# Targeting NELFE Splicing with Antisense Oligonucleotides in Hepatocellular Carcinoma

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**Abstract**

Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer, and is currently one of the fastest increasing types of cancer in the United States. However, its heterogeneity causes major challenges in finding clinical treatments. Negative Elongation Factor E presence has shown to connect with growth of tumors. This paper explores how antisense oligonucleotides (ASOs) can alter the splicing, expression, and stability of the NELFE gene, and how it can be leveraged to decrease harmful NELFE levels in cancer cell. Using HepG2B, Huh1, and Huh7 human cell lines, this research attemps to turn a natural genetic vulnerability into a precision treatment tool via ASO-induced exon skipping, harnessed therapeutically to potentially slowing cancer progression. Lower NELFE disrupts the NELF complex, potentially impairing transcriptional pausing in cancer cells. Moreover, our created i9 and e10 ASOs successfully targeted intron 9 or exon 10 of NELFE and mimicked a naturally occurring single nucleotide polymorphism (SNP) that drives exon 10 skipping to reduce NELFE protein expression.

**Keywords:** Hepatocellular Carcinoma; Antisense Oligonucleotides; Negative Elongation Factor

# Contents

# 1 Introduction

Liver cancer is one of the most rapidly increasing types of cancer in the United States. Currently it is the 6th most common cancer in the world and has the third highest mortality rate with a 5-year survival rate of 21% [1]. It is typically caused by cirrhosis, which is the scarring of liver tissue, often a result of chronic hepatitis virus infection or prolonged alcohol use, both of which are common in diagnosed patients.

The most common type of liver cancer is hepatocellular carcinoma (HCC)[2]. HCC originates in hepatocytes, which are the predominant cell type in the liver, making up to 80% of its mass. Hepatocytes are round in shape, containing a nucleus and an abundance of cellular organelles associated with metabolic and secretory functions. These cells play a pivotal role in liver inflammation and are responsible for carbohydrate, lipid and protein metabolism. Most notably, hepatocytes aid in the detoxification processes and in the activation of immune cells, helping to maintain liver homeostasis.

HCC is a highly heterogeneous malignancy, primarily due to the wide range of underlying risk factors. These include chronic viral hepatitis Hepatitis B Virus or Hepatitis C Virus (HBV or HCV), long-term alcohol use, and metabolic syndromes, all of which can lead to prolonged liver inflammation. Over time, chronic inflammation promotes the development of fibrosis and eventually cirrhosis, significantly increasing the risk of malignant transformation. During this process, hepatocytes accumulate genetic and epigenetic alterations, and the liver microenvironment undergoes major changes that support tumor growth and immune evasion.

Interestingly, HCC patients harboring a naturally occurring single nucleotide polymorphism (SNP) exhibit lower levels of NELFE mRNA in tumor tissues compared to wild-type patients, suggesting that alternative splicing events linked to this SNP may suppress NELFE function.

An accumulation of molecular alterations contributes not only to heterogeneity between patients but also within individual tumors, or even between distinct tumor nodules in the same patient. Such heterogeneity is a major obstacle in the clinical management of HCC. Unlike many other cancers, no singular driver gene or pathway has been identified in HCC, making it difficult to develop effective targeted therapies. Although certain mutations are commonly observed—such as TERT promoter mutations (60%), TP53 (30%), and CTNNB1 (30%, part of the Wnt/-catenin pathway)—there is no universal genetic signature across all patients. Frequently altered pathways include the p53 tumor suppressor pathway, the Wnt/-catenin pathway, and telomerase activation, but these are not consistently targetable across all tumors.

The intratumoral heterogeneity increases the likelihood of cancer recurrence, drug resistance, and tumor dormancy, all of which contribute to the poor prognosis and limited

efficacy of existing therapies. Given the global dysregulation observed at both genetic and epigenetic levels, recent studies have turned attention toward RNA-binding proteins (RBPs) as potential key mediators of HCC development and progression. RBPs are involved in virtually every stage of gene expression—from transcription to RNA splicing, transport, translation, and degradation—and their dysregulation may have widespread consequences on oncogenic signaling networks.

One such RBP that has shown connection to the progression of HCC is the Negative Elongation Factor E (NELFE) [3]. NELFE is one of the top three most differentially expressed RBPs in HCC and is significantly upregulated in tumor tissues [3]. NELFE regulates transcription by pausing RNA Polymerase II. It also plays a central role in promoting MYC oncogenic signaling, which is frequently activated in liver cancer. Moreover, abundance of NELFE is correlated with tumor growth for other cancers as well. It stabilizes oncogene E2F2 mRNA in gastric cancer to accelerate proliferation and metastasis [4] [5], decreases stabilization of tumor suppressor NDRG2 mRNA in pancreatic cancer promoting EMT and metastasis [6], and interacts with oncogene SLUG in breast cancer to drive EMT and cancer stemness [7].

To address the functional importance of NELFE in HCC, we investigated a targeted therapeutic approach using antisense oligonucleotides (ASOs). ASOs are short, synthetic, single-stranded oligonucleotides that can specifically bind to RNA molecules to alter their splicing, stability, or translation [8]. In this study, we designed ASOs that target splice junctions within the NELFE pre-mRNA, specifically the intron 9–exon 10 and exon 10–intron 10 boundaries as shown in Figure 1. By disrupting proper splicing of NELFE transcripts, we aim to reduce its expression and investigate the impact on downstream oncogenic pathways in HCC cells.

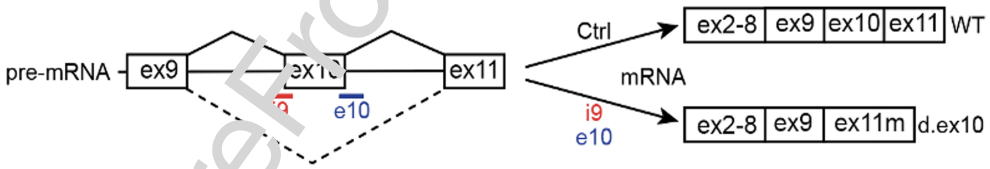


Figure 1: ASOs targeting human NELFE intron 9- exon 10, exon 10-intron 10 splice junctions.

The goal of this study is to analyse how ASOs affect NELFE isoform production, mRNA stability, and protein levels, potentially offering new therapeutic angles for HCC. We aim to turn a natural genetic vulnerability (HCC SNP) into a precision treatment tool via ASO-induced exon skipping, so that it can be harnessed therapeutically and administered as microbubble ASOs to reduce NELFE levels and potentially slow liver cancer progression.

## 2 Methodology

The overall methodology is shown in figure 2.

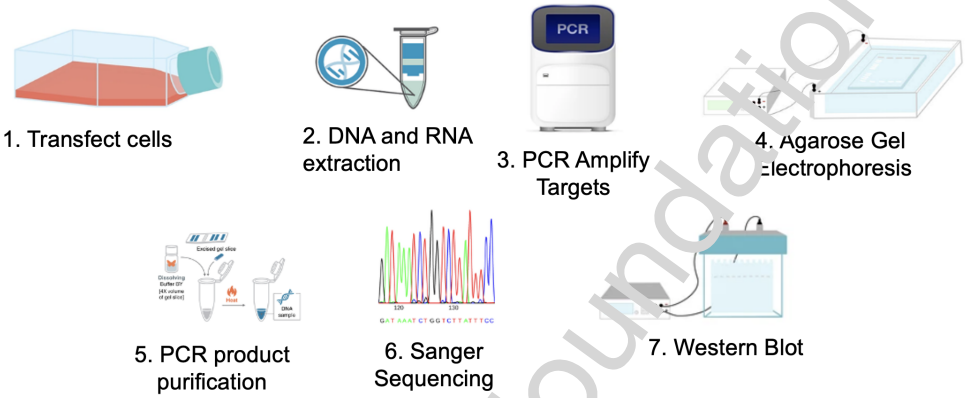


Figure 2: General workflow for studying the skipping of exon 10 utilizing both ASOs and CRISPR-Cas9 system.

### 2.1 Human Cell Lines

The three human cell lines used in this experiment are the **Hep3B, Huh1, Huh7** cell lines. All cells were kept in a 37°C humidity incubator with 5% CO2. Cells were cultured only up to 15 passages from freeze thaw.

**Hep3B** is derived from liver tissue in an 8-year-old Black patient with liver cancer, displaying typical adherent culture properties *in vitro*. Importantly, Hep3B has **3 NELFE copies** and harbors an **integrated Hepatitis B Virus (HBV) genome**. The cell line expresses high levels markers such as alpha-fetoprotein (AFP), hepatitis B surface antigen (HBsAg), albumin and transferrin protein, and is highly tumorigenic (capable of forming tumors) in immunodeficient mice. Hep3B is also **p53 null**. P53 is a tumor suppressor protein that regulates cell cycle arrest, DNA repair, and programmed cell death. It gets activated in response to DNA damage or stress and prevents the cell from dividing until it's repaired.

**Huh1** contains multiple copies of **HBV** as well, with **3 NELFE copies**. It is originally derived from primary liver tumor of an Asian male patient with HBs-antigen carrier and is known for reflecting aggressive tumor behavior. The cell line expresses cancer stem cell markers (e.g., CD133). It has high metastatic potential and often used *in vivo* tumorigenicity models.

**Huh7** is derived from a liver tumor in a Japanese male patient in 1982. It has **2 NELFE copies** and is known for its ability to support **hepatitis C virus (HCV)** replication. Huh7 has high transfection efficiency, produces AFP, and expresses liver-specific enzymes, making it one of the most widely used HCC cell lines. It contains **wildtype p53**, although some sources report mutations due to subclonal variability, having partially dysfunctional p53.

### 2.2 ASO RT-PCRs

ASO RT-PCRs were done with the following: Primestar Max 2x, NELFE SNP F2, NELFE SNP R2, GAPDH F, GAPDH R, Taq 2x, G1testF, SNP Seq R, MYC SYBR G F4, MYC SYBR G R4, 10uM long NELFE SNP F2, plenti-3c-R. All were ran on either 1.5% or 2.0% agarose gel for 30 minutes. The final gels were then imaged with a ChemiDoc and exported to Adobe Illustrator for processing.

### 2.3 Western Blots

Western blots were done with three 15 minute washes after primary incubation, and three 15 minute washes after secondary incubation. ECL max was then added for imaging. Blots were run on 4-12% Bis-Tris gels at 180V for 1.5hrs in 1x MES running buffer. Dry transfer at 23 V for 6 min. Blocked 30 minutes in 5% milk. Primary antibodies were incubated overnight in 5% milk. Secondary antibodies were incubated 30min in 5% milk.

## 3 Results

### 3.1 i9 and e10 ASOs alter NELFE splicing, decreasing NELFE expression and impacting broader NELF complex

3.5uM ASO was electroporated into cells. 48 hours later the cells were collected for RNA isolation (cDNA synthesis, PCR, etc.), giving the cells time to recover and be healthy after electroporation and be within a time range of seeing the affect of the ASO.

The control is a scramble control, meaning it has a set of random 25 bases that should not be binding specifically to anything.

I9 has larger effect on exon 10 skipping. In figure 3, both Huh1 and Huh7 cell lines show isoform switching (D.ex-10), meaning that the ASOs are effectively modulating splicing of the NELFE transcript and inducing exon 10 skipping.

In Figure 4, the western blot analysis of Huh7 cells transfected with ASOs targeting NELFE

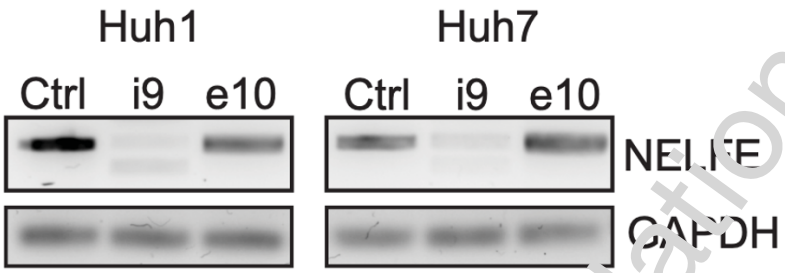


Figure 3: ASO RT-PCR Results showing control, i9, and e10.

exon 10 shows reduced NELFE protein in both i9 and e10 conditions. This is accompanied by a decrease in NELFB and NELFC expression, suggesting destabilization of the NELF complex. NELFA decreases. GAPDH confirms equal loading. The first GAPDH (third row from the top) is for the NELFA and NELFC blots. The second GAPDH (last row) is for the NELFB and NELFE blots. These findings support the model that ASO-induced splicing leads to NELFE loss at the protein level and impacts the broader NELF complex. Losing one unit of the NELF complex causes loss of the complex [9].

### 3.2 Degradation of D.ex-10 isoform in Huh7 should be faster than WT NELFE mRNA. E10 seems more stable than I9 ASO

To track the half-life of our ASOs we used Actinomycin D, a transcriptional inhibitor that binds DNA at the transcription initiation complex and blocks RNA polymerase II from elongating new transcripts. Once transcription is shut off, we are able to track how long an existing RNA lasts (its half life). The goal is to see if the D.ex-10 isoform degrades faster than the WT NELFE mRNA, thus determining if stability of isoforms is the cause of lower RNA.

We test to see if full length or d.ex10 isoforms have different mRNA half lives. If the half-lives are different, it may explain the presence of reduced protein. However, if there is no difference, the reduction could be due to less pre-mRNA being made into mRNA or the mRNA undergoing translation associated decay or the RNA makes a very unstable protein.

MYC is a family of regulator genes and proto-oncogenes that code for transcription factors. It initiates and maintains cancer through both tumour cell-intrinsic mechanisms and host immune and TME-dependent mechanisms [10]. GAPDH serves as the loading control and stable mRNA control for the PCR and MYC serves as the short half life control.

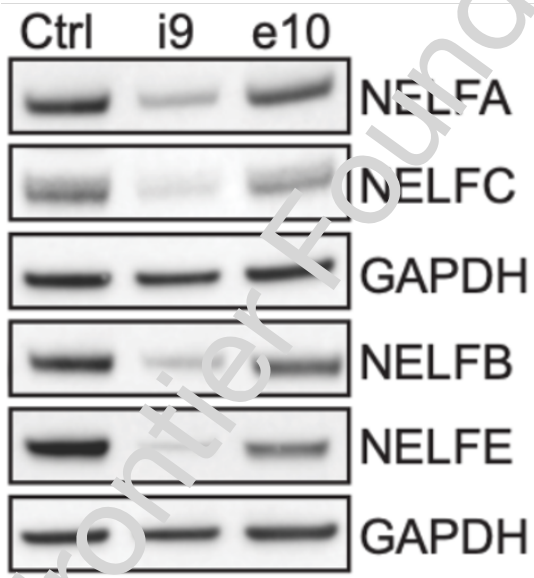


Figure 4: Western Blot showing impact of Huh7 transfected ASOs on NELFA, NELFC, GAPDH, NELFB, and NELFE

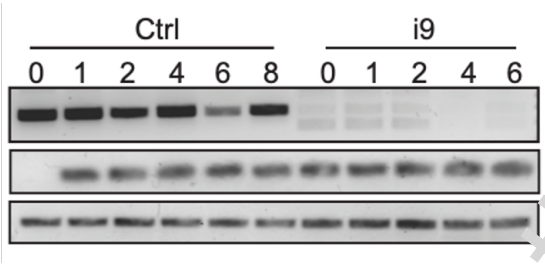


Figure 5: Actinomycin D PCR results control and i9 for Huh7 Cell line


Figure 6: Actinomycin D PCR results control and e10 for Huh7 Cell line

In figure 5, the Huh7 cells are transfected with the ASOs targeting NELFE exon 10. The Actinomycin D shows that the control gets somewhat fainter as hours increase, and the change in i9 FL(top band) and Ex-10 (lower band) density over time seems consistent between both.

In figure 6, e10 does not show a decrease in band strength compared the control over time. If Actinomycin D works, we expect MYC to be gone by 20-40 minutes. [11]

## 4 SNP present in #26 and #75 HEK SNP clones

In Figure 7 both forward and reverse sequencing show a clear G to A mutation. Figure 8 shows a G to A mutation with SNP present but the sequence is slightly dirty with double peaks and indels. Figure 9 shows the RT-PCR where clones are picked for sequencing.

The presence of the natually occuring SNP in the clones suggests that we may be able to turn a natural genetic vulnerability into a precision treatment tool via ASO-induced exon skipping, harnessed therapeutically to reduce NELFE levels and potentially slow cancer progression.


(a) Clone #26 Sequencing results

(b) Clone #75 Sequencing results

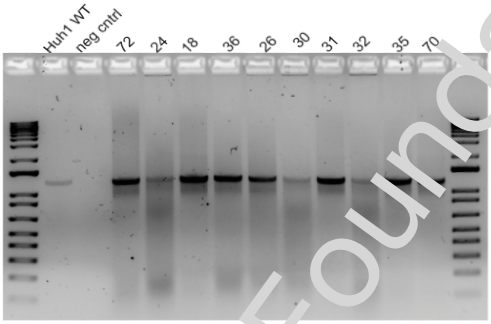


Figure 7: HEK SNP Clones PCR

## 5 Conclusion

Increased NELFE expression is closely associated with the progression of hepatocellular carcinoma (HCC), highlighting its potential role as an oncogenic driver in liver cancer.

To mimic this naturally protective splicing pattern, we designed ASOs targeting the exon 10 splice junctions of NELFE. These ASOs effectively induce exon 10 skipping, resulting in the production of an alternatively spliced NELFE transcript. This disruption significantly impacts the NELF complex, which is essential for RNA Polymerase II transcriptional pausing. ASO-i9 and ASO-e10 were particularly effective in driving exon 10 skipping and caused a marked reduction in wild-type NELFE protein expression.

The downregulation of NELFE by these ASOs is likely to impair the stability of the NELF complex, which may in turn disrupt transcriptional regulation in HCC cells, contributing to reduced tumorigenic capacity.

Collectively, this work highlights a novel splice-switching therapeutic strategy targeting NELFE in HCC. By leveraging naturally occurring splicing patterns and modulating NELFE expression, ASOs may offer a promising route to disrupt oncogenic transcriptional programs and develop more precise treatments for this highly heterogeneous and difficult-to-treat cancer. The created ASOs have an effect on NELFE RNA and protein in

cell lines with different etiologies (HBV vs HCV) and NELFE copies (2 & 3), indicating it may be useful broadly in HCC.

This research can be expanded in the future by delivering different ASOs to liver tumors in vivo using microbubbles, and experimenting with different Mechanical Index ultrasound waves. Furthermore, we can continue to assess ASO effectiveness in syngeneic mouse model *in vivo* , optimize Actinomycin D mRNA decay assay, and validate potential HEK SNP clones.

## 6 Acknowledgments

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