COORDINATIVE CONTROL OF G2/M PHASE OF THE CELL CYCLE BY NON-CONDING RNAS IN HEPATOCELLULAR CARCINOMA

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【Abstract】

Objective: To iInvestigate the interaction of non-coding RNAs (ncRNAs) in hepatocellular carcinoma

Methods:: We compared tThe differential ncRNAs and mRNAs expression profiles was compared betweenof hepatocellular carcinoma and paracancerous adjacent normal tissues by microarray and RT-PCR. The relationship between different ncRNAs and mRNA was analyzed by using bioinformatics tools. A regulatory model of ncRNAs in HCC was proposed developed.

Results: : A total of 1704 differentially expressed lncRNAs, 57 miRNAs, and 2093 mRNAs were identified by microarray analyseis. There is a co-expression relationship between two ncRNAs ((miRNA-125b-2-3p and lncRNA P26302)). Microarray Bioinformatic analysis demonstrated CDK1 and CyclinA2 as potential targets of miR-125b-2-3p and PLK1 as potential target of lncRNAP26302. All three gene are important components in the G2/M phase of cell cycle. Subsequently real-time PCR studies confirmed these microarray results.

Conclusion: MiR-125b-2-3p and lncRNAP26302 may affect the G2/M phase of the cell cycle through the regulation of their respective target genes. This study shows evidence ofa role of ncRNAs in pathogenesis of hepatocellular carcinoma at molecular level, providing a basis for the future investigation aiming at early diagnosis and novel treatment of hepatocellular carcinoma.

【 **Key** 】： Hepatocellular Carcinoma Cells(HCC), microarray, miRNA, LncRNA, mRNA, Pathway

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer (PLC), accounting for more than 85%-90% of all PLC. Among the world's most common malignancies, the incidence of HCC is ranked the 6th, and the mortality rate is the 3rd (Torre et al. 2015). The high mortality rate is mainly due to the strong invasive and metastatic capacity of the hepatocellular cancer cells. Upon diagnosis, HCC is often at advanced stage (Wang et al. 2013)。Therefore, in order to improve the prognosis and treatment of liver cancer, people have been working on the cellular and molecular ~~biological~~ mechanisms leading to malignant transformation of hepatocytes. Early diagnosis is the key to effective treatment of liver cancer. It is important to find effective early-diagnosis markers of liver cancer. In recent years, a large number of studies have suggested that non-coding RNA（ncRNA） is related to the ~~occurrence~~ development of ~~tumors~~HCC, providing a new research direction for the diagnosis and treatment of liver cancer. (Mattick & Makunin 2006).

As more and more non-coding RNAs are studied, it is noted that different non-coding RNAs have different regulatory capabilities for the development of liver cancer. MicroRNA (miRNA) and Long non-coding RNA (lncRNA) are the two most representative forms. For example，miR-122 is a miRNA specifically expressed in the liver, which is overexpressed in hepatocellular carcinoma cell lines HepG2 and HepB3, and promotes apoptosis and inhibits proliferation of hepatoma cells in biological processes. (Datta et al. 2008). In addition, miR-122 may also target the activity of p53 through the Cyclin G1 gene to affect the sensitivity of HCC to doxorubicin (Hsu et al. 2012). In the study of Tsang (Tsang et al. 2015)，lncRNA HOTTIP was identified as the most significantly up-regulated lncRNA in human HCCs, even in early stage of HCC formation. Functionally, knock-down of HOTTIP attenuated HCC cell proliferation in vitro and markedly abrogated tumourigenicity in vivo. In addition, knock-down of HOTTIP also inhibited migratory ability of HCC cells and significantly abrogated lung metastasis in orthotopic implantation model in nude mice. Furthermore, they identified miR-125b as a post-transcriptional regulator of HOTTIP. Ectopic expression of miR-125b reduced HOTTIP-coupled luciferase activity and suppressed the endogenous level of HOTTIP. LncRNA HULC is highly expressed in HCC，while it has a binding site of miR-372. Therefore, HULC can competitively bind miRNAs, making miRNAs lose their ability to target genes, thereby reducing the tumor suppressor effect of miR-372 (Wang et al. 2010)。Researchers names the ncRNAs which competitively bind to miRNA as competitive endogenous RNA（ceRNA） (Salmena et al. 2011)。

This concept has enriched the central dogma of molecular biology and provided a useful tool for analyzing the molecular mechanism of tumors.

During the study of a single ncRNA, it was found that the effect of certain non-coding RNAs on tumorigenesis was accompanied by corresponding changes in other types of ncRNA. In this regard, we hypothesize that ncRNAs between different species may play a regulatory role in the development of tumors by coordinatively acting on the same target gene or upstream and downstream genes on the same signaling pathway. In order to explore whether there are other regulatory mechanisms of lncRNA and miRNA in HCC pathogenesis (Shafei et al. 2018, I am not sure this reference belongs to here), we compared the expression level of lncRNA, miRNA and mRNA between hepatocellular carcinoma and normal counterparts. Bioinformatics prediction, correlation analysis and pathway evaluation were used to identify the potential important ncRNA and its target genes.

Materials and Method

1. Materials

In this study, 12 pairs of primary hepatocellular carcinoma (HCC) tissues and their adjacent tissues 2cm away from cancer tissue were selected (Chen et al. 2016). All specimens were obtained from the First Affiliated Hospital of Guangxi Medical University. Then tissue samples were processed within 30 minutes after the tumor was isolated. They were immediately frozen in liquid nitrogen and stored at -80 degrees Celsius. The patients that specimen sourced were all undergoing the first operation on the primary disease and none had received radio-chemotherapy. Histopathology confirmed as HCC after surgery 。The use of all specimens was reviewed by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, and patients and their families had informed consent for participating in scientific research. （The use of all specimens was reviewed by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. (Ethical Application Ref: 伦审 2013-KY-国基-085).）

2. RNA Prepartion

Total RNA in tissue blocks was extracted using Trizol (Invitrogen, USA). Total RNA was further purified respectively using the NucleoSpin® RNA clean-up kit (740.948.250) and the mirVanaTM miRNA Isolation Kit (AM1561). The prepared total RNA will be used for the next experiment.

3. miRNA microarray

We performed microarray analysis of miRNAs using Affymetrix GeneChip miRNA arrays (Santa Clara, CA, USA). In short, polyA polymerase uses the

Genisphere FlashTag HSR kit to label 1 mg of total RNA from the tissue. This was followed by hybridization of RNA with Affymetrix miRNA arrays. After hybridization, the standard Affymetrix ribbons were stained, washed, scanned, and transformed with AGCC software (Affymetrix® GeneChip® command console® software) and the GeneChip® Scanner 3000. Using .CEL files as source files, we use the Expression Console software provided by affymetrix to perform row data preprocessing: including RMA normalization, whether the probe signal is significantly higher than the background signal, and integrate the probe signal into a probe set signal. Three miRNA microarray tests were performed on each sample.

4. lncRNA and mRNA microarray

Jingxin ® human lncRNA + mRNA Expression Microarray (CapitalBio, China) is a kind of screening of lncRNA and mRNA gene chip at the same time，According to the instruction，5μl total RNA extracted from sample was used to synthesize double-stranded complementary DNA (cDNA). Double-stranded cDNA was labeled and hybridized to Jingxin® human lncRNA+mRNA Expression Microarray (CapitalBio, China). The volume of the cDNA product obtained after reverse transcription and purification was concentrated to 14 μL, and cy3-dCTP/cy5-dCTP was added and placed on a PCR machine (reaction at 37°C for 1.5 hours, reaction at 70°C for 5 minutes, retention at 4°C). Then, the cDNA was purified using the Nucleospin ® Extract II (MN, Cat. No. 740609.250) kit, and the fluorescently labeled product was subjected to fluorescence incorporation and nucleic acid quantification using an ultraviolet spectrophotometer. 100 μL of the hybridization solution was applied to the hybrid coverslip, and the hybridization cassette was mounted on the rotor of the Hybridization Oven G2545A and hybridized at 45°C for 12 hours. Scan the cleaned chip with Agilent G2565CA Microarray Scanner to get a hybrid picture. Hybrid images were analyzed and data extracted using Agilent Feature Extraction (v10.7) software. Then we use Agilent GeneSpring software to normalize and analyze the data.

5. RT-PCR Detection

Total RNA was isolated from samples using the Trizol reagent and followed by cDNA synthesis using viral polymerase and random primers. The cDNA producted was amplified and the target gene expression level was normalized to 18s mRNA, an internal quantity control. Experiments were performed in triplicates。

6. Statistical analysis

We analyzed the differentially-expressed genes in the preprocessed data. Three or more biologically replicated data in the sample were analyzed using the SAM (significance analysis of microarray) R package (Tusher et al. 2002). The screening criteria for differential genes were: q -value ≤ 5% and Fold Change ≥2 or ≤0.5 (Clarke et al. 2008) (Yang et al. 2005). The results of differentially expressed genes were subjected to unsupervised hierarchical clustering (Cluster 3.0) and TreeView analysis (Stanford University, Stanford, CA, USA).

Results

1. Affymetrix miRNA microarray was used to establish the MiRNA expression profiles of 12 pairs of human hepatocellular carcinoma tissues and their adjacent normal hepatic tissues. Based on the fold change: ≥ 2 or ≤ -2, $p < 0.05$ for the t- testand as the screening criteria for differential genes, there were 57 differential miRNAs, including 9 up-regulated miRNA and 48 down-regulated miRNA（Figure 1A）. Same samples were subject to study of the gene expression profiles using the Jingxin® human lncRNA+mRNA V4.0 chip. Compared to the adjacent normal hepatic tissues, HCC tissues show significant gene expression levels in 2093 mRNAs, among which 635 were up-regulated and 1,458 were down-regulated（Figure 1B）. There were 1,704 lncRNAs showed significant expression levels, of which 607 were up-regulated and 1,097 were reduced（Figure 1C）.

2. Co-expression analysis constructs signaling pathways

We performed a co-expression analysis of lncRNAs and miRNAs in order to select the co-expressed genes based on an absolute value of correlation coefficient > 0.7 and P-value<0.05. The lncRNAs and miRNAs with a co-expression relationship were used to predict their target genes, which were crosschecked with genes identified by mRNA microarray analysis. We found there are 20 co-expressed lncRNAs and miRNAs, of which only lncRNA P26302 has a fold difference greater than 10 time, so we chose miR-125-2-3p co-expressed with lncRNAP26302(Correlation coefficient= -0.87, Pvalue=0.0001) for further study. Meanwhile, three differentially expressed mRNA which were target genes of miR-125b-2-3p or lncRNA P26302 were identified by mRNA microarray. The target genes for miRNA-125b-2-3p are cyclin-dependent kinases 1(CDK1) and CyclinA2. Polo-like Kinase 1(PLK1) is LncRNA P26302 target genes. Through KEGG Pathway analysis of target genes, we found that the three target genes are co-exist in the same signaling pathway (ko04110 cell cycle)， Figure2. By further querying the KEGG Pathway map, we found that these target genes are in the G2/M phase of the cell

cycle and they are close in the order within the pathway, so we can propose the following signal pathways，（Figure 3）.

3. To verify the accuracy of differentially expressed genes screened by microarrays, we chose to confirmation study of RT-PCR for miR125b-2-3p，lncRNAp26302，CDK1、CyclinA2（CCNA2）and PLK1. The gene expression detected in hepatocellular carcinoma and paracancerous tissues was consistent with the results of the gene chip screening. (Figure 4 ）

Discussion

Non-coding RNAs such as lncRNA and microRNA have been proved to participate in the regulation of gene expression through competition for endogenous RNA networks with mRNA (Kartha & Subbaya 2014). The interaction between these two ncRNAs plays an important role in tumor development (Jiang et al. 2017; Wu et al. 2016). At present, the specific molecular mechanism of the interaction between lncRNA and miRNA is speculated to be within two possibilities:1. because lncRNA and mRNA have a similar structure, miRNA can specifically bind to its 3' UTR and down-regulate lncRNA expression through a mechanism similar to that of regulating mRNA (Shi et al. 2013); 2. studies have confirmed that lncRNA can competitively target miRNAs to inhibit the expression of miRNAs, thereby reducing its inhibitory effect on target genes (Salmena et al. 2011).

During the G2 to M phase transition of the cell cycle, CDK is a set of Ser/Thr kinase systems that correspond to cell cycle progression. Various CDKs are alternately activated along the cell cycle, and phosphorylation of the corresponding substrate allows the cell cycle to proceed in an orderly manner. CyclinA2 is a cyclin that binds to CHEK1 to form a CycA/CDK1 complex. The CycA/CDK1 complex is the main damage monitoring mechanism in S phase. The withdrawal of the cell cycle from mitosis requires Cdk1 inactivation, and the most important mechanism of Cdk1 inactivation is the hydrolysis of mitotic cyclins. In higher eukaryotes, this involves the continuous destruction of type A and type B cyclins, ie, the CycA/CDK1 complex and the CycB/CDK1 complex are successively inactivated. When DNA damage occurs, CycA is first destroyed, resulting in the inactivation of CycA / Cdk1 necessary for the G2 to M transition (Kaspar et al. 2001). Finally, the cell cycle is stopped in the G2 phase. Plk1 is a class of highly conserved serine/threonine protein kinases expressed in eukaryotes. Plk1 is a key gene in cell cycle regulation and is regulated by phosphorylation and protein degradation (Barr et al. 2004) (Catherine & Jonathon 2004). Current studies have found that Plk1 is able to restart mitosis by acting on the CycA/CDK1 complex, from the G2 phase to the M phase. In the G2 phase, the cytokine CycA/CDK1 complex is inactivated due to the phosphorylation of the residues on the adenosine triphosphate binding domain

of Cdk1, while Myt1 kinase phosphorylates the threonine residue Tyr15. Avoid entering the mitosis phase before DNA replication is completed and destroying genome integrity (Takizawa & Morgan 2000). Plk1 is able to phosphorylate and inhibit the activation of Myt1, thereby dephosphorization of Cdk1, and activation of CycA/CDK1 complex initiates mitosis (Nakajima et al. 2003).

To further explore the relationship among the three groups, we performed co-expression analysis of the differential miRNA and lncRNA genes, and analyzed the KEGG enrichment pathway of their respective target genes. We found that miRNA125b-2-3p and lncRNAP26302 expression were correlated in HCC. Moreover, the target genes CDK1 and CyclinA2 of miR-125b-2-3p are in the same gene pathway (the G2/M Phase of the cell cycle) and of the target gene PLK1 of lncRNAP26302. In the normal cell cycle, CycA/CDK1 is the major damage regulation mechanism of S phase (Katsuno & Mak 2009), and CyclinA2 in CycA can promote the synthesis of DNA in S phase, thus promoting the transition of cells over the G2/M phase and into M phase (Nikola et al. 2012). However, when DNA damage occurs, cell cycle regulation enters the DNA damage repair mechanism which inhibits the expression of CycA/CDK1 through the Myt1 gene (Varadarajan et al. 2016), thus allowing the cell cycle to stay in the S phase for cell repair (Chapman et al. 2012; Inger & Gent 2012). In hepatoma cells, the target gene PLK1 of lncRNAP26302 was overexpressed, inhibited the expression of Myt1, and reduced the inhibitory effect of Myt1 on CycA/CDK1. As a result, when DNA damage or mutation occurs in the nucleus of tumor cells, the chromosomes can bypass the DNA damage detection point, and these cells continue to proliferate and divide, thereby promoting the formation of HCC (Perdiguero & Nebreda 2004). In addition, chip detection revealed that low expression of miR-125b-2-3p leads to the rise of the target gene CyclinA2, further promoting the formation of CycA/CDK1. And by differential gene correlation analysis, there is a reverse correlation between miR-125b-2-3p and lncRNAP26302, which indicates that the decreased expression of miR-125b-2-3p in hepatocellular carcinoma may cause overexpression of lncRNAp26302. Therefore, miR-125b-2-3p and lncRNAP26302 may have a coordinative effect on this pathway, which together promote the expression of CycA/CDK1, so as to coordinatively promote the formation of liver cancer.

Besides，we performed RT-PCR validation of differential expression of the genes regulated by the lnRNAs we identified. Among them, the RT-PCR results of hs-mir125b-2-3p, lncRNAp26302(uc003lzi.3), CDK1, CyclinA2 (CCNA2), PLK1, were consistent with their expression on the G2/M Phase of the cell cycle. Therefore, we believe that miRNA 125b-2-3p and lncRNA p26302 likely have coordinative regulatory effect on the G2/M phase in HCC development.

Conclusions

In summary, there are a large number of differentially expressed ncRNAs between HCC tissue and adjacent tissue. Through the expression profiling study of these genes, we propose that mir-125b-2-3p and lncRNA p26302, miR-125b-2-3p and lncRNAP26302 affect the G2/M Phase of the cell cycle through the coordinative regulation of their respective target genes. The gene expression results of this study enriched the understanding of gene expression mechanism, provided support for the ceRNA hypothesis, and provided a more complete understanding of the role of non-coding RNA in the occurrence and development of HCC and the regulatory loop. In the future, functional cell cycle analysis is desired to to demonstrate the role of MiR-125b-2-3p and lncRNAP26302 target gene in cell cycle G2/M phase.

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