



Long intergenic non-coding RNAs in hepatocellular carcinoma—a focus on *Linc00176*

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Liver cancer is a common type of cancer that ranks the second in cancer-related mortality world-wide (1). Hepatocellular carcinoma (HCC) is the predominant type of liver cancer and it usually arises on the background of cirrhosis. Risk factors associated with the incidence of HCC include viral hepatitis (hepatitis B and C viruses), the metabolic syndrome including non-alcoholic fatty liver diseases, autoimmune hepatitis as well as aflatoxin-B ingestion (2,3). The multikinase inhibitor, sorafenib, is the only 1st line medical therapy widely available, with its sister drug regorafenib approved for 2nd line use in selected patients (4,5). These drugs have only a limited impact on life expectancy. Transcriptomic changes accompanying cancer development and progression have been extensively explored in patients with HCC, with subgroups created based on transcription profile and mutational status (6-9). These studies to date have not adequately informed selection of candidate drugs for clinical trials, the vast majority of which have failed to show any survival benefit. Although trial design and toxicities have contributed to failures (8), our lack of global understanding of the tumour biology is likely also to have played a role. Recently, rather than sticking to the central transcriptome dogma of DNA-mRNA-protein, researchers have started to appreciate the many other transcripts out of this workflow that are cancer specific and functional, as alternative sources for the identification of novel candidate therapeutic targets. High-throughput sequencing techniques in combination with advanced computational predicting software and

epigenetic tools have identified novel RNA transcripts and are even capable of predicting specific functions. These novel identified transcripts include “long non-coding RNAs (lncRNAs)”, a term generating more than 13,500 hits in a PubMed search at the end of 2017—three quarters of which were published in the last decade. These include a recent article by Tran and colleagues, published in *Oncogene*, in which the lncRNA *Linc00176* has been interrogated in publicly available datasets. It has been reported as an upregulated transcript in HCC that is worthy of pursuit as a biomarker directed target for cancer therapy (10).

The term lncRNA defines a category of RNA transcripts more than 200 nucleotides in length that don't encode for proteins. These features differentiate lncRNAs from short transcripts like miRNAs, tRNAs or snoRNAs, as well as from mRNAs. lncRNA and mRNA share similarities in the regulation of their transcription and post-transcriptional processing, but lncRNAs are shorter in length and have fewer exons and conserved primary structures compared to protein coding transcripts (11). Integration of the human RNA transcriptome with advanced bioinformatics analysis has indicated that over 60% of transcripts in the so called “MiTranscriptome” of human long polyadenylated RNA transcripts are lncRNAs (12). Located within the intergenic regions and expressed at lower levels than protein coding RNAs, unsupervised clustering of the differentially expressed transcripts has identified the existence of distinct tissue-specific signatures of lncRNAs. Moreover, lncRNAs are differentially expressed between

cancer and normal tissue within the same organ (12). In the liver, RNA sequencing of 60 primary HCC in Chinese patients, matched with tissue from portal vein tumour thrombosis (PVTT) and adjacent non-tumour liver, has identified a deregulated pool of lncRNAs in the primary and metastatic tumours (13). Remarkably, more than 75% of sequenced lncRNAs identified in HCC had not been previously annotated in either the MiTranscriptome (12) or the GENCODE transcriptome (14). In this cohort of Chinese patients with HCC, DNA methylation and copy number variation (CNV) were associated with deregulated lncRNAs pool and the lncRNA transcripts appeared to have regulatory roles in cellular functions, such as the immune response and cell adhesion. The tissue and cancer specificity of lncRNAs, alongside key regulatory roles, implies importance in cancer pathophysiology and highlights their potential as novel cancer-specific targets.

The transcription/export (TREX) mRNA export complex is a master regulator of mRNA biogenesis, being involved in different steps of mRNA transcription, processing and export. The mammalian TREX complex includes THOC1 (hHpr1), THOC2, THOC7, THOC5 (FMIP), THOC6, THOC3 (hTEX1), Uap56, DDX39c and Aly (15). The Hannover research group led by Teruko Tamura has previously shown that THOC5 null mice have drastically reduced numbers of hematopoietic system and myeloid progenitor cells, without any effect in adult kidney, heart and liver (16). The group hypothesised that THOC5 was essential for the maintenance of stem cells/progenitor cells but not for the terminally differentiated cells like hepatocytes. The same group demonstrated elevated levels of THOC5 in HCC tissues and cell lines, alongside an induction of apoptosis and cell cycle deregulation *in vitro*, after THOC5 knock down (17). Hypothesising that THOC5 target genes were important in liver cancer cell survival, Tamura's team went on to study one target gene in particular—namely the lncRNA 00176 (*Linc00176*).

Linc00176 (NR_027686.1) is located on chromosome 20 and its HCC transcript is reported to have 4 exons and be 5,264 nucleotides long. In the HepG2 HCC cell line an alternatively spliced *Linc00176* transcript, lacking exon 1, 1,601 nucleotides from exon 2 as well as 962 nucleotides from exon 4, has been identified (18). Apart from this information, little was known about the role of *Linc00176* in HCC. Tran *et al.* (10) have now explored HCC patients data available from The Cancer Genome Atlas (TCGA) and the ENCODE consortium (18). *Linc00176* alternatively spliced transcripts were present in HepG2, Hep3B, Huh7,

HLE and HLF HCC cell lines, with HepG2 cells showing the highest expression levels. *Linc00176* expression wasn't evident in any normal human tissues (including liver, pancreas, lung, skin, brain, adipose tissue, muscle, heart and bone marrow) or other malignancies (leukaemia, melanoma, breast cancer, neuroblastoma, rhabdomyosarcoma and cervical human cancer cell lines) (10). In human primary HCC data in TCGA, high versus low levels of *Linc00176* expression was significantly associated with both poorly differentiated tumour grade and poorer patient survival.

Studying *Linc00176* putative promoter region (500 nucleotides upstream of the initiation site), Tran *et al.* have integrated human hepatocyte and HepG2 cells DNase-Seq, RNA-Seq, ChIP seq and cap analysis of gene expression (CAGE) data (18) and applied PROMO (ALGGEN, program predicting transcription factor binding sites) software. The team focused on a number of candidate regulatory transcription factors (Myc, MAZ and AP-4). Myc was reported to preferentially regulate the transcription machinery of lncRNAs (19) and Tran *et al.* have expanded this observation *in vitro* by showing that Myc/AP-4 double knock down synergistically depleted *Linc00176* expression (10). Functionally, depletion of *Linc00176* from Huh7 and HepG2 abolished their proliferation and converted them to TUNEL-positive cells after 2 days compared to control cells. Unlike THOC5 depletion-induced apoptosis (17), *Linc00176* reportedly exerts its role through necroptosis via mixed lineage kinase-like (MLKL) (10). The bioinformatics ingenuity pathway analysis (IPA) performed on the genes differentially expressed in HepG2 cells after knock down of *Linc00176* supported this finding, ranking cancer and necrosis as the top deregulated pathways.

Having identified upstream regulators of *Linc00176*, as well as the impact of its knockdown, Tran *et al.* considered the three main mechanisms by which lncRNAs bring about their regulatory functions (20). Some lncRNAs act as physical scaffolds forming flexible macromolecular complex with nuclear matrix, chromatin regulatory and DNA methylation proteins to control the chromatin state. Other lncRNAs recruit chromatin regulatory machinery to specific DNA loci either through their affinity to some regulatory proteins or through 3D proximity-guided localisation to certain gene loci. Alternatively, many lncRNAs shape the nuclear structure in a way that encourage/discourage gene expression. Tran *et al.* went on to demonstrate that *Linc00176* binds to two anti-tumour, Myc-regulated miRNAs, namely miRNA-9 and miRNA-185 (10). Depleting the concentrations of these miRNAs diminished

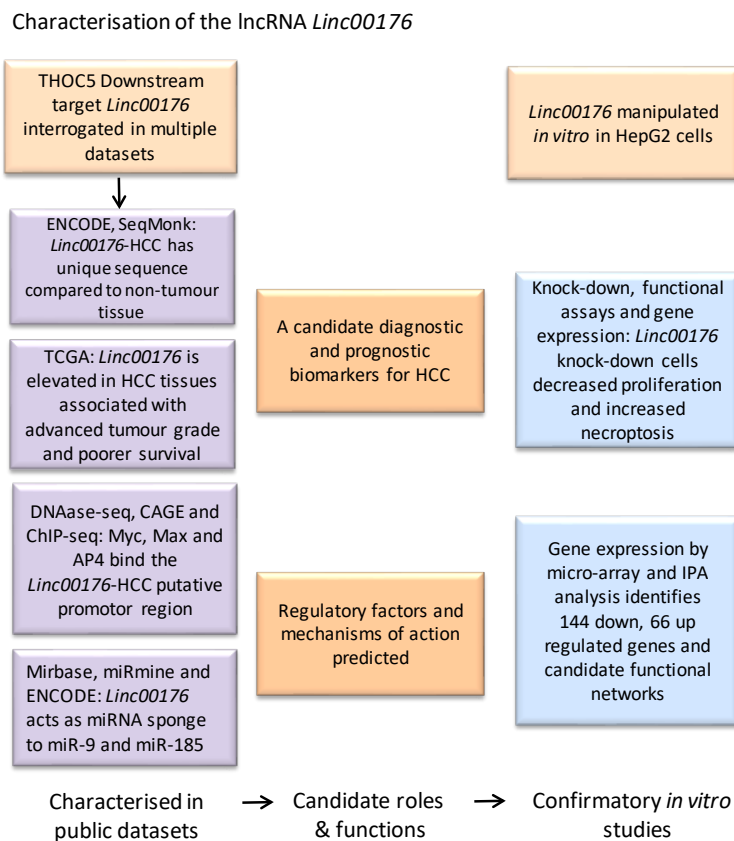


Figure 1 Characterisation of *Linc00176* in hepatocellular carcinoma: flow-chart summarising the bioinformatics, functional and mechanistic analyses of *Linc00176* performed by Tran *et al.*

their anti-proliferative effects on the tumour cells. Moreover, depletion of these two miRNAs using specific inhibitors rescued the proliferation-prohibited effect of *Linc00176* knock down in tumour cell lines.

This work by Tamas' group (10) exploits publicly available datasets, combined with confirmatory and exploratory laboratory studies, in a fashion that brings lncRNAs to the heart of the HCC field. Not only are these transcripts highly specific for HCC, suggesting potential roles in HCC specific diagnosis, their functional roles also raise the potential for intervention to either prevent or treat cancers. *Linc00176* joins other lncRNAs, like previously reported RP11-166D19.1 (13), as a candidate diagnostic biomarker. In addition, this study suggests that targeting of *Linc00176* would not only selectively inhibit the proliferation of tumour cells, it would also favour the development of an anti-tumour niche by increasing the availability of tumour-limiting miRNAs like miRNA-9 and miRNA-185. The development of diagnostic and monitoring biomarker assays, measuring *Linc00176* and its targets in tissues, alongside

the means to target its functional effects, may be worthy of consideration for patients with HCC. There may be other lncRNAs, or combinations, which have relevance to different patients, possibly with cancers arising in different etiological backgrounds. This is a landmark study not just because it highlights the potential importance of *Linc00176*, but because it leads the way in HCC, demonstrating the translational value of systematic interrogation and integration of publicly available data (Figure 1).

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