Short Communication

New long noncoding RNA biomarkers and ceRNA networks on miR-616-3p in colorectal cancer: Bioinformatics-based study

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Background: Cancer development is aided by the role of long noncoding RNAs (lncRNAs) that act as competing endogenous RNAs (ceRNAs) absorbing microRNAs (miRNAs). We aimed to discover a novel regulatory axis in colorectal cancer (CRC) and potential biomarkers based on miR-616-3p. **Materials and Methods:** The gene expression omnibus database was mined for differentially expressed lncRNAs (DELs) and mRNAs. LncRNAs and mRNAs were predicted using the RegRNA and TargetScan databases. A combination of the ciBioPortal and Ensemble databases was used to locate the mRNAs. Cytoscape 3.7.1-built CeRNA networks. A quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to confirm the expression levels of these RNA molecules. Statistical analyses were implemented by GraphPad Prism 9. **Results:** qRT-PCR showed (Linc01282, lnc-MYADM-1:1, and Zinc Finger Protein 347 [ZNF347]) were overexpressed whereas, (salt-inducible kinases 1 [SIK1], and miR-616-3p) were down regulated. **Conclusion:** These results identify unique, unreported lncRNAs as CRC prognostic biomarkers, as well as prospective mRNAs as new treatment targets and predictive biomarkers for CRC. In addition, our study uncovered unexplored ceRNA networks that should be studied further in CRC.

Key words: Colorectal cancer, linc01282, lnc-MYADM-1:1, microRNAs-616-3p, salt-inducible kinases 1, ZNF347

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INTRODUCTION

Colorectal cancer (CRC), often known as CRC, is ranked as the fourth most malignant type of cancer.[1] microRNAs (miRNAs) are conserved single-stranded ncRNAs known to have "18 ± 24 nucleotide length." Many explorations have indicated that miRNAs influence various physiological processes such as invasion, apoptosis, migration, and cell proliferation.[2]

According to several past investigations, miR-616-3p played a substantial regulatory function in multiple cancer signaling pathways. For instance, according to Wu *et al*., the low expression of PTEN, which is one of the direct targets of miR-616-3p, was responsible for the effects of miR-616-3p on metastasis and angiogenesis. We

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hypothesize that the maintenance of phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression might inhibit the angiogenesis generated by miR-616-3p.[3] Furthermore, numerous papers have indicated an abnormal miR-616-3p expression in a wide range of malignancies, involving ovarian, breast, and gastric cancers.[3-6] However, no study has proven that miR-616-3p is connected to CRC. As a result, the mentioned miRNA has been examined as a potential candidate miRNA for future investigation in a new ceRNA network for the first time.

Long noncoding RNAs (LncRNAs), serve essential functions in regulation of apoptosis, cell cycle, differentiation, and other processes.[7] Moreover, LncRNAs can act as competing endogenous RNAs (ceRNAs), absorbing miRNAs and change their target gene expressions.[1] ceRNA has been made in different cancers such as pancreatic adenocarcinoma,[8] breast cancer,[9] and CRC.[10] As a result, ceRNA networks may be able to act as a biomarker for anticipating and treating CRC.[11]

As a result, this research aimed to investigate the new lncRNA biomarkers and also new ceRNA networks in CRC. We used several bioinformatics tools and data analysis obtained from gene expression omnibus (GEO) database. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to corroborate the findings of particular analyses. This technique revealed new potential lncRNA biomarkers in CRC.

MATERIALS AND METHODS

Identification of differentially expressed long noncoding RNAs and mRNAs

To identify the differentially expressed lncRNAs (DELs) and mRNAs, and investigate their functions, a bioinformatics tool was applied. In addition, the GEO database (http://www.ncbi.nlm.nih.gov/geo/) has the gene expression profile dataset no. GSE117830; GPL15314 platform.[12] The dataset consisted of 10 chips: involving five tumor tissues and five normal tissues. Both DEmRNAs and DELs were evaluated using a threshold of ∣ log2 (fold change [FC]) ∣ >2 and ∣ log2 (FC) ∣−2 and adjusted $P \leq 0.001$. R software version 3.5.3 (https://www.r-project.org/) with the package “Limma, Gplot, GGplot, Plyr” was used for further analysis.

Long noncoding RNAs–microRNAs interaction analysis

The RegRNA 2.0 database (regrna2.mbc.nctu.edu.tw/detection.html)[13] suggested the lncRNAs-targeted miR-616-3p (containing the lncRNAs and miRNAs folded RNA predicted structure). When anticipating target sites of miRNA, we used a system score of >160 and a minimum folding free energy of 20. A higher score indicates a stronger capacity to bond.

Identification of microRNA-targeted mRNAs interactions

The RegRNA 2.0 and TargetScan databases were applied to collect information on miRNA-mRNA interactions. The human analog (HNG) codes for mRNAs with pivotal functions in the apoptotic pathway were obtained after searching the Ensemble database (https://asia.ensembl.org/).[14] They were looked at in the cBioPortal database for insights into key mRNAs in CRC.[15]

Survival analysis

Patients with varying gene expressions were subjected to a Kaplan–Meier analysis to determine their likelihood of survival. Statistical significance was evaluated utilizing the log-rank test. According to the median profile of RNA molecules, high- and low-risk categories were considered to classify the patients.

Construction of the long noncoding RNA–microRNA–mRNA network

The lncRNA-mediated ceRNA network in CRC was designed to assist researchers in better understanding the associations between DEmRNAs, lncRNAs, and miRNAs. The gene expression profile dataset no. GSE117830 was used to acquire all of the LncRNAs and mRNAs. LncRNA-miRNA and mRNA-miRNA interactions were discovered with the help of RegRNA 2.0 and TargetScan databases. Using Cytoscape 3.7.1, we built and viewed the co-expression network.

Tissue collection

It is a case–control study. Tissues with CRC were obtained from the patients with the CRC symptoms referred to the Poursina Hakim Gastrointestinal Disease Research Center. Patients gave their written agreement and the research was approved by the Isfahan University of Medical Sciences Ethics Committee. There was 30 CRC tissues and 30 tissues nearby nontumor samples. Patients involved in the study did not receive radiation or chemotherapy before tissue collection.

Quantitative real-time polymerase chain reaction analysis

TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) was exerted to provide total RNA. cDNA was synthesized with the use of a Transcriptor First Strand cDNA Synthesis Kit (Biofact, Korea). BioFact™ 2× RT-PCR master mix was utilized for qRT-PCR analysis using a Rotor gene 6000 system. The 2Ct technique adjusted to GAPDH was used to measure relative gene expression.

Statistical analysis

Statistical analyses were implemented by GraphPad Prism9 software, which was also used to lay out the figures. A <0.05 two-tailed P value was regarded to be statistically critical.

RESULTS

MIR-616-3p was downregulated in colorectal cancer tissues and showed a positive correlation with gender

This research led us to choose miR-616-3p as a prospective miRNA for future investigation into new ceRNA networks. To study the expression of miR-616-3p, qRT-PCR showed downregulation of it in tumor tissues as compared to those in normal samples ($P \leq 0.0059$) with (log2 FC = 0.15) [Figure 1a]. According to Kaplan–Meier analysis, a lower level of miR-616-3p expression may not be related to a poor prognosis of CRC ($P \leq 0.27$) [Figure 2a]. In addition, the miR-616-3p downregulation in tumor tissues was shown to have a favorable link with the expression of female tumors ($P \leq 0.02$). There is no correlation between the other clinicopathological parameters and the miR-616-3p expression [Table 1]. The findings of the survival study performed on all of the RNA molecules investigated in this research are given in Figure 2a-e.

Abnormal expression of salt-inducible kinases 1 and ZNF347 in colorectal cancer tissues

Several bioinformatics methods were used to locate miR-616-3p-targeted mRNAs. SIK1 and ZNF347 showed the most significant degree of differential expression, and association with miR-616-3p in CRC.

The SIKs have been known to handle gene expression and have been inquired previously as a regulator of anoikis and suppression of metastasis in breast cancer. The SIK1 expression indicated a downregulation in the tumor tissues than normal tissues [Figure 1b, 95% confidence interval, 0.2479–1.932; $P \leq 0.013$] and (log2 FC = 0.62). There was no association between the clinicopathological features with the declined expression of SIK1.

ZNF347, is another target, located on chromosome 19. RT-PCR analysis demonstrated that ZNF347 expression was substantially increased in CRC tissues as compared to healthy adjacent tissues ($P \leq 0.014$) and with (log2 FC = 3.46) [Figure 1c]. In addition, other clinical parameters indicated no relationship with ZNF347 overexpression in CRC.

Linc01282 and lnc-MYADM-1:1 were upregulated in colorectal cancer tissues

Dataset no. GSE117830 [Table 2] was utilized to identify DELs in tumor tissues than normal samples. The RegRNA

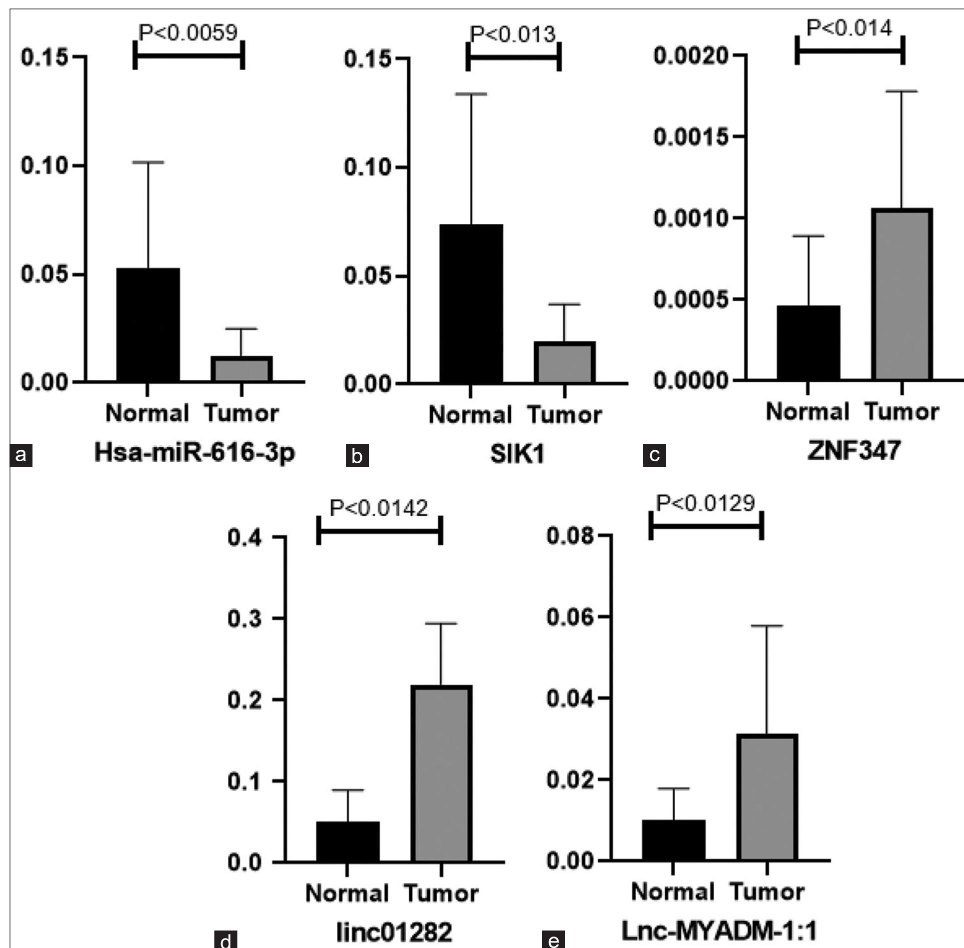

Figure 1: (a-e) Aberrant expression levels of hsa-miR-616-3p, salt-inducible kinases 1, ZNF347, Linc01282, and lnc-MYADM-1:1 in tumor tissues compared with normal samples assessed by quantitative polymerase chain reaction, respectively. SIK 1 = Salt-inducible kinases 1

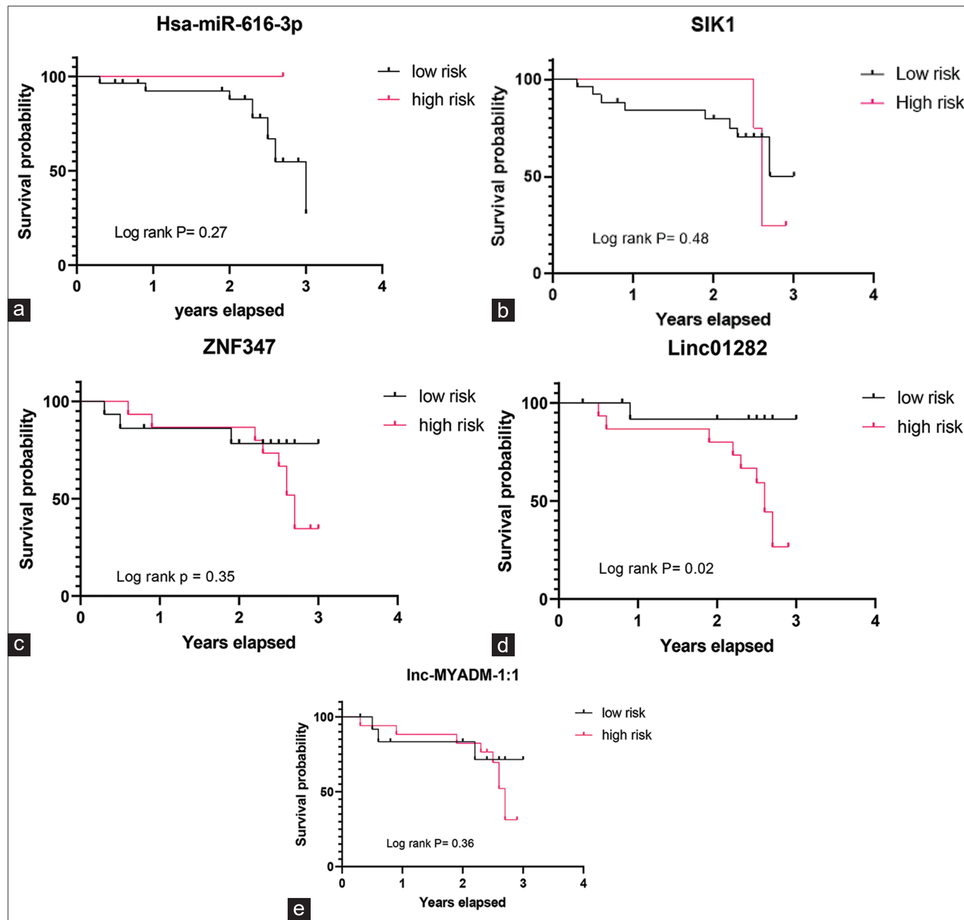

Figure 2: (a-e) Kaplan–Meier survival curves for colorectal cancer patients. Patients were divided into high- and low-risk groups in accordance with the median expression level of RNA molecules. *P* values were estimated using the log-rank test. SIK1 = Salt-inducible kinases 1

Table 1: Correlation between expression of Has-miR-616-3p in colorectal cancer with clinicopathologic feature

Clinicopathological parameters	Number of cases	Has-miR-616-3p		*P*
		Low	High	
Total	30	21	9	0.0059
Age (years)				
≤60	9	5	4	0.3664
>60	21	117	4	
Gender				
Female	15	12	3	0.0209
Male	15	9	3	
Location				
Colon	20	15	5	0.82
Rectum	10	6	4	
Differentiation				
Well/moderately	24	18	6	0.69
Poorly	6	6	0	
TNM stage				
I–II	15	10	5	0.24
III–IV	15	11	4	

TNM=Tumor, node, metastasis

2.0 database was applied to evaluate the interactions between lncRNAs and miR-616-3p. Linc01282 and lnc-MYADM-1:1 were found with the least folding-free energy to perform modulatory functions on the mentioned miRNA.

To portend the folded RNA configuration of the lncRNAs and miRNAs RegRNA2.0 software was implemented. The most commonly forecasted secondary structure is hairpin loops considered to promote the binding of lncRNAs to miRNAs. Consequently, the binding of Linc01282 and lnc-MYADM-1:1 to has-miR-616-3p was demonstrated in Figure 3.

Linc01282 and lnc-MYADM-1:1 by-21.8 and-13.5 minimum folding-free energy were the most DELs in this research (log2FC of >2, <−2 and $P \leq 0.001$). qRT-PCR demonstrated that Linc01282 was substantially overexpressed in the CRC tissues than normal tissues ($P \leq 0.0142$) and (log2 FC = 5.18) [Figure 1d] and its increased expression was far related to a poor prognosis of CRC [Figure 2d]. Plus, the upregulation of Linc01282 illustrated a positive relation to male tumor expression ($P \leq 0.04$).

RT-PCR analysis showed that lnc-MYADM-1:1 significantly overexpressed in the CRC tissues compared

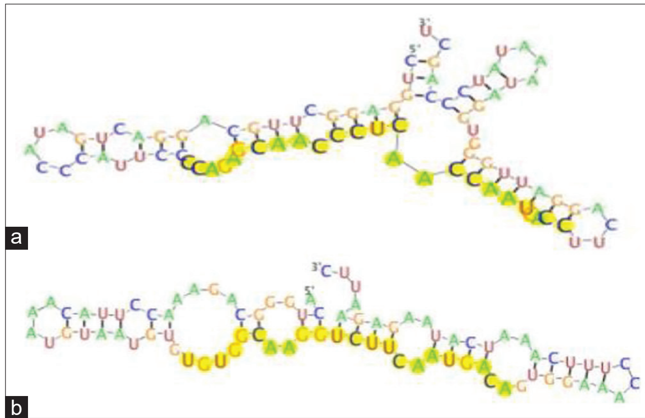

Figure 3: RNA fold reliability data of likely long noncoding RNAs-microRNAs pairs. (a) Linc01282–hsa-miR-616-3p. (b) Lnc-MYADM-1:1-hsa-miR-616-3p

to the adjacent nontumoral tissues ($P \leq 0.0129$) and (log2 FC = 2.09) [Figure 1e] and also has a positive relationship with the position of tumor ($P \leq 0.0284$).

Constructing the long noncoding RNA–microRNA–mRNA network

ceRNA network was finally created by applying related databases to validate the correlation among lncRNA, miRNA, and mRNA markers. A total of 56 common mRNAs were observed interacting with LncRNAs and miR-616-3p [Figure 4].

Table 2: miR-616-3p-targeted long noncoding RNAs classified by expression status

Has-miR-616-3p
Upregulate
AC094104.1, AL354861.2, CARMN, LINC00910, LINC01641, lnc-CCDC39-1:5, lnc-CDC27-2:3, lnc-CIAO1-6:1, lnc-EEF1AKMT1-3:6, lnc-LILRB4-1:2, lnc-MAN1B1-1:1, lnc-MTRNR2L11-2:2, lnc-NABP2-1:1, lnc-PALLD-4:1, lnc-PILRB-1:13, lnc-RPS24-3:24, lnc-SERPIND1-1:62, lnc-SLC24A2-2:1, lnc-SMG6-6:1, lnc-SPTBN2-6:1, lnc-SYN2-6:1, lnc-TAAR6-1:1, lnc-TRIM39-2:1, lnc-VMO1-1:1, lnc-WDR12-3:1, lnc-WFIKKN2-3:1, lnc-ZNF479-13:1, lnc-ZNF716-12:1, lnc-ZNF836-1:8, AC002463.1, AC006004.1, AC008278.1, AC016933.1, AC073316.2, AC103563.2, AL139246.4, AP000356.2, APTR, FAM138B, FAM138E, LINC00305, LINC00350, LINC00700, LINC00842, LINC01205, LINC01271, LINC01282, PRDM16-DT, SNHG14, HSALNT0023219, HSALNT0028808, HSALNT0050876, lnc-AIG1-8:14, lnc-ANKRD20A3-4:4, lnc-BDH1-5:7, lnc-CDH10-6:5, lnc-DDX39A-1:1, lnc-DUPD1-3:1, lnc-EXOSC1-1:1, lnc-FKBP5-1:2, lnc-FNBP1L-2:3, lnc-FRG2-9:1, lnc-FRMD1-3:1, lnc-FTCDNL1-1:1, lnc-GATA3-6:2, lnc-GRM3-2:3, lnc-HIVEP2-1:1, lnc-MARCKS-18:1, lnc-METTL16-3:2, lnc-MYADM-1:1, lnc-PALMD-1:1, lnc-PCDH1-2:1, lnc-PDLIM1-1:5, lnc-PRSS3-2:10, lnc-RAB19-3:1, lnc-RAPSN-2:1, lnc-SFTPA2-1:5, lnc-SLC47A2-1:4, lnc-ST8SIA4-7:1, lnc-STS-10:1, lnc-TGFA-1:1, lnc-TMEM121-6:1, lnc-TSSK3-2:2, lnc-TXNDC17-1:2, lnc-USP6NL-3:1, lnc-VWDE-2:1, lnc-ZNF726-5:1, NONGGOT009232.1, NONHSAT098681.2, NONHSAT123805.2, NONHSAT148170.2, NONHSAT159169.1, NONHSAT165589.1, NONHSAT196116.1, NONHSAT200082.1, NONHSAT214069.1, NONRATT014570.2, PLCE1-AS2:15
Downregulate
lnc-C21orf59-1:1, lnc-PTPN2-4:1, lnc-TRPC4-2:1, lnc-HLA-DRB1-5:1, HSALNT0031418, lnc-LHFPL4-2:1, LY6E-DT, lnc-GJA9-1:2, lnc-MNX1-23:3, lnc-BPY2B-3:1, lnc-ATP12A-1:1

DISCUSSION

The current research uncovered two previously unreported new lncRNAs, which is known to be involved in CRC. Linc01282 is the first one located on chromosome X, has a length of 880 base pairs, and has six exons. In both the qRT-PCR and the bioinformatics analysis, it was demonstrated that Linc01282 was considerably upregulated in the tumor samples over in the nontumor neighboring samples. In addition, overexpression of Linc01282 was more common in men, which suggests extended Linc01282 expression might serve as a biomarker for predicting the outcome of the disease in males and shows poor prognosis in CRC patients. As a result, Linc01282 is worthy of consideration as a possible new option for CRC treatment and may be a novel diagnostic biomarker.

The second lncRNA, called Lnc-MYADM-1:1 situated on chromosome 19 and has a total length of 555 base pairs, divided into three exons. Analysis using qRT-PCR revealed that lnc-MYADM-1:1 was significantly elevated in tumor samples and its overexpression has a positive link with the position of the tumor. It presented that this phenomenon was more widespread in colon than in the rectal region. Therefore, an elevated level of lnc-MYADM-1:1 might be used as a diagnostic biomarker for CRC and the position of the tumor. Similarly, Yu *et al*.[16] hinted that u50535 is a novel lncRNA associated with accelerated metastasis, tumor growth, and frequent increases in CRC tissues. Through its regulation of the CCL20 signaling pathway, u50535 contributes to the progression of CRC carcinogenesis. As a result, two new predictive biomarkers for CRC were uncovered through this investigation. However, further research is required to investigate the precise practical activities of related lncRNAs in CRC.

miR-616-3p expression in CRC has never previously been examined, and the related miRNA expression in CRC samples considerably declined regarding the qRT-PCR examination in this study. This finding suggests that miR-616-3p may be considered a prognostic indicator in CRC. Moreover, downregulation of miR-616-3p was seen to be much more common in females than in males.

ZNF347 and SIK1 are two mRNAs with high affinity to miR-616-3p and involvement in the apoptotic pathway. ZNF347, situated on chromosome 19, was the previously unreported mRNA we found and published in this study for the first time. According to our findings, the expression of ZNF347 was significantly increased in CRC cases than in healthy tissues. Although Kaplan–Meier analysis did not

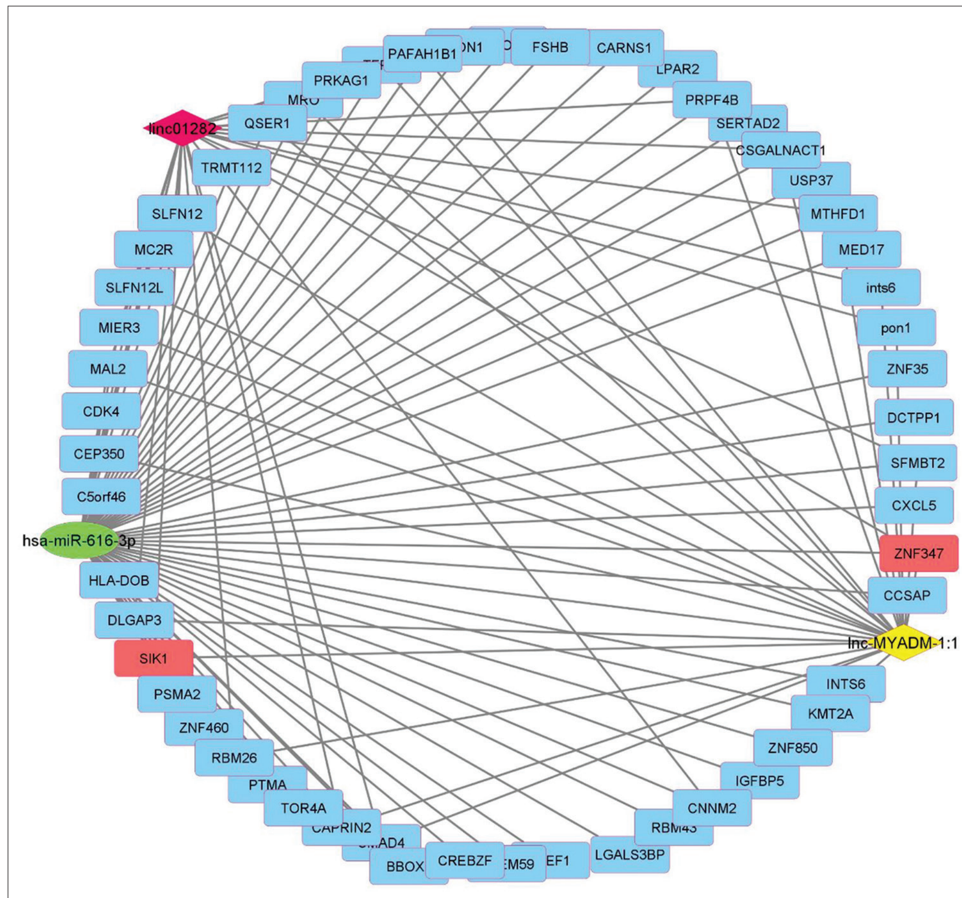

Figure 4: The long noncoding RNAs (lncRNA)–microRNAs–mRNA ceRNA network. Diamond nods indicate lncRNAs, Ellipse nodes indicate miR-616-3p, and rectangle nods indicate mRNAs

show a significant correlation between ZNF347 and poor prognosis, it was upregulated in CRC, and its overexpression may be considered a prognostic signal in CRC.

SIK1, an additional miR-616-3p-targeted mRNA, demonstrated considerable downregulation in CRC tissues over normal samples. In a study with similar findings, Huang *et al*. indicated that an elevated form of miR-17 may advance the development of CRC and act as an anti-tumor miRNA by interacting with SIK1.[17] As a result, the reduced expression of SIK1 may have a potential prognostic role in CRC.

In this investigation, bioinformatics tools have shown that Linc01282 and lnc-MYADM-1:1-targeted miR-616-3p directly. The related miRNA is located in the stem-loop structure of each of these genes. We propose this hypothesis that in lnc-MYADM-1:1/miR-616-3p/ZNF347 axis, overexpression of lnc-MYADM-1:1 suppresses the miR-616-3p expression, increasing the expression of ZNF347.

Overall, we used qRT-PCR to evaluate our bioinformatic analyses and the putative correlations between the many genes implicated in this study and the clinical features and expression patterns of those genes. These results were completely compatible with each other, showing that our approach was reliable in demonstrating possible biomarkers and possible ceRNA networks in CRC. Even though these findings might benefit from a larger sample size, additional molecular tests, and validation using clinicopathological data, we are convinced that our findings provide unique prognostic and therapeutic targets to handle CRC patients.

CONCLUSION

In summary, by applying bioinformatics analysis, we elucidated the correlation between miR-616-3p and related RNA molecules that have strong interactions with each other, leading to the formation of different ceRNA networks. These ceRNA networks may act critical modulatory functions in various signaling pathways. qRT-PCR was used to confirm dysregulated genes in CRC. Overall, our results present that among all the genes investigated in this study, linc01282 may be considered a diagnostic biomarker and may be involved in the regulation and progression of CRC. However, further experimental investigations are needed to completely elucidate the effects of mentioned RNA molecules on the development of CRC.

Ethics approval and consent to participate

The protocols were carried out based on the Ethical Committee and Research Deputy of the Isfahan University of Medical Sciences, Iran for the Care and Use of Laboratory Medical, and were validated by the Institutional Human Care and Use Committee guidelines of the Isfahan University of Medical Sciences, Iran.

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Simin Hemati (M.D) Associate Professor of Radiooncology.

Conflicts of interest

There are no conflicts of interest.

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