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Type

Research paper

Keywords

hepatocellular carcinoma, sex hormones, bioinformatics analysis, long noncoding RNA

Abstract

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Material and methods
In our study, we performed lncRNA sequencing to analyze the transcriptome profiles of the human HCC-derived HepG2.2.15 cell line treated with estradiol (E2), testosterone, or untreated. Datasets were analyzed by Coding-Non-Coding Index (CNCI), Coding Potential Calculator (CPC), and other software based on lncRNA levels, such as GO term enrichment analysis and KEGG pathway analysis.

Results
We identified 1203 lncRNAs and 29565 mRNAs that were expressed differentially. A total of 269 lncRNAs and 9429 mRNAs obtained from HepG2.2.15 cells treated with E2 were upregulated, and 552 lncRNAs and 7196 mRNAs were downregulated compared to the control group. A total of 163 lncRNAs and 7185 mRNAs were upregulated and 219 lncRNAs and 5755 mRNAs were decreased in the testosterone group compared to the untreated group. GO analysis demonstrated that the differentially expressed genes were involved in biological processes, such as cellular processes, cell components, and binding. KEGG functional pathway analysis revealed that the top 20 enriched pathways of antisense target genes included dopaminergic synapse, GnRH signaling pathway, FoxO signaling pathway, and MAPK signaling pathway.

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Running title: Long noncoding RNA profiling in HepG2.2.15

Weichuang Wang [1], Xiangsheng Cai [1], Xiaorong Yang [1,*]

[1] The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, China

[*]Corresponding author: Xiaorong Yang, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, China. Email: Xiaorongayang@126.com.

Abstract

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor and the fourth leading cause of cancer-related death worldwide [1, 2], fifth in males and seventh in females [3]. The cirrhosis secondary to chronic infection with hepatitis C virus (HCV) or hepatitis B virus (HBV) is the most common risk factor of HCC [4]. Epidemiological data indicate that the incidence of HCC is 2 to 11 times higher in men than that in women, suggesting significant gender differences in HCC [5]. It appears that sex hormones may be involved in the occurrence and development of HCC [6]. Evidence has shown that gender disparity can be attributed to a higher prevalence of alcohol abuse, smoking, and hepatitis infection in men than in women [7]. Although significant improvements have been achieved in diagnostic techniques, chemotherapy, molecular targeted therapy, and surgical treatment, the 5-year survival rate of HCC patients are still unsatisfactory due to the recurrence and metastasis of liver cancer [8]. Therefore, a better understanding of the mechanisms involved in HCC occurrence and development is vital to provide novel and accurate information for diagnostic

biomarkers, targeted molecular therapy, and prognosis of patients.

Although more than 90% of the human genome sequence can be actively transcribed, only approximately 2% of the gene sequences are subsequently translated. The remaining transcripts, which do not encode any proteins, are grouped as noncoding RNAs (ncRNAs) [9, 10]. Long noncoding RNA (lncRNA) is a novel member of ncRNA. LncRNAs are more than 200 nucleotides in length and lack protein-coding function. Increasing evidence has suggested that lncRNAs play an essential role in diverse biological processes, including cell proliferation, invasion, metastasis, apoptosis, and autophagy [11-13]. Thus, lncRNAs can promote the development and progression of various human carcinomas, including HCC [14, 15].

Recently, advances in lncRNA microarrays, high-throughput sequencing, and bioinformatics methods have attracted considerable attention in medical molecular biology. For example, Qin G et al. [16] revealed that lncRNA PSTAR binds to hnRNP K and induces its SUMOylation. Thus, lncRNA PSTAR enhances the interaction between hnRNP K and p53, which eventually results in the accumulation and transactivation of p53 in HCC. The downregulation of lncRNA MITA1 strongly inhibits the migration and invasion of hepatoma cells suggesting that MITA1 promotes the epithelial-mesenchymal transition in HCC [17]. Therefore, differentially expressed lncRNAs may be appropriate biomarkers for predicting the tumorigenesis and development of HCC. However, the pathological and biological functions of the majority of lncRNAs remain to be further elucidated.

Furthermore, recent studies have suggested that sex hormones play a critical role in the pathogenesis and development of HCC. For example, 17β-estradiol (E2) can strongly inhibit the malignant growth of HCC cells and tumor progression through the E2/ERβ/MAPK pathway. This pathway mediates the upregulation of the NLRP3 inflammasome [18]. Moreover, the androgen receptor enhances HBV RNA transcription by directly binding to the androgen response elements near the virus core promoter, thereby increasing the HBV viral titer. This activity works together with its downstream target gene HBx to promote hepatocarcinogenesis [19].

In this study, we measured differentially expressed lncRNAs and mRNAs obtained

from HepG2.2.15 cells treated with E2 or testosterone (experimental groups) or untreated (control group), aiming to provide new potential diagnostic and prognostic candidates for HCC.

Materials and methods

Cell culture

The human cell line HepG2.2.15 was a gift from Dr. Xuemei Lu (The School of Life Sciences and Biopharmaceutics of Guangdong Pharmaceutical University, China). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 1% penicillin-streptomycin (Gibco, Invitrogen, USA) and 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) at 37°C in a cell culture incubator with 5% CO_2.

Drug treatment

The HepG2.2.15 cells were plated into 6-well plates. After 24h incubation, cells were treated with 100μM of E2 (E2 group) or 100μM of testosterone (TEST group), and the untreated cells were set as Control group. After 48h incubation, cells were subjected to RNA extraction.

RNA extraction

~~When HepG2.2.15 cells grew to 70%-80% confluency in 6-well plates, they were divided into Control group, E2 group (100 μM E2), and TEST group (100 μM testosterone).~~ Total cellular RNA was isolated from the HepG2.2.15 cells by using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. RNA quantification and quality evaluation were determined using an ultraviolet spectrophotometer (Amoy Diagnostics).

Microarray analysis

Sample preparation, sequencing, and analysis were performed by Genedenovo Biotechnology Co., Ltd. (Guangzhou, China). Total RNA was extracted from the samples, and the ribosomal RNA was removed by Ribo-Zero Plus rRNA depletion kit (Illumina, USA) following the manufacturer's instructions to maximally retain coding RNA and ncRNA. The remaining RNA was randomly fragmented into short pieces, and

then the fragmented RNA was used as a template to synthesize the first cDNA strand with random hexamers. The second cDNA strand was synthesized after adding dNTPs (dUTP instead of dTTP), RNase H, DNA polymerase I, and buffer. The second cDNA strand was then purified with a QiaQuick PCR kit and eluted with EB buffer. The second strand was then degraded by the UNG (Uracil-N-Glycosylase) enzyme after end-repair, the addition of base A and the addition of the sequencing connector. Agarose gel electrophoresis was used for fragment size selection, and the selected fragments were subjected to PCR amplification. The final sequencing library was sequenced by Illumina HiSeq 4000 (Figure 1).

Construction of the mRNA-lncRNA gene co-expression network

The clean data were obtained after filtering the sequencing data. The reads were compared to the reference genome, and the known and novel transcripts were analyzed using Cufflinks to reconstruct the transcriptome. Coding Potential Calculator (CPC)[20], Coding-Non-Coding Index (CNCI)[21], and other software based on lncRNA levels, such as GO term enrichment analysis and KEGG pathway analysis, were used to predict the coding ability of the novel transcripts as described previously [22]. A lncRNA-mRNA co-expression network was finally conducted, which was based on the correlation between differentially expressed mRNAs and lncRNAs. Additionally, structural analysis was performed on the transcripts, including gene structure optimization and variable shear analysis.

Results

LncRNA transcript classification analysis

Recent studies have shown that many lncRNAs have stable secondary structure, high abundance, and subcellular localization, which indicates that these noncoding sequences are likely to be functional [10, 23, 24]. However, the function of lncRNAs is more challenging to determine than that of microRNAs and proteins. At present, it is not possible to speculate on their role-based only on their sequence or structure. However, we can infer the role of lncRNAs relative to the position of the adjacent

protein-coding gene in the genome. According to the biogenesis and genomic locations of the novel lncRNAs relative to the parental protein-coding genes, the novel lncRNAs are divided into five subtypes: intergenic lncRNAs, bidirectional lncRNAs, intronic lncRNAs, antisense lncRNAs, and sense overlapping lncRNAs [25, 26] (Figure 2).

Expression profile of lncRNAs and mRNAs in HepG2.2.15 cells

Three groups (E2-treated, testosterone-treated, and untreated) of HepG2.2.15 cells were chosen to visualize the differentially expressed lncRNAs and mRNAs. Volcano plot analysis was applied to examine the differentially expressed lncRNAs directly and mRNAs obtained from E2-treated, testosterone-treated, and untreated HepG2.2.15 cells (Figure 3). However, hierarchical clustering revealed systematic variations in gene expression among the E2, testosterone, and untreated groups of HepG2.2.15 cells. Figure 4 indicated the differential expression of lncRNAs and mRNAs. Our results demonstrated that 821 differentially expressed lncRNAs (Figure 4A) and 16625 differentially expressed mRNAs (Figure 4B) in untreated HepG2.2.15 cells compared with the E2 group. Of the 821 differentially expressed lncRNAs, 552 were downregulated, and 269 were upregulated. In addition, a total of the 16625 differentially expressed mRNAs, 7196 were downregulated, and 9429 were upregulated. The results also showed 382 differentially expressed lncRNAs (Figure 4A), and 12940 differentially expressed mRNAs (Figure 4B) in the testosterone group compared with the untreated group. Of the 382 differentially expressed lncRNAs, 163 were upregulated, and 219 were downregulated. Moreover, a total of the 12940 differentially expressed mRNAs, 7185 were upregulated, and 5755 were downregulated. These findings indicate that many lncRNAs may play vital roles in the tumorigenesis and development of hepatocellular carcinoma cells after E2/testosterone treatment.

GO and pathway enrichment analyses of differently expressed mRNAs

GO analysis provides GO functional classification terms and GO functional significance enrichment analysis of differentially expressed transcripts. GO analysis reveals distinctive upregulated and downregulated functions that are grouped into the

ontologies' biological processes, cellular components, and molecular features. We determined that significantly differentially expressed transcripts between HepG2.2.15 cells treated with E2 or testosterone and the untreated group were associated with cellular processes (ontology: biological process), cells and cell components (ontology: cellular component) and binding (ontology: molecular function) (Figure 5A and 5B).

Antisense analysis of the lncRNA-mRNA co-expression network

LncRNAs are similar to small RNAs, such as miRNAs and snoRNAs. LncRNAs are also involved in the regulation of many posttranscriptional processes, which are often associated with complementary binding. Some antisense lncRNAs may regulate gene silencing, transcription, and mRNA stability by binding to the positive-sense strand of mRNAs. To reveal the interaction between antisense lncRNAs and mRNAs, we used RNAplex to predict the complementary binding sites between antisense lncRNAs and mRNAs. RNAplex is a software used to find the interactions between two long RNA sequences [27]. This program contains the Vienna RNA package [28], which calculates the minimum free energy based on the thermodynamic structure to predict the best base-pairing relationship. KEGG pathway analysis was performed of antisense target genes with significant enrichment compared to the whole transcriptome. Figure 6 shows the top 20 enriched pathways t, including dopaminergic synapse, GnRH signaling pathway, FoxO signaling pathway, MAPK signaling pathway, etc. RichFactor refers to the ratio of the number of differentially expressed transcripts in each pathway entry to the total number of transcripts. Q value is the P value after multiple hypothesis test corrections and ranges from 0 to 1.

Discussion

HCC is developed most often in patients with cirrhosis caused by HBV and HCV[4, 29]. Infection with ~~hepatitis B virus (HBV)~~ is a significant risk factor for hepatocellular carcinoma (HCC) in developing countries. Epidemiological studies demonstrated that the incidence of HBV-related HCC in males and postmenopausal females is higher than that of other females. Increasing evidence indicates that sex hormones, including

androgens and estrogens, play an essential role in the progression of an HBV infection and in the development of HBV-related HCC [30]. The chronic HCV infection was the major etiology of HCC in the western countries. The development of HCV-related HCC was associated with concurrent liver diseases, lifestyles, obesity, and diabetes [31].

In the past decade, lncRNAs have been considered to be transcriptional noise or erroneous transcription [32]. However, recent studies have shown that lncRNAs play a critical role in the tumorigenesis and progression of cancers through various biological and pathological processes, including cell proliferation, metastasis, invasion, apoptosis, and autophagy [33, 34]. LncRNAs are functional transcripts that can function in a variety of ways. LncRNAs play roles in guiding epigenetic repressors, blocking transcription factor binding sites, acting as protein scaffolds, and sequestering miRNAs [25, 35]. Moreover, much evidence has revealed that lncRNAs are also differentially expressed in HCC.

Epidemiologic studies from different areas of the world have shown that males have significantly increased mortality and a higher prevalence of HCC than females [36]. Estrogen has been implicated in the gender disparity in inhibiting the development and progression of HCC [37]. For example, ER agonists and E2 reduced the growth of HCC cells by inhibiting cell proliferation and promoting cell apoptosis [38]. Moreover, estrogen replacement therapy in menopausal women raises the risks of breast, gallbladder, urinary bladder, and endometrial cancers. However, the therapy reduces the risks of other categories of tumors, such as colon, rectum, and liver carcinomas [39]. These findings indicate that it is feasible to develop estrogen drugs that mainly act on the liver without adversely affecting other tissues such as ovarian, breast, and uterus tissues.

In this study, the differential lncRNA expression profiles in HepG2.2.15 cells treated with E2 or testosterone compared with the untreated group were analyzed comprehensively. Interestingly, we found that 821 lncRNAs and 16625 mRNAs were significantly expressed differentially in untreated HepG2.2.15 cells compared to the E2 group. In addition, 382 lncRNAs and 12940 mRNAs were expressed differentially in the untreated group compared with the testosterone group. Bioinformatic studies using

co-expression network, GO, and KEGG pathway analyses demonstrated that differential expression of lncRNAs and mRNAs are valuable as potential predictive biomarkers. Recently, some types of lncRNAs differential expression, for instance, intergenic lncRNAs, antisense lncRNAs, and lincRNAs, have been identified to have specific functions in cancers [40, 41]. In addition, the differentially expressed lncRNAs were classified into five categories, and among them, the most abundant were intergenic lncRNAs (Figure 2). Intergenic lncRNAs have been proven to regulate the gene expression levels that are genomically distant (trans-acting) or adjacent (cis-acting) through various molecular mechanisms. Therefore, intergenic lncRNAs can be classified as either transcription-dependent or transcript-dependent [26, 42]. Recently, it was found that intergenic lncRNAs possibly play a role as competing endogenous RNAs (ceRNAs). Intergenic lncRNAs can adjust the expression of a miRNA target by sponging the miRNA, which can ultimately promote the progression of HCC [43]. With the rapid development and latest progress in microarray technology, it appears that tumor research has entered a genome era. In this study, we found a novel relationship for E2, lncRNAs, and HCC. These findings indicate that the expression level of numerous lncRNAs is regulated in liver cancers. Some of these lncRNAs may provide a novel avenue for the development of more efficient diagnosis and therapeutic strategies. However, this study also has some limitations. The HepG2.2.15 is a most studied cell line for liver cancer research. Nevertheless, other hepatocellular carcinoma cell lines are needed to be considered in the further studies. Further research will be critical to understanding the gender differences observed in HCC.

Conclusion

In this study, a total of 1203 lncRNAs and 29565 mRNAs were identified expressed differentially. A total of 269 lncRNAs and 9429 mRNAs obtained from HepG2.2.15 cells treated with E2 were upregulated, and 552 lncRNAs and 7196 mRNAs were downregulated compared to the control group. A total of 163 lncRNAs and 7185 mRNAs were upregulated and 219 lncRNAs and 5755 mRNAs were downregulated in the testosterone group compared with the untreated group. GO analysis demonstrated

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Author contributions

W.C.W. and X.R.Y. designed the study. W.C.W. and X.S.C. performed the experiments and analyzed the data. W.C.W. and X.R.Y. prepared the manuscript. All authors critically reviewed the manuscript and approved the final version submitted for publication.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure legends

Figure 1 Experimental flow chart and schematic diagram of database construction.

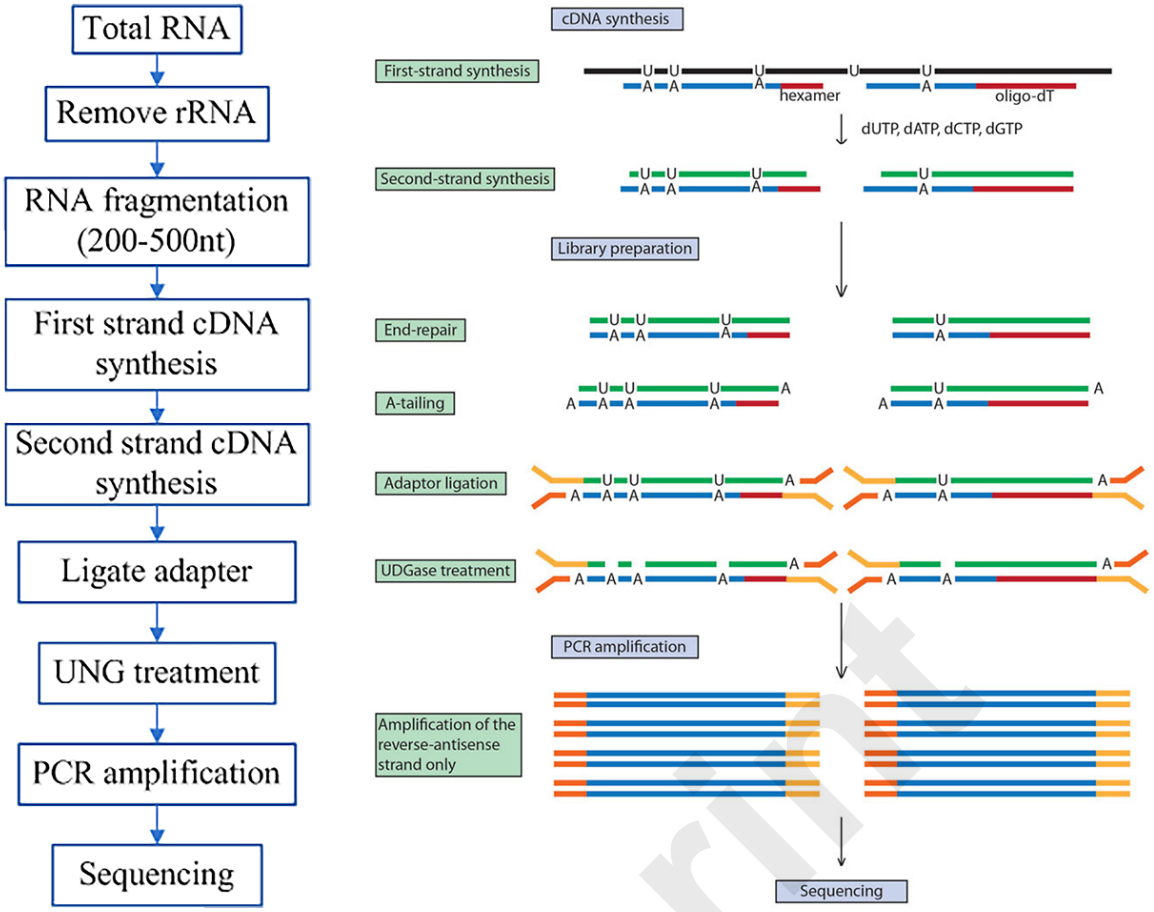
Figure 2 Statistical map of transcript types of the differentially expressed novel lncRNAs.

Figure 3 The volcano plots illustrate the distributions of the data in the lncRNA profiles of the E2 (A) or testosterone (B) group and mRNA profiles of the E2 (C) or testosterone (D) group.

Figure 4 Cluster dendrogram of differential expression of lncRNAs (A) and mRNAs (B) in HCC cells treated with E2, or testosterone, or untreated (control group).

Figure 5 GO classification histogram of differentially expressed transcripts between E2- (A) or testosterone-treated (B) HCC cells and untreated HCC cells. GO analysis of mRNAs according to biological processes, cellular component, and molecular function ontologies.

Figure 6 The top 20 significantly enriched KEGG pathways of antisense target genes. The larger the RichFactor, the higher the path enrichment. The closer Q value is to zero, the more significant the enrichment is.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Ligate adapter
UNG treatment
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Sequencing
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oligo-dT
dUTP, dATP, dCTP, dGTP
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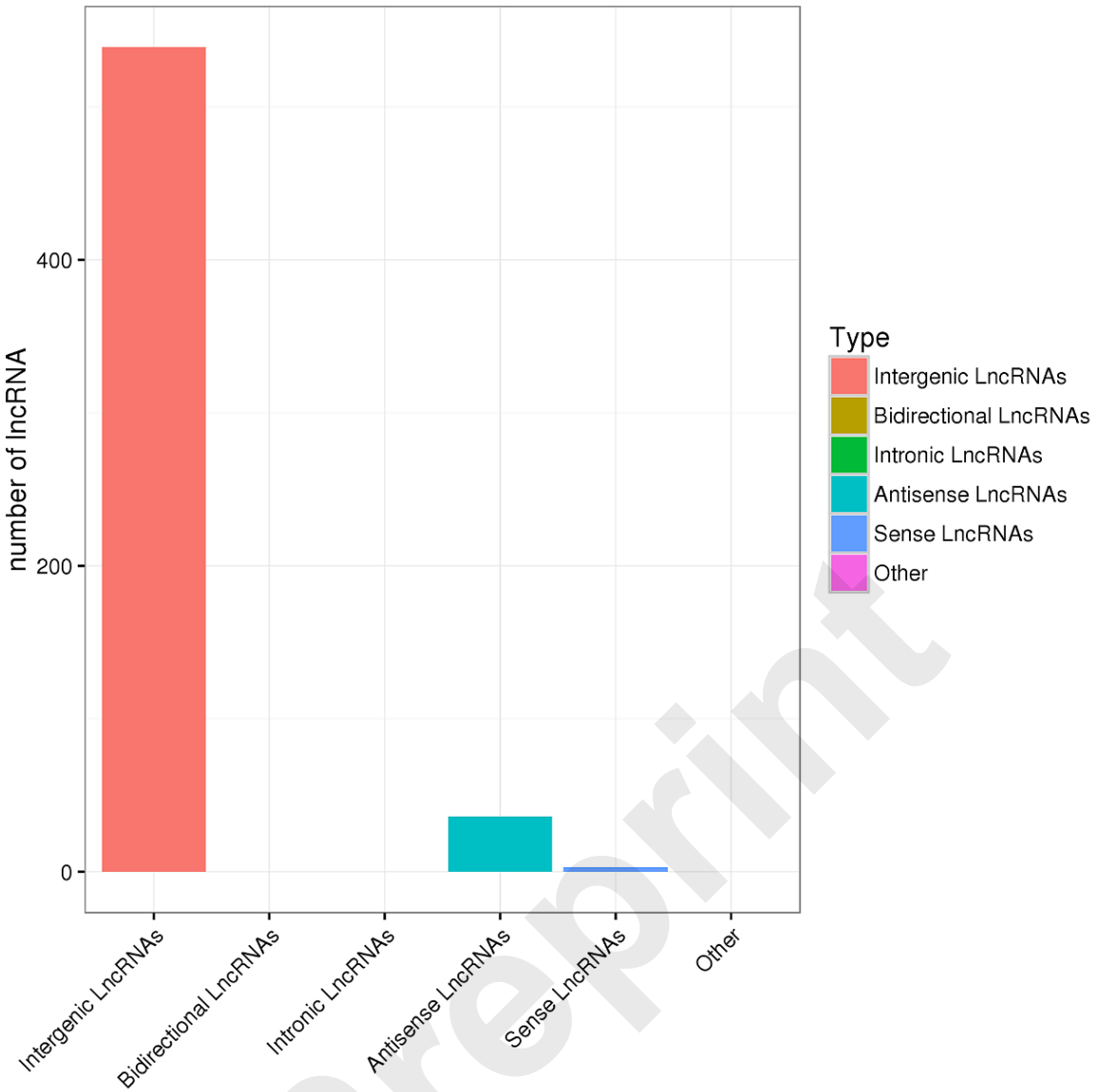
A-tailing
Adaptor ligation
UDGase treatment
PCR amplification
Amplification of the reverse-antisense strand only
Sequencing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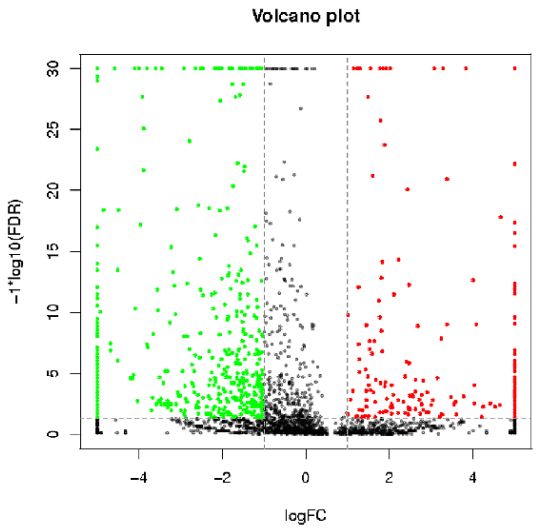

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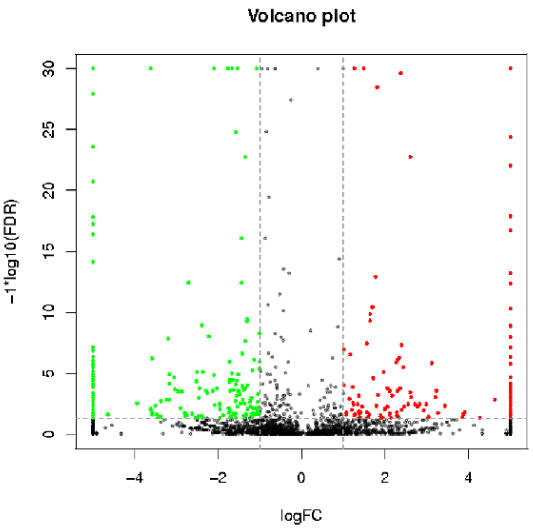

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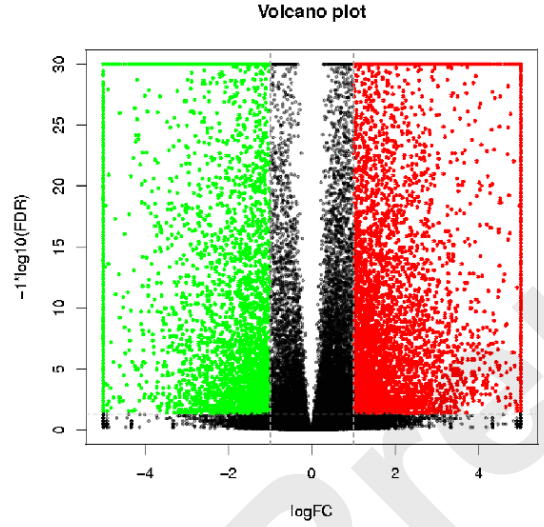

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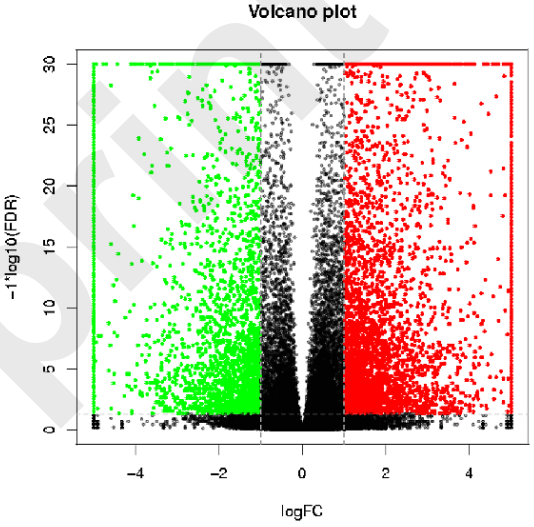

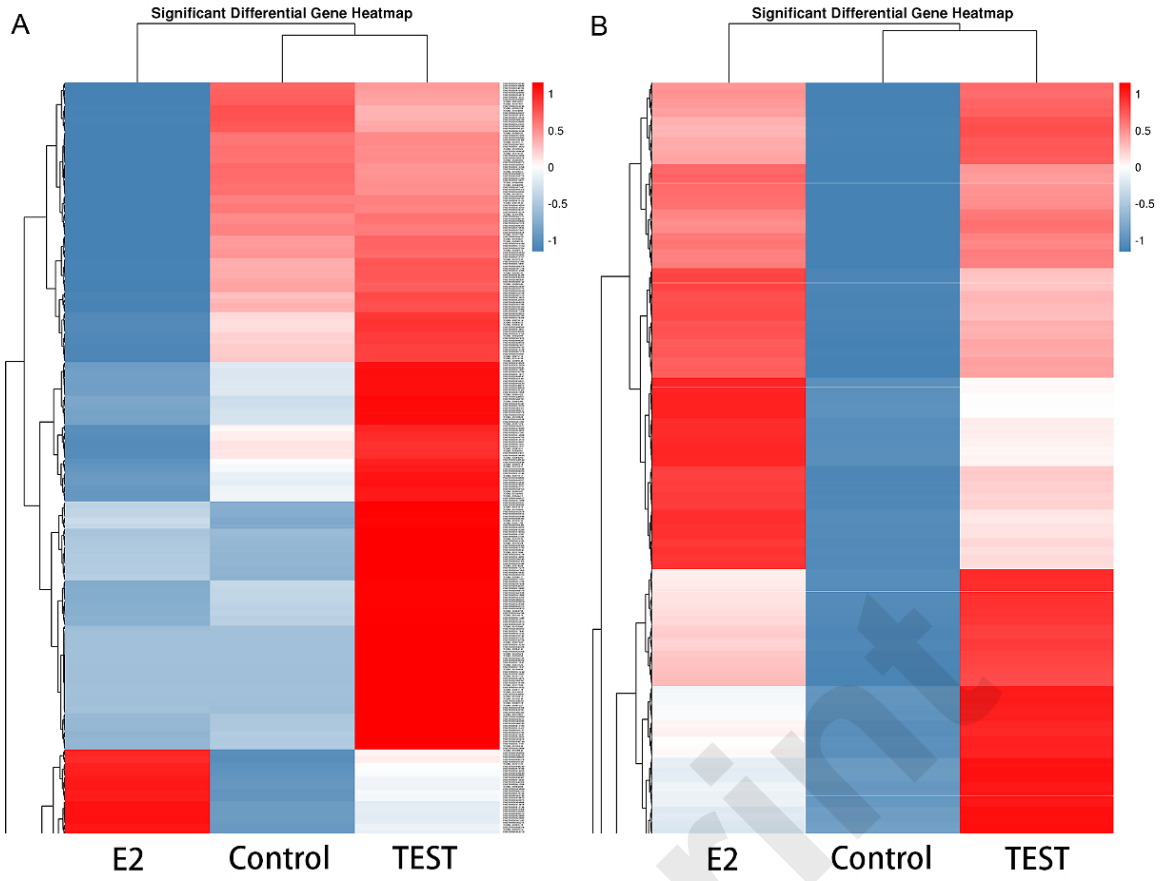
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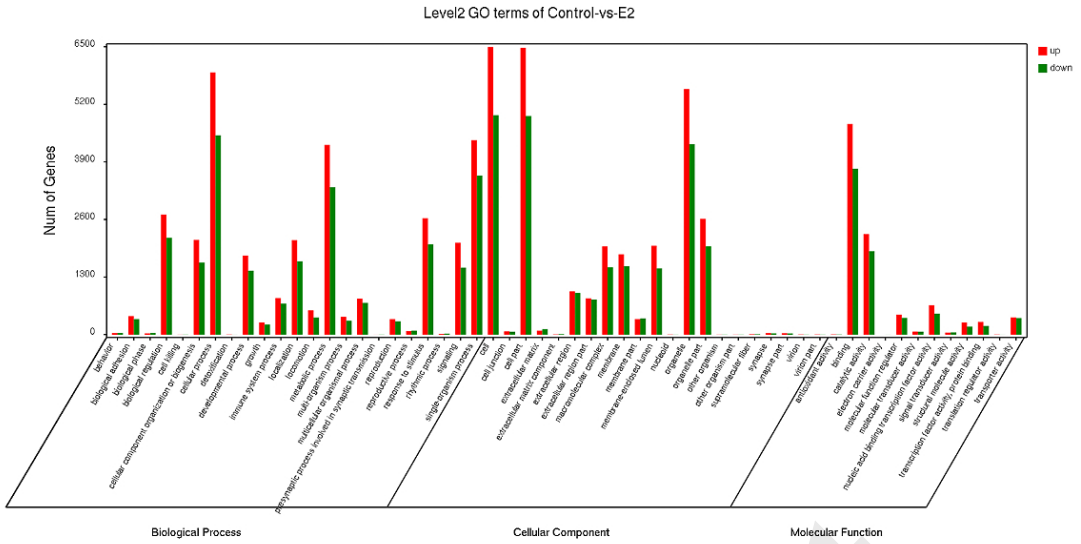
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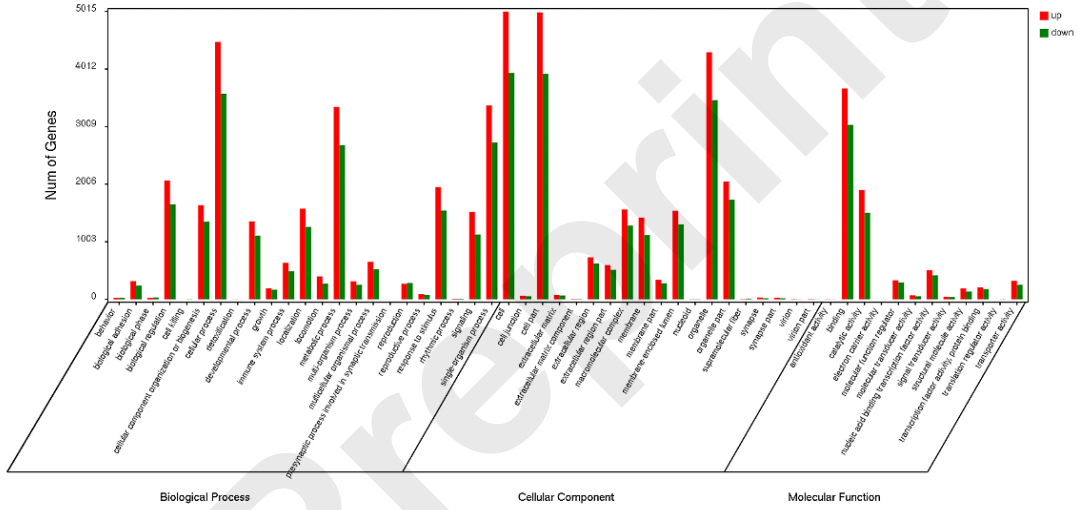
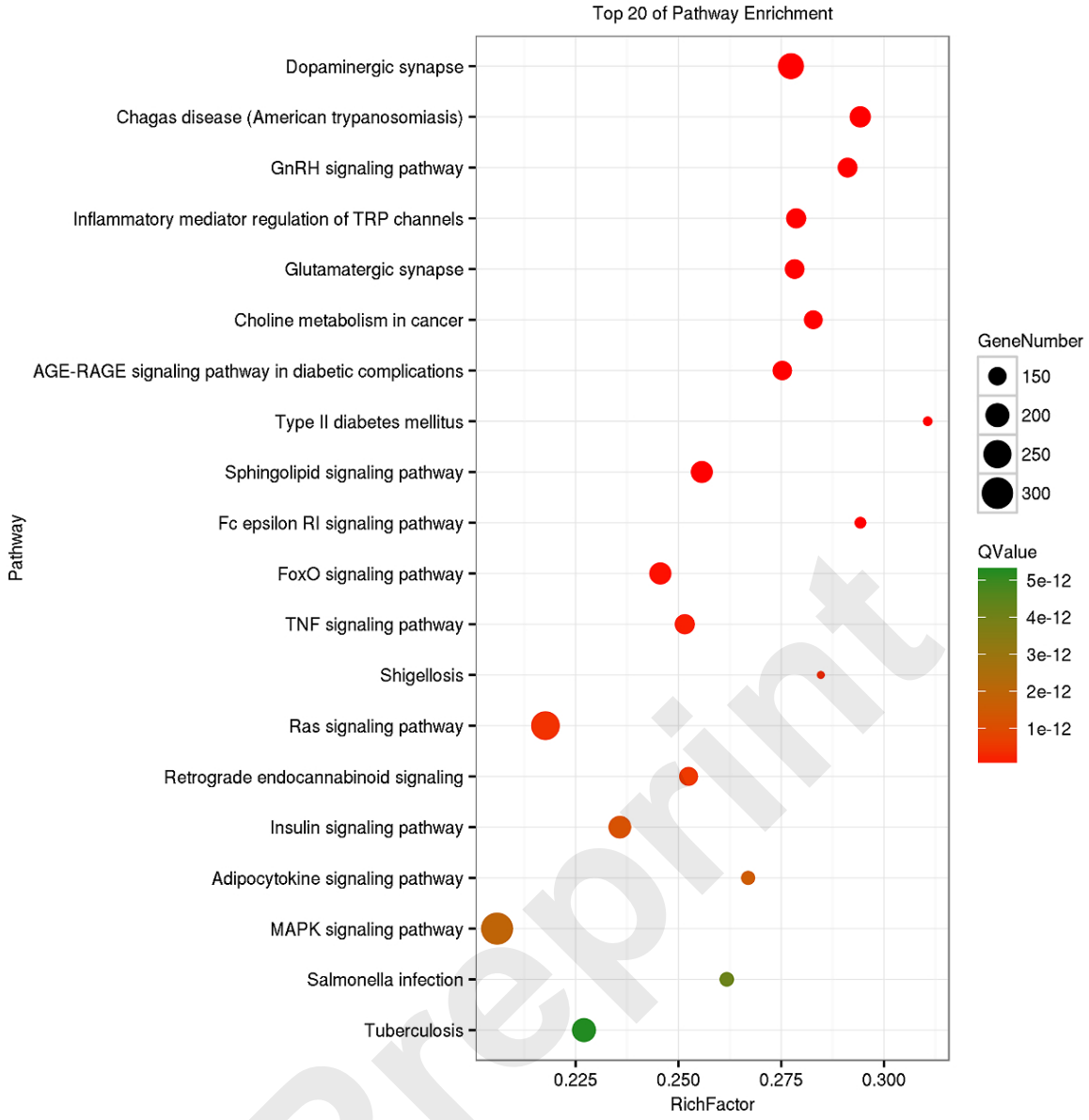
down
Biological Process
Cellular Component
Molecular Function

B


Level2 GO terms of Control-vs-TEST
Num of Genes
up
down
Biological Process
Cellular Component
Molecular Function


Top 20 of Pathway Enrichment
Dopaminergic synapse
Chagas disease (American trypanosomiasis)
GnRH signaling pathway
Inflammatory mediator regulation of TRP channels
Glutamatergic synapse
Choline metabolism in cancer
AGE-RAGE signaling pathway in diabetic complications
Type II diabetes mellitus
Sphingolipid signaling pathway
Fc epsilon RI signaling pathway
FoxO signaling pathway
TNF signaling pathway
Shigellosis
Ras signaling pathway
Retrograde endocannabinoid signaling
Insulin signaling pathway
Adipocytokine signaling pathway
MAPK signaling pathway
Salmonella infection
Tuberculosis
Pathway
0.225
0.250
0.275
0.300
RichFactor
GeneNumber
150
200
250
300
QValue
5e-12
4e-12
3e-12
2e-12
1e-12