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EVALUATION OF SNORNA LEVELS IN HCC: A STUDY EXPLAINING THE ROLE PLAYED BY SNORA74D IN HCC DEVELOPMENT.

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Abbreviations

HCC: Hepatocellular Carcinoma

SnoRNA: small nucleolar RNA

NAFLD: Non- alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

AFP: Alpha-fetoprotein

sdRNA: snoRNA derived RNA

ScaRNA: Cajal body specific RNA

SNARE: N-ethylmaleimide –sensitive factor attachment protein receptor

lncRNA: long non-coding RNA

ASO: Antisense oligo nucleotide

CRC: Colorectal cancer

TGIRT-seq: Thermostable Group II Intron Reverse Transcriptase RNA sequencing

BrdU: 5-bromo-2'-deoxyuridine

Abstract

Among non-coding genes small nucleolar RNA are recently getting attention in the field of prognosis and diagnosis of cancer including the Hepatocellular Carcinoma. The biological roles of snoRNA in HCC are, but no limited to, involvement in initiation, proliferation, metastasis, apoptosis, and cell cycle. Here in this study we are focusing on getting insight into the relationship between snoRNA and HCC. To this end we received sequenced tissue samples from patients with NASH and HCC. After performing initial bioinformatics analysis we found potential set of candidate snoRNAs i.e. SNORA74D, SNORA80D, and SNORA5C which were differentially expressed. We further verified these potential targets with qPCR analysis and found only SNORA74D to be significantly upregulated in HCC samples in comparison to NASH. In order to determine the mechanistic role we depleted HepG2 cell line from SNORA74D and found its potential role in cell survival and proliferation. TGIRT sequencing was also performed on SNORA74D depleted HepG2 samples to determine the prospective downstream effectors and regulators. Multiple protein coding genes and noncoding RNA are significantly dysregulated by knockdown of SNORA74D. Of note, we also characterized and discovered the presence of different isoforms of SNORA74D both in tissue samples and cell lines. The presence of different type of isoforms in cancer tissues compared with normal tissues further suggest potential involvement of SNORA74D in cancer development and progression.

Introduction

Hepatocellular carcinoma:

Primary liver cancer ranks as the sixth most prevalent malignancy globally and stands as the third leading cause of cancer related deaths making up around 906,000 new cases and 830,000 fatalities in 2020 [1]. According to the estimations over 1 million people will be affected by liver cancer annually. This would pose a significant health challenge and societal burden. The predominantly prevalent histological subtype of primary liver cancer is Hepatocellular carcinoma also known as HCC. Around 90% of the total primary liver cancer cases are attributed to HCC whereas around 10-15% of the cases account for Cholangiocarcinoma also known as CCA [2]. Population-based interventions indicate that the rate of liver cancer proliferation remains closely aligned with mortality, implying that a significant proportion of individuals diagnosed with HCC succumb to it [3].

HCC is a significant health concern usually attributed to excessive alcohol consumption, chronic infection with hepatitis C virus or hepatitis B virus and non-alcoholic fatty liver disease also abbreviated as NAFLD [4]. NAFLD could span from simple steatosis to non-alcoholic steatohepatitis also known as (NASH). Additional risk factors involved in advancement of the disease include exposure to aflatoxins in case of HCC and inflammation [5]

A significant challenge is faced by researchers in developing a better diagnosis and treatment because of complex heterogeneity of liver cancer [5]. The European Association for the Study of Liver (EASL) along with several other organizations such as National Comprehensive Cancer Network (NCCN) and American Association for the study of liver disease (AASLD), have published a set of

guidelines to standardize the assessment and treatment approaches. Similarly to other cancers, early diagnosis of HCC leads to a better prognosis and this could be achieved by vigilant monitoring of individuals with high risk. This would involve patients who are carriers of hepatitis B virus and individuals with cirrhosis due to any underlying cause. These high risk individuals are encouraged to receive AFP screening and ultrasounds every six to twelve months. Increase in AFP and presence of a hepatic nodule larger than a 1cm is considered fit to further evaluation. According to available data for US, the five year survival rate of individuals diagnosed with HCC have seen a slight improvement of 26%. This has been achieved by closely monitoring the high risk patients such as those with hepatitis B or C virus infection, along with clinical interventions for patients diagnosed with HCC at an early stage [3].

Traditional treatment methods for HCC include trans-arterial chemoembolization, resection of liver, liver transplant and local ablation with radiofrequency. Some emerging new therapeutic strategies for HCC such as the use of immune checkpoint inhibitors, monoclonal antibodies and tyrosine kinase inhibitors are seen contributing to the patient survival significantly [2]. However, despite these substantial advancements the overall efficacy of treatments remains less than satisfactory. For successfully combating the disease it is necessary to better understand it and develop methodologies for its efficient diagnosis and treatment.

SnoRNAs:

Small nucleolar RNA also abbreviated as snoRNA date back to the initial years of RNA study. But they were ignored by researchers around the globe for the most part of the history until it was revealed by the genome sequencing analysis that the total numbers of snoRNAs in humans are way beyond what was initially expected. The

term snoRNA was coined in 1982 following a number of foundation studies by many researchers including some prominent cancer researchers i.e. Bob Weinberg. More than 2000 snoRNA have been annotated in human genome. SnoRNAs represent a distinct category of small and stable non-coding RNAs, typically ranging from 60 to 200 nucleotides and are present within nucleolus [6]. The initial discovery of snoRNA rightfully pointed out the probable function of snoRNA within the nucleolus since it was their discovery site. Traditionally snoRNAs are recognized for their role in guiding the chemical modifications of ribosomal RNAs, transfer RNAs and also small nuclear RNAs [7, 8]. They were reported to do post transcriptional modification of ribosomal RNA (rRNA) by forming robust complexes with the ribonucleoproteins. However, the sheer number of known snoRNA and better understanding the functional diversity of non-coding RNA have pushed researchers into digging deep into investigating other possible functions of snoRNA within the cell. The current studies suggest the potential involvement of snoRNA in the nucleus and even the cytoplasm.

In recent years with the advancements in high throughput sequencing of RNA, several differentially expressed snoRNAs involved in various disorders were identified. SnoRNAs due to this have gained a lot of attention of researchers and hence have been established by now as significant contributors for maintaining normal physiological function. They are also been found to be significant regulators of immune system that suggest their impact on human health and diseases i.e. cancer biology. Their impact on carcinogenesis can be attributed to single snoRNA or a group of snoRNA working together to assert a significant affect. [9]. Since, it is becoming increasingly evident that snoRNA have broader biological functions through non- canonical pathways then recognized previously [10] further research

into snoRNA can uncover a novel avenue of tumorigenesis and therefore provide additional insight into diagnosis and treatment of HCC.

The structure and biogenesis of SnoRNA:

SnoDB is a database for snoRNA. According to this database, a total of 2064 snoRNAs have been identified. snoRNAs contain highly conserved sequences and specific secondary structures [6]. Originally this term was only used for those molecules that were strictly present in nucleolus however in subsequent years it has evolved to also contain group of other related RNA structures that operate outside the nucleolus [11].

Traditionally these can be divided into two main types depending on the common sequences, structure and the presence of the type of core proteins. [12]. But with the advancement in the discovery methods, numerous other variants have been identified therefore, the class snoRNA should now be considered to include a spectrum of molecules that contain these motifs. For example a specie of long non-coding RNAs (lcRNA) that use the C/D box motif for stabilization as an alternative to 5' cap and polyadenylation was found by researchers [13-15]. Furthermore, it has been growingly reported that the snoRNAs can be subjected to further processing in order to produce snoRNA derived RNA (sdRNAs), sometimes trimmed down to only the C/D box structures[16], or trimmed down to the size of microRNA (miRNA) and piwi-interacting RNA (piRNA) [17, 18].

That being said snoRNAs can be categorized majorly into two types: Box C/D snoRNA known as SNORD and box H/ACA snoRNA (SNORA). The SNORD family contains a conserved sequence box C (RUGAUGA) and box D (CUGA). SNORD makes a kink-turn motif that helps in guiding the modification of the target RNA by forming a snoRNP complex with NOP56, NOP58, Snu13 and fibrillarin

proteins. Whereas, on the other hand the H/ACA box snoRNA contain an H box (ANANNA) and a three nucleotide ACA box making a hairpin structure. H/ACA snoRNA carry out pseudouridine modification at the target positions. H/ACA box snoRNA form a snoRNP complex with other types of core proteins i.e. dyskerin, Nhp2, NOP10 and Gar1[19].

A specific subset of snoRNA is also named as Cajal body specific RNA (scaRNA) due to their subcellular localization (nucleus), presence of distinct C/D and H/ACA box and corresponding structure and functions.

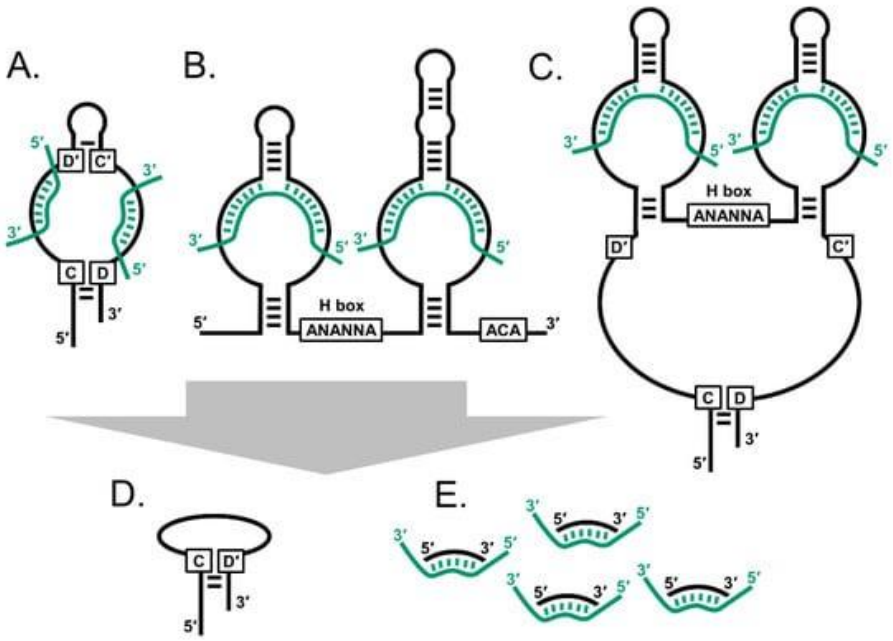

Figure 1:

SnoRNAs can be of three types (A) C/D box (B) H/ACA box (C) Small Cajal body-specific RNAs known as scaRNAs. SnoRNA derived specie include (D) (C/D) box

like snoRNAs and (E) snoRNA derived RNAs (sdRNAs). “Black strands: snoRNA; green strands: rRNA, spliceosomal snRNA and mRNA” [11]

SnoRNAs are usually transcribed from intron regions of the protein coding genes or may be present in the introns of the snoRNA host gene that are either protein coding or code for larger lncRNA. Some examples of such host genes include GAS5 (growth arrest specific transcript 5), small nucleolar host gene 1-32 and ZNFX1 antisense RNA 1 also abbreviated as ZFAS1.Special selective splicing helps in the production of these snoRNAs but there is a small group of snoRNAs that are also derived from independent transcripts [20].

Functions of SnoRNAs

SnoRNAs typically cause modification of rRNAs, tRNAs and small nuclear RNAs. SnoRNAs with specific sequences that align with the target RNA in the antisense orientation are called guide snoRNA whereas, other snoRNA who lack any apparent complementarity with the target RNAs are termed as orphan snoRNA. Orphan snoRNA account for 17% of the total snoRNA [19]. Although the orphan snoRNA are not able to guide modification at specific target sites but they can still target potential RNA and proteins targets indicating that they still might have some regulatory functions in non-canonical pathways [21].

In addition to RNA modification, snoRNAs are also involved in complex biological processes. For example studies have demonstrated their involvement in opening of chromatin, telomerase synthesis and processing of pre rRNA and pre mRNA [22, 23]. Moreover, as mentioned above snoRNAs can also serve as precursors for miRNAs, snoRNA derived RNAs and piwi-interacting RNAs.

Over the years gradually the role of snoRNA in cancer has been explored and their role as oncogenic or tumor suppressor regulators has been unfolded recently. In myeloid leukemogenesis, snoRNA can disrupt the protein translation process by causing ribosomal RNA modifications [24]. Additionally a subset of snoRNAs binds and activates poly (ADP-ribose) polymerases -1, leading to subsequent induction of DNA damage repair, ribosomal DNA transcription and biogenesis of ribosomes, thereby promoting tumorigenesis. Moreover, there have been many studies who have identified snoRNAs to be present as a significant component in the upstream or downstream of multiple tumor signaling pathways that ultimately govern the fate of the cell. For instance, in breast cancer, many upregulated snoRNAs can serve as oncogenic regulators by binding to fibrillarin and blocking P53 activation for example SNORD50A and SNORD50B act as Oncogenes, and their deletions result in release of GMPs and their translocation into nucleus which then can recruit U7 ultimately binding to p53 causing a decrease in ubiquitination of p53, thereby inhibiting tumor progression [25, 26]. The overexpression of SNORA71A in lung cancer stimulates epithelial – mesenchymal transition (EMT) by modulating the MAPK/ERK pathway. In ovarian cancer, ectopic expression of SNORA72 activates stem cell transformation through the Notch1/c-Myc pathway [27]. Furthermore, snoRNAs also have the potential to act as downstream molecules of p53 and MYC pathway, contributing to the tumorigenesis. Additionally, a group of snoRNA derived RNAs (sdRNAs) are linked to certain characteristics of tumor-immune microenvironment and help in cancer progression by functioning similarly to microRNAs [28].

1. snoRNAs as regulator of rRNA maturation:

   SNORAs and SNORDs are known to be typically residing in nucleolus where they make complexes with RNPs and modify rRNAs. On the other hand

scaRNAs typically are known to localize in cajal bodies where presumably they modify U1-U6 [29].

Speaking about the functionality, C/D box is required by the SNORDs to form a hairpin loop and attach to the complementary sequence in rRNA, interacting with RNPs to cause post transcriptional methylation at the target site on rRNA. Similarly H/ACA box snoRNA make complex with their respective RNPs resulting in pseudouridylation at the respective target site of rRNA. Recently in an effort to consolidate the record for total number of known snoRNA it was determined that at least 246 SNORAs, 461 SNORDS and 21 scaRNAs existed in the human genome. Moreover, due to gene duplication around 2064 separate snoRNA genes are thought to be present [11, 30]. Due to this huge number it is safe to assume that there are many more snoRNA than required to modify known targets of rRNA implying that they would have functions other than ribosome modifications.

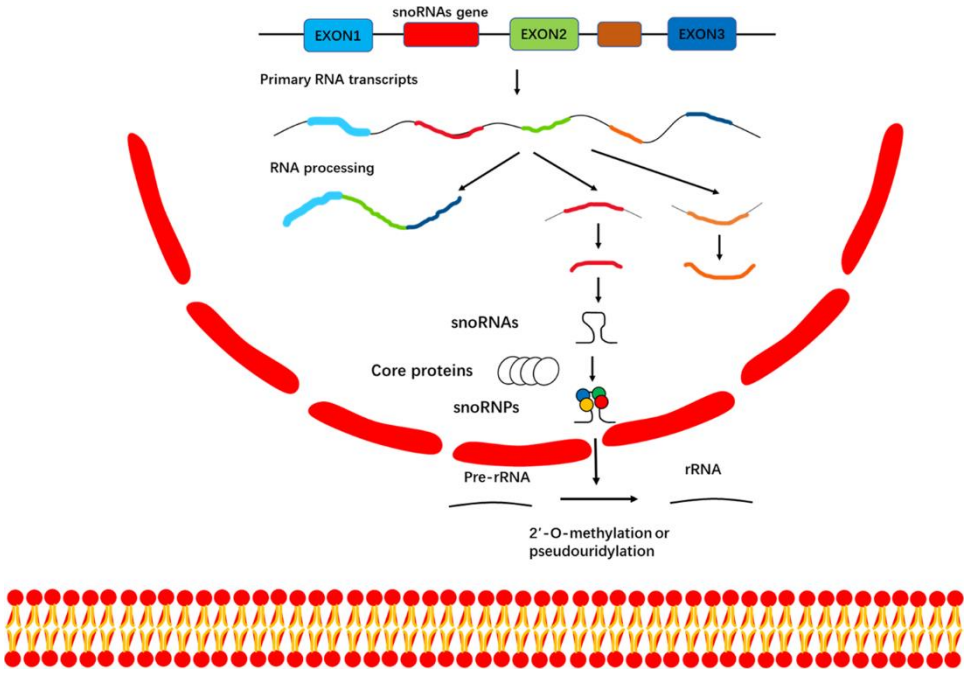

Figure 2:

SnoRNAs encoded in the introns are transcribed by RNA polymerase II. They bind with ribonuceloproteins to form snoRNPs. The structure of rRNA is modified to induce stability in it through 2'-O-methylation and pseudouridylation [31].

2. Functions of snoRNAs beyond ribosomes:

Multiple studies focusing on cancer biology, chromatin studies and human developmental genetics, suggest the evidence of snoRNA being involved in doing more than modifying nuclear RNA. Some genetic conditions like Angelman syndrome and parder-Willi syndrome have been key topics for study in human genetics. The reason being that they although result from the same deletion at the same imprinted locus but they manifest differently depending on the parent of origin. Turns out this remarkable difference is because of snoRNA itself. As with conditions caused by deletions in the genome, initial attempts were made to identify the region critical for this disease [32]. Initial studies suggested that multiple genes might be involved in contributing to this disease however, as further research reduced the critical altered region for PWS and AS, the focus of researchers shifted towards SNHG14. SnoRNA host gene 14 occupies a sub locus called as SNURF-SNURPN. This region encodes for 29 copies of SNORD116 and 47 copies of SNORD115 and a certain number of single copy SNORDs i.e. SNORD64, SNORD108, SNORD109a and SNORD109b [33]. Several mechanistic studies pointed out at first that SNORD115 family was responsible for the disease but later a group of researchers examined patients with small deletions

and concluded that it was actually the loss of SNORD116 family that was the critical event in manifesting this genetic disease [23, 34]. The mouse models were generated to test this observation further. Although the phenotypes generated by the loss of SNORD116 were subtle but changes were observed in diet hormones and cognitive defects [35, 36]. Whereas, the similar model made with deletion of SNORD115 was less affected by the deletion [37]. Another study later was able to achieve a better change in phenotype (body weight) by specifically targeting the deletion of SNORD116 to the hypothalamus [38].

Despite the SNORDs being known for causing PWS and AS, the exact mechanism of it remains unclear particularly due to their lack of complementarity to rRNAs. However, early work on SNORD115 suggested its potential to alter splicing of mRNA that encodes for 5-hydroxytryptamine receptor 2C [37]. Moreover, some molecular studies with knockout of SNORD116 in mice indicated an altered DNA methylation pattern in neuronal genes during diurnal cycle [39]. Hence, the evolving understanding of these SNORDs indicate their involvement in functions outside the nucleolus. Similar observations have been reported for orphan snoRNAs with implications for cancer.

Immunoprecipitation studies performed by Schubert et al. identified specific RNA specie involved in compaction of chromatin in drosophila cells. Many snoRNAs were among the detected molecules [40]. Another SNORD i.e. SNORD27 has been reported by Falaleeva et al. to have a functional role within a novel complex. It is reported to compete with U1 snRNPs for altering the splicing of mRNA that encodes for E2F7 (E2F Transcription factor 7). E2F7 has been stated to be involved in retinal cancers and both head and neck

cancers [41]. Interestingly, another SNORD i.e. SNORD88C was found to also similarly cause alteration in mRNA splicing for fibroblast growth factor 3 also known as FGFR3. This alteration contributes to cancer progression among various cancer types [42]. Notably, the alteration mechanism involves shorter sdRNAs derived from SNORD88C, suggesting that this could be one of the ways by which snoRNAs extend their impact. A group of researchers (Ender et al.) analyzed small RNAs associated with immunoprecipitated human AGO1 (Argonaute RISC Component 1). AGO1 is part of a RISC complex that plays role in RNA silencing. Although the majority of specie they identified in their observation were miRNA, a fraction of them were derived from snoRNAs [43]. An unbiased sequencing of human small RNAs was performed by Kawaji et al. They also showed multiple classes of 20-40nt specie originating from noncoding RNA that includes snoRNAs [17]. This observation was further verified by other studies too [18]. Kalantari et al. quite recently demonstrated the presence of RISC complex both inside and outside of nucleus, this could potentially allow sdRNAs to forms complexes within nucleus and the cytoplasm [44].

Additionally, cytoplasmic mRNA in addition to nuclear RNA are also now recognized to have methylation as a frequent form of modification. Numerous reports state its function to be regulatory in nature [45, 46]. One such noteworthy type of methylation is adenosine N6-methylation (m6A). It is catalyzed by the methyltransferase-like protein 3 and 14 (METTL3/METTL14). Certain localization studies located METTL3 in nucleolus along with FBL, where they were validated to help in co-modification of rRNA [47]. Another type of mRNA methylation is known as ribose 2'-O-methylation (2'OMe). Effect of snoRNA on 2'OME was studied

by Elliot et al. They used a knockout mouse model of SNORD50A for this purpose. They observed if this altered 2'MOE modification of mRNA for peroxidasin. Their study concluded that the methylation was indeed altered thereby affecting the stability and translation of the mRNA [48]. All the above mentioned studies collectively demonstrate that snoRNAs may participate in a broad spectrum of molecular functions beyond their involvement in ribosome modification.

SnoRNAs and Cancer

Early evidence for role of snoRNA in cancer biology came from several resources. Individuals with dyskeratosis congenita face an elevated cancer risk due to mutations in genes associated with snoRNA functions in nucleolus and Cajal bodies, including DKC1, which plays a role in pseudouridylation [49]. Host gene called as SNHG5 harbors SNORD50. It is mapped to a chromosome breakpoint which is involved in human B-cell lymphomas. Later, it was also observed to be frequently deleted in prostate cancers [50]. In non-small cell lung cancers (NSCLC), Liao et al. used GeneChipR oligo arrays to prove the oncogenic role of SNORA42 [51, 52]. Separate other researches indicated the contribution of SNORD78 to the disease. [53] In a subclass of T cell lymphomas, Valleron et al. employed RT-qPCR to analyze a panel of 80 different snoRNAs, revealing a significant association between SNORD71 and the outcome of the disease [54].

With the increasing availability of genome wide expression analysis and RNA-seq technologies, it has been used by several researchers to study snoRNA in cancer. Schulten et al. conducted a gene expression analysis in breast cancer metastasis in the brain and compared it to non- metastatic breast cancer by making use of microarrays. Among the differentially expressed genes, 13 were protein coding, 6

were SNORAs and 1 was observed to be scaRNA, indicating a strong support for the link of snoRNA with metastatic progression of cancer [55]. Gao et al. analyzed 178 non-small lung cancers (NSCLC) using a combination of RNA-Seq and RT-PCR, detecting a total of 458 snoRNAs, with 29 showing increased expression in cancer tissues when compared to normal tissues. A separate testing of cohorts allowed them to develop a prognosis predictor based on three snoRNAs i.e. SNORA47, SNORA68 and SNORA78 [56]. In another study Crea et al. performed RNA-Seq analysis on paired primary and metastatic prostate cancers. The study resulted in identification of 21 snoRNAs that were found to have correlation with metastasis particularly highlighting SNORA55 as being a probable driver gene [57].

In a pioneering study of pan-can analysis, Gong et al. used the bioinformatics approach to mine for small RNA in the Cancer Genome Atlas (TCGA) database covering 31 human cancer types. They were able to identify around 400 to 500 snoRNAs expressing in each cancer type. Almost all of these snoRNA mostly demonstrated an increased expression level in comparison to normal tissue. Among this list of snoRNA 203 of them had association with clinical stage of the disease, 355 had association with patient survival and this included 229 SNORDs, 108 SNORAs and 18 scaRNAs [58]. Later, Chow et al. performed a similar pan-caner analysis of TCGA data, focusing on small sdRNAs and observed a clear relationship with the clinical outcome across cancers [59]. Another study performed by Uzunova et al. used a combination of RNA-Seq and RT-PCR in a cohort of 106 patients with prostate cancer to reveal that sdRNAs derived from SNORD44, SNORD81, SNORD78 and SNORD74 to be upregulated. Among these, SNORD78 was demonstrated to be associated with metastatic disease [60]. In conclusion it can be stated that snoRNA hold a significant prognostic value and are very likely to be

active contributors of cancer progression and metastasis. The following table contains few known snoRNAs involved in Cancer:

snoRNAs	Class	Expression level	Function	Cancer	Intersection molecules and/or pathway
SONRA42/MBI-43	H/ACA box	Increased	OG	NSCLC, CRC	P53
SNORD78/U78	C/D box	Increased	OG	NSCLC, PCa	
U50/SNORD50A/RNU50	C/D box	Decreased	TS	BRCA, PCa	Ras-ERK1/ERK2
U3/SNORD3@	C/D box	Increased	OG	BRCA	p53
U8/SNORD118/LCC	C/D box	Increased	OG	BRCA	p53
RNU44/SNORD44/U44	C/D box	Decreased	TS	BRCA	
RNU43/SNORD43/U43	C/D box	Decreased	TS	BRCA	
SNORD48/U48/RNU48	C/D box	Decreased	TS	BRCA	
SNORD29/U29/RNU29	C/D box		OG	BRCA	YB-1
SNORD34/U34/RNU34	C/D box		OG	BRCA	YB-1
SNORD67/HBII-166	C/D box		OG	BRCA	YB-1
SNORD33/U33/RNU33	C/D box		OG	BRCA	YB-1
ACA44/SNORA44	H/ACA box		OG	BRCA	YB-1
SNORD114-1/14q(II-1)	C/D box	Increased	OG	APL	Rb/p16
SNORD112-114	C/D box	Increased	OG	APL	
SNORD35A/RNU35/RNU35A/U35	C/D box	Increased	OG	AML	AES and DDX21
SNORD74/U74/Z18	C/D box	Increased	OG	AML	AES and DDX21
SNORD14D	C/D box	Increased	OG	AML	AES and DDX21
SNORD43/RNU43/U43	C/D box	Increased	OG	AML	AES and DDX21
SNORA21/ACA21	H/ACA box	Increased	OG	CRC	
SNORA15/ACA15A	H/ACA box	Increased	OG	CRC	
SNORA41/ACA41A	H/ACA box	Increased	OG	CRC	
SNORD33/U33/RNU33	C/D box	Decreased	TS	CRC	
SNORA18L5	H/ACA box	Increased	OG	HCC	stress-RPs-MDM2-P53
SNORD126/MIR1201/MIRN1201	C/D box	Increased	OG	HCC	PI3K–AKT
ACA11/SCARNA22	H/ACA box	Increased	OG	HCC, MM	PI3K–AKT, RP genes
SNORD76/U76	C/D box	Increased /Decreased	OG/TS	HCC,GBM	Wnt/-catenin pathway
SNORA47/HBI-115	H/ACA box	Increased	OG	HCC	
SNORD113-1/14q(I-1)	C/D box	Decreased	TS	HCC	ERK1/2 and SMAD2/3
SNORA55/ACA55	H/ACA box	Increased	OG	PCa	
SNORD59A/U59/RNU59	C/D box	Decreased	TS	PCa	
SNORD82/RNU82/U82/Z25	C/D box	Decreased	TS	PCa	
SNORD116/HBII-85	C/D box	Decreased	TS	PCa	
SNORD117/U83	C/D box	Decreased	TS	PCa	
SNORD24/RNU24/U24	C/D box	Decreased	OG	ACC	miRNAs
SNORA74B/U19-2	H/ACA box	Decreased	TS	GBC	AKT/mTOR
SNORD47/RNU47/U47	C/D box	Decreased	TS	GBM	
SNORD94/U94	C/D box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORA70/DXS648E/RNU70/U70	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORD10/mgU6-77	C/D box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORA13/ACA13	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORA38/ACA38	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORA79/ACA65A	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORA46/ACA46	H/ACA box	Increased	OG	Osteosarcomas	Mutant p53, Est2
SCARNA9/Z32/mgU2-19/30	SCARNA	Increased	OG	Osteosarcomas	Mutant p53, Est2
SNORA23/ACA23	H/ACA box	Increased	OG	PDAC	SYNE2
SNORD35B/RNU35B/U35B	C/D box	Decreased	TS	HNSCC	
SNORD114-10/14q(II-10)	C/D box	Decreased	TS	OC	

Table 1:

The table contains a list of known snoRNA involved in cancer (OG, oncogene; TS, tumor suppressor; BRCA, breast Cancer; PCa, prostate cancer; APL, acute promyelocytic leukemia; MM, multiple myeloma; GBM, glioblastoma; ACC, adenocarcinoma; GBC, gallbladder cancer; PDAC, pancreatic ductal adenocarcinoma; HNSCC, head and neck squamous cell carcinoma; OC, ovarian cancer)[61].

The role of snoRNA in immune response against cancer

Cancer cells are good at avoiding the immune system and this ability of theirs has recently become the central focus in the field of oncology. Certain proteins that shield the non-cancerous cells from immune system are overexpressed on the surface of cancer cells and on immune cells which helps them evade the cytotoxic immune response. Although chemotherapy and radiation therapy are used to treat cancers, some antibody based drugs that target the programmed cell death protein 1 (PD-1), programmed cell death protein ligand 1 (PD-L1) and cytotoxic T lymphocyte associated protein 4 (CTLA-4) have demonstrated to be a superior approach to these therapies for various cancer types [62]. Warner et al. performed a study on acute lymphoid leukemia (ALL), acute myeloid leukemia (AML) together with a selection of normal white blood cell lineages, found a significant difference in expression of snoRNA levels both in cancers and between the lineages from which the cancer originated. Interestingly, the detected signatures were dominated by snoRNA belonging to SNURF-SNURPN and DLK1-DIO3 loci. These dysregulated sites appear to be a dominant feature of blood cancer hence, reiterating that snoRNAs are important regulators of lymphocyte activity [63]. A study performed by Zhong et al. demonstrated that sdRNA derived from SNORD63 may control the stability of Interleukin-4 (IL-4) mRNA [64]. Furthermore, Motzer et al. defined kidney cancers

into molecular subtypes and then measured their response to anti PDL1 therapy. Using the non-hierarchal clustering all the included 823 cancers were grouped into seven subsets. The subset defined by marked snoRNA expression was reported to have a good response rate to anti PDL1 therapy [65]. These findings collectively suggest that snoRNA expression plays crucial role in immune response against cancers, lymphocyte function and the oncogenic mechanism promoting progression to metastatic disease.

SnoRNA Host Genes and Cancer

Considerable interest has been taken by researchers in studying the snoRNA host genes themselves. The expression levels of these host genes have been observed to be upregulated generally in cancers and are often demonstrated to hold prognostic implications. It has been reported that snoRNA host genes retain introns in their mRNA and ship snoRNA sequences into the cytoplasm but further studies on this process are required to fully comprehend the mechanism of action. GAS5, initially cloned in 1988 from cDNA library to represent growth- arrested NIH/3T3 mouse fibroblast, was later recognized as snoRNA host gene. Its expression causes an inhibition of growth of human T lymphocytes in multiple cell lines [66-68]. Another such gene is ZFAS1 which was cloned early and encodes for SNORD12, SNORD12A and SNORD12B. It is expressed in mouse mammary glands and in breast cancers. Knock down of ZFAS1 promoted proliferation in breast cancer cells and was silenced in ductal carcinoma in situ [69]. However, subsequent studies have indicated that ZFAS1 has varying significance in other cancer types also i.e. lung, gastric, liver and colorectal cancers, promoting tumor growth and metastasis. Importantly, multiple studies have suggested the idea that ZFAS1 may act as a sponge or decoy for other miRNA species [70, 71]. This concept is observed as a common theme for many host genes as a group but it nevertheless remains an open

question as to why the expression of snoRNA is linked to miRNA sponging. The expression of another host gene called as SNHG1 has also been associated with worst outcome. It acts as a miRNA sponge promoting epithelial-mesenchymal transition (EMT) [72]. Similarly SNH3 was found to increase cell progression and sponge miRNA in liver, osteosarcoma and colorectal cancers [73]. Furthermore, SNHG5 acts as miRNA sponge in melanomas, leukemia, breast and gastric cancers and promote proliferation and migration. Continuation of this theme was observed with similar findings being reported for SNHG20, SNHG7, SNHG15, SNHG6, SNHG16 and SNHG12 [11].

MEG8 (Maternally expressed 8) is located within another imprinted locus DLK1-DIO3. It is responsible for muscle phenotype in sheep and 2 distinct conditions in humans [74-76]. 9 copies of SNORD113 and 31 copies of SNORD114 are contained in MEG8 thus making it comparable to the SNURF-SNURPN locus in nature. As many other host genes MEG8 is also been found to promote EMT in lung and pancreatic cancer and correlate with poor prognosis in liver cancer [77-79].

When examining the potential contribution of snoRNAs in cancer progression, it's crucial not to underestimate their canonical role in rRNA modification. Ribosome function is essential for cell growth and is upregulated in cancers [80]. mTOR signaling pathway coordinates the growth signals from Ras and other pathways with ribosome control. This is the key driver of lymphocyte expansion making it a significant oncogenic pathway in cancer[81]. 5'TOP mRNA are named such because of the oligopyrimidine tract in their 5'UTRs helping them in recognizing ribosome and moreover, they also encode many ribosomal proteins. An analysis of snoRNA host genes revealed that they too are typically 5'-TOP mRNAs, implying that snoRNAs are regulated by the mTOR pathway [82]. Consistent with this, GAS5 expression in T lymphocyte cell lines altered their sensitivity to rapamycin [83].

When liver cancer cells having deletion of SNORA24 were injected into mice, it resulted in tumors that were in accordance with oncogenic RAasG12V and altered ribosome activity [84]. Consequently, a noticeable pattern emerges, indicating that the expression of snoRNA host genes typically influences cancer progression and metastasis. However, it is important to note here that many studies are based on NGS analysis and therefore focus on only functions of snoRNA host genes and their relevance to cancer. Poly-A sequencing does not allow to see dysregulation in snoRNA along with snoRNA host genes. Therefore, we feel more research is needed to establish a functional link between the snoRNA and their respective snoRNA host genes giving special importance to their biogenesis and role in tumor proliferation. This would help in clarifying the definitive functional roles played by the snoRNA and the respective host genes.

SnoRNA in Hepatocellular carcinoma and associated diseases

Multiple studies over the years have shown that snoRNA are dysregulated in liver cancer. Yang et al. studied The Cancer Genome Database and observed a general upregulation of snoRNAs in 372 HCC and 50 non-tumor tissues. 54 snoRNA were found to be upregulated and 14 snoRNA were downregulated, belonging to the pathways of cell cycle, DNA replication and ribosome function [85]. 2 HCC cohorts from Gene Expression Omnibus database were analyzed by Liang et al. thereby identifying 54 snoRNAs that were differentially expressed [86]. High throughput small RNA sequencing was used for 6 HCC tissue with their corresponding non-cancerous liver tissue by Wang et al. to identify 10 upregulated snoRNAs and 7 down regulated snoRNAs [87].

From an etiological standpoint, liver cancer can be attributed to various diseases, such as chronic infection with HCV and HBV, altered lipid metabolism, alcoholic

and non-alcoholic liver disease. Some of these diseases associated with HCC involve snoRNAs

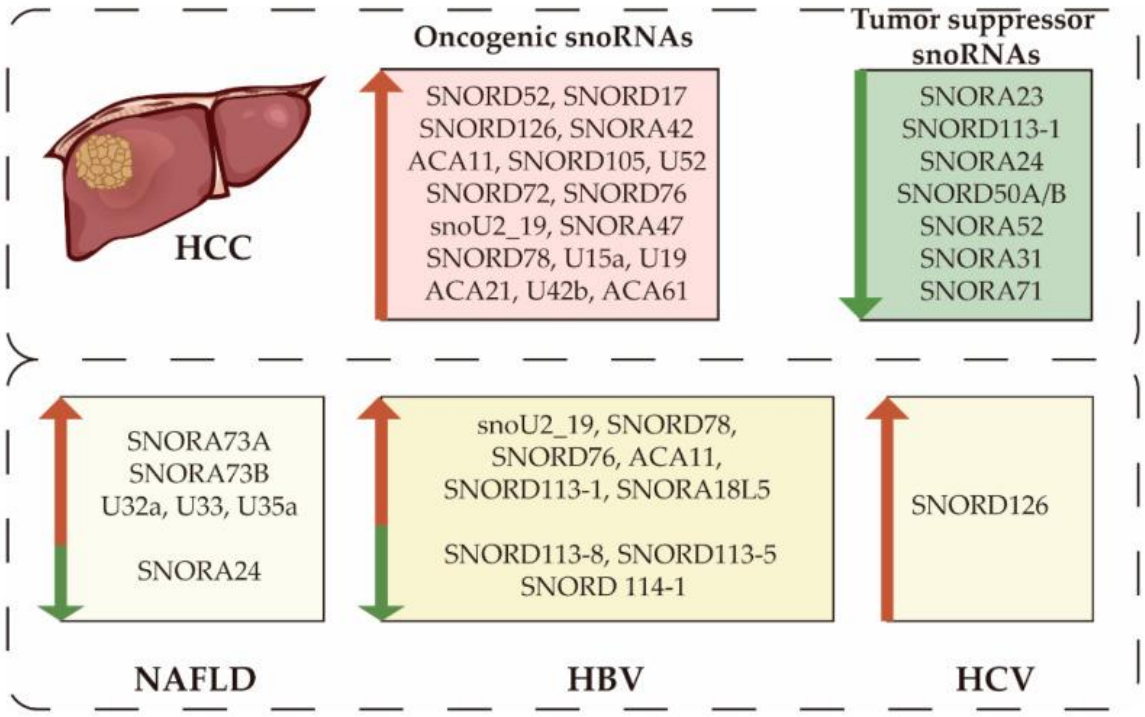

FIGURE 3

The figure shows different snoRNA involved across HCC and the associated diseases [2].

SNORA18L5 duplication is responsible for increased risk of HBV- related HCC and SNORD126 is involved in promoting HCC infection [88, 89]. Other snoRNAs such as U32a, U33 and U35a play a crucial role as mediators in metabolic stress and their deficiency leads to resistance against lipotoxicity [90]

The progression of hepatocellular carcinoma is a long and multi stage process that is usually initiated with a chronic liver injury followed by inflammation and

advances subsequently into cirrhosis, ultimately leading to liver cancer [91]. A study performed by Koduru et al. by accessing the publicly available small RNA sequencing data from the National Institute of Health's short read archive, revealed that 3 snoRNAs i.e. SNORD15-31, SNORD121B and SNORA37 exhibit negative regulation across four distinct liver conditions. The samples in this study included 9 healthy livers, 14 cirrhotic tissues, 9 samples from low and 6 samples from high grade dysplastic nodules and 6 early and 20 advanced HCC tissue. The variety of sample type made sure that the selected snoRNAs have potential involvement in overall trajectory of liver injury. However, no significant differences were observed among the different pathological processes but that could be attributed to overall small sample size [92]. There is definitely a need to conduct more studies at each individual pathological stage of the disease to attain further information.

Dysregulated snoRNAs in HCC and their clinical significance

Despite the current treatment options, HCC has a very high rate of recurrence and mortality. Therefore, it is important to find new biomarkers for early diagnosis, prognosis and tumor classification of HCC. Altered expression of snoRNA can be considered as potential biomarkers to predict recurrence and survival time. For now the methods used for detection of snoRNA include real time PCR, microarrays, RNA sequencing and other diagnostic methods based on PCR or sequencing. Yang et al. identified a set of 9 snoRNAs as independent prognostic factors leading to construction of a prognostic risk model. In this model patients were divided into low and high risk groups. The validation assay suggested that the risk of deaths in HCC patients was higher in patients belonging to high risk groups than the low risk groups. These snoRNA include SNORA7, SNORA24, SNORA63, SNORD19B, hTR, SNORD36C, U3_chr8-2, U3_chr9 and U44 [85]. Zhuang et al. constructed a prognostic model based on 7 snoRNAs for the risk of relapse. The selected snoRNA

list was obtained from analyzing data from 283 HCC patients and adjacent normal tissue [2]. Many studies exploring snoRNA in HCC revealed clinical importance of these molecules. For example SNORA52, SNORA31 and SNORA71 appeared to be downregulated in HCC and exhibited a significant correlation with size of tumor, number of lesions, capsular invasion, stage and degree of tumor. Lower expression levels of these snoRNAs in HCC was linked to shorter disease free survival and reduced overall survival rate [93-95]. Conversely, SNORD76 and SCARNA22 were upregulated in HCC and these elevated levels were linked to HBV infection, BCLC stage, histological grade and portal vein tumor thrombosis [96, 97]. These differentially expressed snoRNA hold potential as prognostic indicators.

Numerous studies have consistently demonstrated the presence of stable snoRNAs in various biological fluids such as cell-free saliva, plasma, serum, urine and tissue samples [98-100]. The potential for snoRNAs as non-invasive liquid biomarkers for diagnostic and prognostic purposes have been affirmed by several investigations. A study found 38 snoRNAs to be enriched in extracellular vesicles of 4 liver cancer cell lines. 9 out of these snoRNAs exhibit a significant elevated expression levels [101]. In a study performed on plasma from patients with HCC it was observed that among the differentially expressed genes, a large portion belonged to snoRNAs [102]. While the abundance and diversity of these snoRNAs did not conclusively establish their uniqueness to HCC, their prevalence suggested potential as diagnostic biomarkers.

Role of snoRNA in liver carcinogenesis

Multiple studies have demonstrated the role of snoRNAs as either a tumor promoter or suppressor in hepatocellular carcinoma.

snoRNA	Chromosomal Location	Host Gene	Role in HCC	Expression	Sample Size, HCC/Control	Targets
SNORD52	6p21.33	SNHG32	Oncogene	Up	80/80	CDK1
SNORD17	20p11.23	SNX5	Oncogene	Up	175/175	NPM1, MYBBP1A
SNORD126	14q11.2	CCNB1IP-1	Oncogene	Up	30/30	hnRNPK
SNORA42	1q22	KHDC4	Oncogene	Up	60/60	P53, p21
ACA11	4p16.3	NSD2	Oncogene	Up	92/92	-
SNORD105	19p13.2	PPAN-P2Rγ11	Oncogene	Up	712/801	PPAN
SNORD72	5p13.1	-	Oncogene	Up	46/46	ID2
SNORD76	1q25.1	GAS5	Oncogene	Up	66/66	Fibronectin, vimentin
snoU2_19	4, 7	-	Oncogene	Up	80/80	β-catenin
SNORA47	5q13.3	ZBED3	Oncogene	Up	60/60	-
SNORA24	4q26	SNHG8	Tumor suppressor	Down	91/91	18S rRNA
SNORD50A SNORD50B	6q14.3	SNHG5	Tumor suppressor	Down	-	K-Ras
SNORD113-1	14q32.31	MEG8	Tumor suppressor	Down	112/112	ERK1/2, SMAD2/3
SNORA23	11p15.4	IP07	Tumor suppressor	Down	-	28S rRNA

Table 2

The table contains a list of known snoRNAs involved in HCC [2].

It has also been verified that snoRNA have the potential to affect certain pathophysiological processes including initiation and progression of tumor, drug resistance and metastasis [84, 86, 104].

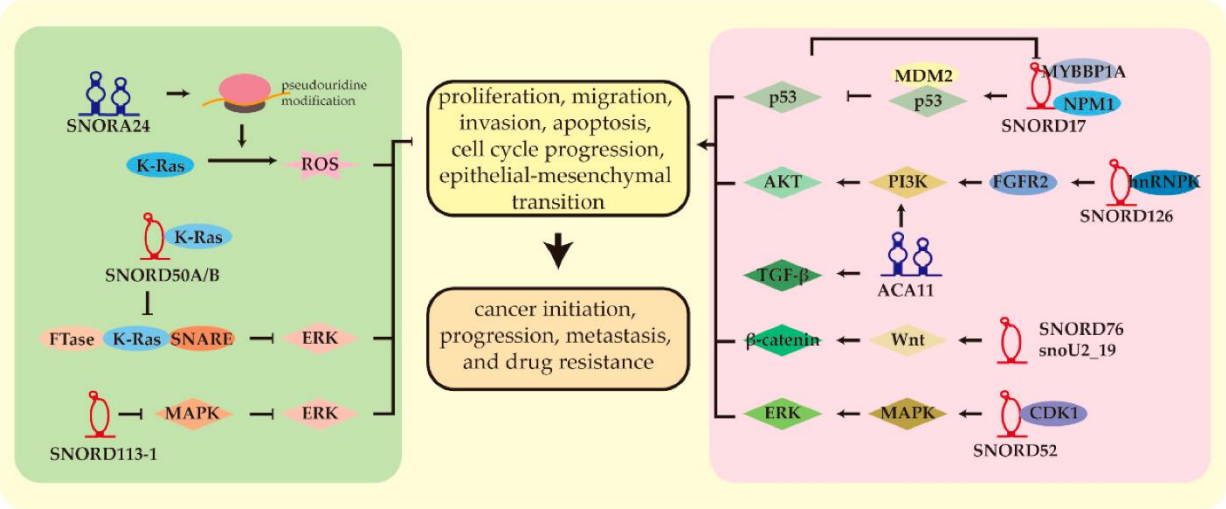

Figure 4

Mechanism of snoRNAs in HCC: Altered expression of snoRNA in HCC could result in different pathophysiological changes. This involves modulating signaling pathways and ultimately affecting cancer initiation progression and metastasis [2].

1. Canonical functions of snoRNA in HCC

   SnoRNAs play a crucial role in the modification of nucleotides in rRNA and disturbances in ribosomal activity have been shown to convert normal, healthy cells into neoplastic cells [105]. In context of HCC, it raises the question of whether dysregulation of snoRNAs might impact the initiation and progression of cancer by having an effect on ribosomes. McMahon et al. addresses this hypothesis and shared useful insights into the matter.

Oncogene-induced cellular senescence (OIS) represents a vital cellular defense mechanism triggered in response to the arrest of malignant neoplasms, particularly in cells expressing activated oncogenes aiming to prevent malignant progression. In a study by McMahon et al. primary human skin fibroblasts were stimulated by overexpressing the activated oncoprotein RASG12V, leading to an overall increase in snoRNA expression, concomitant with a reduction in protein synthesis levels [84]. This is a process through which cells undergo OIS to counteract oncogene –induced malignant transformations. Among the snoRNAs examined, SNORA24 exhibited upregulation during RAS induced senescence but significant down regulation in HCC tissues.

In a mouse liver model, RAS-induced senescence was activated without concurrent tumorigenesis. However, when SNORA24 was downregulated using LNA, the progress of senescence was impeded while malignant transformation ensued synergistically. The decreased level of SNORA24 played an important role in initiation and progression of tumor and also evasion from tumor suppressive defenses conferred by OIS. SNORA24 primarily facilitated pseudouridine modifications at two sites (uridine 609 and uridine 863) in the 18S rRNA of the small 40S ribosomal subunit [106, 107]. SNORA24 impacts various aspects of mRNA translation, which is a complex and dynamic process encompassing initiation, elongation and termination [108].

Knocking down SNORA24 in HCC cells altered several processes related to mRNA translation [84]. The absence of SNORA24 guided modifications led to dynamic changes in ribosomes, enhancing their ability to select tRNA and

exhibiting a preference for different tRNA states during decoding in comparison to control cells. While SNORA24 seemed dispensable for global protein levels, its deficiency compromised translation fidelity in a codon specific manner. The Ψ609 modification by SNORA24, positioned in the decoding center of the ribosome, directly participated in recognizing codon-anticodon interactions during the decoding process [109]. The defects in ribosomes results in disruption of precision of mRNA decoding, ultimately leading to errors in decoding numerous codons i.e. stop codons.

To summarize, the loss of SNORA24 with its impact on accuracy of translation, under the combined influence of efficient aa-tRNA selection, conformational dynamics and decreased decoding precision, led to specific errors in OIS related mRNA translation in HCC. This allowed liver cells to evade RAS-induced senescence and continue malignant transformation, contributing to the development of HCC.

2. Noncanonical role of snoRNAs in HCC

While snoRNAs that lack any complementarity with conventional rRNA targets are incapable of regulating post transcriptional modifications, research has shown that they exert their function by binding to non-traditional targets. Numerous studies have thoroughly investigated the molecular mechanisms underlying the progression of hepatocellular carcinoma that involve both snoRNAs and snoRNPs.

SNORD17, previously identified as diagnostic and prognostic marker in plasma exosomes of cervical cancer, has been identified as an oncogene in various human tumors, including hepatocellular carcinoma [86, 110, 111].

SdRNAs derived from SNORD17 were observed to be linked with the levels of CD8+ tumor infiltrating lymphocytes [59]. In HCC, SNORD17 demonstrated an increase in proliferation capacity, enhanced lung metastatic capacity, cell cycle progression in vitro and resistance to apoptosis [86]. The underlying mechanism involves the crucial role of p53 proteins, where SNORD17 regulates the p53 pathway by specifically binding to nucleophosmin1 (NPM1) and Myb-binding protein 1A (MYBBP1A).

NPM1 involved in ribosome biogenesis, primarily accumulates in nucleolus [112]. The translocation of NPM1 can be prompted by certain cellular stresses including DNA damage and inhibition of proteasome. In the nucleoplasm, NPM1 binds to human murine double minute 2 (MDM2). MDM2 is an oncogene that functions as an ubiquitin ligase for proteasomal degradation of p53. This relocalization of NPM1 competes with MDM2 for blocking its domain with E3 ligase activity thereby causing dissociation of MDM2-p53 complexes, ultimately inhibiting p53 degradation. Another nuclear protein that can redistribute from nucleoli to nucleoplasm under nuclear stress is called as MYBBP1A. In the nucleoplasm, it interacts with p53 and enhances p300-mediated p53 acetylation by promoting tetramerization of p53 causing an increased activation of p53 [113]. In hepatocellular carcinoma NPM1 and MYBBP1A both translocate to the nucleoli co-localizing with SNORD17 [86]. Overexpression of SNORD17 leads to formation of a complex between SNORD17 and truncations generated from NPM1 and MYBB1A. To summarize the elevated levels of SNORD17 in HCC cause the NPM1 and MYBB1A to remain anchored in the nucleoli resulting in reduced stability and transcriptional activity of p53. This decline in p53 in turn enhanced the expression of SNORD17, establishing a reciprocal regulation between p53

and SNORD17. This creates a positive feedback loop that contributes to tumorigenesis.

SNORD52 exhibited a significant upregulation in HCC and demonstrated an inverse correlation with poor prognosis [2]. In vitro and in vivo experiments revealed it to have pro oncogenic effects on both biological behavior and function [114]. Up-frameshift 1 is a HCC suppressor gene that works as an upstream signaling molecule for SNORD52. Low expression levels of this gene results in decreased level of nonsense mediated decay [115]. Upon transcription of SNORD52, the premature termination codon and exon junction complex are generated, yet they remain unrecognized or degraded over time, ultimately leading to SNORD52 upregulation. Upregulated SNORD52 was observed to interact with cyclin-dependent kinase 1 (CDK1). CDK1 is an oncogene that drives the cell cycle transition of S/G2 and G2/M in HCC [116]. The study also indicated that SNORD52 caused enhanced phosphorylation and stability of CDK1 and reduced ubiquitination and proteasomal degradation, consequently elevating its protein levels.

A study on pancreatic cancer highlighted the common occurrence of somatic loss involving both SNORD50A and SNORD50B across various malignancies. In hepatocellular carcinoma at least 20% of patients have these deletions which are further linked to unfavorable clinical outcomes. The activation and plasma membrane enrichment of K-Ras is required whereas its binding to farnesyltransferase (FTase) for prenylation is a limiting process [109]. Additionally, translocation of K-Ras towards the plasma membrane requires its binding to soluble N-ethylmaleimide –sensitive factor attachment protein receptor (SNARE) proteins.

SNORD50A and SNORD50B both functioning as endogenous inhibitors bind to KRAS tightly, weakening its interaction with FTase and SNARE proteins, and thereby inhibiting its pro-cancer activities. The frequent deletion of SNORD50A/B releases the shared binding sites for K-Ras resulting in activation of KRAS and the downstream MAPK/ERK pathway [117].

SNORD126 has been reported to be upregulated in HCC and also linked to unfavorable prognosis. This upregulation causes a significant increase in proliferation of tumor cells and resistance to vinblastine, etoposide and cisplatin [104, 118]. SNORD126 forms a complex with heterogeneous nuclear ribonucleoprotein K. This complex is then subsequently recruited to promoter region of fibroblast growth factor receptor 2 facilitating its transcription leading to activation of the PI3K/AKT pathway.

High methylation of CpG islands in the upstream region of SNORD113-1 leads to it not being expressed in HCC [119]. This is further correlated with poor clinical outcomes. Despite its deletion lacking a noticeable impact on overall mRNA expression at the transcriptional level, it suppresses HCC tumorigenesis through TGF-β and MAPK/ERK pathways. Conversely SNORA42 also linked with poor prognosis, is markedly upregulated in HCC [120]. It plays a crucial role in development of HCC by inhibiting apoptosis and promoting proliferation, migration and invasion. SNORA24 interferes with p53 pathway to accelerate cell cycle progression.

SCARNA22 is also highly expressed in HCC. It is reported to use PI3K/AKT pathway for promoting cell growth, migration and invasion [96]. Meanwhile, cell cycle progression and EMT is promoted by activation of its downstream

factor cyclin D1. Individuals with high SCARNA22 levels are susceptible to recurrence and short overall survival. ROC curve analysis indicated SCARNA22 to be a potential biomarker for HCC.

On the other hand SNORA23 is downregulated in HCC and have been reported to be a tumor suppressor both in vitro and in vivo [121]. The highly prevalent oncogenic activation of PI3K/AKT pathway in various cancers result in downregulation of SNORA23 in HCC. The reduced expression of SNORA23 impedes its binding to 28S rRNA leading to a reduction in 2'-O-methylation. Additionally, SNORA23 promotes tumorigenesis by reducing phosphorylation of 4E binding protein 1 which is a downstream regulator of PI3K/AKT/mTOR pathway.

Altered susceptibility to HCC is observed due to single nucleotide polymorphism in SNORD105 with the GG genotype conferring a lower risk compared to AA genotype due to lower expression of SNORD105 [122]. HCC cells with overexpression of SNORD105 exhibit increased viability and migration ability. LncRNA-LALR1 is upregulated in HCC and is reported as a tumor promoter. It binds to SNORD72 to increase its expression [123]. High expression levels of SNORD72 contribute to HCC by improving the stability of mRNA of DNA-binding inhibitors 2. SNORD76 and snoU2_19 promote cell cycle progression, proliferation and invasion by activation of Wnt/β-catenin pathway [87, 97]. SNORA47 along with SNORD76 promotes EMT, facilitating HCC progression [124].

SnoRNA involved in virus induced hepatocarcinogenesis

The leading cause of Hepatocellular carcinoma continue to be infections with hepatitis B (HBV) and hepatitis C (HCV). From 1990 to 2015 HBV infections accounted for 30% of HCC related deaths whereas, 21% of deaths were caused by HCV infections [125]. Both HBV and HCV infections have the potential to progress to chronic hepatitis, consequently resulting in cirrhosis and ultimately developing HCC. Several mechanisms are involved in contributing to HBV induced HCC. In patients with HCC with a pre-existing HBV infection, it is common observation that HBV genes integrates with the host genomic DNA. This integration leads to formation of chimeric HBV-human transcripts and induces genome instability, ultimately causing the expression and activation of cancer related genes, driving tumorigenesis [126]. Moreover, HBV protein is tumorigenic and contributes in HCC development [127]. In contrast to HBV, HCV is not directly involved in HCC development and majority of HCV associated HCC occur in context of cirrhosis [128].

Numerous studies have explored the clinical correlation between snoRNA and hepatitis virus infections in HCC. In comparison to patients with no HBV infection, those with HBV exhibit high levels of snoU2_19, SNORD78, SNORD113-1, SNORD76, SCARNA22 and reduced expression of SNORD114-1, SNORD113-5 and SNORD113-8 [87, 96, 97, 119, 129]. A genome wide association study conducted by Cao et al. on 1583 HCC patients with HBV and 1540 HBV patients without HCC revealed low frequency duplication on chromosome 15q13.3 [88]. This duplication region encodes for no protein coding gene but it contains SNORA18L5. The expression of this snoRNA was found proportional to the gene copy number. The upregulated expression of H3K4me3 in its promoter region also

increased SNORA18L5. This increased expression then causes accumulation of mature 28S and 18S rRNAs leading to hyperactive ribosome biogenesis. Moreover, similar to SNORD17 described above, SNORA18L5 promoted HCC in a p53 dependent manner. SNORA18L5 binds to ribosomal proteins RPL5 and RPL11 in the nucleolus making them absent in the nucleoplasm. This leaves MDM2 free of any complex and hence causes release of more MDM2, increasing the ubiquitination and degradation of MDM2-mediated p53.

Another study explored the snoRNAs' role in susceptibility of HCV infection [89]. High-throughput small RNA sequencing of HCV samples identified changes in snoRNA expression levels with 40 upregulated and 13 down regulated snoRNAs among which SNORD126 was the most significantly down regulated both in vitro and in vivo. It was further revealed that SNORD126 facilitated the entry of HCV into host gene dose dependently whereas not having any effect on viral replication and release. The cell surface distribution and expression of Claudin-1, an HCV entry factor, is promoted by SNORD126 via activation of PI3K/AKT pathway. Any step in this pathway can reverse the HCV susceptibility due to SNORD126 overexpression. The downregulation of SNORD126 in HCV infection but upregulation in HCC suggest dynamic expression alterations during pathogenesis [104]. SNORD126 decrease in early infection and resists the external infection but after completion of this process, the expression level of SNORD126 may increase for cancer initiation and progression.

SnoRNAs in NAFLD and NASH

Presently, viral infection by HCV and HBV remain the primary reason for HCC. Advancements in antiviral therapies and widespread adoption of HBV vaccination

is helping in decreasing the overall prevalence of it. On the other hand metabolic diseases i.e. obesity, type II diabetes and nonalcoholic steatohepatitis (NASH) are anticipated to play a more prominent role in HCC in the years to come [130]. A shift in epidemiology of HCC has been observed from being predominantly associated to viral hepatitis to an increasing prominence of NASH where non-alcoholic fatty liver disease (NAFLD) emerging as the fastest growing cause of HCC globally [131]. NAFLD covers a range of different pathophysiological processes including nonalcoholic fatty liver to steatohepatitis which can progress to NASH cirrhosis and NASH associated HCC. NAFLD initially presents as hepatic steatosis, advancing to severe inflammation and causing damage to hepatocytes [132]. NAFLD global prevalence is 25%. Approximately 1.5% to 6.45% of these adults develop NASH [133]. Annually, the patients with NASH can develop HCC in 5.59 per 1000 persons-years and notably, half of these individuals have the potential to develop HCC without progressing through cirrhosis [134]. A systematic analysis of data from its inception till 2022, around 15.1% of total HCC cases are attributed to NAFLD and this proportion is on the rise [135]. Although the total reported cases maybe lower than what are being reported for HBV or HCV but the high burden of NASH and NAFLD would contribute an upsurge in HCC cases associated with these metabolic diseases.

SNHG3 is a long non-coding RNA (lncRNA) that is upregulated and has pro oncogenic effects across multiple cancer types including HCC [136]. It is a host gene for two snoRNAs i.e. SNORA73A and SNORA73B. These snoRNAs are involved in maintaining cellular cholesterol homeostasis in cholesterol esterification and transport [137]. The mutant cell line 2E4, highly resistant to lipid induced oxidative stress and cell death, has disrupted loci encoding for SNORA73 and SNHG3.

Lipotoxicity is an early event of NASH and through a series of experiments it was concluded that SNORA73 plays a role in response to lipotoxicity [138].

2E4 cells in comparison to wild type cells exhibited reduced production of reactive oxygen species and increased levels of the critical glutathione and NADPH upon exposure to lipotoxic free fatty acid palmitate. Further experiments showed that loss of SNORA73 causes an increase in mitochondrial metabolism, glutathione biosynthesis and oxidative phosphorylation without affecting pentose phosphate pathway or aerobic glycolysis. Moreover, SNORA73 loss improved the cell's ability to handle the increased capacity of fatty acid substrates. Similar improvement in resistance to lipotoxicity and other metabolic alterations were observed in a liver steatosis mouse model induced by high fat diet. Although SNORA73 is crucial for modification and processing of rRNAs, the data obtained after altering SNORA73 levels revealed no changes in abundance of rRNA [139]. It is important to note here that mTOR pathway played a central role in this process. Studies indicated that impaired rRNA production could activate the mTOR pathway, influencing the cellular metabolism [140, 141]. Therefore, the defective rRNA processing resulted from SNORA73 loss acted as an initiator, activating the mTOR pathway to regulate metabolism as a countermeasure to lipotoxicity.

Another study highlighted similar role played by 3 snoRNAs (U32a, U33 and U35a) [88]. In lipopolysaccharide-induced liver, these snoRNAs are activated. Knocking out the expression of these snoRNAs helped in protecting the cells from oxidative stress, endoplasmic reticulum stress and reactive oxygen species in both in vitro and in vivo settings. SNORA24, as mentioned earlier, displayed a strong association with lipid deposition [84]. SNORA24 is downregulated in HCC and is linked with decreased survival. Concurrently, SNORA24 expression was reported to be

inversely correlated with lipid content. Knocking down SNORA24 in human cell line HUH7 increased lipid droplet formation. Similarly, a dramatic accumulation of lipids was observed in mouse HCC models, induced by combination of RAS and LNA-24. Collectively these findings suggest the potential role of SNORA24 in triggering aberrant lipid metabolism and promoting tumor formation and progression.

SnoRNA present novel therapeutic opportunities

Considering the emerging evidence that snoRNA play a role in modifying cancer progression and metastasis one can wonder if they can be therapeutically targeted? Several new technologies provide significant hope now. For instance, adenovirus mediated delivery of SNORD44 inhibited growth of CRC aligning with the rapamycin treatment in CRC cell line xenografts. Another such case was the antisense oligonucleotide (ASO) mediated downregulation of SNORA23 which negatively impacted the tumor growth, dissemination and liver metastasis in pancreatic ductal adenocarcinoma xenografts. Therefore, it is safe to say that snoRNA could prove beneficial either as targets for drug development in cancers or as therapeutic agents in their own right.

Since snoRNA clearly play an important role in cancer initiation, progression and metastasis, further studies are needed to determine novel snoRNA involved in development of HCC. Although as mentioned above, many snoRNA have been linked to HCC progression and poor prognosis but all these correlations are only relative. Researchers were able to determine the cause-and-effect relationship for only a few of them. Therefore, it is not only important to find new snoRNA in context of HCC, but also more effort is required in determining the possible functions and the pathway adopted by these snoRNAs to carry out those functions

ultimately leading to the progression of the disease. Moreover, it would help in better understanding the process of development of Hepatocellular carcinoma. These new snoRNAs would also have the potential to be used as targets or help in drug development.

AIMS and Objectives

Hepatocellular carcinoma is one of the deadliest cancers with incidence rate increasing globally. This silent killer is usually detected at a late stage and at that point, not many treatment options are available for the patients. Therefore, it is the need of the hour to find new biomarkers that can efficiently detect HCC and also can help in better understanding the disease. More in depth information about HCC would assist in better management and treatment of the disease.

Among non-coding genes small nucleolar RNA are recently getting attention in the field of prognosis and diagnosis of cancer including the hepatocellular carcinoma. The biological role of snoRNA in HCC are, but no limited to, involvement in initiation, proliferation, metastasis, apoptosis, and cell cycle. Therefore, here in this study we are focusing on finding novel snoRNA that are significantly dysregulated in HCC. Although many snoRNA have been linked to HCC prognosis and patient survival, very few have been fully researched to determine the type of function they perform within the cell. Hence, we further aim on getting better insight into the relationship between snoRNA and HCC.

To this end we received sequenced tissue samples from patients with NASH and HCC. We analyzed the data to determine a set of snoRNAs that were upregulated in HCC samples as compared to NASH. Following are the set of steps we aimed to perform after receiving the data:

- Analyzing RNA seq data to determine novel set of snoRNA.
- Validating and selecting the dysregulated snoRNA in liver tissue samples
- Determining any potential targets for SNORA74D via bioinformatics analysis
- Development of cellular model with knock down (KD) of SNORA74D

    - qPCR analysis to see impact of dysregulation of SNORA74D on expression of predicted gene targets.
    - Identify the phenotypic changes (cell viability, proliferation, cell cycle, migration/invasion, colony formation)
    - TGIRT sequencing of RNA samples obtained from cells to determine new mRNA targets for SNORA74D
- Manipulating cancer mechanisms to see the effect on SNORA74D
    - ER stress
    - Oxidative stress
- Investigating the presence of isoforms of SNORA74D
    - Demonstrating presence of isoform of SNORA74D in cellular model (HepG2), tissue samples and other cell lines.

Methodology

Analyzing RNA sequencing data to determine novel set of snoRNA:

RNA sequencing was employed to examine the gene expression profiles of primary HCCs and NASH tissues. Using the Illumina HiScanSQ platform and a paired-end strategy (2 X 80 bases), whole transcriptome sequencing generated a total of 370 gigabase, with an average of 95 X 106 reads per sample and an average depth of coverage of ~60X. Following BCL to FASTQ conversion (bcl2fastq), quality control, and adapter trimming (AdapterRemoval), the paired-end reads were aligned to the human reference genome HG19 using TopHat/Bowtie. Transcript abundance was calculated using the Python function htseq-count, with the Ensembl72 release serving as the gene feature annotation. Normalization, expressed as counts per million, was performed using a suite of R Bioconductor packages such as edgeR. Complete analysis was performed to determine a set of snoRNA differentially expressing in the dataset.

Validating and selecting the dysregulated snoRNA in Liver tissue samples:

NASH and HCC liver tissue samples were kindly provided by the collaborators. RNA was extracted from these samples by using the miRNeasy kit. Manufacturer's protocol was followed for the RNA extraction. RNA was quantified by NanoDrop Spectrophotometer. GoScript™ Reverse Transcription System was used for efficient conversion of RNA to cDNA for snoRNA. Manufacturer's protocol was followed to make cDNA. 1ug RNA was converted into cDNA and then used for qPCR. Primers for SNORA5C, SNORA74D, SNORA80D were used to amplify each snoRNA. Fold change was quantified for each snoRNA. A paired t test was performed to determine

significant change in fold change expression of these selected genes when compared to HepG2 cell line in both NASH and HCC samples. GAPDH was used as a house keeping gene.

Determining any potential targets for SNORA74D via bioinformatics analysis

Genes from the RNA seq data were used to see if there was any linear correlation between them and SNORA7D. It was deduced that certain genes could potentially have some sort of positive correlation with SNORA74D. SnoGPS was then used to determine any potential binding of SNORA74D with the selected genes.

Development of cellular model with knock down (KD) of SNORA74D:

ASOs were used for knocking down SNORA74D levels in HepG2 cell line. Lipofectamine 3000, Lipofectamine 2000 and RNAimax were tested for their efficiency to get the most decrease in expression of SNORA74D. Lipofectamine 2000 was selected among the 3 transfecting agents for its potential to cause least toxicity and better knock down with the ASOs. The protocol was optimized to use 3ul of lipofectamine 2000 whereas multiple concentrations of ASOs were checked i.e. 30nM and 50nM.

Determining impact of dysregulation of SNORA74D on expression of predicted gene targets via qPCR

The expression level of the predicted targets (USP33, RAP1A, ERP29, GCHFR, FLOT2, CYP20A1, and YIPF6) for SNORA74D were checked by performing qPCR analysis. MRPL19 and YWHAZ were used as housekeeping genes. The following Protocol was used for this purpose:

cDNA	10ng
SYBER Green	5ul
Forward and Reverse primer mix	2ul
water	Upto 10ul total reaction

TGIRT sequencing of RNA samples obtained from HepG2 cells with Knockdown of SNORA74D

48h post transfection, the cells were trypsinized and collected for RNA extraction. RNeasy Mini kit was used according to the manufacturer's instructions for this purpose. 500ng of the extracted RNA was converted into cDNA by using following protocol:

RNA	500ng
dNTPs (10mM)	1ul
Random Primer	1ul
Nuclease-free H2O	Upto 5ul

This mix is denatured for 5min at 65°C and then promptly put on ice. Meanwhile another mix is prepared with the following components:

10X M-MuLV buffer	2ul
M-MuLV RT (200 U/µl)	0.5ul
RNase Inhibitor (40 U/µl)	0.25ul
Nuclease-free H2O	Upto 5ul

Around 5ul of this mix is added to each RNA sample that was denatured in the earlier step. These tubes are then incubated at 25°C for 5min. Then the sample are incubated at 42°C for 60min. In the last step the enzyme is inactivated at 60°C for 20min. The cDNA was used to perform qPCR to confirm SNORA74D expression. The remaining cDNA was stored at -20°C for long term storage.

Following protocol was used to perform qPCR reaction:

cDNA	10ng
SYBER Green	5ul
SNORA74D forward and reverse primer mix	2ul
water	Upto 10ul total reaction

Upon confirmation of knock down in expression of SNORA74D, the RNA samples were cleaned and sent for TGIRT sequencing at RNA platform, Sherbrook University.

Analyzing TGIRT seq data:

All RNA abundance datasets were generated using a succession of bioinformatics tools regrouped in a reproducible Snakemake workflow. All details about parameters and tools used can be found in the Snakemake workflow at https://github.com/etiennefc/TGIRT_Seq_pipeline.git, but are also briefly described below. The datasets generated are of high depth and quality for each sample. Paired-end reads were first trimmed using Trimmomatic v0.36 (with the

following parameters: ILLUMINACLIP:<fastaWithAdaptersEtc>:2:12:10:8, TRAILING:30, LEADING:30, MINLEN:20, all other parameters at default values) to remove adapters and low-quality reads. FastQC v0.11.5 was used before and after trimming to assess the quality of the reads. Trimmed reads were aligned to the human genome assembly GRCh38 (hg38, v87) using the aligner STAR v2.6.1a [53] (with the following parameters: --runMode alignReads, --outSAMunmapped None, --outSAMtype BAM SortedByCoordinates, --outFilterScoreMinOverLread 0.3, --outFilterMatchNminOverLread 0.3, --outFilterMultimapNmax100,--winAnchorMultimapNmax100,--alignEndsProtrude 5 ConcordantPair, all other parameters at default values). The index needed to align reads to the human genome was generated using STAR v2.6.1a (with the following parameters:--runMode genomeGenerate and --sjdbOverhang 74).

Counts were attributed to genomic features using CoCo v0.2.1p4 (with the following parameters: cc -countType both -strand 1 --paired, all other parameters at default values), using our custom annotation (.gtf file available at https://zenodo.org/record/4570182/files/hg38_Ensembl_V101_Scottlab_2020.gtf. Normalized counts in TPM were obtained from the output of CoCo. Only snoRNAs with an abundance greater than 1 TPM in at least one sample, thus referred to as "expressed snoRNAs", were included in this study to filter out low abundance snoRNAs. The differential expression analysis was conducted using DESeq2 (v1.34.0) with default parameters (Love et al., Genome Biology, 2014) to compare the pooled ASO samples to control NC5 conditions.

Identification of any phenotypic changes in the cells upon knocking down SNORA74D

The HepG2 cells were observed under a microscope for any phenotypic changes in them. The viability of cells was observed by counting the total number of alive cells after 48h post transfection. A clear discrepancy in no of alive cells was observed in wells transfected with ASO as compared to the control.

BrdU ASSAY

5000 HepG2 cells were transfected with ASO (30nM) and with control NC5 (30nM) in 96well plate and grown for 48h. After 48h the media was removed. 50ul of 0.75mM BrdU was added to each well and incubated for 1h30min at 37C. After incubation cells were washed with 70ul of PBS.

A mix of 50ul of PBS and 50ul of 7.4% Paraformaldehyde was added. The cells were incubated for 20min. After the incubation the cells were washed with 70ul PBS. 50ul of Trition-X100 was then added to the cells and incubated for 5min. The cells were then washed again with 70ul PBS.

The next step was to block with 70ul of 2%BSA and incubate for 45min at RT. The cells were washed with 70ul PBS and then 50ul of DNase I was added to them and kept in incubation for 1h at 37C. This was followed by another step of washing with 70ul PBS. Later, 50ul of primary antibody mix was added and the cells were left to incubate for 1h at RT. The cells were then washed twice with PBS (70ul) and 50ul of secondary antibody was added to the wells and left to incubate for 45min in the dark. This was followed by washing the cells again with 70ul PBS twice. At the end the cells were suspended in 100ul PBS and read with a microscope.

PI stain

5000 HepG2 cells were cultured in 96 well plate and transfected with ASO and control after 24h of plating. PI staining was performed 48h post transfection. Staining mix was prepared (4ml for 60 wells). 62ul Hoechst, 4ul Calcein and 8ul of PI was added in 4ml of media. 50ul of this mix was added into each 96well and incubated for 30min. The plate was then observed under a microscope immediately. Columbus software was used to analyze the plate.

xCELLigence assay

It was performed by using xCELLigence system. It used specially designed microtiter plated containing interdigitated gold microelectrodes to noninvasively monitor the viability of cultured cells using electrode impedance as the readout. The procedure carried out is as follows:
The machine was turned on and the software was used to create a new experiment. All the required information was filled, and the layout of the experiment was created. Furthermore, the steps were created for blank, seeding and transfection and time was set accordingly. 50ul of warm media was added to the plates according to the layout and were placed in the system and step1 (blank) was performed. Subsequently, the plates were taken out of the system and 50ul of media containing HepG2 cells was added into the respective wells. The plates were then incubated for 45min at RT inside a cell culture hood. After completion of incubation the plates were moved back into the system to perform step2 (seeding). The plates were left in the system for 24h for the cells to attach well evenly. Next day transfection solutions were prepared in the media and 50ul of each solution was added in its respective

well according to the designed layout. After the addition of transfection media, the plates were placed back in the system and the experiment continued to run for 48h to 72h and the growth of cells was regularly monitored.

Manipulation of cancer mechanisms (ER stress & oxidative stress) to see effect on SNORA74D:

1. **ER stress**

   Tunicamycin was used for causing ER stress to the HepG2 cells. After testing multiple drug concentrations, 5ug/ml was selected as a right dosage. HepG2 cells were plated 24h prior to drug administration. The cells were collected after 12h of treatment. RNA was extracted from these cells by using the mRneasy mini kit. cDNA was made by using M-MULVRT enzyme and qPCR was performed to determine any possible effect on SNORA74D.

2. **Oxidative stress**

   Staurosporin was used to cause oxidative stress in HepG2 cells. Multiple concentrations for staurosporin were tested (10nM, 20nM and 50nM) and their respective affect was observed in initiating an oxidative stress in HepG2 cells. Therefore, HepG2 cells were plated 24h prior to treatment with staurosporin. The cells were collected 24h post treatment. RNA was extracted, cDNA was made, and qPCR was performed to determine any effect on SNORA74D expression.

Investigating the presence of isoforms of SNORA74D

1. Discovery and Identification of presence of isoform of SNORA74D in HepG2 and other cell lines.

The bed graph obtained from already present TGIRT seq data from multiple cell lines was kindly provided by Scott lab in Sherbrooke University.

HepG2 cells were grown and were collected over a period (24h, 48h, 72h, 96h, 120h and 144h). RNA was extracted from HepG2 cells by using RNAeasy Mini kit. 500ng of RNA was converted into cDNA by using an earlier mentioned protocol and was sent for ddpcr analysis at RNA platform, Sherbrooke University. The long isoform primer was designed in a way to only bind to their specific target isoform. The canonical form of SNORA74D primer could bind to all 3 isoforms. The short isoform primer was able to bind to both short and long isoforms. Therefore, final concentration of long and short isoform of SNORA74D was subtracted from total canonical form to get the actual amount of canonical SNORA74D isoform. Moreover, total short isoform amount was subtracted from long isoform of get its actual amount.

2. Identification of presence of isoform of SNORA74D in HCC tissue and normal tissue.

cDNA from HCC and the surrounding NASH tissue samples was used to perform ddPCR. The bed graphs for normal tissues were obtained from Scott lab in Sherbrooke University.

Results

RNA sequencing data analysis:

Analysis of the RNA sequencing data indicated the presence of both C/D box and H/ACA snoRNA. It appears that C/D box are frequently downregulated whereas H/ACA are comparatively upregulated in patients with HCC in comparison to NASH.

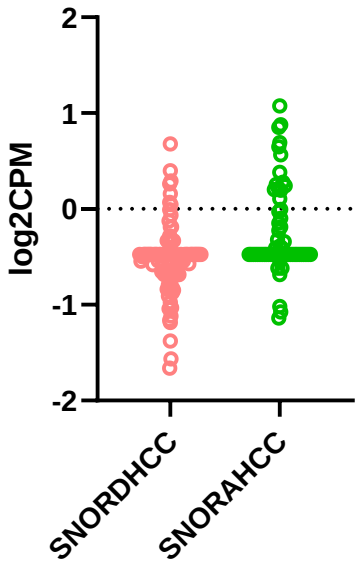

Figure 5: H/ACA and C/D box snoRNAs are differentially expressed in HCC as compared to NASH. H/ACA and C/D box snoRNAs display distinct abundance patterns in HCC and NASH. The abundance of H/ACA and C/D snoRNAs as determined by Whole transcriptome seq was compared using a scatter dot plot. C/D snoRNAs are shown in red and H/ACA snoRNAs are shown in green.

Furthermore, in the next step the DESeq2 analysis was performed to obtain a set of differentially expressed genes among the cohorts of NASH and HCC patients. The genes that were significantly dysregulated among the patient cohorts were then selected. This set of selected snoRNA included SNORA80D, SNORA74D, and SNORA5C.

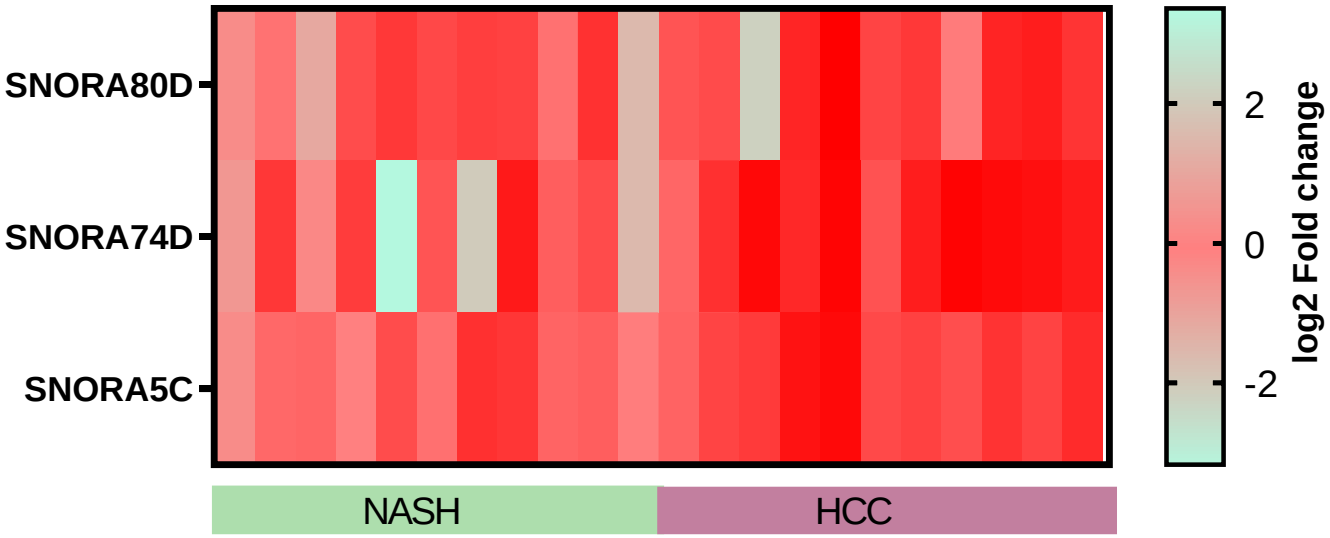

Figure 6: SnoRNAs exhibiting the most significant fold change difference (p value < 0.05) in HCC compared to NASH are depicted in a horizontally clustered heat map.

The next step was to validate this difference in expression levels for SNORA74D, SNORA5C and SNORA80D on liver tissues using qPCR.

Validating the dysregulated snoRNA in liver tissue samples:

23 HCC tissue samples and their adjacent NASH tissue samples were selected from an independent series with confirmed NASH Diagnosis. The samples were used in pairs in order to make sure they represent the samples used for sequencing. RNA

was extracted and cDNA was made using the GoScript. qPCR analysis was performed for SNORA5C, SNORA74D and SNORA80D by using SYBER Green mix. HepG2 is a cell line derived from liver and is a good representative model of liver and thus cDNA from it was used as control. GAPDH was used as a housekeeping gene.

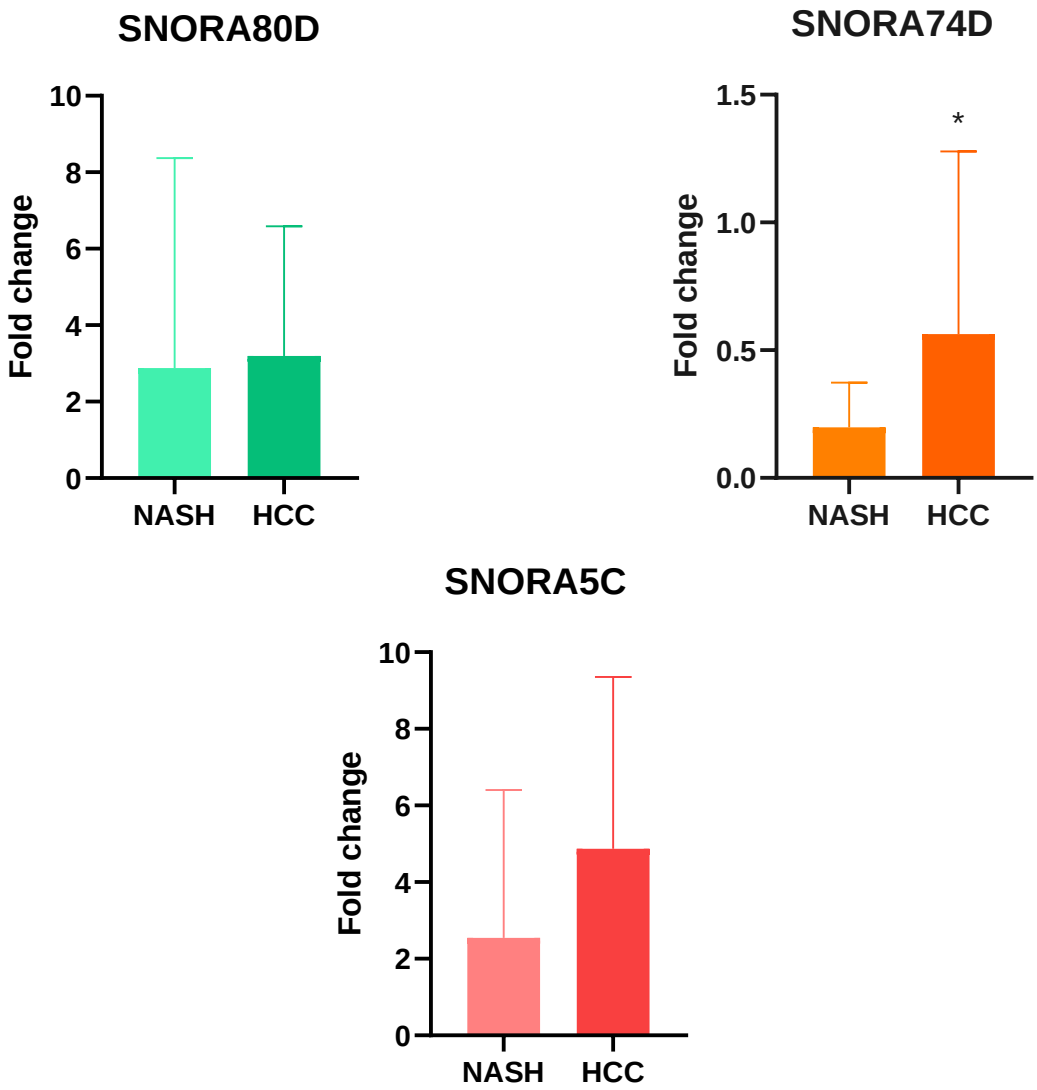

Figure 7: The above graphs illustrate SNORA74D as the only one to be significantly upregulated in tissue samples with HCC in comparison to surrounding

NASH tissue. This signifies its potential relation to development of HCC and therefore, was selected for further study. Statistical analysis was done via two-tailed t-test using 95% CI. (***p value <0.001; **p value < 0.01; p value < 0.05).

HCC is a very complicated disease and therefore the variation among the replicates can be attributed to inter-individual variation among the sample types. Although higher fold change value for SNORA5C was observed in qPCR results but because we were only able to validate the dysregulation of SNORA74D with significance thus, we decided to proceed with SNORA74D for further experiments.

Determining potential targets for SNORA74D via bioinformatics analysis

RNA seq data was analyzed to create a list of 17 genes that had significant correlation with SNORA74D. SnoGPS was used to determine a list of potential targets for SNORA74D among those selected genes. SnoGPS is a web tool that has been used by researchers to identify novel pseudouridylation-guide snoRNA in yeast and mammals. It combines computational algorithms, sequence analysis and structural analysis to identify snoRNA genes and predict their interactions with target RNA. Following is the list of the potential target genes:

Sr.no	Gene
1	USP33
2	RAP1A
3	ERP29
4	GCHFR
5	FLOT2
6	CYP20A1
7	YIPF6

Table 3: The list of mRNA that had the potential to bind to SNORA74D and were known to be involved in cancer.

Development of cellular model with knockdown (KD) of SNORA74D:

Several transfecting agents were used to determine the best for getting the highest knockdown of SNORA74D. It was observed that Lipofectamine 2000 has the best efficiency when compared with Lipofectamine 3000 and RNAimax. NC5 was used as a scramble sequence to use as a control whereas two ASOs namely ASO1 and ASO2 were tested for knocking down SNORA74D. The cells were collected after 48h of transfection.

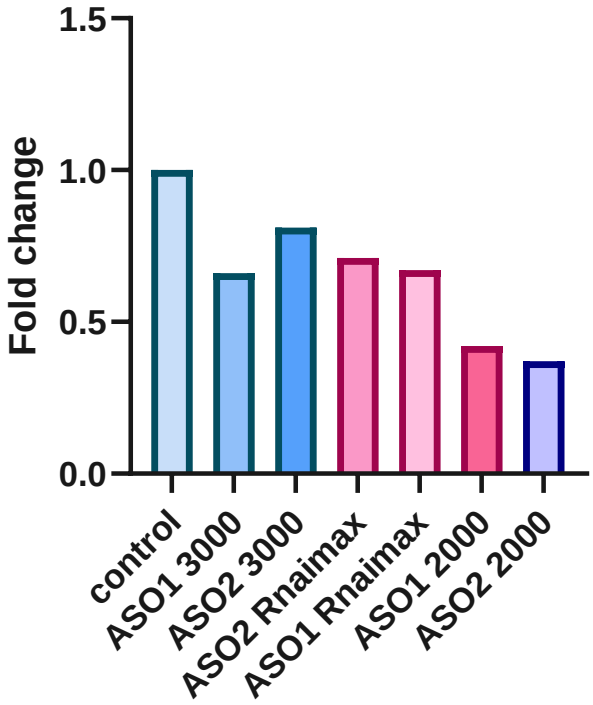

Figure 8:

The graph depicts the amount of knockdown obtained by using the two ASOs in comparison with the scramble sequence. It can be observed that Lipofectamine 3000 was able to get knockdown of approximately 35% by ASO1 and 20% by ASO2. On the other hand, knockdown by using RNAimax appeared to be approximately 33% by ASO1 and 30% by ASO2. Lipofectamine 2000 was selected among the 3 since it appeared to give a consistent knockdown of around 58% by ASO1 and 63% by ASO2 when compared to NC5 scramble sequence.

In the next step multiple concentrations of ASO were tested to determine the dosage that gives a consistent knockdown without causing too much toxicity to the cells with using Lipofectamine 2000 as a transfecting agent. The following graph shows the knockdown obtained by using the two ASO concentrations of 30nM and 50nM at 48h. The same respective concentrations of scramble sequence were used to serve as a control.

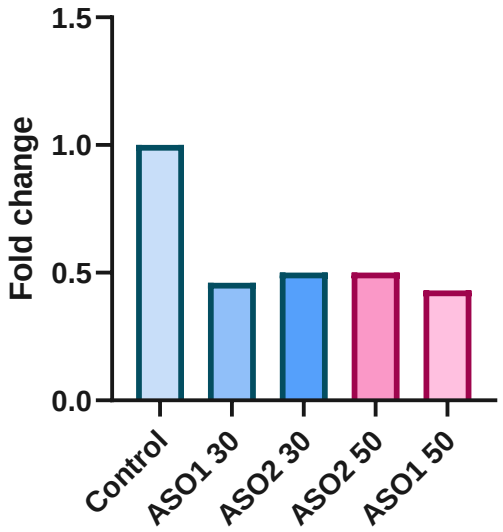

Figure 9:

The above graph depicts the knockdown observed by using two different concentrations of ASOs. As when both ASOs were used in 30nM dosage, we received around 64% and 50% of knockdown in HepG2 cells by ASO1 & ASO2 respectively. On the other hand, by using 50nM concentration, nearly 57% and 50% of knockdown was observed by ASO1 & ASO2 respectively while using NC5 (control) in 30nM and 50nM concentration respectively.

Three biological replicates were obtained with both the concentrations of 30nM and 50nM. It was observed that higher amount of ASO sometimes gave an inconsistent knockdown and was causing more toxicity to the cells. Therefore, RNA from cells with knockdown of SNORA74D by using ASO1 and ASO2 (30nM) was selected for sequencing. The following graph shows the knockdown levels for SNORA74D with respective ASOs. These results were obtained from 3 biological replicates.

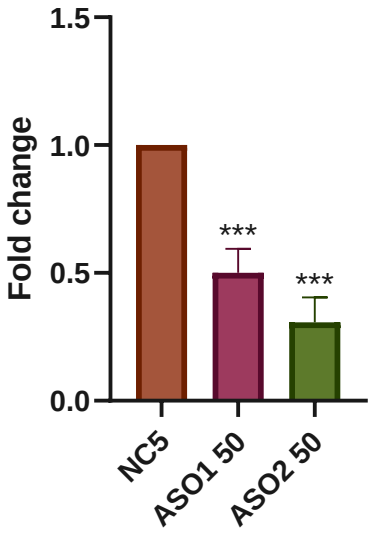

Figure 10:

The above figure shows a knockdown when transfecting cells with 50nM of ASOs. It was observed that the higher amount of ASO was causing too much toxicity to the cells. Therefore, it was decided to not use this amount for any future experiments. Statistical analysis was done via two-tailed t-test using 95% CI. (****p value <0.001; ***p value <0.001; **p value < 0.01; p value < 0.05).

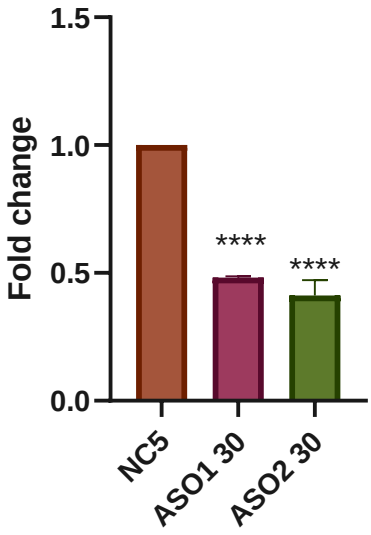

Figure 11:

The above figure shows a consistent knockdown of SNORA74D with 30nM concentration of ASOs. The cell toxicity was also comparatively lower for this concentration of ASOs. These samples were then selected and sent for sequencing. Statistical analysis was done via two-tailed t-test using 95% CI. (****p value <0.001; ***p value <0.001; **p value < 0.01; p value < 0.05).

The impact of dysregulation of SNORA74D on expression of predicted gene targets:

USP33, RAP1A, ERP29, GCHFR, FLOT2, CYP20A1, and YIPF6 were few targets that were predicted by SnoGPS to have some form of interaction with SNORA74D in one or another capacity. Therefore, qPCR analysis was performed to determine any potential effect on these targets upon knocking down the expression of SNORA74D.

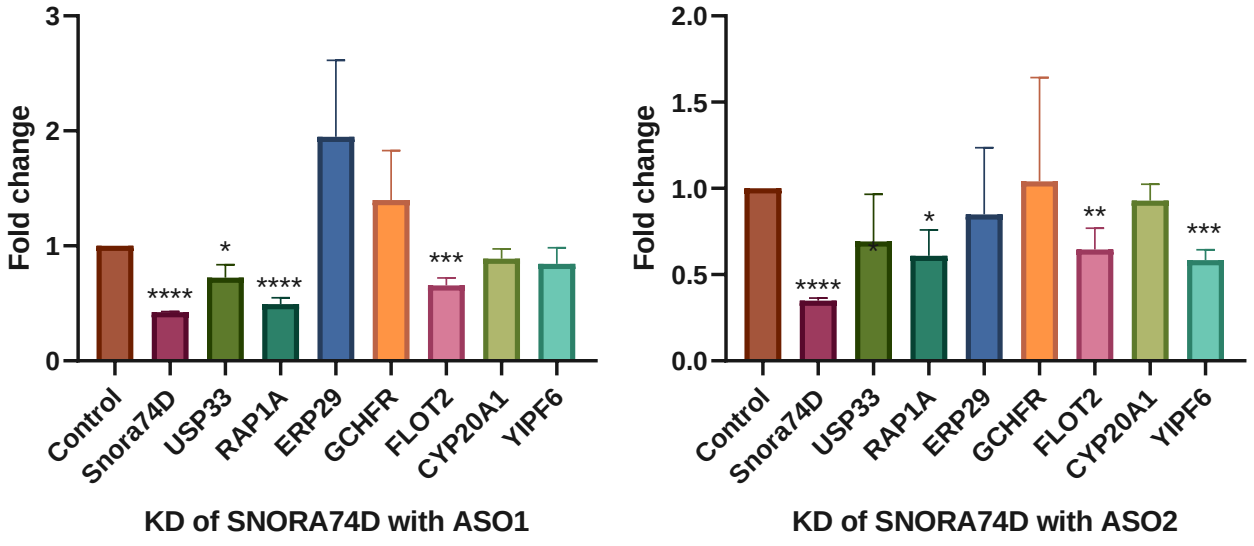

Figure 12:

Analysis revealed that KD of SNORA74D with ASO1 and ASO2 caused a decrease in expression of RAP1A, FLOT2, USP33 and YIPF6. These results were obtained with 3 biological replicates. Statistical analysis was done via two-tailed t-test using 95% CI. (****p value <0.001; ***p value <0.001; **p value < 0.01; p value < 0.05).

TGIRT sequencing of RNA samples with KD of SNORA74D:

The RNA samples from cells transfected with ASO1 & ASO2 and scramble NC5 with 30nM concentration were sent for TGIRT sequencing. Unfortunately, due to some technical issues only one replicate of ASO1 and three replicates each of ASO2 and control samples were used for analysis. The principle component analysis (PCA) plot was made for all the treated and control samples (Figure 13). PCA allows the analyst to look patterns and clusters within the data. This helps in identifying any outliers. Outliers would be data points that deviate significantly from the overall pattern.

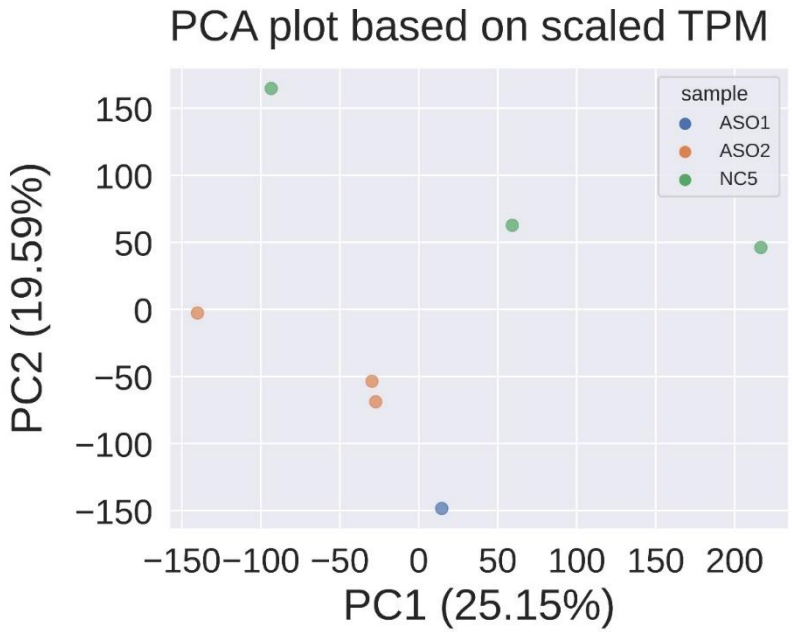

Figure 13:

The above PCA plot indicates no outliers among the analyzed samples as NC5 samples make a distinct cluster and so does the ASO samples therefore, making all these samples fit for further analysis.

BedGraph is used to visualize the quantitative data i.e. signal intensities or scores along specific genomic coordinates. The bedGraph for all the samples including all 3 replicates for ASO1, ASO2 treatment and control samples were studied.

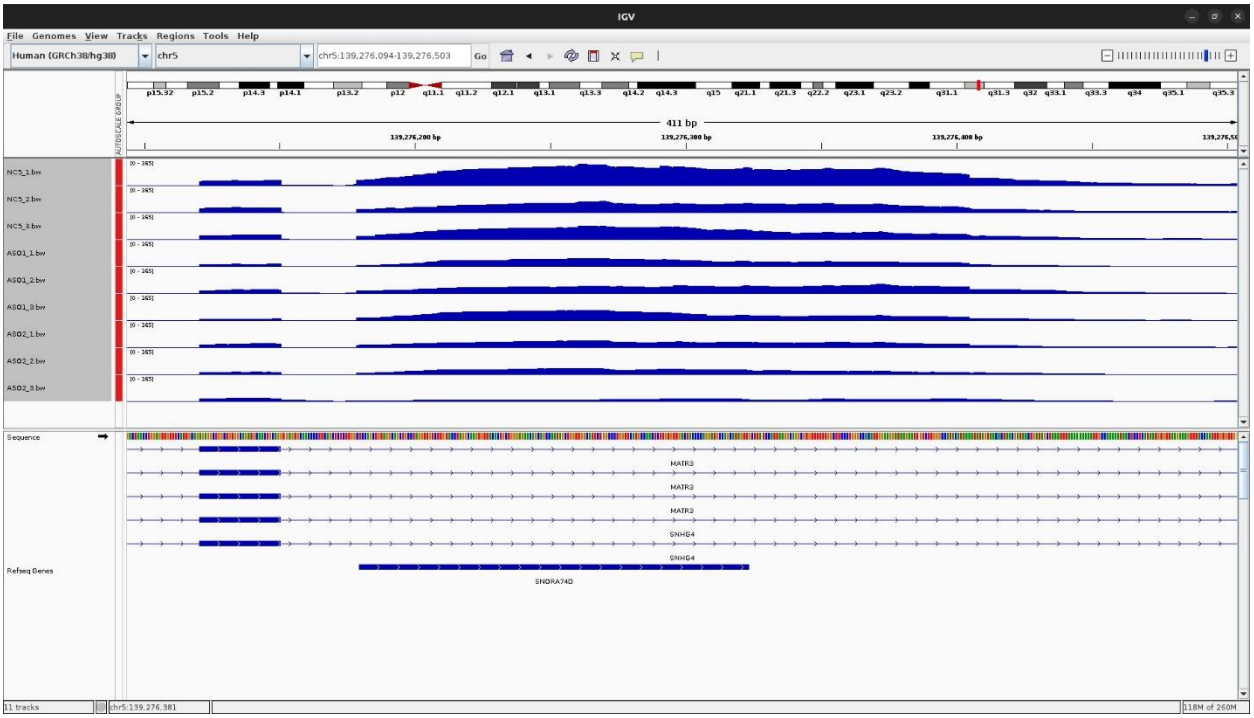

Figure 14:

The first 3 of above bedGraphs show higher height or signal intensity indicating higher expression of SNORA74D whereas, the remaining 6 bed graphs are of samples treated with ASO having a lower graph height. It is apparent that the ASO

treatment worked and SNORA74D expression is reduced in HepG2 cells treated with ASOs in comparison to control samples.

Volcano plot is used to visualize and interpret the differential analysis results. It enables the researchers to identify the genes that exhibit statistically significant changes between the treated and non-treated conditions. The volcano plot was made for genes obtained after DESeq2 analysis for samples treated with ASO and control to see the total number of genes that were significantly dysregulated.

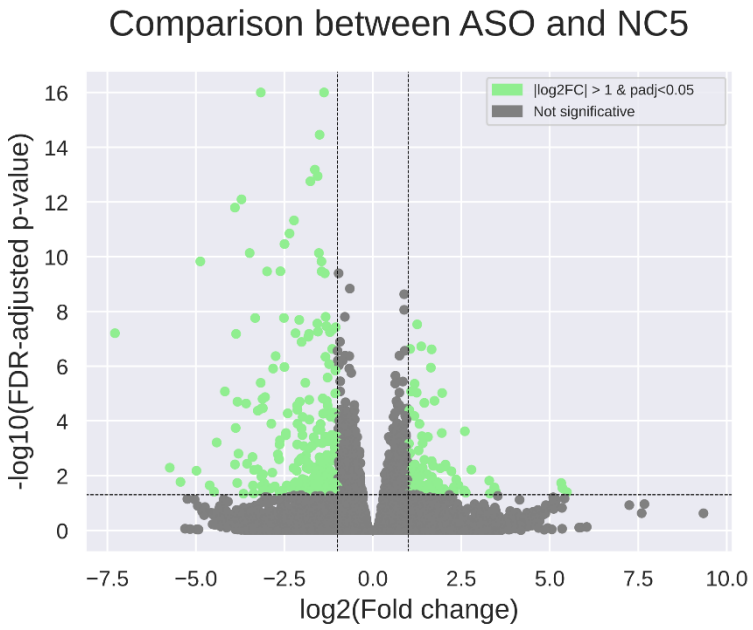

Figure 15:

The above volcano plot shows a significant dysregulation of genes.

Another interesting thing to look for in the obtained data was the type of genes dysregulated. Therefore, another volcano lot was generated to identify the biotype of RNA significantly dysregulated among the treated samples in comparison to control.

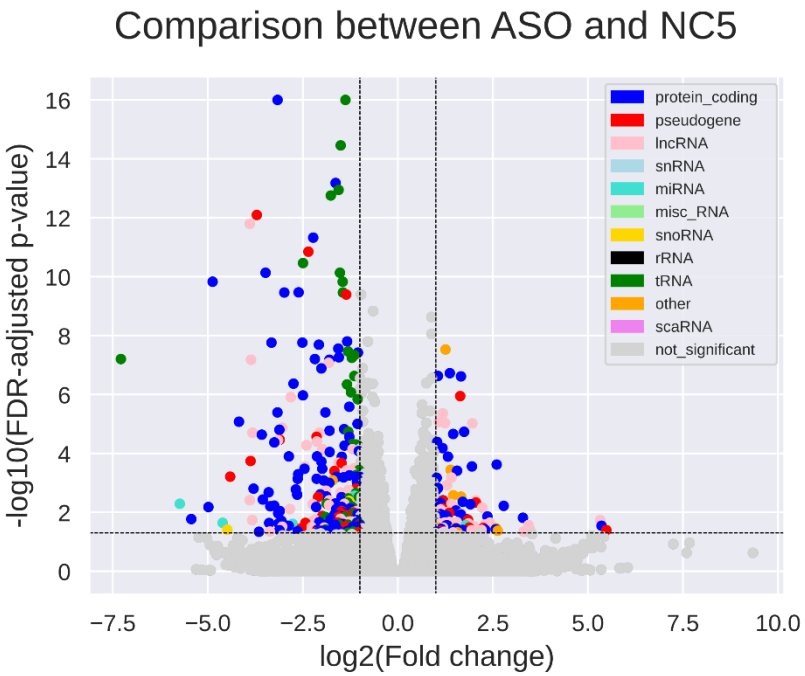

Figure 16:

The above volcano plot shows significant downregulation (Padj> 0.05) of some protein coding genes as well as upregulation in expression of other protein coding genes. Other type of RNA significantly dysregulated include lnRNA, snRNA, miRNA, snoRNA, rRNA, tRNA as evident from the plot. The biological significance of these genes, however, needs to be further evaluated.

SnoRNAs play an important role in maintaining the structural and functional integrity of various RNA molecules i.e. tRNA. If there are any modifications required in tRNA sequence, snoRNAs can guide the necessary modification. Due to their involvement in monitoring the integrity of tRNA molecules, any dysregulation of snoRNAs can affect the translation and protein synthesis. It is worth mentioning here that the specific snoRNAs involved in tRNA modification can vary and the specific details of these interactions are still an active area of research. Since, it is

clear from above figure that a lot of tRNA are downregulated upon KD of SNORA74D, it can be speculated that SNORA74D has some direct or indirect effect on tRNA stability and expression. However, for the purpose of this study we focused on protein coding genes that were significantly dysregulated to perform Gene enrichment analysis by using DAVID. Gene enrichment analysis is a computational method used to specify if some biological functions, process, or pathway are overrepresented in the genes of interest. This helps in gaining insight into the biological relevance of a group of genes by comparing them to a background set of genes. Genes that were significantly dysregulated and had TPM value of more than 1 were used as genes of interest. All the expressed genes with TPM value of more than 1 were used as a background. Functional annotation enrichment analysis concluded that genes were enriched with significance for specific features i.e. presence of glycoproteins and disulphide bonds. It indicates potential involvement of proteins in glycosylation and having functions associated with cell communication or interaction with extracellular environment. Moreover, proteins appeared to have enrichment of EGF like domain feature.

Annotation cluster	Enrichment	Gene count	P-value	Benjamini
UP_KW_DOMAIN	EGF-like domain	5	6.5E-4	9.7E-3
UP_KW_PTM	Disulphide bond	9	2.5E-3	3E-2
UP_KW_PTM	Glycoprotein	10	4.6E-3	3E-2

Table 4:

The table shows the results for functional annotation enrichment analysis performed on the genes of interest in the background of all expressed genes in the data.

Gene_id	Gene_name	Gene_biotype	log2Fold Change	pval	padj
ENSG00000186115	CYP4F2	protein_coding	2.35615	0.000161	0.01386
ENSG00000149968	MMP3	protein_coding	1.812905	0.000218	0.017259
ENSG00000131482	G6PC	protein_coding	1.660422	2.89E-10	2.43E-07
ENSG00000148057	IDNK	protein_coding	1.655762	0.000132	0.011813
ENSG00000168010	ATG16L2	protein_coding	1.527122	8.71E-05	0.008687
ENSG00000146678	IGFBP1	protein_coding	1.458708	0.000768	0.042118
ENSG00000110244	APOA4	protein_coding	1.455991	4.73E-08	2.2E-05
ENSG00000170271	FAXDC2	protein_coding	1.367138	2.12E-10	1.89E-07
ENSG00000061656	SPAG4	protein_coding	1.264102	0.000437	0.028102
ENSG00000183747	ACSM2A	protein_coding	1.185164	2.95E-05	0.003918

Table 5:

The above table shows a list of genes that were significantly upregulated upon KD of SNORA74D

Gene_id	Gene_name	Gene_biotype	log2 Fold Change	pval	padj
ENSG00000158683	PKD1L1	protein_coding	-3.32509	1.33E-11	1.74E-08
ENSG00000215529	EFCAB8	protein_coding	-3.11679	3.12E-08	1.58E-05
ENSG00000205111	CDKL4	protein_coding	-2.18409	6.15E-11	6.26E-08
ENSG00000100053	CRYBB3	protein_coding	-2.01301	6.77E-07	0.00019
ENSG00000074181	NOTCH3	protein_coding	-1.80498	5.3E-06	0.001007
ENSG00000158023	CFAP251	protein_coding	-1.80173	6.71E-11	6.63E-08
ENSG00000165917	RAPSN	protein_coding	-1.79839	3.38E-08	1.7E-05
ENSG00000123977	DAW1	protein_coding	-1.76595	0.000216	0.01717
ENSG00000124882	EREG	protein_coding	-1.65766	0.000436	0.028102
ENSG00000102890	ELMO3	protein_coding	-1.56811	2.23E-11	2.77E-08

Table 6:

The above table shows a list of genes that were significantly downregulated upon KD of SNORA74D

Some of these genes from Table 5 and 6 belong to both pro and anti-apoptotic or proliferative gene categories. This could potentially indicate either dual role of SNORA74D or that the cell tries to counter the effects caused by SNORA74D. Therefore, it is vital to look for any changes in phenotype upon KD of SNORA74D.

Changes in cell viability observed after Knock down of SNORA74D:

Some cell death was observed in cells transfected with 30nM concentration of NC5 (scramble) and ASO1 & ASO2 versus the control. This can be attributed to the stress caused due to toxicity by lipofectamine 2000 and ASO itself. But since this was the only transfecting agent that resulted in a good consistent knockdown, we decided to use it. It was further observed that cells transfected with scramble sequence died comparatively more whereas the cells with decreased expression of SNORA74D survived better. Therefore, further experiments were performed to explore this effect.

BrdU assay:

BrdU (5-bromo-2'-deoxyuridine) is a synthetic nucleoside that is commonly used in molecular biology and cell biology research to label and detect newly synthesized DNA in cells. When cells are exposed to BrdU, it gets incorporated into the newly synthesized DNA during the S phase of the cell

cycle. The BrdU stain provides a way to track and identify actively dividing cells. Columbus software was used to analyze the cells stained with BrdU at 48h post transfection with ASOs.

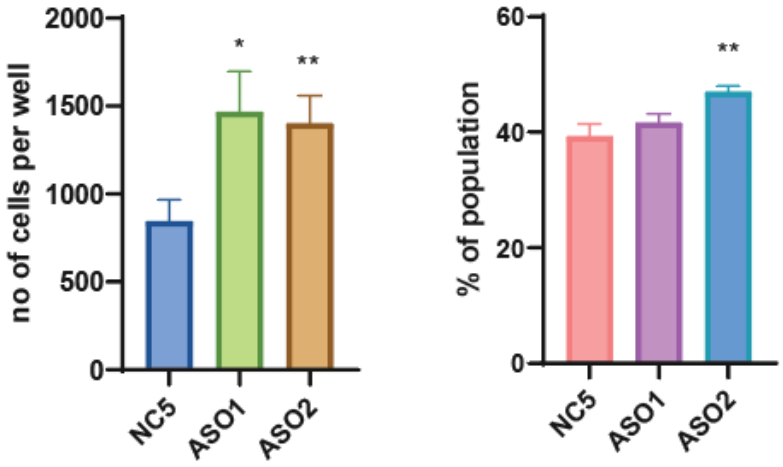

Figure 17:

Left: In comparison to cells with ASOs treatment & treatment with only transfecting agent (control), cells transfected with the scramble sequence exhibited the lowest total cell count. This could be either due to higher incidence of cell death in NC5 or lower growth potential. Statistical analysis was done via two-tailed t-test using 95% CI. (****p value <0.001; ***p value <0.001; **p value < 0.01; p value < 0.05).

Right: We stained the cells with BrdU to check the number of actively growing cells in each well. Cells transfected with ASO2 were significantly seen to stain more with BrdU. This indicates that a higher population of cells were growing in the well with knockdown of SNORA74D. Moreover, a difference in amount of cell growth in cells transfected with ASO1 is also observed but it is not significant. Statistical analysis was done via two-tailed

t-test using 95% CI. (****p value <0.001; ***p value <0.001; **p value < 0.01; p value < 0.05).

PI staining:

Propidium iodide (PI) is a fluorescent dye commonly used in molecular biology and cell biology to stain DNA. PI is impermeant to alive cells but can penetrate cells with compromised cell membranes, such as dead or damaged cells. Therefore, PI staining is often employed to assess cell viability and to distinguish between alive and dead cells in a population.

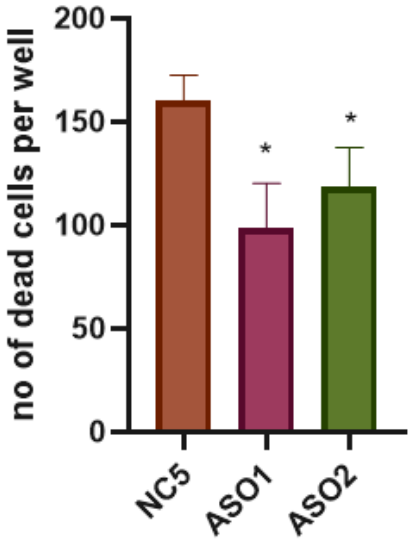

Figure 18:

PI staining revealed that a higher population of cells died in wells transfected with scramble sequence in comparison to ASOs. This further indicates that cells with knockdown of SNORA74D can better survive the stress caused by transfection. Statistical analysis was done via two-tailed t-test using 95% CI. (****p value <0.001; ***p value <0.001; **p value < 0.01; p value < 0.05).

xCELLigence assay:

xCELLigence is a real-time, label-free cell analysis technology that is primarily used for dynamic and continuous monitoring of cellular growth. 5000 HepG2 cells were transfected with scramble and ASOs to establish a KD of SNORA74D. No significant morphological changes were observed upon KD with ASOs 48h post transfection. These cells were then observed under xCELLigence and the results were recorded.

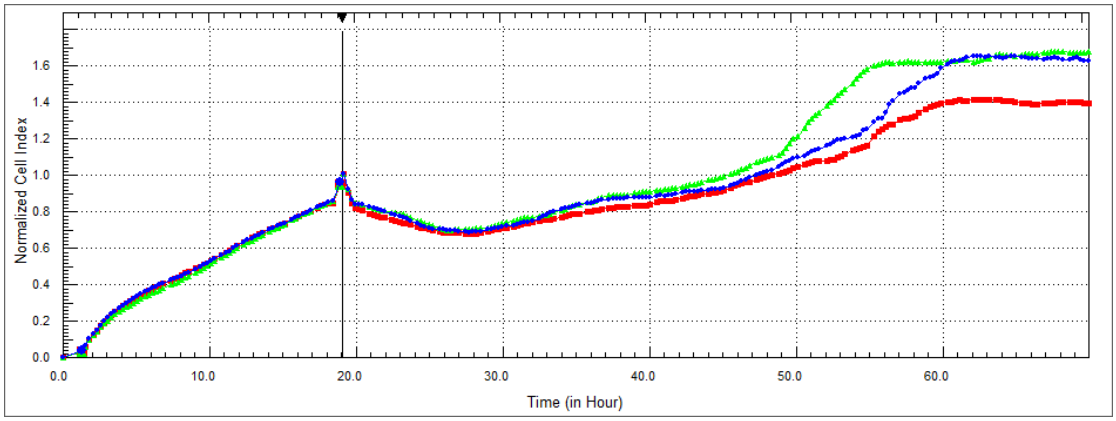

Figure 19:

The growth of cells after successful knockdown in SNORA74D: Red curve indicates the cells transfected with control (NC5) or scramble sequence. Green and Blue curve lines indicate cells transfected with ASO1 and ASO2 respectively. The lines represent an average of 3 replicates. The cells with low expression of SNORA74D appear to have better cell survival and growth.

The results of these experiments indicate a definitive effect on cell viability by KD of SNORA74D. We stated earlier in the results of TGIRT seq that KD of SNORA74D affected both pro and anti-apoptotic genes, but we see the cells surviving better in the absence of SNORA74D therefore, we can speculate that the overall affect caused by anti-apoptotic genes was greater and hence helped the HepG2 cells survive the stress induced by transfection.

In the literature we can find many snoRNAs linked to the prognosis of the diseases but a lot more research is required to understand the molecular functions performed by these snoRNAs within a cell ultimately contributing to the disease progression. Therefore, here I tried to see if I can see a difference in expression of SNORA74D upon inducing stress in the cells to replicate the stress of tumor microenvironment. The experiments performed were however single replicate and thus need further validation.

ER stress

Tunicamycin 5ug/ml was used for causing ER stress to the HepG2 cells. GAPDH, ACTB, MRPL19 were used as housekeeping genes. Snora81 was used as a control snoRNA to test if the effect observed on SNORA74D was a generalized effect on all snoRNA or specific for SNORA74D. The results showed a successful initiation of ER stress as can also be seen in the graph below:

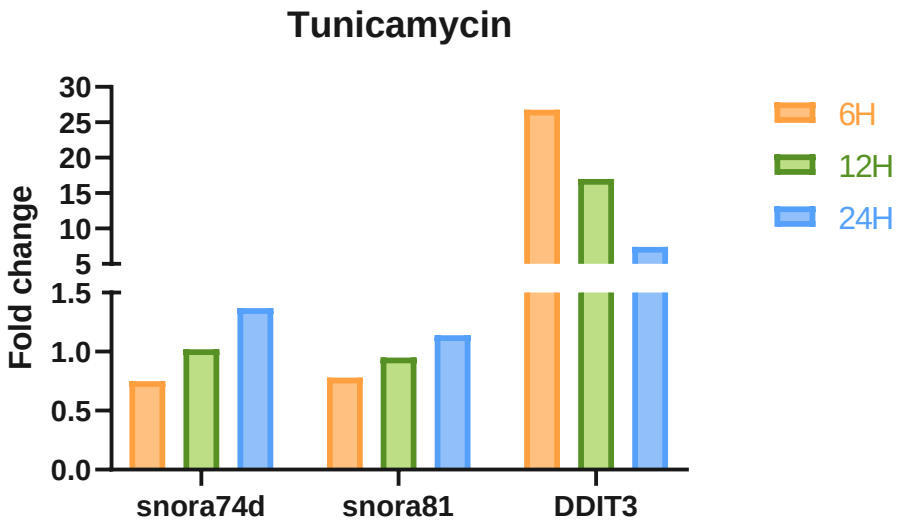

Figure 20:

This graph illustrates the upregulation of DIDT3 gene as compared to untreated control cells at 3-time intervals (6H, 12H and 24H). It is a hallmark of ER stress indicating successful development of ER stress. SNORA74D was seen to increase along with other snoRNA after 24H therefore, indicating that the upregulation could be the result of a collective increase in transcription machinery upon reduction of overall ER stress.

Oxidative stress

Various concentrations of staurosporine (10nM, 20nM and 50nM) were used to cause oxidative stress in HepG2 cells. The cells were collected after 24h of treatment. GAPDH, ACTB, MRPL19 were used as housekeeping genes. Untreated cells were used as control for the experiment.

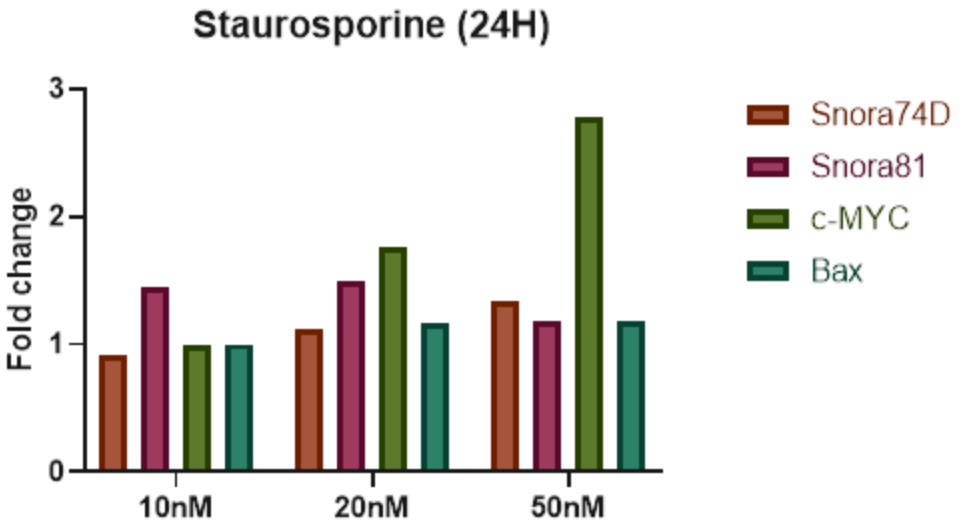

Figure 21:

This graph shows a slight increase in expression of SNORA74D but so does an increase in SNORA81 (control snoRNA) is observed. This makes us believe that oxidative stress could cause the overall expression of snoRNA to be increased. However, this is the result of a single experiment therefore there is a need for it to be validated via multiple replicates.

Discovering multiple isoforms of SNORA74D

The bedGraph analysis of multiple cell lines indicated the presence of a longer extension of SNORA74D in addition to the canonical sequence. This can be observed in figure 22.

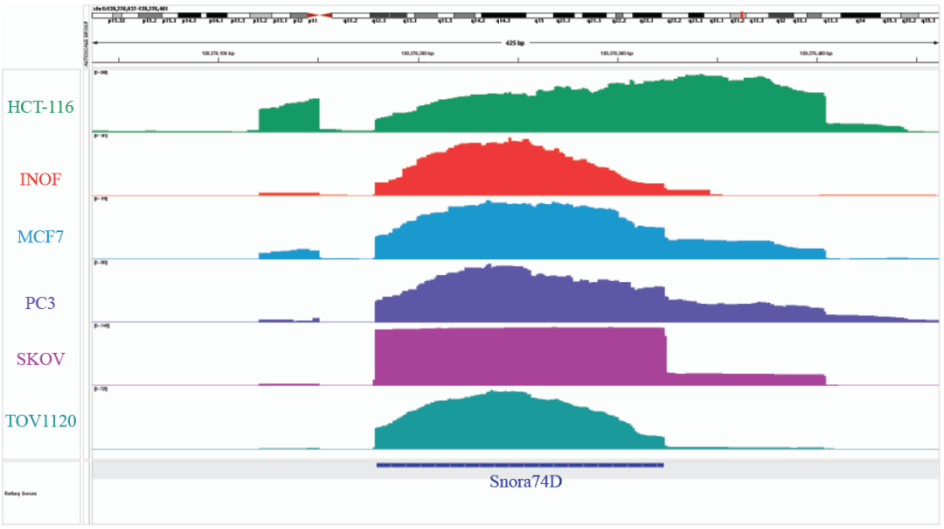

Figure 22:

The presence of reads beyond the sequence of SNORA74D in the above bedGraph shows the presence of extended form of SNORA74D in some cell lines while other cell lines such as INOF and TOV112D appear to not have this extension.

This observation of presence of multiple isoforms was very interesting as this has never been reported for SNORA74D. This prompted us to see if the cell line we used for KD of SNORA74D also had the presence of these isoforms.

Identification of presence of isoform of SNORA74D in HepG2:

ddPCR showed that among the canonical SNORA74D sequence, long extension of SNORA74D and short extension of SNORA74D, only long form existed in HepG2 cell line grown over a period as presented in Table 7. Primers were specifically designed to bind with individual forms therefore providing a clear picture of their presence.

	SNORA74D	SNORA74D (long extension)	SNORA74D (short extension)	SNHG pre-mRNA
HepG2 24H	0	153.3	30	30.7
HepG2 48H	0	391.2	51	80.8
HepG2 72H	0	418.2	35	72.8
HepG2 96H	0	716.3	96	63.7
HepG2 120H	3	119	13	15
HepG2 144H	0	298.4	61	41.6

Table 7:

This table shows the total amount of each isoform present in the cell line. SNORA74D original sequence doesn't appear to be present in HepG2 cell line. The amount of short extension equals to pre-mRNA approximately so it can be suggested that it also doesn't exist independently. Therefore, leaving only the long form to independently exist in HepG2 cells.

This is quite interesting because it raises multiple questions regarding why the known form is not present in HepG2 and if there is a precursor product relationship between these isoforms? Moreover, the presence of SNORA74D isoforms in HepG2 cell line sparked our interest to investigate if we can identify these isoforms in normal and tumor samples.

Identification of presence of isoform of SNORA74D in normal tissue:

The presence of all 3 forms of SNORA74D were investigated in normal tissue samples. The following bedGraphs show that apart from breast and testis, no normal tissue including liver had the extended version of SNORA74D sequence.

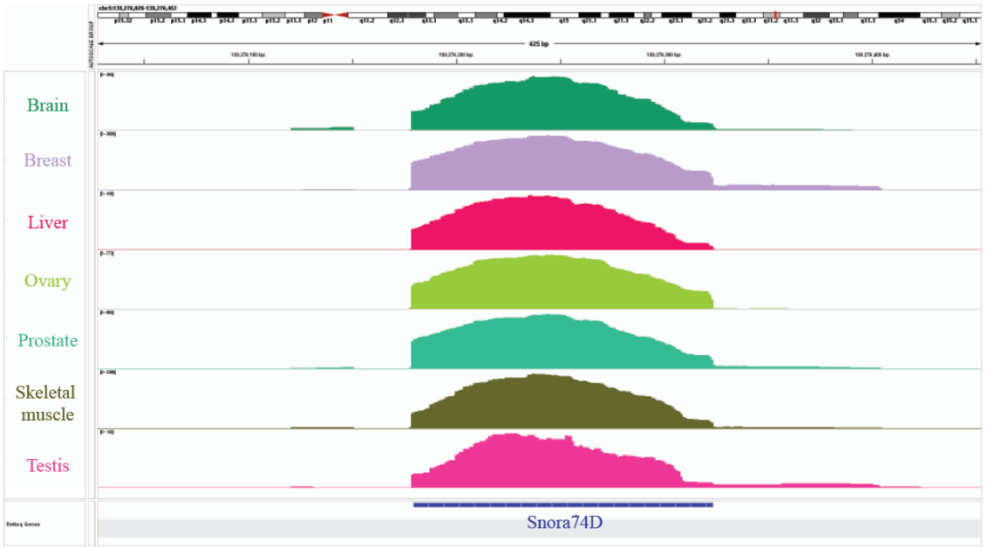

Figure 23:

The bedGraph shows the presence of extended form of SNORA74D in breast and testis while other normal tissue samples expressed only the canonical form of SNORA74D.

Detection of the isoform of SNORA74D in liver cancer tissue:

The presence of all 3 forms of SNORA74D were investigated in HCC samples. ddPCR was performed on cDNA from both the HCC tissue and the surrounding NASH tissue. The results are presented below:

Samples	SNORA74D long extension	SNORA74D short extension	SNORA74D (canonical)
Sample 1 NASH	7.3	2	11.1
Sample 1 HCC	39.6	40.2	47.2
Sample 2 NASH	14.5	8.7	7
Sample 2 HCC	154	110	0
Sample 3 NASH	22.2	9	7.6
Sample 3 HCC	116.8	75.2	12

Table 8:

The above table represents the isoforms in both the HCC tissue and the corresponding NASH tissue.

The presence of these isoforms in the HCC tissue as well as the NASH tissue indicate their potential involvement in the process of development of HCC. Therefore, it is important to perform further research on these isoforms and understand their function and biogenesis.

Discussion

The study of snoRNA is vital for better understanding the initiation and progression of HCC. It would help in unraveling the molecular mechanisms that are not only associated with HCC development but also can serve as potential targets for therapeutic interventions. The snoRNAs are usually dysregulated in HCC just as in the case of any other cancer. Finding novel snoRNAs that are significantly dysregulated between the cancer and the paired NASH tissue was the primary objective of this study.

To this end we received the RNA seq data from 11 patients with NASH and HCC. The analysis of this data revealed a set of snoRNA that were significantly upregulated in HCC in comparison to NASH. We selected those snoRNAs and further tried validating this observation on HCC tissue samples and NASH samples via qPCR. The results of that qPCR revealed that SNORA74D was the only snoRNA that was significantly upregulated in HCC tissue samples. This solidified our interest in further researching on SNORA74D.

This study was further focused on determining the potential targets of SNORA74D and identifying the underlying mechanism of action for it in HCC. The RNA seq data was re-analyzed to determine a set of genes that have significant correlation with SNORA74D. SnoGPS was used as a Bioinformatics tool for determining the potential targets of SNORA74D among the selected genes. We received 7 genes at the end of this analysis.

Furthermore, cellular model with KD of SNORA74D was developed. The effect of KD of SNORA74D on the target genes was then analyzed by qPCR. The qPCR demonstrated that majority of these target genes were influenced by the KD of

SNORA74D. However, for further validation we sent the RNA for TGIRT sequencing. The purpose of this sequencing was to not only identify any potential targets of SNORA74D in mRNA but also among non-coding RNA. TGIRT seq analysis revealed that all the potential target genes are not significantly dysregulated upon KD of SNORA74D. However, there are many other protein coding genes and non-coding RNA i.e. tRNA and snRNA that were significantly dysregulated in the samples. This is interesting because it shows that the knockdown of SNORA74D impacted directly or indirectly a number of different processes inside the cell and hence it can be safely assumed that SNORA74D is important for the disease progression. Nevertheless, further research is required to identify the specific mechanisms affected by SNORA74D in the cell and how SNORA74D affects the HCC development and progression by utilizing those pathways.

To understand the phenotypic changes in the cell upon KD of SNORA74D we observed the changes in cell viability. It was evident by looking under the microscope at the wells transfected with ASO and the control, that comparatively more no of cells died in wells transfected with control ASO than the cells with the KD of SNORA74D, who were observed to be surviving better. Although at this point one can think that since SNORA74D was upregulated in HCC then its KD should impact the cells in a way that it reduces the proliferation and survival of the cells. But this observation can be explained by the fact that cancer is a very complex disease. On one side the cells are proliferating in cancer but on the other side the cells are dying also due to extreme stress and inflammation often undergoing necrosis. Massive necrosis is also a hallmark of HCC. That is how the liver must lose all its function at one point. The swelling and rupturing of cells lead to further inflammation thereby contributing to tumor microenvironment. Moreover, aberrant apoptosis is also a prominent feature of chronic liver disease [142]. HCC exist in

chronic hepatitic liver where hepatocyte apoptosis is frequently observed. Hepatocyte apoptosis is also involved in inducing compensatory liver regeneration which is believed to contribute in tumor initiation. Additionally, increase in apoptosis causes a rise in oxidative stress which may promote further tumor progression independent of compensatory liver regeneration. Therefore, apoptosis can not only have role in removing the mutated cells but also it is now believed to assist in tumor progression by impacting the tumor microenvironment [143].

Therefore, for determining if the cell death was significantly higher in control samples when related to ASO we performed PI staining. The results obtained after analyzing the data indicated that indeed higher no of cells were dead in control wells and significantly less cells died in wells treated with ASOs. We further performed Xcelligence assay to determine the difference in cell survival upon KD of SNORA74D. The results of this assay showed that indeed the cells transfected with ASO had better survival and were better able to overcome the stress of transfection. This further proves that KD of SNORA74D impacted the cell survival by either downregulating the pro-apoptotic genes or upregulating the pro-survival genes. This was also observed in TGIRT seq data as the top most significantly upregulated and downregulated genes are both anti-apoptotic and pro-apoptotic. Therefore, we assume that either SNORA74D has a dual role, or it is impacting more than one processes of the cells which collectively helps in tumor proliferation and survival and its knockdown also then initiates a cascade of events that help in cellular survival.

To see if the KD impacted the growth of the cells, we performed BrdU assay and determined that indeed more no of cells were alive in wells transfected with ASO in comparison to control. Moreover, cells in the wells transfected with ASO2 were significantly dividing more than control. This indicates that the cells in control well

were under stress, and they were either dying more or replicating very slowly making the total no of cells in wells with KD of snora74D to be higher than control upon end of treatment at 48h.

Further in the study we tried to observe effects on SNORA74D upon inducing stress i.e. ER stress and oxidative stress. The qPCR did not show a significant change in SNORA74D expression upon introduction of these stresses. These results should be considered preliminary as they were performed near the time of thesis submission and thus need to be further verified and explored to better understand what type of stress causes an upregulation of SNORA74D.

During this study we also identified the presence of more than one isoform of SNORA74D. This is a very interesting observation as this is the first time it's being reported. We were able to see the canonical and extended form of SNORA74D in normal tissue, cell lines and HCC tissue. What is worth noting here is that according to our initial observations, there is no extended form in normal liver tissue but both extended and canonical forms are present in HCC tissues. This makes us curious in further exploring these isoforms and their function. We would like to further work on determining their potential involvement in HCC.

Conclusion

The data obtained in this study clarify the stance of SNORA74D to be significantly dysregulated in HCC tissue in comparison to NASH. This dysregulation was further verified by qPCR. Furthermore, TGIRT seq analysis indicated the potential involvement of SNORA74D in modifying multiple tRNAs thereby affecting the translation in a cell. Particularly, multiple protein coding genes were also observed to be dysregulated which potentially could be the result of either of them being direct targets of SNORA74D or they could be the result of SNORA74D impacting the translation machinery of the cell. It was also reported by the study that knockdown of SNORA74D impacted the overall cell survival. Finally, multiple isoforms of SNORA74D were reported in this study that gives a very interesting direction to this study since different types of isoforms are observed in normal and HCC tissue. We now wonder if these isoforms have some precursor product relationship or if one of the two isoforms is more involved in cancer initiation and progression. This would help in better understanding HCC and develop better therapeutic targets in the future.

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