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LncRNAs in liver cancer: what we know in 2014.

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Abstract

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Abstract

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer with an estimated over half million people diagnosed annually. Due to the difficulty in early diagnosis and lack of effective treatment options, the 5-year survival rate of HCC remains between 6 and 11%. The identification of better diagnostic and prognostic biomarkers, along with new therapeutic agents is of paramount importance. Long non-coding RNAs (lncRNAs) are abundantly transcribed, participating in nearly every single step of genomic regulation ranging from transcription, post-transcription to chromatin modification. While lncRNAs such as XIST and H19 were discovered in early 90's, the investigation of lncRNAs at a functional level has remained a fledgling area until recently. Currently, it is estimated that over 30000 lncRNAs are detectable across the human genome. Furthermore, a well organised intrinsic network between lncRNAs and other ncRNAs such as microRNAs has been suggested. Dysregulation of lncRNAs has been found in a range of cancers including HCC. Well-characterised lncRNAs have been shown to be specifically expressed in HCC and suggested as an independent risk factor for poor prognosis. Moreover, it was discovered that the regulation of lncRNAs increased the sensitivity of HCC patients to chemo-therapy. This review focuses on the recent findings of lncRNAs in HCC development and progression, with particular attention on epigenetic regulation, as a central molecular mechanism in hepatocarcinogenesis. In addition, in silico analysis was conducted to demonstrate potential intrinsic linkage between lncRNAs and microRNAs in HCC pathogenesis. While lncRNAs were previously considered as "background noise" in human genomics, their investigation may represent a new milestone in the understanding of HCC providing highly sought after novel diagnostic and therapeutic management approaches for this lethal condition.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer with an estimated over half million people diagnosed annually [1]. While extensive efforts have been carried out in both research and clinical management, mortality rates remain the third highest for neoplastic death in the world. This is mainly due to difficulty in early diagnosis and lack of effective treatment options. Patients that present at clinics with HCC are usually at an advanced stage of their disease. This results in limited suitability for liver resection and transplantation. Therefore identifying new diagnostic and prognostic biomarkers, along with therapeutic agents for HCC is of paramount importance.

With the development of new biological technologies such as deep sequencing and DNA tiling arrays, biomedical scientists are able to investigate gene expression in great detail unravelling the pathogenesis of human diseases. Subsets of the transcriptome that are actively transcribed, however lack protein coding ability are collectively termed as non-coding RNAs [2]. MicroRNAs are a group of evolutionarily conserved non-coding RNAs (19-24 nucleotides) that regulate target genes at a post-transcriptional level, competitively binding to the 3' untranslated regions (3'UTRs) of messenger RNAs (mRNAs). This biological process leads to either the inhibition of mRNA translation or the destruction of mRNA [3]. In fact, microRNAs are now considered to be key players essential in cellular processes such as proliferation, embryogenesis, and apoptosis. They are also robust biomarkers in cancer research with a utility in diagnostics, prognostics and therapeutic intervention. The role of microRNAs as diagnostic, prognostic and therapeutic agents in HCC has been reviewed elsewhere [4]. Long non-coding RNAs (LncRNAs) offer many of the same properties with respect to being stable biomarkers, capable of withstanding multiple freeze thaw cycles and regulating multiple genes; nonetheless, compared to microRNAs the study of LncRNAs is in its infancy.

2. Long non-coding RNAs

While LncRNAs such as XIST and H19 were discovered in early 1990, detailed investigations of their expression and function have only recently emerged [5,6]. Due to their similarity to other non-coding RNAs, no distinct definition can specifically include all biochemical features that are associated with LncRNAs. In order to distinguish LncRNAs from other ncRNAs, LncRNAs are commonly separated from small ncRNAs such as microRNAs at a cut-off point of 200 nucleotides in length. Most LncRNAs are transcribed by RNA polymerase II followed by general RNA processes including 5' terminal methylguanosine capping, splicing and 3' end polyadenylation[7,8]. It has been suggested that over 30000 LncRNAs can be detected across the human genome

(Arraystar Human LncRNA Array v3.0) [9]. While previously considered “background noise” within the transcriptome, recently an *in silico* computational analysis revealed that lncRNAs are actively involved in cell differentiation, immune cell activation and differentiation [10]. In addition, mounting evidence has demonstrated that they are pivotal gene regulators at various levels, and their dysregulation is closely related to human diseases including cancer [11,12]. For example dysregulation of the lncRNAs BCAR4, PCA3 and HULC are associated with breast cancer, prostate adenoma and HCC, respectively [13-15]. Whether or not the dysregulation of lncRNAs is the causative factor or merely a symptomatic consequence remains to be clarified.

LncRNAs are diversely expressed across various genes. They regulate their target genes at the transcriptional, post-transcriptional and translational levels [7]. Based on their expression location within a gene, lncRNAs can be further divided into sense/antisense (same/complementary sequence on the same or opposite strand of a transcript), bidirectional (located at the opposite strand near the transcription initiation site of a transcript), intronic (embedded totally within an intron of a transcript) and intergenic lncRNAs (located between the genomic interval of two transcripts) [16,17]. In recent years, increased attention has revealed that the biological functions of lncRNAs range from X chromosome activation and genomic imprinting (Xist and H19, respectively) to gene regulation, cell cycle regulation, protein scaffolding and chromatin modification. Nevertheless, further research is still required in order to fully elucidate their functions.

LncRNAs can act as an antisense strand with complementary sequences to their target genes. This results in their target gene inhibition or reduction in activity (**Figure 1**). For example, lncRNA HULC may downregulate microRNA372 and the tumour suppressor gene p18 leading to the promotion of HCC cell proliferation [18,19]. LncRNAs can also act as a protein scaffold either recruiting histone modification proteins to influence expression of their target genes or competitively binding to essential proteins initiating conformational change that can activate or inhibit expression of target genes. Recently, a study using photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) revealed that lncRNAs interact with microRNAs, suggesting an intrinsic crosstalk exists between ncRNAs in the development and progression of cancer [20]. In fact, accumulating evidence has demonstrated dysregulation of microRNAs by lncRNAs and *vice versa* in hepatocarcinogenesis [18,21,22].

While lncRNAs are often less conserved across species, their tissue and cell specificity make them excellent potential candidates in cancer diagnosis and prognosis. Recent evidence indicated that lncRNAs may be used as potential prognostic biomarkers associated with therapeutic response in cancers [23-25]. In this context, there is no doubt that the importance of lncRNAs will revolutionise the traditional concepts of the transcriptome and the pathogenesis of liver cancer.

3. LncRNAs and HCC

While there is an increasing interest in lncRNAs, to date only a handful has been investigated in HCC. Herein we review the current knowledge regarding lncRNAs in the development and progression of HCC.

Highly up-regulated in liver cancer (HULC)

HULC was the first hepatocyte specific lncRNA identified in 2007 [26]. The expression of HULC is upregulated 33-fold in human HCC tissue compared to normal liver tissues, whereas the level of HULC in other tumorigenic tissues such as lung, colon and prostate is very low in comparison. Interestingly, HULC is only slightly upregulated in cirrhotic liver tissue, suggesting its specificity in hepatocarcinogenesis. In fact, HULC is reported to be elevated not only in HCC, but also during intrahepatic metastasis [27].

Hepatitis B (HBV) and hepatitis C (HCV) still remain the predominant etiological factors in the onset and development of HCC. However, the molecular processes involved in the conversion of an infectious liver disease to cancer remains to be fully elucidated. The HBV X protein (HBx) has been reported to elevate HULC expression in HBV patients with or without the presence of a liver tumour (**Table 1**). This is consistent with *in vitro* studies whereby HBx was shown to increase HULC expression levels both in normal liver cells (L-O2) and liver cancer cells (HepG2) (**Table 2**). These data point towards an important role for HBx in the onset and development of HCC [19]. The mechanism involves HULC-mediated downregulation of p18 (a regulator of cell cycle and tumour suppressor gene p53) giving rise to increased proliferation of HCC cells. Overexpression of HBx was shown to increase the activity of the HULC promoter region, which is critical for transcription initiation. It has been demonstrated that a cyclic-AMP responsive element binding protein (CREB) binding site is located at the core promoter of the HULC gene and its inhibition can dramatically reduce promoter activity, thus influencing transcription initiation [18]. Mounting evidence has illustrated that a close relationship exists between CREB and HBV in the pathogenesis of HBV-HCC [28,29]. Therefore, HBx may induce the expression of HULC *via* CREB.

Cumulative evidence has pointed towards a closely related interaction between lncRNAs and microRNAs [20]. It has been illustrated that HULC has a conserved binding site for microRNA372, thus suggesting that it can mediate an antisense effect on microRNA372 (miR-372). Such an event could dramatically influence the activity of miR-372 and its target genes, for example cAMP-dependent protein kinase catalytic subunit beta (PRKACB). Once activated by protein kinase A (PKA), PRKACB translocates to the nucleus further activating CREB by phosphorylation [18]. Coincidentally, phosphorylated CREB is significantly elevated in a rat model of HCC [30].

Importantly, in liver cancer cells the HULC promoter is more sensitive to histone modification than normal hepatocytes; this may in turn result in the aberrant expression of HULC and its target genes including miR-372. Recently, HULC was shown to be destabilised by a specific member of IGF2 mRNA-binding proteins; this study may provide a novel insight for the targeting of HULC in HCC therapeutics [31].

Hox transcript antisense intergenic RNA (HOTAIR)

HOTAIR is a lncRNA located on chromosome 12q13.13. HOTAIR has been particularly studied as an oncogene involved in the promotion of metastasis by means of chromatin modification events and is also a promising biomarker that can indicate poor prognosis in breast cancer and colorectal carcinoma [32,23]. Notably, a higher expression level of HOTAIR has been strongly correlated with liver metastasis in colorectal cancer patients. Multivariate analysis estimates that colorectal carcinoma patients with a high HOTAIR expression level have an increased relative risk in overall survival (RR = 5.62, $p = 0.008$). In fact, it has been recently demonstrated that HOTAIR expression in HCC tumours was significantly higher than in the adjacent non-tumour tissues, especially in HCC patients with lymph node metastasis (**Table 1**) [33,34]. The mechanistic role of HOTAIR in HCC remains to be unravelled. However, it has been shown that HOTAIR may positively regulate the expression levels of multiple metastatic genes including vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9). This may partly explain its high expression in HCC patients with lymph node metastasis and its essential role in tumour migration and invasion (**Table 2**).

HOTAIR is a well-known protein scaffolding lncRNA binding to the polycomb repressive complex 2 (PRC2) and lysine-specific demethylase 1 (LSD1) via its 5' and 3' regions respectively. This can also result in the recruitment of enzymes involved in histone modification, leading to the repression of essential tumour suppressor genes [12]. It was found that the inhibition of HOTAIR in liver cancer cells (BEL7402 and HepG2) can significantly reduce cell proliferation and sensitise cells to treatment with standard chemotherapy drugs such as Cisplatin and Doxorubicin in a dose dependent manner (**Table 2**). Moreover, HOTAIR has also been reported as an independent biomarker for HCC recurrence-free survival after liver resection or transplantation, a finding that is consistent with other studies on breast and colorectal cancer (**Table 3**). In view of these findings, HOTAIR is likely to represent an important functional and prognostic factor in liver cancer.

H19

The H19 gene is located in chromosome 11p15.5 that encodes a lncRNA that has a pivotal role in human embryogenesis. H19 was identified as having a role in cancer tumour suppression using embryonal tumours [35]. It has been reported that the expression level of H19 is normally at a low level during adulthood. However, it has been shown to be aberrantly up-regulated in liver cancer, particularly HBV-induced HCC [36]. The mechanism underlying this aberrant expression was demonstrated to be due to a gain of function (a process of converting the silenced copy of the gene to an actively expressed gene). This can lead to a shift from monoallelic expression to biallelic expression leading to the upregulation of H19 in HCC. This imbalance between H19 and its closely related paternally imprinted gene, insulin-like growth factor 2 (IGF2) was proposed to have a role in HCC development and progression in late 90's [37]. *In vitro* the expression of H19 has been shown to be elevated in several liver cancer cell lines under various conditions, especially hypoxia [38]. Moreover, H19 knockout CD-1 nude mice subcutaneously implanted with Hep3B liver cancer cells have an 82% reduction in both mean tumour volume and weight, revealing the essential role that H19 plays in HCC tumour development (**Table 2**). However, a recent study reported that H19 was generally elevated in non-tumour tissue of less than 2 centimetres apart from the HCC tumour tissue site compared with the intratumoral section [22]. Furthermore, a low ratio of H19 expression in intratumoral tissue versus non-tumour tissue was associated with invasiveness and decreased disease-free survival (**Table 3**). Taken together, these data point towards roles for H19 in HCC development and metastasis. While the mechanism by which H19 contributes to the pathogenesis of HCC is still unclear, the results from several recent studies certainly suggest that H19 is important in HCC onset and progression. Many lncRNA genes are located in the same chromosome cluster with other important cancer related genes. The cell cycle inhibitor P57^{Kip2} is located in the same cluster as H19 and was demonstrated to be downregulated in HCC tissue compared with normal liver tissue. This suggests that H19 may promote HCC *via* directly disrupting specific cell cycle processes [38]. Moreover, H19 can be aberrantly regulated by the oncogene, c-Myc, through either histone acetylation or hyper/hypo-methylation in differentially methylated regions (DMR) during hepatocarcinogenesis. These data clearly illustrate a pivotal mechanistic role for epigenetics.

Maternally expressed gene 3 (MEG3)

The MEG3 gene is located at the DLK1-MEG3 locus of chromosome 14q32.3. MEG3 functions as a tumour suppressor gene and a regulator of other well-known tumour suppressor genes including p53 and its target genes. A recent study observed that MEG3 was down-regulated in 81% of HCC human samples (**Table 1**) [21]. HCC cells

transfected with MEG3 had decreased anchorage-dependent growth, elevated retention between G0/G1 phases in the cell cycle process and induction of cell apoptosis (**Table 2**). Thus MEG3 may interfere with the cell cycle process by directly upregulating cell cycle regulators including p21. The expression of MEG3 is often lost in many cancer tissues as a result of gene deletion, hypermethylation of its promoter or hypermethylation of DMR [39,40]. Concomitantly, the expression of MEG3 has been demonstrated to be increased in HCC cells (HepG2 and Huh7) treated with the methylation inhibitor (5-Aza-dc) and the inhibition of DNA methyltransferase (DNMT1 and DNMT3B) (**Table 2**).

High expression in HCC (HEIH)

HEIH is a newly identified lncRNA suggested to be central in hepatocarcinogenesis. The expression of HEIH was recently shown to be significantly elevated in HCC patients, especially with liver cirrhosis (**Table 1**) [41]. In addition, HEIH was found to be highly associated with HCC recurrence and an independent prognostic factor in overall patient survival (**Table 3**). Concurrently, data from *in vitro* studies reveal that the basal expression of HEIH is higher in liver cancer cell lines (HepG2 and Huh7) and that targeting HEIH for inhibition reduces overall cell proliferation. Interestingly, the expression of HEIH in HCC has been demonstrated not to be affected by either histone acetylation or DNA methylation, indicating that not all lncRNAs are epigenetically regulated in tumorigenesis [41]. lncRNAs can act as an intermediate protein scaffold, recruiting functional enzymatic proteins. This may lead to the upregulation or downregulation of target genes. It has been shown that the interaction of HEIH with the enhancer of zeste homolog 2 (EZH2) (an essential subunit of PRC2 complex) was required to down-regulate central cell cycle regulators such as p21 in HCC. Importantly, EZH2 has been demonstrated to have an important role in liver cancer metastasis *via* the negative regulation of microRNAs that have a specific tumour suppressor function, such as microRNA139-5p, microRNA125b, microRNA101, let-7c and microRNA200b [42]. In order to investigate interactions between HEIH and other target genes, studies utilizing the cancer co-expression network revealed that HEIH expressions can be correlated to two lncRNAs and 16 protein-coding genes, some of which are still functionally uncharacterised.

Down-regulated expression by HBx (Dreh)

Over 400 lncRNAs are either up-regulated or down-regulated in HBx transgenic mice [43]. Notably, the lncRNA Dreh was significantly down-regulated in HBx-transgenic mice. *In vitro* studies have shown that liver cells transfected with HBx have lower levels of Dreh, resulting in the induction of cellular proliferation and invasion (**Table 2**) [43]. In

addition, mice injected with liver cells that express Dreh were observed to have a reduced tumour weight and significantly less metastasis. The human form of Dreh (hDreh) (located on chromosome 5) is markedly reduced in HCC patients; its down-regulation is accompanied with decreased overall survival rate. On occasion lncRNAs may interact with proteins to induce conformational changes and deactivate their specific target proteins⁷. In this context it has been demonstrated that Dreh could act as a tumour suppressor gene through its interaction with the filament protein, vimentin. This interaction was noted to induce changes in the cytoskeleton structure of liver hepatoma cells. Vimentin is an essential metastatic marker that is often overexpressed in HCC metastasis [44].

Microvascular invasion in HCC (MVIH)

MVIH is a newly identified lncRNA residing in the same chromosomal region as the ribosomal protein S24, yet independently transcribed [45]. The expression of MVIH is reported to be almost 4-fold elevated in HBV-HCC patients. It has been demonstrated that MVIH expression positively correlates with microvessel invasion, tumour growth and intrahepatic metastasis (**Table 1 and 2**). These data suggest that MVIH may represent an independent prognostic factor, with high levels predicting poorer recurrence-free survival and overall survival in post-operative HCC patients, and be useful in the surveillance of patients (**Table 3**) [45]. It was hypothesized that MVIH may promote angiogenesis by interacting with the anti-angiogenesis protein phosphoglycerate kinase 1 (PGK1) produced by tumour cells [46]. The concentration of PGK1 in conditioned medium from liver cancer cells (Huh7 and HCCLM3) transfected with MVIH was markedly reduced compared with negative controls [45]. Moreover, human umbilical vein endothelial cells (HUVECs) in conditioned medium from liver cancer cells showed an enhanced formation of capillary-like structures, indicating metastatic potential. Mice subcutaneously implanted with liver cancer cells that had been transfected with the MVIH gene had increased microvessel density within the tumour. Importantly, the upregulation of MVIH was reported to be inversely correlated with PGK1 levels in serum and positively associated with microvessel density in HCC patients.

Low expression in tumour (LET)

Recently, the lncRNA LET was reported to be decreased in HCC, colorectal cancers and squamous-cell lung carcinomas, indicating suppression may be associated with intra- or extra-hepatic metastasis [47]. In fact, the level of LET has been shown to be reduced in both HBV-HCC and HCC patients and its low expression correlates with tumour micro-metastasis and encapsulation (**Table 1**). Mice injected with liver cancer

cells overexpressing the LET gene exhibited diminished tumour cell growth, hepatic and abdominal metastasis (**Table 2**). Hypoxia is a pivotal characteristic associated with the tumour microenvironment. In this context, the level of LET is dramatically down-regulated, leading to stabilisation of nuclear factor 90 (NF90) proteins and its target gene hypoxia induced factor (HIF-1 α). LET has a 1:1 stoichiometry with NF90 in normoxic conditions and over-expression of LET can repress NF90. In fact, LET can dramatically reduce the stability of NF90 by up to 66% in liver cancer cells (**Table 2**). It was demonstrated that the down-regulation of LET under hypoxic conditions may be under the control of histone deacetylation. Decreased histone 3 (H3) and histone 4 (H4) were observed in the LET promoter region, but no other areas across LET gene. The elevation of histone deacetylase 3 (HDAC3) under hypoxia was demonstrated to increase the expression of HIF-1 α through the downregulation of LET. Moreover, the suppression of LET was shown to be recovered with the HDAC inhibitor Trichostatin A (TSA) even under hypoxic conditions.

Metastasis associated lung adenocarcinoma transcript 1 (MALAT1)

MALAT1 is an lncRNA located on chromosome 11q13.1 that is widely expressed in many tissues [48]. Chromosomal translocation is a condition where regions within two chromosomes are broken and rearranged in a different way. This abnormality is often observed with the aberrant expression of MALAT1, and is associated with an increased genomic instability leading to its up-regulation in many cancers including HCC [49-51]. While MALAT1 was initially identified as a biomarker for poor prognosis in non-small cell lung cancer, a recent study reported supraphysiological levels of MALAT1 in HCC patients that have undergone liver transplantation that concurrently revealed a higher risk factor for tumour recurrence [52,53]. Inhibition of MALAT1 in a preclinical liver cancer cell line (HepG2) effectively reduced cell viability, motility, invasiveness and increased their sensitivity to apoptosis.

Several more lncRNAs have been newly identified to have crucial roles in HCC. These include: 1) chemotherapy drug associated lncRNA, Adriamycin resistance associated (ARA) in HCC cell proliferation [54]. 2) Ultraconserved lncRNA (ucRNA), TUC339 acting as a messenger within hepatocarcinogenesis microenvironment [55]. 3) Potential biomarker of HCC disease progression and outcome, HOTTIP/HOXA13 [56]. 4) Histone deacetylase inhibitor (Trichostatin A) dependent lncRNA in HCC treatment, uc002mbe.2 [57]. While lncRNAs are pivotal biological components in hepatocarcinogenesis, there is no doubt that their intensive study will give rise to more fruitful outcomes for HCC patients in the coming years.

4. Epigenetic regulation of lncRNAs in HCC

Due to the abundance, diversity and complexity of lncRNAs, the dissection of their role in cancer pathogenesis is an active area of research. In this regard, how lncRNAs function at an epigenetic level has been much studied (recently reviewed in Mercer & Mattick 2013 [7]). lncRNAs are shown to be associated with more than 12 chromatin regulating proteins, targeting genes by methylation and acetylation [58]. lncRNAs that are epigenetically dysregulated in HCC are described in detail in the following section.

Methylation

The well characterised lncRNA, HOTAIR, adversely regulates multiple genes, in particular cell cycle regulators of hepatocarcinogenesis through its interaction with chromatin regulating proteins such as PRC2 and LSD1. This can lead to trimethylation and demethylation of H3K4, H3K27. Both HOTAIR and HEIH have been shown to enhance HCC progression by interacting with the essential component EZH2 in the PRC2 complex [59,41]. Moreover, the expression of MEG3 has been shown to be downregulated in HCC as a consequence of methylation in its promoter region. MEG3 can be upregulated by methylation inhibitor 5-Aza-dc and microRNA29 *via* the inhibition of DNMT1 and DNMT3B [21].

Acetylation

The downregulation of the tumour suppressor lncRNA LET under hypoxic conditions has been suggested to occur as a result of H3 and H4 deacetylation by HDAC3. This was shown to occur across the promoter region of LET and recovered to a normal level with the application of the HDACi TSA, even under hypoxia [47].

Methylation and acetylation

While lncRNAs can regulate their target genes *via* epigenetic modifications in HCC, their aberrant expression has also been demonstrated to occur as a result of abnormal histone modifications (**Figure 2**). Specific locations within the HULC promoter region has been reported to be highly sensitive to acetylation (H3 and H4) and methylation (H3K4) [18]. The interaction between CREB and its binding site at the HULC promoter region was shown to be essential in HCC onset and progression. Furthermore, overexpression of HULC may downregulate miR-372, the negative regulator of CREB. CREB is an essential player bridging Hbx with oncogenes such as HULC and yes-associated protein (YAP) in the development of HBV-HCC [60]. While the overexpression of lncRNA H19 has been demonstrated in HCC, it was noted that H19 may activate miR-200 family members including miR-200a, miR-200b, miR-200c, miR-141 and miR-429 leading to subsequent suppression of genes involved in epithelial-mesenchymal transition (EMT) such as zinc finger E-box binding homeobox 1/2

(ZEB1/2). This activation of miR-200 occurs as a result of an interaction between H19 and the histone modification protein complex hnRNP U/PCAF/RNA PolII *via* the RNA binding member hnRNP U. This lncRNA-protein complex may elevate miR-200 family expression by increased H3 acetylation at the promoter region, causing a marked suppression in the metastatic potential of HCC [22]. In addition, the differentiated methylated region of the lncRNA H19 gene has been reported to be either hyper-methylated or hypo-methylated in HCC [61].

Taken together, these results reveal an essential role for epigenetic regulation in upstream and downstream effects of lncRNA events that have been observed in the pathogenesis of HCC.

In silico analysis of the lncRNAs and microRNAs intrinsic crosstalk in hepatotumorigenesis

Bioinformatic resources have revealed that lncRNAs can interact with not only proteins, but also with RNAs, miRNAs and possibly DNAs [7]. It is noteworthy that several lncRNA database programs including NRED, lncRNAdb database and NONCODE v3.0 have been generated to enable interrogation of the expression, sequences, functions and association of lncRNAs with other molecules. In addition, the newly founded lncRNADisease database can be used to predict diseases associated with lncRNAs and their neighbour genes [11]. We performed *in silico* analysis by using miRcode, a network that searches for potential target sites between miRNAs and lncRNAs in order to illustrate the potential influence of lncRNAs. The numbers of miRNAs associated with lncRNAs including H19, HOTAIR, MALAT1 and MEG3 are between 27 and 150, thus indicating a comprehensive association can occur between them. This computational analysis revealed that microRNAs (miR-130ac/301ab/301b/301b-3p/454/721/4295/3666) are associated with H19, HOTAIR and MEG3 at chromosome 11, 12 and 14 respectively (**Figure 3**). Interestingly, miR-130b has been shown to be overexpressed in HCC cancer stem cells with the CD133 (+) marker [62]. Concomitantly, the miR-130 family has been demonstrated to be upregulated under hypoxic conditions, this in turn elevates HIF-1 α . These data indicate the importance of such miRNA-lncRNA interactions in hepatotumorigenesis (**Figure 3**). There is no doubt that when the miRcode database and other networks are expanded upon, further intrinsic linkages between lncRNA and microRNA will be revealed. These aspects will help in understanding the molecular mechanisms and influences that underscore HCC likely pointing towards better therapeutic strategies and diagnostic tools for this incurable disease.

6. Future challenges and opportunities

LncRNAs are no longer considered backstage participants of biological processes. In fact, their discovery may represent a new era in the understanding of the pathogenesis of several disease states, in particular cancer [63]. Without doubt, the identification of new lncRNAs will continue in parallel with the development of modern scientific and computational technologies. While lncRNAs are abundant and diverse, to date the function of the vast majority of lncRNAs remains unclear. The impact that miRNAs are having in cancer biology is also vast. It has been suggested that there might be a well-organised intrinsic network between lncRNAs and other ncRNAs such as miRNAs [20]. Investigation of such networks may lead to a clearer view of the complex molecular mechanisms in hepatocarcinogenesis and could lead to specific targeted therapies and diagnostics for HCC. Difficulty in early diagnosis and metastasis are central reasons that the mortality rate in HCC remains high. The abundance of lncRNAs suggest they may be ideal biomarkers for liver diseases associated with HCC (Table 3). On the other hand, the combination of using both lncRNAs and miRNAs will likely generate interesting avenues of basic and clinical research. Furthermore, there is strong evidence that the upregulation and downregulation of many lncRNAs are associated with HCC. Therefore, targeting these lncRNAs in combination with other therapeutic agents could have potential for HCC[64].

7. Conclusions

LncRNAs participate in most steps in genomic regulation from transcription, post-transcription to chromatin regulation. While lncRNAs have gained increasing interest in recent years, their behaviour in HCC has led to an increased focus by many international leading laboratories. There is no doubt that the identification of novel lncRNAs and their investigation in HCC will continue at a rapid pace.

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Figure legend

Figure 1: Antisense regulation of lncRNA in HCC

LncRNAs containing complementary sequences to target genes can act as antisense inhibiting their target genes including microRNAs and protein coding genes. For

example, lncRNA HULC may inhibit tumour suppressor genes such as miR-372 and p18 in HCC.

Figure 2: Epigenetic mechanism of lncRNA in HCC

Histone modification of promoter regions in many lncRNA results in their aberrant expression; thus recruiting histone regulation proteins to modify the histone structure of their target genes. For example, interaction between phosphorylated CREB and CREB binding sites near the promoter region of the HULC gene upregulates the expression of HULC. Specific locations within the HULC promoter region are highly sensitive to acetylation (H3 and H4) and methylation (H3K4), this in turn makes HULC promoter region more susceptible to phosphorylated CREB from interaction. In addition, overexpression of dysregulated HULC inhibits CREB repressor miR-372, further exacerbating the onset and progression of HCC (especially in HBV-HCC).

Figure 3: Computation analysis of lncRNA-microRNA interaction in HCC pathogenesis

Utilising the miRcode program, a potential prediction between lncRNAs and microRNA in HCC pathogenesis has been postulated. The dysregulation of lncRNAs H19, HOTAIR and MEG3 may upregulate miR-130 family or vice versa, especially under hypoxic conditions in HCC tumours, in turn elevating HIF-1 α and VEGF to facilitate the development of HCC. Meanwhile, overexpression of the miR-130 family in hepatocytes induces the formation of cancer stem cell, thus initiating hepatocarcinogenesis.

Table 1 Preclinical studies of lncRNAs in HCC

lncRNA	Characteristic and outcome of study	References
HULC	HBx upregulated HULC in both normal liver cells (L-02) and liver cancer cells (HepG2). Silencing HULC reduced tumour size, weight and frequency of formation in mice.	19
HULC	HULC upregulated in liver cancer cells (Hep3B**) and no expression was found in normal liver cells (HL-7702).	18
HOTAIR	Silencing HOTAIR in liver cancer cells (BEL7402) reduced proliferation by 50% (p<0.05) and inhibited growth of cells even after 6 days. Silencing HOTAIR downregulated metastasis genes VEGF and MMP9.	33
HOTAIR	Silencing HOTAIR reduced cell viability in HepG2 after 72 hours. Silencing HOTAIR increased response of HepG2 to apoptosis stimulation from TNF- α and chemo-drug Cisplatin and Doxorubicin on a dose-manner. Silencing HOTAIR decreased invasive behaviour of HepG2.	34
HOTAIR	Introducing HOTAIR into liver cancer cells elevated proliferation above control cells without HOTAIR.	65
H19	H19 upregulated in many liver cancer cell lines. Hypoxia significantly induced H19 expression in Hep3B. Silencing H19 in mice implanted with Hep3B reduced mean tumour volumes and weight by 82%. In one of the experiments, no mice developed tumour in the liver.	38
H19	H19 inhibited invasion capacity of HCC cell line SMMC7721. Silencing H19 upregulated tumour metastasis mesenchymal markers including N-cadherin, Snail1, Vimentin and Twist1. H19 induced microRNA-200 family members in a tumour suppressing manner.	22
MEG3	HCC cells transfected with MEG3 had decreased anchorage-dependent growth, accumulation in G0/G1 phase and increased apoptotic cells. Inhibition of DNA methyltransferase (DNMT1 and 3B) and methylation inhibitor (5-Aza-dc) elevated MEG3 expression in HepG2 and Huh7 cells. Knockout of MIR-29a/b1 in mice significantly reduced GTL2 (mouse form of MEG3).	21
HEIH	The expression of HEIH was significantly higher in HepG2 and Huh7 cells. Knockdown HEIH reduced cell proliferation in HepG2 and Huh7. Silencing HEIH induced cell cycle arrest by upregulating P21, P16 and P27. Knockdown of HEIH in mice inhibited xenografts developments.	41
DREH	DREH was down-regulated in HBx-transgenic mice. Liver cells (BNL-CL2 and Hepa1-6) transfected with HBx had reduced expression of DREH. Silencing DREH induced liver cell proliferation and migration. Mice injected with cells transfected with DREH had significantly reduced tumour weight and metastasis.	43
MVIH	Mice subcutaneously implanted with liver cancer cells (HCCLM3) transfected with MVIH had significantly increased tumour volume and intrahepatic metastasis.	45
LET	Mice injected with hepatoma cell lines (SMMC-7721 and HCCLM3) transfected with LET had reduced xenograft, hepatic invasion and	47

Table 2 Clinical studies of LncRNAs in HCC

LncRNA	Characteristics and outcome of study	References
HULC	mRNA level of HULC positively correlated with HCC tissue (n=33) and HBX-positive non-tumour liver tissue (n=19).	19
HULC	HULC upregulated in all 14 HCC samples compared with adjacent non tumour liver tissues.	18
HOTAIR	48/63 (76%) HCC patients had higher levels of HOTAIR in tumour tissue than adjacent non-tumour tissue (P<0.05). HCC patients with lymph node metastasis had significantly higher expression of HOTAIR (P=0.003).	33
HOTAIR	32/50 (64%) HCC samples had higher HOTAIR expression than adjacent non-tumour tissue. 65.7% HCC samples with high HOTAIR had recurrence of HCC.	34
H19	H19 expression was higher in non-tumour tissue < 2 cm apart from HCC tissue (L) than HCC intratumoral tissue (T) from 33 HCC patients. H19 expression was significantly lower in HCC invasive samples than non-invasive ones from 80 HCC samples. Low H19 T/L ratio was significantly correlated with intrahepatic metastasis (P=0.02).	22
MEG3	Expression of MEG3 was reduced in 81% of HCC samples compared to adjacent cirrhotic tissue. MEG3 staining was intensively positive in non-neoplastic liver compared to HCC tissues.	21,40
HEIH	Transcript levels of HEIH were significantly higher in HCC samples compared to corresponding non-tumour liver tissues (P=0.010). Expression of HEIH was higher in HBV cirrhotic liver than healthy liver (P=0.014). HEIH was highly expressed in HCC patients with cirrhosis (P=0.032). No correlation between HEIH and age, gender, tumour size and HCC stage.	41
hDREH	The transcript levels of hDREH in HBV-HCC patients were significantly down-regulated compared with non-cancerous hepatic tissue from the same patients (P<0.0001).	43
MVIH	MVIH was upregulated 3.654 fold in HBV-HCC patients (P=0.00205). Higher expression of MVIH was associated with increased microvessel invasion (P=0.016) and advanced tumour node metastasis (TNM) stage (P=0.009).	45
LET	The expression of LET was lower in HBV-HCC and HCC tissue compared to normal hepatic tissue from same donor. Low LET expression correlated with tumour micrometastasis and encapsulation.	47

Table 3 Diagnostic and prognostic studies of LncRNAs in HCC

LncRNA	Characteristic of study	Outcome	References
HULC	Diagnostic	HULC expression in blood samples of 3 out of 4 HCC patients were between 10 and 30 times higher than healthy and cirrhotic individuals.	26
HOTAIR	Prognostic	HCC patients with low HOTAIR expression were estimated to have approximately 55% 3-year recurrence-free survival after hepatic resection compared to 22% in patients with low HOTAIR level ($P<0.05$).	33
HOTAIR	Prognostic	HCC patients with low levels of HOTAIR that underwent liver transplantation and resection were estimated to have significantly higher 3-year recurrence-free survival rate than patients with high HOTAIR, especially for the ones who met Milan criteria, who could have 100% survival rate.	34
H19	Prognostic	HCC patients with Low H19 T/L ratio were estimated to have shorter disease-free survival ($P=0.004$).	22
HEIH	Prognostic	HEIH was significantly associated with HCC tumour recurrence after 18 months ($P=0.009$). Multivariate analysis revealed that HEIH was an independent prognostic factor for overall survival ($P=0.014$).	41
hDREH	Prognostic	Kaplan-Meier analysis revealed that HCC patients with low levels of hDREH were markedly correlated with reduced recurrence free survival ($P=0.002$) and overall survival ($P=0.039$).	43
MVIH	Prognostic	HCC patients with high levels of MVIH that underwent hepatectomy were estimated to have poorer recurrence free survival ($P<0.001$) and overall survival ($P=0.007$). Early stage HCC patients with high levels of MIVH who underwent hepatectomy were correlated with shorter recurrence free survival ($P=0.003$) but not overall survival ($P=0.061$).	45

Figure 1

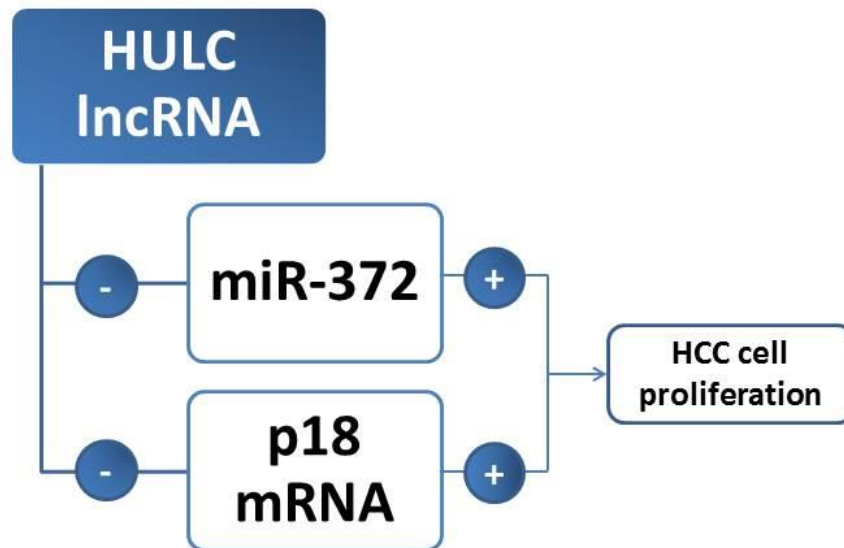


Figure 2

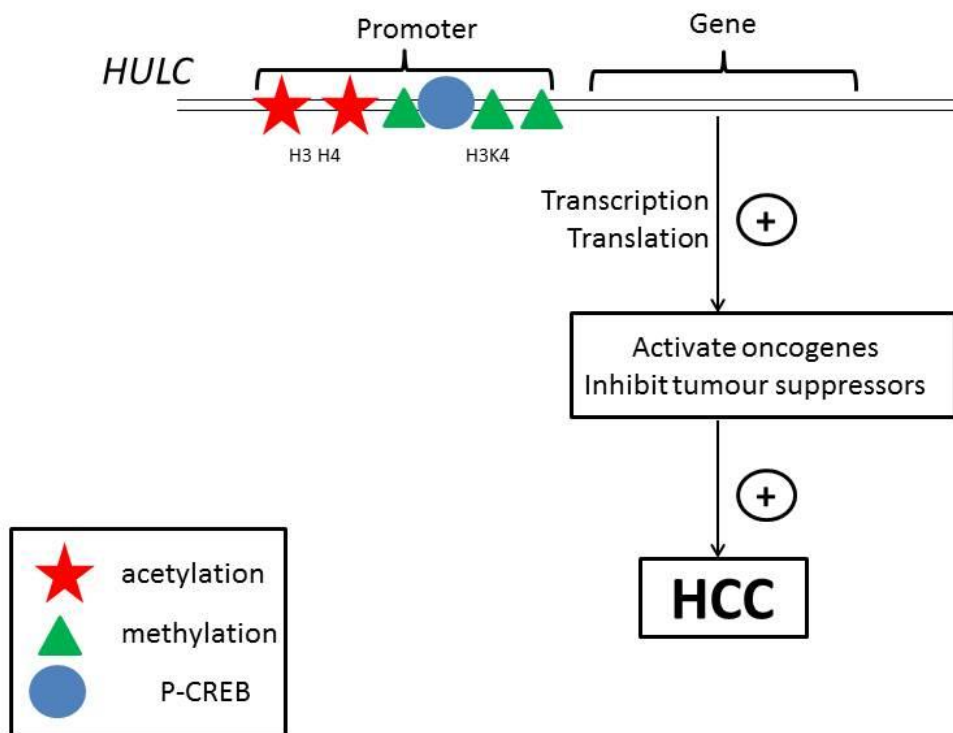


Figure 3

