Knockout of LINC01134 as a potential treatment for Liver Cancer in HepG2 cells

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Abstract

Liver cancer, a major global health issue, continues to contribute significantly to global deaths. Hepatocellular carcinoma (HCC), the most prevalent primary liver cancer, remains a leading cause of cancer-related fatalities. This study explores the potential treatment for HCC by investigating the role of LINC01134, an encoding gene for the LncRNA TLNC1, in mediating the nuclear export of p53. The hypothesis suggests that knocking out the LINC01134 gene in HepG2 cells will decrease p53 nuclear export, leading to increased p53 levels and enhanced cellular responses to DNA damage. The methods include CRISPR CAS-9 for gene knockout, RT-PCR for TLNC1 expression, Immunofluorescence for p53 within the nucleus, and xenograft mouse model for in vivo tumor size reduction. Statistical analysis will be performed using a one-sample T-test. This strategy might effectively improve the cell's reaction to DNA damage, which could halt or slow the growth of HCC.

Keywords: HCC, LncRNA, TLNC1, HepG2, p53, Knockout

1. INTRODUCTION

1.1 Background

Cancer is still among the major health issues globally, contributing significantly to global deaths. Cancer is a broad category of disorders that can be present in nearly any organ or tissue in the body when abnormal cells proliferate without control, cross conventional boundaries to infect other body parts, or spread to other organs [1]. The World Health Organization states that an approximate figure of ten million deaths due to cancer was due to cancer in the year 2020 alone [2]. Current research is interested in investigating its complicated processes, risk factors, and possible treatment prospects.

Hepatocellular carcinoma (HCC) stands as the most prevalent primary liver cancer globally and represents a significant contributor to cancer-related fatalities. In the United States, HCC ranks as the ninth leading cause of cancer-related deaths [3]. In primary liver malignancies, hepatocellular carcinoma (HCC) accounts for 75–85% of cases [4]. Despite progress in preventive measures, screening procedures, and advancements in diagnostic and therapeutic technologies, both the incidence and mortality rates of HCC continue to increase. The development of cirrhosis, regardless of its origin, remains the primary risk factor for HCC. Both Hepatitis B and C serve as independent risk factors for cirrhosis development [5]. Diagnosis typi-

cally occurs without histological confirmation. Screening strategies involve radiological tests, including ultrasound, computerized tomography, magnetic resonance imaging, and serological markers like α-fetoprotein at six-month intervals [6]. Various treatment options are available, yet only orthotopic liver transplantation (OLT) or surgical resection offers a curative potential [3].

As a sentinel of genome stability and an intricate barrier against tumorigenesis, p53 is central in coordinating cellular reactions to DNA injury. It performs multiple functions, such as DNA repair, cell cycle arrest, and apoptosis [7].

p53, a 53-kilodalton (kD) protein, becomes active when the equilibrium of normal cell stability is disrupted, triggered by factors like DNA damage, scarcity of nutrients, exposure to high temperatures, viral invasion, pH alterations, oxygen deprivation, and activation of oncogenes. [8]Its main role is to sustain the integrity of the genetic material by overseeing various processes, including halting the cell cycle, managing DNA replication and repair, orchestrating programmed cell death, and controlling energy metabolism.[9] The abundance of p53 protein is typically kept at a low level during periods of minimal stress, thanks to a delicate interplay between its production and breakdown. This equilibrium is crucial since excess p53 can be detrimental to cells, while insufficient levels can promote cancer development. The regulation of p53 occurs meticulously in response to diverse cellular stresses,

involving adjustments at the levels of transcription and translation, as well as through multiple posttranslational modifications, such as phosphorylation, acetylation, ubiquitination, neddylation, simulation, and methylation.[10] Notably, the levels of p53 are controlled by specific ubiquitin ligases, notably HDM2 (also known as MDM2, for mouse double minute 2) and Pirh2 [11]

HepG2 represents the most utilized human hepatoma cell line in pharmaco-toxicological investigations. Originating from liver biopsies of a 15-year-old Caucasian male afflicted with a well-differentiated hepatocellular carcinoma (Aden et al., 1979), this cell line is characterized by its non-tumorigenic nature and robust proliferation. This enables its successful cultivation in extensive-scale culture systems. When cultivated on a solid substrate, they exhibit epithelial characteristics, and in specific growth environments, HepG2 cells can become polarized, leading to the development of structures resembling bile canaliculi between neighboring cells [12].

The nuclear pore complex (NPC) comprises an unchanging group of approximately 30 distinct proteins known as nucleoporins. It is an entryway for the movement of substances between the cytoplasm and the nucleus.[13] TPR (translocated promoter region) is a constituent of the NPC, believed to be situated along internal nuclear filaments [14].

LncRNA is an RNA that lacks protein-coding capabilities, but its presence is very crucial to gene expression modification, chromatin remodeling, and epigenetics that control cell growth, differentiation, and diseases.[15]However, several recent studies have demonstrated that lncRNA is presented as deregulated in many human cancers and other complex diseases; this offers promise for useful diagnostic biomarkers and drug targets [16].

For instance, one such genetic entity is the LncRNA TLNC1, which has LINC01134 as its encoding gene. The LINC01134 gene has been linked with regulatory mechanisms controlling nuclear export pathways, among other cellular functions. TLNC1 has been discovered as an essential component of the nuclear export mechanism involving the p53 protein, a crucial tumor suppressor protein.[17] TLNC1 interacts with the translocated promoter region (TRP) of the nuclear pore complex, exporting p53 out of the nucleus and significantly decreasing its concentration within the nucleus. [4]

This study investigates molecular interactions controlling the progression of HCC, especially those related to TLNC1-mediated export of p53 into the nucleus. The results may open up new therapy targets against TLNC1 and p53 interactions, thus suggesting a new perspective for exploring possible treatments in the fight against HCC.

1.2 Hypothesis

I predict that the removal of the LINC01134 gene coding for lncRNA TLNC1 by CRISPR in HepG2 cells will decrease the nuclear export of p53, leading to increased p53 protein levels within the nucleus and enhanced cellular responses to DNA damage, ultimately inhibiting cancer progression.

2. Methods and Materials

The knockout will be carried out by CRISPR CAS-9, and TLNC1 levels will be tested by RT-PCR. Nuclear p53 level will be examined through Immunofluorescence and tumor size reduction by xenograft mouse in vivo. Negative control is the knockout of a random LncRNA instead, and positive control is treatment with Taxol.

2.1 CRISPR Cas-9

A guide RNA (gRNA) will be designed based on the sequences of the LINC01134 gene. The Ribonucleoprotein complex system. The Preassembled Cas9 and gRNA will be used directly and transfected into the HepG2 Cell line. A sample from this edited cell line will be tested with polymerase chain reaction (PCR) after 4 hours, 12 hours, and 24 hours for the presence of LINCO01134 to verify the knockout. Functional assays may also be utilized to further verify this.

2.2 RT-PCR

Total RNA is extracted from the control and experimental HepG2 cell samples. Then, reverse transcription (RT) converts the extracted RNA into complementary DNA (cDNA). A no-reverse transcriptase control is included to account for potential genomic DNA contamination. Specific primers for TLNC1 that flank the target sequence of interest are designed. Ensure the primers are specific to TLNC1 and do not cross-react with other related sequences. PCR amplification is then conducted using the cDNA samples and the designed TLNC1 primers. A standard PCR reaction with appropriate controls and experimental samples is set up. After that, the PCR products are separated using agarose gel electrophoresis. The amplified DNA fragments are visualized under UV light to confirm the expected band size for TLNC1. Quantify the TLNC1 expression levels using image analysis software or a quantitative PCR instrument. Normalize the TLNC1 expression levels to the expression of a housekeeping gene, β-actin, to account for variations in sample loading and cDNA synthesis efficiency.

2.3 Immunofluorescence

Before the procedure, cells are fixed, their membranes permeabilized, and non-specific binding sites are blocked.

Primary antibodies specific to p53 are then applied to the cells, allowing them to selectively bind to the target protein. Following this, cells are treated with fluorescently labeled secondary antibodies that recognize the primary antibodies, creating a visible signal. A nuclear stain like DAPI may be used to highlight the cell nucleus. The fluorescence microscope is then utilized to visualize and capture images of the stained cells, allowing for precise examination of p53 localization within the nucleus. Image analysis software can quantify fluorescence intensity, providing insights into the relative abundance of p53 within the cell nucleus.

2.4 Xenograft in vivo

NCID Immunodeficient mice are chosen and moderated to be similar in weight and height. HCC tumor cells are injected into the selected site in the mice, ensuring consistency in the cell number and injection technique among all the mice. Then, the tumors can be established and grow to a measurable size, ensuring that all mice have palpable tumors of similar size before initiating the treatment. Mice are randomly assigned into experimental groups, including a control group and one or more treatment groups receiving the knockout treatment. Measure the tumor size regularly using calipers or MRI throughout the treatment period. Record the measurements at specified time points, including tumor volume or dimensions.

2.5 Statistical Analysis

A one-sample T-test will be used for the statistical analysis of the experiments 2.2 to 2.4 to determine the effectiveness of the knockout. The t-value will be calculated using the formula for the selected t-test, considering the means, standard deviations, and sample sizes of the control and experimental groups for each experiment. Calculate the degrees of freedom for the t-distribution based on the sample sizes of the two groups. Then, Identify the critical t-value from the t-distribution table or using statistical software based on the chosen significance level ($\alpha = 0.05$) and the degrees of freedom. Finally, the calculated t-value will be compared with the critical t-value to determine whether the observed difference between the means of the control and experimental groups is statistically significant. The presence of statistical significance for each experiment is recorded as + in the table below and – if there is no statistical significance.

3. Results

Table 1. The possible combination of experimental results and its significance to the hypothesis

Possible observations	CR1	CR2	CR3	CR4	CR5	CR6	CR7	CR8
TLNC1 reduced/absent?	+	+	+	+	-	-	-	-
Nucleus P53 present/increased?	+	+	-	-	+	+	-	-
Was tumor size reduced?	+	-	+	-	+	-	+	-
Supporting Hypothesis?	yes	partial	partial	no	no	no	no	no

CR1: TLNC1 levels reduced or completely inhibited, P53 levels within the nucleus have increased, and tumor size in mice has been reduced or tumor growth rate has been reduced. This supports the hypothesis.

CR2: TLNC1 levels reduced or completely inhibited, and P53 levels within the nucleus have increased. However, tumor size has not been reduced. This could indicate that unknown pathways in vivo may disrupt this pathway.

CR3: TLNC1 levels are reduced or completely inhibited, and tumor size in mice has been reduced, or the tumor growth rate has been reduced. However, the tested cell line's P53 levels within the nucleus have not increased or are absent, which means there may be pathways missing in vitro that are significant to the TLNC1 pathway.

CR4: TLNC1 levels reduced or completely inhibited. However, P53 levels within the nucleus have not increased or are absent, and tumor size in mice has not been reduced, or the tumor's growth rate has not been reduced. This suggests that TLNC1 is not significant for the development of HCC, and it may be a part of a more significant mechanism, while it is not a keystone in this pathway.

CR5,6,7: TLNC1 levels have not been reduced or completely inhibited. However, P53 levels within the nucleus have increased in CR5 and 6. Tumor size in mice has been reduced, and the tumor growth rate has been reduced in CR5 and 7. This suggests there has been an error with the controls, and the experiment needs recalibration as the effect has been showing without treatment effects. It could also be the act of treatment that has had some unintended effect on other HCC pathways that has caused these unexpected results.

CR8: TLNC1 levels have not been reduced, P53 levels within the nucleus have not increased or are absent, and tumor size has not been reduced. This indicates no rela-

tionship between the LINC01134 gene and HCC development or p53 concentration.

4. Discussion

In support of the initial hypothesis (CR1), the reduction or inhibition of TLNC1 levels is correlated with decreased cell proliferation and a subsequent reduction in tumor size in the mice model, indicating the potential significance of TLNC1 in HCC progression. This supports the notion that TLNC1 plays a substantial role in the regulatory pathways associated with HCC development, highlighting its potential as a viable therapeutic target for HCC treatment.

However, CR2, CR3, and CR4 results suggest a more complex relationship between TLNC1 and HCC. The reduction or inhibition of TLNC1 levels is associated with increased p53 concentration in the nucleus and reduction in tumor size in CR2 and CR3, respectively. CR2 proves that the p53 protein may not be the main regulator of HCC development, or other mutations in HCC can mask its effects. CR3 shows that TLNC1 may directly affect HCC without facilitating the nuclear export of p53 protein. The lack of effects on p53 and tumor development in CR4 implies the involvement of intricate mechanisms beyond TLNC1 in HCC pathogenesis, independent of the p53 regulatory pathway. These findings emphasize the necessity of considering the broader molecular landscape and potential compensatory pathways that may interact with TLNC1 in influencing HCC development.

The unexpected outcomes observed in CR5, CR6, CR7, and CR8 raise concerns regarding the experimental procedures, suggesting potential issues with control groups or unintended off-target effects. More specifically, it can suggest that there could have been an error during the knockout procedure, which led to no gene or a different gene being knocked out, as the production of TLNC1 itself was not affected at all. These discrepancies highlight the need for meticulous recalibration of the experimental protocols and a thorough reevaluation of the experimental setup to ensure the reliability and accuracy of the observed results.

Conclusion

Targeting the specific genetic pathway associated with p53 regulation, this approach may hold promise in enhancing the cell's ability to respond to DNA damage, thereby potentially suppressing cancer progression. Further experimental validation and in-depth mechanistic studies are warranted to ascertain the feasibility of LINC01134 knockout as a treatment for HCC, offering valuable insights into developing novel therapeutic strategies for cancer treatment.

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