Clinical Significance of Long Non-Coding *RNA* *HCG18*, *OIP5-AS1*, *FGD5-AS1* and *LINC00657* Expression in Human Gastric Cancer

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Abstract

Background

Gastric cancer (GC) is a major health problem and the third cause of cancer-induced deaths globally. Long non-coding RNAs (lncRNAs) are new cellular regulators in cancers whose contribution to GC carcinogenesis is still unknown to a large extent. This study aimed to investigate expression levels of bioinformatically ranked lncRNAs in GC tissues.

Methods

Using a bioinformatic approach, lncRNAs supposed to be involved in GC tumorigenesis were prioritized. Further, the top-ranked lncRNAs including *HCG18*, *OIP5-AS1*, *FGD5-AS1*, and *LINC00657* were selected for experimental validation. Moreover, qPCR was used to validate bioinformatics findings in 35 GC and 35 paired adjacent normal gastric tissue samples (ANGTs). Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) were generated to assess the diagnostic values of the lncRNAs.

Results

lncRNA-*HCG18* showed to be the top-ranked lncRNA involved in GC, as identified by bioinformatics analysis. Additionally, *HCG18*, *OIP5-AS1*, *FGD5-AS1*, and *LINC00657* expression levels significantly increased in GC tissue samples compared to ANGTs. The area under the curve (AUC) of *HCG18*, *OIP5-AS1*, *FGD5-AS1*, and *LINC00657* were 0.80, 0.74, 0.73, and 0.71, respectively. Considering the findings, it was concluded that *HCG18*, *OIP5-AS1*, *FGD5-AS1*, and *LINC00657* lncRNAs may contribute to GC tumorigenesis.

Conclusion

This study also found that the bioinformatic approach could be applied as an efficient approach to finding candidate lncRNAs relevant to GC progression.

Introduction

Gastric cancer (GC) is regarded as the third oncological cause of death worldwide. Although great efforts have been made to reduce the GC progression, the mortality rate of GC is still high [1]. Nowadays, GC is a major clinical challenge because this is usually asymptomatic in early-stage and diagnosed at late-stage characterized by a poor prognosis as well as limited treatment options [2]. Accordingly, detailed mechanisms of GC tumorigenesis are still unclear. Hereupon, comprehending how these mechanisms work might contribute to the formation of novel diagnostic and prognostic biomarkers [3]. Long noncoding RNAs (lncRNAs) are greater than 200 nucleotides in length and possess no protein-coding

potential. They have been identified as novel gene regularizers acting via multiplex methods, including transcriptional modulation, microRNA competition, histone modification, and chromatin remodeling [4]. The present documents suggested that lncRNAs are involved in the control of diverse biological procedures, including metabolism, differentiation, cellular senescence, apoptosis, survival, and tumorigenesis [5, 6]. MicroRNAs (miRNAs) are also the regulators of gene expression which are a family of endogenous non-coding short RNA molecules [7]. It is acknowledged that numerous lncRNAs serve as competing endogenous RNAs (ceRNAs) and sponging specified microRNAs [4]. This kind of lncRNAs competes with miRNAs for binding to the target and lessening miRNA-mediated inhibitory effects on gene expression. Therefore, the lncRNAs create a regulatory axis to control the gene expression [4]. Figure 1 illustrates the roles of *HCG18*, *OIP5-AS1*, *FGD5-AS1*, and *LINC00657* in the tumorigenesis process to show how these lncRNAs influence different steps in this process [8–12]. In literature related to GC, manifold lncRNAs are known to be aberrantly expressed, and in their abnormal expression, they remarkably act on the malignant characteristics of GC by altering miRNA expression [8, 13–15]. In considering such findings, it is likely to assume that lncRNAs can regulate tumor progression; hence, the investigation of specific roles of GC-related lncRNAs may propose an efficient target for anticancer therapy. Therefore, this study strived to (I) prioritize lncRNAs involved in GC by bioinformatic resources and (II) investigate the expression levels of candidate lncRNAs in clinical samples.

Materials And Methods

In-silico analysis

A general schema of the bioinformatic pipeline is depicted in Figure 2 [16]. lncRNAs can target miRNAs and then indirectly affect mRNAs, so it is possible that the dysregulation of lncRNAs that have further target miRNAs indirectly affects more mRNAs and more cellular/molecular pathways than lncRNAs with fewer miRNAs targets. Therefore, a bioinformatic method was designed to centralize the study on lncRNAs with more target genes as fundamental regulators in GC.

At first, the expression profiles of GC differentially expressed (DE) lncRNAs and miRNAs were downloaded from the Cancer RNA-Seq Nexus (<http://syslab4.nchu.edu.tw>) [17] and dbDEMC 2.0 online web-servers (<https://www.picb.ac.cn/dbDEMC/>) [18], respectively. Indeed, the expression profiles of DE-lncRNAs in the Cancer Genome Atlas-Stomach Adenocarcinoma (TCGA–STAD) dataset downloaded from the Cancer RNA-Seq Nexus web-server were explored [17]. This dataset was generated through RNA-seq experiments and included 431 transcriptome profiles comprising 35 noncancerous gastric tissues and 396 gastric tumor samples. Using a tool implemented in the Cancer RNA-Seq Nexus web-server [17], the TCGA-STAD dataset was analyzed to identify DE genes in GC (GC–DE lncRNAs).

At the next step, two aforementioned datasets were cross-referenced with a bulk dataset including comprehensive interactions of lncRNA-miRNA obtained from starBase 2.0 (<http://starbase.sysu.edu.cn>) [19] and “lncRNA-miRNA implicated in GC” dataset created. Finally, lncRNAs were listed in a created

dataset, ranked according to the numbers of target miRNAs, and top-ranked outputs were considered as “lncRNAs-implicated in GC.”

Patient samples

The expression levels of lncRNAs were determined in 70 gastric tissue samples including gastric tumors (n=35, 20 diffuse subtypes and 15 intestinal subtypes) and paired adjacent normal gastric tissues (ANGTs) (n=35, histologically normal samples with at least three cm distance from tumor).

Tissues were attained during surgery prior to receiving chemo/radiotherapy. Then, samples were immediately frozen and transferred to the laboratory for later assessments. Samples were stored at -70°C until expression analysis. All specimens were examined by a pathologist to prove the pathologic signs. The study protocol was approved by the Ethical Committee (IR.BMSU.REC.1398.281) in Baqiatallah Medical University. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

RNA extraction and quantification of lncRNAs

Total RNAs from tissue samples were exploited using the AccuZol™ total RNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. cDNA from RNA was synthesized by the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan). The qPCRs were conducted with SYBR Green Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) and according to the standard program on Rotor-Gene Q instrument (QIAGEN, Germany). The GAPDH gene was used for the normalization of expression data. Moreover, a non-template control reaction was used for every primer set to certify reaction specificity. The primer sequences have been presented in Table 1.

Statistical analysis

The quantitative variables were presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Graph-pad Prism 7 (RRID: SCR_002798). The lncRNA expression data were examined for normality with the Shapiro–Wilk test. The paired t-test was used to compare the lncRNA expression between two defined groups. ROC curve analysis (ROC) was then performed to measure the diagnostic value of lncRNAs, using MedCalc Version 18.5 (RRID: SCR_015044). A P-value $\leq$ 0.05 was considered to be statistically significant.

Results

In-silico results

The lncRNA-miRNA pairs in which target miRNAs showed differential expression in the GC, were identified. More than 1700 discrete lncRNA-target miRNA pairs were discovered. Thereafter, lncRNAs were prioritized based on the number of their target miRNAs. Table 2 shows the top 10 ranked lncRNAs found to be involved in GC (Supplementary File S1 for a complete list).

3.2. Transcript Quantities of lncRNAs

For experimental assays, the study was limited to investigating those ranked-lncRNAs that have not been vastly explored regarding GC tumorigenesis. Therefore, MALAT1, KCNQ10T1, and lncRNA NEAT1 which had been investigated broadly had to be excluded. Taking into consideration all the above-mentioned limitations, the present study assessed the expression levels of HCG18, OIP5-AS1, FGD5-AS1, and LINC00657. The results revealed that expression levels of HCG18, OIP5-AS1, FGD5-AS1, and LINC00657 are significantly upregulated in the GC group compared with the ANGTs group as determined by paired t-test (Table 3, Figure 3).

Correlation between the four-lncRNA signature and clinicopathological features of gastric cancer patients

Patients' demographic data are presented in Table 4. The correlation was examined between clinicopathological variables and expression levels of HCG18, OIP5-AS1, FGD5-AS1, and LINC00657 in the GC group. Table 5 shows the summary of the results. Accordingly, no meaningful difference was observed between HCG18, OIP5-AS1, FGD5-AS1, and LINC00657 expression levels in the GC group concerning patient's age and sex ($P > 0.05$). The statistical analyses also revealed no significant correlation of studied lncRNAs with *H. pylori* infection except for OIP5-AS1, which was near-threshold p-Value and marginally significant ($P = 0.06$).

In the case of HCG18, results showed a significant upregulation of this gene expression in advanced-grade samples ($P=0.04$), but its upregulated expression had no notable correlation with tumor size, lymph node metastasis (LNM), or *H. pylori* infection ($P > 0.05$). The results also revealed that OIP5-AS1 is obviously upregulated in LNM positive specimens ($P=0.01$). Moreover, it was detected that the OIP5-AS1 expression level is upregulated in tumor samples with higher developed grades (I +II vs. III+IV, $P = 0.01$) as well as tumors with a larger size ($P=0.007$). Regarding FGD5-AS1, although remarkable upregulation was found in both LNM positive samples ($P = 0.008$) and tumor tissues greater than 5 cm in size ($P = 0.01$), there was no significant difference regarding cancer grade.

Moreover, results showed that LINC00657 significantly overexpresses in tissue samples with more advanced clinical-grade ($P = 0.01$) and those with positive LNM ($P = 0.003$); however, no significant difference regarding tumor size was observed.

Evaluation of Diagnostic Power of lncRNA

To determine if the expression of four investigated lncRNAs has the potential to discriminate between healthy and unhealthy subjects, a receiver operating characteristic (ROC) curve was illustrated. Analyses of ROC curve outputs demonstrated that HCG18 has the greatest diagnostic power (area under the curve (AUC) = 0.80, P -value <0.0001). OIP5-AS1, LINC00657, and FGD5-AS1 are ranked thereafter (AUC values= 0.74, 0.73 and 0.71; P values= <0.0001 , 0.0004 and 0.0005, respectively) (Figure 4). The detailed evaluation of ROC curve parameters is presented in Table 6.

Discussion

In this research, an integrative bioinformatics approach was applied to choose lncRNAs involved in GC. It is commonly agreed that lncRNAs can regulate tumor development by sequestering miRNAs (microRNA), releasing their downstream target mRNAs, and prompting mRNAs to exert functions [14, 15]. These findings imply that aberrantly expressed lncRNAs exert an important act on the malignant trait of cancer by changing miRNA (microRNA) expression. Taking this into consideration, lncRNAs were arranged according to the number of their target miRNAs. Then, in order to focus the study on gastric cancer-related non-coding RNAs precisely, at first, candidate lncRNAs were limited to those which are GC differentially expressed based on the Cancer Genome Atlas-Stomach Adenocarcinoma (TCGA-STAD) data. Secondly, the targeted miRNAs were cross-referenced with GC differentially expressed miRNAs in the dbDEMC 2.0 database. As seen in Table 2, HCG18 has been recognized as the top-level lncRNA involved in GC, with 69 different target miRNAs. To further examine the bioinformatics findings, the expression levels of selected lncRNAs, including HCG18, OIP5-AS1, FGD5-AS1, and LINC00657 were analyzed. The obtained results revealed that these four lncRNAs are obviously upregulated in GC specimens. In some recent investigations, various dysregulated oncogenic lncRNAs have been also reported in human cancers [8, 20, 21].

HCG18 is characterized as a 2430-bp lncRNA on chromosome 6p22.1. Recently, it has been known as a novel cancer-related lncRNA which repeatedly upregulates and acts chiefly as an oncogene in different cancers including colorectal cancer and nasopharyngeal carcinoma [8, 13]. For example, Li et al. verified that HCG18 is involved in regulating the MTDH gene (a potential oncogene in colorectal cancer) by sponging miR-1271 [13]. To date, the implication of HCG18 in GC advancement is still unclear. Results revealed that HCG18 significantly overexpresses in GC samples (ANGT vs. GC, $P = < 0.0001$). Although these results demonstrated a meaningful increase of HCG18 expression in samples with advanced tumor grade ($P = 0.04$), no significant correlation was detected between HCG18 expression level and LNM positivity or size of the tumor. Likewise, a recent study has revealed that HCG18 is overexpressed in GC tissues, which supports this work results and indicates that HCG18 acts as a tumor promoter in GC [22]. The involvement of HCG18 in determining metastatic features and tumor invasion has already been reported [22]. The above-mentioned contradictions could be described partly by both biological pathway redundancies in tumor biology as well as tumor heterogeneity. Some recent investigations have further shown that lncRNA HCG18 promotes cancers by modulating PI3K/Akt, Wnt/ β -catenin, and Hedgehog signaling pathways [8, 22].

OIP5-AS1 is a novel and promising lncRNA that is located on chromosome 15q15.1. According to the results, OIP5-AS1 is upregulated in GC samples (GC vs. ANGTS, $P = 0.0015$), especially in higher grades, larger tumors, and LNM positive samples which highlights its function in tumor progression. It has been also proposed that over-expression of OIP5-AS1 considerably promotes the tumorigenicity and proliferation of GC cells; however, its clinical importance in GC has remained unknown. Results of the current work agree with those of the previous studies by Bai et al. and Tao et al. They declared that OIP5-AS1 is overexpressed in GC tissues and cell lines [21, 23]. In a recent investigation, the analysis of cell

cycle on osteosarcoma revealed that silencing of OIP5-AS1 could give rise to the apoptosis of tumor cells, prevention of the cell cycle at G0/G1 phase, and consequently inhibit tumor growth [9]. Accordingly, various studies suggested the oncogenic role of OIP5-AS1 in multiple human cancers including hepatoblastoma and cervical cancer [24, 25].

In this study, it was demonstrated that the expression level of FGD5-AS1 is remarkably upper in GC specimens than ANGTS. Meantime, the upregulated FGD5-AS1 was meaningfully associated with larger tumor size and LNM positive specimens. Dysregulation of FGD5-AS1 has been also reported in various types of malignancies [10, 20, 26]. Additionally, it has been discovered that FGD5-AS1 is aberrantly upregulated in invasive colorectal cancer and FGD5-AS1 knockdown significantly weakens cell invasion and migration potential. Moreover, certain evidence has suggested that significant upregulation of FGD5-AS1 could promote colorectal cancer invasion and metastasis through upregulating CDCA7 (a cancer cell metastasis mediator) [10].

In addition, the LINC00657 dysregulated expression has already been reported in various tumors [12, 27, 28]. However, the direction of dysregulation has remained conflicting. An investigation on colon cancer indicated that LINC00657 could promote the expression of CAPN7 gene and thus inhibit cell invasion [28]. In contrast, pancreatic ductal adenocarcinoma is characterized by LINC00657 overexpression in tissue specimens [12]. Atypical expression of this lncRNA begins cell transformation toward malignancy and tumorigenesis by activating RhoA/ROCK1 pathway [29]. In the current study, it was found that expression of LINC00657 is considerably upregulated in GC samples compared with normal ones. In addition, LNM positive samples and also higher-grade tumors increased LINC00657 expression proposing the correlation between this lncRNA and the risk of developing tumors.

Conclusions

In this research, we showed that applying a bioinformatic approach could beneficially prioritize lncRNAs involved in GC. In addition, the up-regulation of HCG18, OIP5-AS1, FGD5-AS1, and LINC00657 was found in tumor tissues compared with ANGTS. Furthermore, the ROC curve outputs revealed that four candidate genes could differentiate gastric samples from normal gastric tissue with high accuracy. However, it should be pointed out that the results of this study were based on a relatively limited number of subjects and clinicopathological variables; thus, confirmation is required by analyzing larger cohorts.

Declarations

Author Contributions: A.Z., HZ, analyzed the data; A.Z., H.Z., data curation; A.Z., H.Z., wrote the manuscript; H.B., E.R. performed the experiment; M.H., H.B., H.Z., writing—review and editing M.H., H.B., H.Z, supervision, M.H. project administration.

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Ethics approval and consent to participate

The study was approved by Baqiatallah University of Medical Sciences Ethics Committee (IR.BMSU.REC.1398.281) and performed in compliance with Helsinki declaration.

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Competing interests

The authors declare that they have no financial competing interests

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