Long non-coding RNAs and hepatocellular carcinoma (Review)

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Abstract. Recent advances in next-generation sequencing technology in transcriptome analysis have helped identify numerous non-coding RNAs. The long non-coding RNA (lncRNA) is commonly defined as an RNA molecule with a length of 200 bp-100 kbp that lacks protein-coding potential. LncRNAs play a critical role in the regulation of gene expression, including chromatin modification, transcription and post-transcriptional processing. It has been confirmed that dysregulation of lncRNAs is associated with a number of human diseases, particularly tumors. In this study, we focused on the most extensively investigated lncRNAs in hepatocellular carcinoma (HCC). The biological functions and molecular mechanisms of the majority of lncRNAs have yet to be investigated. The improved knowledge on lncRNAs in HCC may help identify lncRNAs that may be used as novel prognostic markers and therapeutic targets.

Contents

1. Introduction

With the development of deep sequencing and DNA tiling array technology, an increasing number of investigators are focusing on non-coding RNAs (ncRNAs) that are not translated into protein and the number of articles on ncRNAs is increasing exponentially (1-3). It was estimated that ~70% of the human genome is pervasively transcribed; however, protein-coding genes account for <2% of the human genome (4). Consequently, abundant ncRNAs are transcribed from the human genome, including small interfering RNAs (siRNAs), microRNAs (miRNAs), PIWI-interacting RNAs and long ncRNAs (lncRNAs) (5-7). Extensive studies have been conducted on siRNAs and miRNAs and their molecular functions have been elucidated (8-10). However, our understanding of the involvement of lncRNAs in diseases remains limited. Recent studies revealed that lncRNAs play a pivotal role in the regulation of gene expression, such as chromatin modification, transcription and post-transcriptional processing (11-13).

In this study, we aimed to focus on some critical, well-known lncRNAs in hepatocellular carcinoma (HCC) and briefly outline their known functions and possible underlying molecular mechanisms, as well as their potential application as therapeutic targets or biomarkers.

2. Characteristics of lncRNAs

The most common definition of lncRNA is an RNA molecule with a length of 200 bp-100 kbp that lacks protein-coding potential (14). However, this simplistic definition may be associated with several problems (15). For example, the cut-off of 200 nucleotides was factitiously selected and was not based on functional meaning. In addition, lncRNAs may contain an open reading frame longer than 100 amino acids. However, polypeptides shorter than 100 amino acids may also be functional in organisms and are not by-products of canonical proteins. These problems suggest our limited understanding of lncRNAs and how difficult it is to determine a definition.

The majority of lncRNAs are transcribed by RNA polymerase II and then undergo co-transcriptional modifications, including polyadenylation and pre-RNA splicing (16). LncRNAs participate in several biological processes, such as epigenetic regulation, transcriptional and post-transcriptional regulation, processing of small RNAs and other regulatory functions (17-20).

Establishing a widely accepted classification of lncRNAs is a challenging task. There are currently five broad categories of lncRNAs, namely i) sense or ii) antisense, when overlapping one or more exons of another transcript on the same or opposite

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strand, respectively; iii) bidirectional, when the expression of lncRNA and a neighboring coding transcript on the opposite strand is initiated in close genomic proximity; iv) intronic, when the lncRNA is derived from an intron of a splicing transcript; and v) intergenic, when it lies in the genomic interval between two genes (21,22).

To facilitate the search for lncRNAs, the currently available online databases including ncRNAs are listed in Table I. These databases were retrieved from GenBank annotations or from published manuscripts. Some of these databases have been experimentally verified, others are merely computational predictions or have been annotated as ncRNAs according to the predicted length of their open reading frames.

Table I. Public ncRNA databases.

Website	Name
http://biobases.ibch.poznan.pl/ncRNA/	
http://www.noncode.org	Noncode
http://research.imb.uq.edu.au/rnadb	RNAdb
http://www.ncrna.org	fRNA
http://jsm-research.imb.uq.edu.au/nred/cgi-bin/ncrnadb.pl	NRED
http://www.lncrnadb.org	LncRNAdb
http://rfam.sanger.ac.uk	Rfam

ncRNA, non-coding RNA.

3. Dysregulation of lncRNAs in HCC

The number of studies on lncRNAs is soaring unexpectedly (23-25). In this study, we aimed to summarize the potential functions and molecular mechanisms of the most widely investigated lncRNAs in HCC.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). MALAT1, also referred to as nuclear-enriched abundant transcript 2, is a nuclear lincRNA >8,000 nt that is transcribed from chromosome 11q13 (26). The highly preserved level of MALAT1 in numerous species indicates its functional significance (25). MALAT1 has been shown to modulate primary transcripts transcriptionally or post-transcriptionally (27,28).

Lai *et al* (29) reported that MALAT1 was increased *in vitro* and *in vivo*. In addition, analysis of multivariate data demonstrated that MALAT1 level was an independent prognostic factor for HCC recurrence. More importantly, the higher expression of MALAT1 was also associated with shortened patient disease-free survival following liver transplantation. These results suggest that treatment by targeting MALAT1 may be of clinical value by selectively eliminating diffuse or residual cancer cells following surgery. Silencing of MALAT1 by siRNA in HepG2 cells may significantly decrease cell viability, inhibit motility and invasiveness and sensitize cells to multi-stimuli-induced apoptosis.

HOX antisense intergenic RNA (HOTAIR). Another lncRNA implicated in cancer metastasis is referred to as HOTAIR, is encoded in antisense direction from the HOXC gene cluster and acts *in trans* to regulate HOXD genes through interaction and recruitment of the polycomb repressive complex 2 (PRC2) to induce transcriptional silencing (30). Notably, pull-down assays with PRC2 revealed a direct and specific interaction with HOTAIR, which was an unexpected novel finding.

High levels of HOTAIR are mostly correlated with poor patient survival rate and tumor recurrence (31-33). Ishibashi *et al* (34) reported that high expression of HOTAIR was identified in primary HCCs in 13 of 64 patients. Patients with high HOTAIR expression exhibited significantly poorer prognoses and a larger primary tumor size compared to those with low HOTAIR expression. Furthermore, application of human HOTAIR to liver cancer cells demonstrated that HOTAIR induced a more rapid proliferation when compared to control samples.

Geng et al (35) demonstrated that knockdown of HOTAIR in the Bel7402 HCC cell line led to the reduction of matrix metalloproteinase-9 and vascular endothelial growth factor proteins, which are critical for cell motility and metastasis. Their study also revealed that there was a obvious association between HOTAIR level and lymph node metastasis. Thus, HOTAIR lincRNA may be a potential biomarker for the presence of lymph node metastasis in HCC. Yang *et al* (36) reported that the HOTAIR level in HCC was higher compared to that in adjacent non-cancerous tissues. Additionally, a high level of HOTAIR was an independent prognostic marker for predicting HCC recurrence in liver transplantation patients. Furthermore, patients with a higher level of HOTAIR exhibited significantly shorter recurrence-free survival. Similar to breast cancer, the inhibition of HOTAIR by siRNA in a liver cancer cell line decreased cell viability and invasion, sensitized cancer cells to tumor necrosis factor α-induced apoptosis and improved the sensitivity of cancer cells to cisplatin and doxorubicin.

H19. Oncofetal H19 had been extensively investigated in cancer biology, even before research was focused on lncRNA (37,38). H19 is a paternally imprinted gene which locates at chromosome 11p15.5. H19 is highly expressed during embryonic development, but is repressed right after birth in most tissues (39,40). H19 is highly expressed during tumorigenesis and was shown to possess tumorigenic properties in all types of tissues (37, 41-43).

Matouk *et al* (44) demonstrated that H19 was excessively produced in a human HCC, even more than the traditional HCC marker α-fetoprotein. Therefore, an approach based on H19 knokdown may be an efficient method of targeting tumor cells. The results of that study suggested that HCC tumors transfected with H19 siRNA exhibited significant inhibition of tumor growth and, in certain cases, there was complete inhibition of tumor formation. There was a 82% decrease of mean tumor weights and mean tumor volumes in the two transfected cell lines. By contrast, Zhang *et al* (45) demonstrated that H19 was downregulated in intratumoral HCC tissues (T) when compared to peritumoral tissues (L). In addition, the Kaplan-Meier analysis revealed that HCC patients with low T/L ratios exhibited a worse prognosis compared to those with high ratios. H19 inhibited HCC metastasis and epithelial-to-mesenchymal transition. More

importantly, H19 stimulated the miR-200 family by inducing histone acetylation, thus inhibiting the rate of tumor metastasis in advanced-stage HCC. The results of that study provided novel insights to the mechanism underlying the modulation of the level of small ncRNAs by lncRNAs. Yoshimizu *et al* (46) demonstrated that H19 regulated the timing of appearance of SV40-induced hepatocellular carcinoma using *in vivo* murine models of tumorigenesis. Therefore, it was concluded that H19 exerts a tumor suppressor effect in mice.

In summary, the above contradictory results indicate that the precise functions of H19 remain to be further investigated.

Maternally expressed gene 3 (MEG3). Another extensively investigated lncRNA is MEG3, which locates on chromosome 14q32. MEG3 belongs to the DLK1-MEG3 imprinting locus, containing at least three paternal protein-coding genes and abundant maternal ncRNAs (47,48). The gene expression in this locus is firmly regulated by at least two differentially methylated regions (DMRs), namely the intergenic DMR and the MEG3-DMR (49).

MEG3 plays a crucial role in cell development and growth (50,51). In humans, MEG3 is highly expressed in normal tissues and downregulation of MEG3 has been identified in a number of human tumors (50,52). Additionally, upregulation of MEG3 inhibits *in vitro* tumor cell proliferation. Consequently, it was concluded that MEG3 is a tumor suppressor gene (53).

Braconi *et al* (54) reported that MEG3 was decreased by 210-fold in HCC tumor tissues compared to non-malignant hepatocytes. Enforced expression of MEG3 in HCC cells notably inhibited cell growth and increased apoptosis. Furthermore, hypermethylation of MEG3 promoter was detected by methylation-specific PCR and the level of MEG3 was increased with siRNA treatment of DNA methyltransferase (DNMT) 1 and 3b. More importantly, miR-29, which is able to regulate DNMT 1 and 3, increased the level of MEG3. These findings highlighted the interrelationship between two classes of non-coding RNAs, namely miRNAs and lncRNAs, and the epigenetic modulation of gene expression.

In addition, Anwar *et al* (55) suggested that the DLK1-MEG3 locus was continually deregulated in HCC. Knockdown of DNMT1 in HCC cells resulted in a reduction of MEG3-DMR methylation and a subsequent increase in the MEG3 level.

Microvascular invasion in HCC (MVIH). MVIH is located in the intron of the RPS24 gene and encodes a protein that belongs to the S24E family of ribosomal proteins (56). Despite the location of MVIH within the RPS24 gene, these two genes are transcribed separately, indicating that MVIH may play an independent role in biological behavior (57).

Yuan *et al* (58) reported an lncRNA termed MVIH that was increased in tumor tissues compared to the corresponding non-cancerous tissues and was associated with microvascular invasion of HCC. Furthermore, MVIH was considered to be an independent risk factor predictive of poor recurrence-free survival. The authors of that study also observed that MVIH was able to boost tumor growth and intrahepatic metastasis *in vivo*. The suppression of phosphoglycerate kinase 1 secretion by MVIH resulted in activation of angiogenesis *in vitro* and *in vivo*.

Highly upregulated in liver cancer (HULC). HULC is a~500-nt lncRNA located on chromosome 6p24.3 (59). Compared to non-neoplastic liver tissues, high expression of HULC was observed in HCC tissues (60). Xie *et al* (61) found that HULC lncRNA was detected with higher frequency in the plasma of HCC patients when compared to healthy controls. Higher HULC detection rates were also observed in the plasma of patients with higher Edmondson grades or with hepatitis B virus (HBV)-positive status. These findings suggested for the first time that the level of HULC in the plasma may be used as a novel, non-invasive biomarker for the diagnosis and/or prognosis of HCC. Interestingly, in other tumors, no obvious difference was observed in the expression of HULC between the tumor and normal tissues (62). In addition, high level of HULC was detected in metastatic liver nodules from colon cancer, but not in the primary colorectal carcinoma specimen and corresponding normal tissues. High level of HULC was also detected in liver metastases but not in lymph nodes, demonstrating its specificity to any malignant cells located in the liver (62).

Wang *et al* (63) provided evidence that HULC was a self-amplifying, auto-regulatory loop through inhibition of miRNA-372. There was a binding site of cAMP response element-binding protein (CREB) in the proximal promoter region of HULC (from -67 to -53 nt). Activation of the protein kinase A pathway was implicated in the upregulation of HULC. HULC may function as an endogenous 'sponge', downregulating the expression and activity of miR-372. Suppression of miR-372 resulted in reducing the translational inhibition of its target gene, cAMP-dependent protein kinase catalytic subunit beta, which in turn increased the phosphorylation of CREB. This regulatory loop demonstrates the latent interactions between lncRNAs and miRNAs and increases the complexity of the gene regulation network.

Liu *et al* (64) indicated that the variant genotypes of rs7763881 were significantly correlated with reduced HCC risk in a dominant genetic model. However, there was no significant association between single-nucleotide polymorphisms and HBV clearance. Therefore, the authors concluded that the variant genotypes of rs7763881 in HULC may contribute to the decreased susceptibility to HCC in HBV chronic carriers.

Du *et al* (65) revealed that HULC participated in HBx-mediated HCC. The expression of HULC was positively associated with the level of HBx in clinical HCC samples. Moreover, the luciferase reporter gene assay and chromatin immunoprecipitation assay indicated that HBx improved the activation of the HULC promoter via CREB. Finally, it was confirmed that the upregulation of HULC by HBx increased the proliferation of HCC cells through inhibiting p18. Taken together, these findings provide novel insight into the roles of lncRNAs in HBx-related HCC.

Other lncRNAs in HCC. Recent studies revealed that several other lncRNAs are dysregulated in HCC. Huang *et al* (66) reported that downregulated expression by HBx (termed lncRNA-Dreh) was able to decrease HCC growth and function as a tumor suppressor in the progression of HBV-related HCC. It was further observed that lncRNA-Dreh altered the normal cytoskeleton structure to suppress tumor metastasis by targeting the intermediate filament protein vimentin.

In addition, lncRNA-Dreh was significantly associated with poor prognosis of HCC. In summary, these results may provide a strategy for the development of lncRNA-based targeted methods for the treatment of HBV-related HCC. Yang *et al* (67) revealed that the level of lncRNA high expression in HCC (termed lncRNA-HEIH) in HBV-related HCC was markedly associated with recurrence and was an independent prognostic biomarker for survival. The authors of that study also manifested that lncRNA-HEIH played a pivotal role in G0/G1 arrest. It was further demonstrated that lncRNA-HEIH was correlated with enhancer of zeste homolog 2 (EZH2) and that this correlation was required for the inhibition of EZH2 target genes. Taken together, these results reveal that lncRNA-HEIH is an oncogenic lncRNA that promotes tumor development and, thus, it may act as a critical regulatory factor in HCC progression. Yang et al (68) indicated that uc002mbe.2 exhibited the most notable changes among these differentially expressed lncRNAs in HCC cell lines upon trichostatin A (TSA) treatment. Moreover, the TSA-induced uc002mbe.2 expression was positively associated with the apoptotic effect of TSA on HCC cells. Furthermore, a reduction in the level of uc002mbe.2 significantly decreased TSA-induced apoptosis of Huh7 cells, consequently unmasking that the TSA-induced apoptosis of HCC cells is uc002mbe.2-dependent and the low expression of uc002mbe.2 may be associated with liver carcinogenesis.

4. Conclusions

High-throughput sequencing has helped demonstrate the dysregulation of numerous lncRNAs in HCC. However, our current knowledge of lncRNAs compared to miRNAs is limited. Further investigation is required to elucidate the biological functions and molecular characteristics of lncRNAs in HCC.

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