly dysregulated lncRNAs and mRNAs. MAGI2-AS3 and ZEB2 were found to play key roles in LUSC. Furthermore, we mapped these signaling pathway based on its role as a miRNA sponge to predict the binding of miRNA to MAGI2-AS3 and ZEB2.

Conclusion

We concluded that MAGI2-AS3/ZEB2 loop exhibited tumor-suppressor activity in LUSC by inhibiting miR-374a-5p and miR-374b-5p and modulating the downstream signal transduction through a ceRNA network.

1. Introduction

Lung cancer has the highest morbidity and mortality rates among all cancer types worldwide. According to GLOBOCAN 2018 statistics(1), lung cancer was the most frequent cause of cancer-related death in most regions globally (14/20 world regions). In males, lung cancer accounted for the highest proportion of all estimated new cases and deaths in 2018. By contrast, lung cancer is the second most common cancer among females, followed by breast cancer. In the past two decades, lung cancer became the most common cancer in China(2). The WHO categorizes lung cancer into the two following major classes based on its biology, therapy, and prognosis: non-small-cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for more than 80% of all lung cancer cases and includes two subtypes: nonsquamous (including adenocarcinoma, large-cell carcinoma, and other subtypes) and squamous cell carcinoma(3). Lung adenocarcinoma (LUAD) is the most common type of NSCLC, accounting for 40% of lung cancer cases. Lung squamous cell carcinoma (LUSC) represents 25% to 30% of lung cancer cases, and it tends to originate from cells located in the airway epithelium(4).

Lung cancer therapies include surgical resection, chemotherapy (neoadjuvant chemotherapy and adjuvant chemotherapy), immunotherapy, and targeted therapy. Significant progress in targeted therapy and immunotherapy for NSCLC has been made. Thus, the death rates for lung cancer patients have been declining(3). Although LUAD and LUSC are both subtypes of NSCLC, there are significant differences in the targeted therapies used to treat them. Targeted therapies for specific LUAD molecular subtypes are well-established, but those for LUSC are still under development. Furthermore, *EGFR* (27%) and *KRAS* (32%) are the most commonly mutated genes in LUAD but not in LUSC (<9% and 3%, respectively)(5). Therefore, targeted therapy is limited to non-LUSC histological subtypes containing actionable driver mutations. To further improve survival outcomes, a better understanding of the molecular mechanisms underlying LUSC carcinogenesis is essential for effective disease prevention or treatment.

In recent decades, the roles of long non-coding RNAs (lncRNAs) in cancer have received increasing attention. lncRNAs exhibit a variety of biological functions in cancer, including the regulation of epigenetics, DNA damage, cell cycle progression, microRNAs, and signal transduction pathways(6, 7). lncRNAs are involved in NSCLC pathogenesis by modulating fundamental cellular processes, such as proliferation, cell growth, apoptosis, migration, stem cell maintenance, and epithelial to mesenchymal transition. Furthermore, lncRNAs represent very promising biomarkers in early-stage NSCLC patients and may become particularly useful in noninvasive screening protocols. For example, lncRNAs may be used as predictive biomarkers of the patient response to chemotherapy and targeted therapies(8). Our research team focuses on the emerging roles of RNA-RNA crosstalk in tumor-related processes. It has become increasingly clear that numerous miRNA-binding sites exist on a wide variety of lncRNAs, leading to the hypothesis that lncRNAs containing miRNA-binding sites can communicate with and regulate messenger RNA (mRNAs) or microRNAs by specifically competing for shared miRNAs, thereby acting as competing endogenous RNAs (ceRNAs)(9).

To establish if lncRNAs play a role in the progression of LUSC, we constructed a global triple RNA network based on the ceRNA theory using data from The Cancer Genome Atlas (TCGA) database(10). Important hub lncRNAs were identified in the lncRNA-microRNA-mRNA network, and new subnetworks were formulated that centered on these lncRNAs. These data provide valuable insight into the molecular progression of LUSC and contribute to the identification of potential pathogenic mechanisms. Furthermore, these findings may contribute to improvements in the diagnosis and prognosis of LUSC patients and help with the identification of putative drug targets.

2. Materials And Methods

2.1 Data collection and preprocessing

The data for analysis were downloaded from the TCGA database website using the GDC tools provided by the TCGA itself (<https://portal.gdc.cancer.gov/>). The downloaded data consisted of raw non-normalized quantitative gene expression data (count form data), microRNA isoform expression data, and general clinical data, including the age, gender, survival status, and tumor stage of patients, to further

validate our results. The downloaded data were collated using the “GDCRNATools” package(11). Ethical approval was not necessary for our study because the expression profiles were downloaded from the public TCGA database, and no new experiments in patients or animals were conducted.

2.2 Screening of differentially expressed genes (DEgenes)

The “edgeR” package(12) of the R program was used to screen differentially expressed mRNAs (DEmRNAs), lncRNAs (DElncRNAs), and miRNAs (DEmiRNAs) between primary tumor and solid normal tissue samples in the count form expressing data. Values of $|\text{Log}_2\text{FC}| > 2$ and $\text{FDR} < 0.01$ were used as the criteria to identify significant differences in gene expression.

2.3 Constructing ceRNA networks

A ceRNA network was constructed from DEmRNAs, DElncRNAs, and DEmiRNAs using the “gdcCEAnalysis” function of the “GDCRNATools” package. The co-expression network of DEmRNAs, DElncRNAs, and DEmiRNAs was built using Cytoscape software (Version: 3.8.0)(13).

2.4 Functional enrichment analysis and gene set enrichment analysis (GSEA)

To further study the function of hub mRNAs, gene ontology (GO) and KEGG pathway enrichment analyses were conducted using the “enrichDAVID” function of the “clusterProfiler” package(14) in the R program. Values of $P < 0.05$ were used as the cutoff criteria. The “gseGO” and “gseKEGG” functions of the GSEA “clusterProfiler” package of the R program were used to identify the potential functions of the identified lncRNAs and mRNAs. GSEA was conducted to detect whether previously defined biological processes (BPs) were enriched in the gene rank derived from hub lncRNAs and mRNAs between the two groups. Terms with an $\text{FDR} < 0.01$ were identified for all enriched DEGs.

2.5 Survival analysis

The “gdcSurvivalAnalysis” function in the “GDCRNATools” package of the R program was used to conduct a survival analysis of the hub lncRNAs and mRNAs.

$P < 0.05$ was considered significant.

3. Results

3.1 DEmRNAs, DElncRNAs, and DEmiRNAs in LUSC samples

The RNA expression profiles-labeled TCGA-LUSC and corresponding clinical information were downloaded from the TCGA database website. According to the GDC data dictionary, samples with “01B” at the end of the sample ID are formalin-fixed samples with low sequencing quality, which were removed from our study (five samples were removed). One duplicate sample (sample ID: TCGA-21-1076-01A) was also removed. To remove significant outlier samples, correlation and cluster analyses were conducted.

The IDs of outlier samples included TCGA-98-A53C-01A, TCGA-98-A53D-01A, TCGA-77-A5FZ-01A, TCGA-60-2697-01A, TCGA-85-A513-01A, TCGA-NC-A5HJ-01A, TCGA-56-8623-01A, TCGA-37-4129-01A, TCGA-43-2581-01A, TCGA-22-1017-01A, TCGA-85-A4PA-01A, and TCGA-56-7730-01A. Finally, 533 samples were selected for analysis, including 485 “PrimaryTumor” samples and 48 “SolidTissueNormal” samples. The count form expression data were normalized using the “edgeR” package in the R program, and the “calcNormFactors” function was used to calculate the naturalization factor of the expression matrix. Genes with CPM values of <1 in more than half of the samples were removed, resulting in 15432 genes finally included in the differential expression analysis (Figure S1). A total of 1853 DEgenes were identified (Figure S2), including 1682 DEmRNAs and 130 DElncRNAs. Of the 1682 DEmRNAs, 954 genes were upregulated, and 728 were downregulated (Figure S3). Of the 214 identified DEmiRNAs, 137 miRNAs were upregulated, and 77 were downregulated (Figure S4).

3.2 Construction of the ceRNA network and identification of hub lncRNAs and mRNAs

A ceRNA network based on the identified DEmRNAs, DElncRNAs, and DEmiRNAs was constructed and visualized using Cytoscape software (Figure 1). The lncRNA-miRNA-mRNA network contained seven lncRNA nodes, 33 miRNA nodes, and 61 mRNA nodes. The correlations between lncRNA and mRNA expression levels were also analyzed, with $r > 0.6$ and $p < 0.05$ as the cutoff points to identify significant correlations. We obtained 16 pairs of three lncRNAs (MAGI2-AS3, AC079160.1, and SNHG1) and 15 mRNAs (ZEB2, STARD13, RECK, CYBRD1, TNS1, E2F7, CENPF, KIF11, KLF9, DEPDC1, CDC25A, POLQ, PERP, PITX1, and BCL11A) with significant correlations (Table S1). The results showed that MAGI2-AS3 expression had the highest correlation with ZEB2 expression ($r = 0.813$, $p = 5.17e-127$) (Figure 2A). Thus, we identified MAGI2-AS3 as the hub lncRNA in the ceRNA network. Furthermore, MAGI2-AS3 expression was significantly correlated with the expression of STARD13 ($r = 0.787$, $p = 1.19e-113$), RECK ($r = 0.73$, $p = 5.91e-90$), CYBRD1 ($r = 0.68$, $p = 6.04e-74$), TNS1 ($r = 0.673$, $p = 9.73e-72$), and KLF9 ($r = 0.641$, $p = 3e-63$). By searching the GeneCards database (<https://www.genecards.org/>)(15) for gene information, we found that the genes significantly related to MAGI2-AS3 expression were mainly associated with the occurrence, proliferation, metabolism, and migration of tumors.

The analysis of TCGA-LUSC data revealed low ZEB2 expression (LogFC = -2.18, $p = 5.02e-83$, FDR = $1.29e-81$). We then validated ZEB2 expression at the transcription level using the Oncomine database (<https://www.oncomine.org/>). The results showed that ZEB2 was expressed at low levels in a variety of tumor tissue samples (Figure S5). Furthermore, ZEB2 exhibited significantly low expression in other LUSC samples (Figure 3)(16-18).

3.3 Survival analysis

All identified DEmRNAs, DElncRNAs, and DEmiRNAs were subjected to survival analysis using $P < 0.05$ as the significance criterion. The results showed that MAGI2-AS3 (HR = 1.43, CI: 1.09–1.88, $p = 0.0102$) and ZEB2 (HR = 1.47, CI: 1.12–1.94, $p = 0.0055$) expression had a significant effect on the survival of LUSC patients (Figure 2B, C). Furthermore, low MAGI2-AS3 and ZEB2 expression levels were beneficial to the 5 year survival of LUSC patients.

3.4 Predicated function of hub mRNAs within the ceRNA network

GSEA was conducted on the identified DE mRNA set, focusing on the BP subclass. The results showed that “regulation of signal transduction,” “positive regulation of signal transduction,” “regulation of cell migration,” “regulation of locomotion,” and “regulation of cell motility” were the top five significantly enriched processes (Figure S6). ZEB2 was simultaneously enriched by multiple functions (Figure 4).

The protein-coding genes contained in the ceRNA network were used for GO and KEGG pathway enrichment analyses. The DAVID database(19, 20) was used as the basis for these analyses. The 10 most significant BP, CC, and KEGG pathway terms are shown in Figure S7. These genes are widely involved in tumor-related functions and pathways.

4. Discussion

The molecular mechanisms that contribute to LUSC remain unclear, which hinders early diagnosis, prognosis predictions, and therapeutic target selection in LUSC. Using LUSC samples collected in the TCGA database, we identified potential DE genes and DE lncRNAs. We then constructed a ceRNA network by combining these data with microRNA expression profiles. Additionally, the detailed post-transcriptional regulatory mechanisms of genes involved in the pathological process of LUSC were analyzed, and hub lncRNAs and genes were screened. The present study found that MAGI2-AS3 might be a hub lncRNA involved in LUSC progression. It formed ceRNA networks with miR-374 family members (miR-374a-5p and miR-374b-5p), which are known to participate in cell signal transduction, cell metabolism, and cell migration in LUSC. These processes play an important role in the occurrence, progression, and metastasis of LUSC.

According to the ceRNA hypothesis, lncRNAs can act as molecular sponges of miRNAs and effectively occupy their binding sites, thereby releasing target mRNAs and increasing their post-transcriptional levels. The present study showed that MAGI2-AS3 expression was significantly positively correlated with ZEB2 expression; both of which were downregulated in LUSC samples. Through the construction of the ceRNA network and corresponding analysis, it was shown that MAGI2-AS3 or ZEB2 could act as a molecular sponge of miR-374 family members (miR-374a-5p and miR-374b-5p). The downregulation of MAGI2-AS3 might increase the available binding sites of miR-374 and promote the degradation of downstream mRNAs, thereby leading to the downregulation of ZEB2, and vice versa.

Recently, it was confirmed that MAGI2-AS3 functioned as a sponge of miR-374a/b-5p *in vitro*(21). The present study verified that MAGI2-AS3 overexpression could inhibit the colony-forming ability of LUSC cells and significantly reduce cell metastasis and invasion. Bioinformatics analysis of prognostic biomarkers for human bladder cancer(22) showed that MAGI2-AS3 was downregulated in bladder cancer samples, and it was identified as the hub lncRNA in the ceRNA network. The tumor-suppressive role of MAGI2-AS3 has also been confirmed in multiple studies related to breast cancer(23-25). In a study on hepatocellular carcinoma (HCC) (26), the tumor-suppressive role of MAGI2-AS3 and targeted regulatory relationship between MAGI2-AS3 and miR-374b-5p were also confirmed. A previous study on epithelial

ovarian cancer (27) also confirmed that MAGI2-AS3 played a tumor-suppressive role, which was achieved by targeting miR-374a/b. However, these studies did not further determine the downstream targets of miR-374a/b-5p in the ceRNA network using reliable experiments. For example, He, J, et al. predicted that MAGI2-AS3 might regulate the function of the downstream CADM2 axis via miR-374a/b-5p using only an online database. The study of Yin, Z. et al. showed that MAGI2-AS3, miR-374b-5p, and SMG1 constituted the ceRNA network. This group suggested that SMG1 acted as a molecular sponge of miR-374b-5p and modulated the expression of MAGI2-AS3, thereby participating in the regulation of HCC progression. The results of Du, S. et al. demonstrated that MAGI2-AS3 regulated PTEN, the direct target of miR-374a, by acting as a molecular sponge of miR-374a and thus participating in the regulation of breast cancer cell metastasis and invasion mediated by the PTEN signaling pathway. Although PTEN is an important tumor suppressor and plays an important regulatory role in many tumors, it is inactivated in LUSC(5). Moreover, miR-374a/b-5p does not interact with or regulate PTEN in LUSC cells(21). The results of the present study showed that MAGI2-AS3 expression was significantly reduced in LUSC samples and that MAGI2-AS3 was involved in the ceRNA network together with miR-374a/b-5p, which is consistent with previous studies. However, no differential expression of SMG1, PTEN, or CADM2 was found in the LUSC samples in our study. Therefore, we consider that the MAGI2-AS3-mediated regulation of cell functions via acting as a molecular sponge of miR-374a/b-5p was achieved through other targets.

The miR-374 family has been shown to be involved in many physiological and pathological processes. This family has been well studied in digestive system tumors but not in other tumors. It is speculated that this family may regulate cell differentiation and proliferation and be associated with the occurrence of tumors(28). In the present study, microRNAs targeting ZEB2 were investigated using the TargetScan online database (http://www.targetscan.org/vert_72/), and the results revealed that the miR-374 family targeted and regulated ZEB2 (Figure S8). Based on the results of the present study, MAGI2-AS3, the miR-374 family, and ZEB2 constitute a ceRNA regulatory network and participate in the regulation of LUSC progression.

We suggest that ZEB2 plays very diverse roles in LUSC. ZEB2 expression was low in tumor tissues, suggesting that ZEB2 may play a tumor-suppressive role and that a decrease in ZEB2 expression promotes tumor progression. However, the results of survival analyses demonstrated that low ZEB2 expression was a good prognostic factor for patients with LUSC and contributed to improvements in the 5 year survival rate. This seemingly contradictory result stimulated great interest by our research team. It has been proposed that mRNAs and proteins encoded by the same sequence of a gene may have different biological effects in certain situations. ZEB2 is a practical example that supports this hypothesis(29).

The protein encoded by *ZEB2* is a member of the Zfh1 family of two-headed zinc finger/homeodomain proteins that function as DNA-binding transcriptional repressors(15, 30). It has been confirmed that ZEB2 is widely involved in various tumor-related pathological processes, including proliferation induction, differentiation arrest, apoptosis suppression, and angiogenesis(29). ZEB2 promotes tumor progression and invasion by controlling epithelial-to-mesenchymal transition (EMT) in epithelial cancers(31). ZEB2

has been identified as a molecular switch of EMT(29). In prostate cancer (PCa), miR-200c-3p was confirmed to inhibit EMT by targeting ZEB2 and thus reducing the migration and invasion of PCa cells (32). Similarly, miR-145 and ZEB2 form a two-way negative feedback loop that achieves complex functional regulation during PCa invasion and migration and plays a key regulatory role in different pathological processes in the bone metastasis of PCa(31). ZEB2 is also confirmed to be a necessary factor for tumor metastasis in melanoma cells, and the ZEB2-mediated regulation of EMT is one of the most essential mechanisms for the metastasis and ectopic growth of melanoma(33). Furthermore, in a study on triple-negative breast cancers (TNBCs), the researchers confirmed that p53 promoted the transcription of miR-30a-3p and miR-30a-5p, which directly target ZEB2, to inhibit the invasion and distant metastasis of tumor cells. This mechanism is related to the ability of ZEB2 to regulate EMT. Moreover, the inactivation of p53 could affect the biological behavior of TNBCs(34).

Therefore, ZEB2 plays an important role in regulating the invasion and migration of tumor cells. This conclusion may explain the contribution of low ZEB2 expression to the good prognosis of LUSC patients. However, compared with paracancerous normal tissues, we found that ZEB2 mRNA was downregulated in the LUSC samples. According to previous studies, the downregulation of ZEB2 expression promotes the apoptosis of tumor cells but inhibits their invasion and migration. Therefore, we believe that the downregulation of ZEB2 mRNA in LUSC samples achieved the post-transcriptional regulation of other molecules at the mRNA level. TP53 and PTEN, two important tumor-related molecules, are commonly inactivated in LUSC(5). Thus, although TP53 and PTEN are important components of the ZEB2/miR-374 regulatory axis in other tumors(24, 34), they likely do not contribute to the regulatory mechanism of the ZEB2/miR-374 axis in LUSC. The ceRNA regulatory network composed of ZEB2, MAGI2-AS3, and miR-374 family members might play key roles in extensive post-transcriptional regulation. ZEB2 might target the miR-374 family and competitively bind to miR-374, thereby modulating MAGI2-AS3 expression and further regulating the function of downstream molecules. Downregulated ZEB2 mRNA expression may increase the available competitive binding sites on miR-374, thereby enhancing its targeted regulation of other downstream factors. Moreover, MAGI2-AS3 may be directly targeted and regulated by miR-374, resulting in the subsequent downregulation of MAGI2-AS3 expression and induction of tumor progression in LUSC. However, low MAGI2-AS3 expression was also associated with a good prognosis of LUSC patients, indicating that MAGI2-AS3 may have more unknown functions in LUSC. We speculate that the ceRNA network composed of MAGI2-AS3, miR-374, and ZEB2 might have a complex two-way regulatory effect on different biological functions in various stages of tumor development. Additionally, as a transcription factor, ZEB2 might also directly target miR-374/MAGI2-AS3 or other downstream molecules, such as SMADs, to participate in cell signal transduction and more complex regulatory mechanisms.

We believe that the ceRNA regulatory network composed of MAGI2-AS3, miR-374, and ZEB2 has significant research potential. In the future, our team plans to further study the relevant molecular mechanisms involved in the occurrence and progression of LUSC to explore potential therapeutic targets and improve the efficacy of LUSC treatments.

Declarations

Ethics Approval and Consent to Participate:

Not applicable.

Consent for Publication:

Not applicable.

Availability of Data and Materials:

The data that support the findings of this study are available in TCGA database at <https://portal.gdc.cancer.gov/projects/TCGA-LUSC>.

Conflict of Interest:

The authors declare that they have no conflict of interest.

Funding Statement:

No funding was received for this study.

Author's Contribution:

Doctor CH and Professor JL made contribution to conception and design of the study. Doctor ZW made contribution to collection and analysis the TCGA data and the interpretation of the results. Doctor CH and Doctor QW worked together to draft the manuscript. Doctor JianjunL produced the figures. Professor JL revised the manuscript critically for important intellectual content. And Professor JL agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

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Figures

Figure 1

Competing endogenous RNA(ceRNA) network constructed bioinformatically for MAGI2-AS3, PVT1, H19, SNHG1 et.al. with there targets microRNA and there mRNA targets in LUSC.

Figure 2

(A) The scattergram showed correlation analysis between expression of MAGI2-AS3 and ZEB2 expression. (B) Survival analysis of MAGI2-AS3. (C) Survival analysis of ZEB2. Blue lines represent low expression and red line represent high expression.

Figure 3

Validation the expression of ZEB2 on transcription level by Oncomine database. (A) Results from Bhattacharjee, A. et.al[16]. (B) Results from Garber, M.E. et.al[17]. (C) Results from Hou, J. et.al[18].

Figure 4

(A) The cnetplot of GSEA result and (B) the heatmap of GSEA result.

Supplementary Files

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- [FigS8.miR374PositionZEB2.tif](#)
- [FigS7.GoandKEGG.eps](#)
- [FigS6.GSEABPtop5.eps](#)
- [FigS5.OncomineSummaryresult.tif](#)
- [FigS4.miRNA.eps](#)
- [FigS3.VolofDEG.eps](#)
- [FigS2.DEGheatmap.pdf](#)
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