The Hepatitis delta virus:

Potential interference in alternative splicing of the p53 tumor suppressor gene

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Introduction

The hepatitis *delta* virus (HDV) is among the smallest human virions, nevertheless, it represents one of the most virulent forms of hepatitis which often leads to fulminant hepatocellular carcinoma[1]. This small virus does not encode its own replication machinery and, thus, requires the help of a cellular host to insure its propagation[5]. Recent studies have shown evidence that several splicing factors are affected in presence of HDV replication as the virus usurps the cell's transcriptional tools. A preliminary high-throughput Ribonucleic acid (RNA) sequencing analysis in infected cells suggested a significant change in the alternative splicing of several genes, particularly that of the p53 gene. Moreover, the p53 gene encodes a tumor suppressor protein that plays a key role in cell cycle regulation.

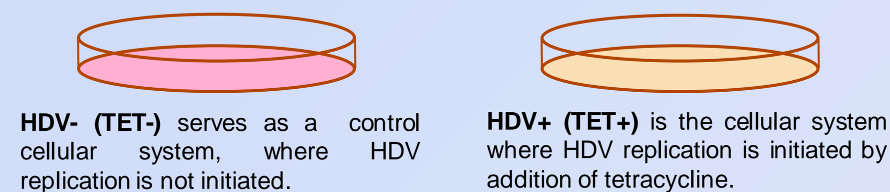
Therefore, it is hypothesized that the HDV can promote liver tumor development by affecting p53 alternative splicing. In order to verify this hypothesis, HDV replication was first initiated in a cellular system under the control of tetracycline-inducible delta proteins δAg[3]. Afterwards, quantitative polymerase chain reactions (qPCR) will be performed to identify the p53 messenger RNA splice variants produced in presence of HDV replication in the cellular system. Consequently, the alterations undergone by the p53 alternative splicing will be revealed. The results of the present research will contribute to decipher the complex mechanisms underlying the interactions between the HDV and cellular host-components involved in tumor development.

Methodology

Objective: Verify by qPCR that the expression levels of p53 splice variants are changed in presence of HDV replication in a 293-Ag & 293-HDV cellular system.

1 Induce HDV replication with tetracycline in a 293-Ag & 293-HDV cellular system

After being maintained for a few generations, a sufficiently confluent cellular system is induced with tetracycline (TET). Tetracycline provokes the expression of the δAg proteins present in the cell medium which are necessary to HDV replication.



HDV- (TET-) serves as a control cellular system, where HDV replication is not initiated.

HDV+ (TET+) is the cellular system where HDV replication is initiated by addition of tetracycline.

2 RNA extraction

TRIzol RNA extraction is used to isolate RNA from the cellular systems.

1. **Cellular Lysis**: TRIzol is added to the cells. TRIzol is a ready to use reagent which maintains RNA integrity while disrupting and breaking down cells and their components.
2. **RNA separation**: Addition of chloroform and centrifugation allows the isolation of RNA in the superior aqueous phase.
3. **RNA precipitation**: Isolation of the aqueous phase and addition of isopropyl alcohol enables recovery of RNA by precipitation.
4. **RNA resuspension**: After centrifugation, the supernatant is disposed of and ethanol is added to remove remaining salts. After another centrifugation, the aqueous phase is removed and the RNA pellet is left to dry. The RNA pellet is then resuspended in distilled water.

4 Quantitative polymerase reaction (qPCR)

The polymerase chain reaction (PCR) is a technique used to amplify a target gene region using a set of specific primers.

Table 1. PCR primer sequences and amplicon sizes. Primer specificity was tested and amplicon sizes were determined using the NCBI BLAST tool.

Primers	Sequence	Amplicon size (bp)
GAPDH	(F) CTGTTCGACAGTCAGCCGCATC (R) GCGCCCAATACGACCAAATCCG	110
18s	(F) CGGACAGGATTGACAGATTGATAGC (R) TGCCAGAGTCTCGTTCGTTATCG	118
HDV	(F) CCCTCGGTAATGGCGAATG (R) CCCAGTGAATAAAGCGGGTT	250
Δ40p53[4]	(F) CAGCCAAGTCTGTGACTTGCA (R) GTGTGGAATCAACCCACAGCT	99
Δ133p53[4]	(F) ACTCTGTCTCCTTCCTCTTCCTACAG (R) GTGTGGAATCAACCCACAGCT	102
αp53[4]	(F) AACCACTGGATGGAGAATATTTCAC (R) CAGCTCTCGGAACATCTCGAA	71/204/131
βp53[4]	(F) AACCACTGGATGGAGAATATTTCAC (R) TCATAGAACCATTTTCATGCTCTCTT	90

By this method it is possible to detect that:

i) Cellular and viral cDNA were obtained after reverse transcription;

ii) Target p53 splice variant sequences are identifiable.

Once PCR reactions have been optimized, the primers of the p53 splice variants can be used in quantitative PCR (qPCR). During qPCR, the amplification of the target cDNA region by PCR is quantified by fluorescence intensity using a fluorescent reporter molecule. The measurement of this fluorescence signal while the amplification is progressing (real-time qPCR) allows to calculate the initial template quantity.

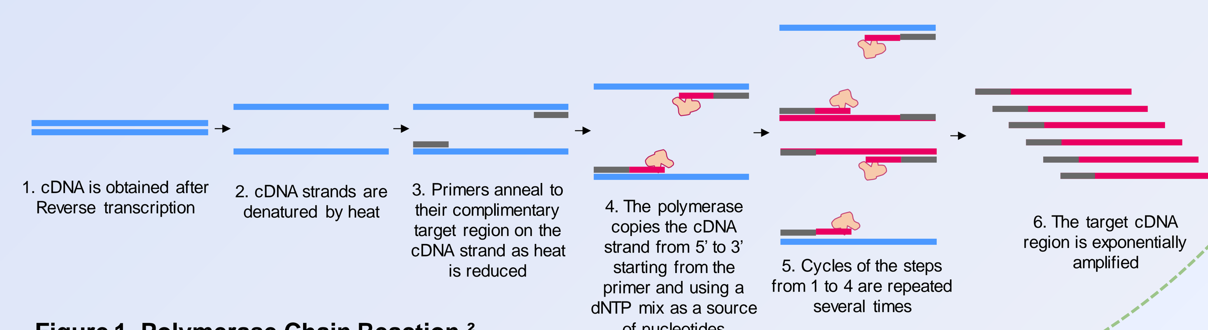
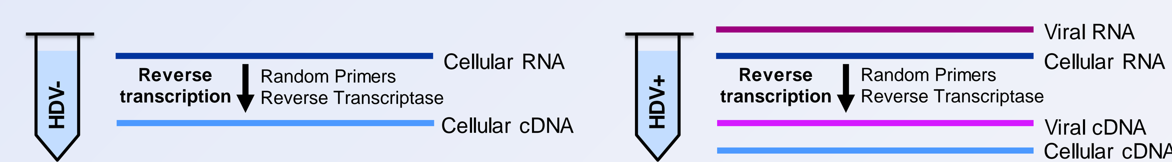


Figure 1. Polymerase Chain Reaction.[2]

3 Reverse transcription

The reverse transcriptase enzyme generates the complimentary desoxyribonucleic acid (cDNA) of an RNA template.



HDV- sample: The cellular cDNA obtained includes the p53 splice cDNA expressed in absence of HDV replication.

HDV+ sample: The cellular cDNA obtained includes the p53 splice cDNA expressed in presence of HDV replication.

Results

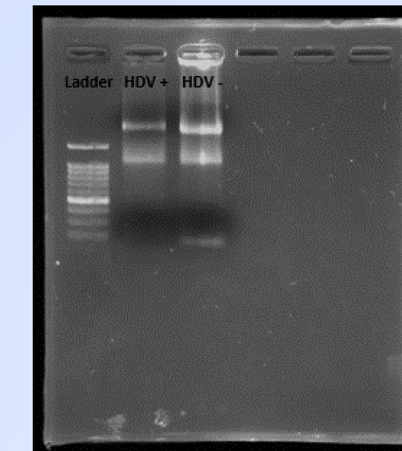


Figure 2. 1.5% Agarose gel verifying RNA integrity following TRIzol extraction. SYBR Green was used to stain samples. These results were obtained after a second RNA extraction. The first RNA extraction was performed when cells weren't sufficiently confluent which resulted in poor yields of RNA. Nanodrop measures revealed a 771,8 ng/μL RNA concentration for the HDV+ sample and a 1418,5 ng/μL RNA concentration for the HDV- sample.

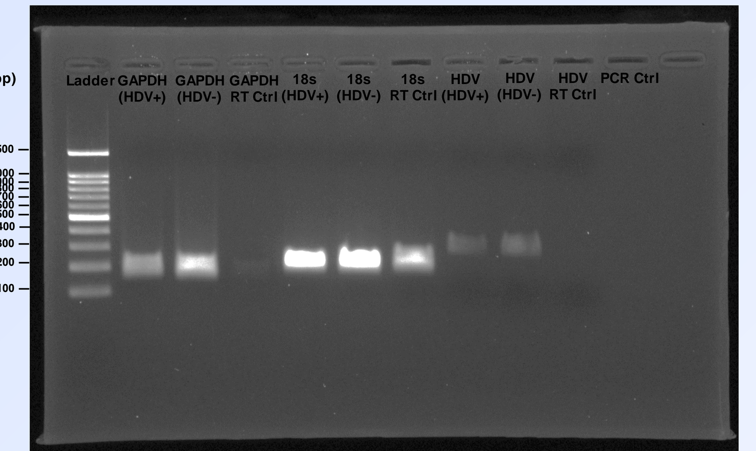


Figure 3. 1.5% Agarose gel verifying by PCR that cellular and viral cDNA were obtained after reverse transcription. SYBR Green was used to stain samples. GAPDH, 18s and HDV primers were used in the PCR reactions. As the first PCR reactions didn't display anything on agarose gel, it was first conluded that no cDNA was obtained by reverse transcription. After several PCR trials and troubleshooting, one of the reagents was found to be problematic. These are the results of the 6th PCR after replacement of the aforementioned reagent.

Current conclusion

- RNA was obtained following a second TRIzol RNA extraction from a tetracycline induced 293-Ag & 293-HDV cellular culture (Figure 2).
- Amplification by GAPDH primers confirmed cellular cDNA was obtained after reverse transcription (Figure 3).
- Amplification by HDV primers revealed poor yields of viral cDNA were obtained after reverse transcription such that HDV replication was not well initiated in the cellular system (Figure 3). Thus, HDV replication induction by tetracycline should be repeated.

Next steps

- Induction by tetracycline will be performed once again, followed by RNA extraction and reverse transcription. PCR using GAPDH and HDV primers will confirm obtention of cellular and viral cDNA.
- The PCR reactions using the p53 splice variants primers will be optimized.

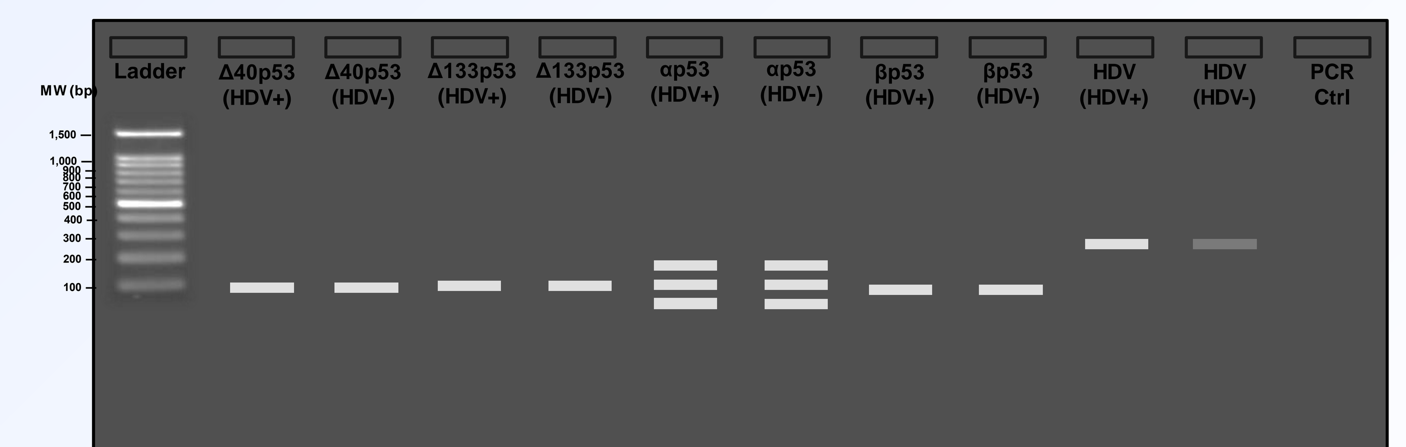


Figure 4. Agarose gel illustrating expected results of optimized PCR using p53 splice variant primers. This prediction is made using the amplicon sizes determined by BLAST on NCBI (see Table 1).

- A qPCR will then be performed using the optimized PCR reactions to quantify the expression of the different p53 splice variants in both the HDV+ sample and HDV- sample. This will enable comparison of levels of p53 splice variants' expression in presence and in absence of HDV replication.

References

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Acknowledgments

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