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Extended Abstract

Combinatorial Approach to Target Human Metapneumovirus Using shRNA and Ribozyme

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Abstract:

We aimed to restrict the propagation of human metapneumovirus (hMPV) in host cells by designing a chimeric construct encoding N gene specific shRNA linked through an intracellular cleavable linker to a ribozyme targeted to the same gene. Constructs expressing shRNA or ribozymes were also designed to evaluate their individual effects. The RNA expressed from the chimeric construct had the capability to cleave the target mRNA corresponding to N gene of hMPV under *in vitro* condition. Introduction of chimeric construct along with the N gene encoding DNA into A549 cells led to approximately 70% downregulation in the level of target RNA. When the construct expressing only shRNA was used, approximately 35% reduction in the level of target RNA was observed while the construct having only the catalytically active ribozyme caused approximately 45% inhibition in the RNA level. Significant knockdown of viral protein was also observed in the presence of the chimeric construct while the individual shRNA and ribozymes were less effective. Thus, we have demonstrated that the shRNA and ribozyme targeted to the nucleoprotein gene of hMPV acted synergistically to downregulate the gene and this combinatorial approach could thus be effective in reducing the replication of hMPV in infected cells.

Introduction:

Human metapneumovirus (hMPV) is reported to be one of the major causes of respiratory infections, especially among children, elderly persons and adults with abnormal medical conditions like diabetes (Barrera-Badillo et al., 2020). hMPV, being a RNA virus, mutates itself at a rapid rate making it difficult to be neutralized by the host immune system and hence is one of the major public health problem globally. Approximately 5-15% of severe bronchitis and pneumonia in young children is caused by this virus (Viazov et al., 2003; Williams et al., 2004). Currently, any vaccine or antiviral drug is not available to counter hMPV infection and therefore, there is a need to develop novel antiviral strategies that can restrict the replication of hMPV. Among the nine open reading frames in viral genomic RNA, the first one encodes for nucleoprotein (N) which has an important role in safeguarding the viral RNA and assisting in the viral replication (van den Hoogen et al., 2002; Derdowski et al. 2008). Therefore, intracellular downregulation of N protein could be chosen for inhibiting the replication of hMPV in infected cells. Nucleic acid based approaches which include small interfering RNAs (siRNAs), ribozymes, DNAzymes, antisense oligonucleotides and aptamers are available to interfere with viral gene expression in a sequence specific manner (Asha et al., 2019). Ribozymes having hammerhead catalytic motif have been used by several investigators as a promising virus specific therapeutic molecule. It consists of 22 nt long catalytic motif flanked by 8 nt long hybridising arms on both sides which are specifically complementary to the target RNA. Attempts have successfully been made to inhibit the replication of influenza virus using viral gene specific ribozymes (Motard et al., 2011). RNA interference (RNAi) can

also be triggered by introduction of preformed siRNAs, shRNAs or *via* vectors expressing the shRNAs specific to the target genes. We had earlier made a successful attempt to use this technology to inhibit influenza virus in mammalian cells (Kumar et al., 2010).

In this study, we designed multiple shRNAs and ribozymes targeted to N gene of hMPV and selected the effective combinations of shRNA and ribozyme to design the chimeric constructs. The constructs were assessed in A549 cells for their efficacy in downregulating the N gene expression. The most effective construct could restrict the level of N gene RNA by approximately 70% and use of construct expressing individual shRNA and ribozymes could be used to modulate the extent of gene inhibition. An appropriate method of vector delivery may further be used to evaluate the chimeric construct under *in vivo* condition.

Results:

Target site selection and generation of novel siRNA-ribozyme chimeric construct: Secondary structures corresponding to N gene mRNA were generated using M-Fold program and the target region (nt 301-317) present in the single stranded loop region was selected to construct hammerhead ribozyme. The ribozyme was constructed by synthesizing eight bases complementary to the N gene on either side of the target site GUU (308-310) along with the central conserved catalytic domain (Fig. 1) (Goila and Banerjea, 2004). DNA encoding the complete ribozyme sequence was amplified and cloned in pcDNA vector to yield the plasmid, p-Rz-308. siRNA sequences specific for N gene mRNA was obtained using siDirect software and shRNA targeting the single stranded loop region in the N gene were designed. The chimeric construct was generated by synthesizing an oligonucleotide consisting of shRNA sequence at its 3 end followed by cleavable linker and ribozyme sequence towards the 5 end (Kumar et al., 2010) (Fig. 2). We designed individual constructs to determine the efficacy of ribozyme and shRNA component of the chimeric constructs. All the oligonucleotides were cloned in pcDNA vector. N gene of hMPV was also cloned in the same vector to generate pcDNA-N plasmid which served as the source for the target of chimeric and individual constructs.

Chimeric construct mediated modulation of N gene expression: A549 cells were transfected with 0.5 µg of pcDNA-N alone and in combination with 5 µg of chimeric and individual constructs. We observed significant reduction in the level of N gene RNA by real time RT-PCR using RNA isolated from transfected A549 cells. The real time RT-PCR was performed in duplicate using SYBR Green from Bio-Rad and the level of nucleoprotein RNA in different set of experiments was compared by calculating the ΔΔCt value. The nucleoprotein RNA level in cells transfected with pcDNA-N was taken as reference whose value was “1” and the RNA levels in experiments done with all other combinations were compared with it. We observed approximately 70% decrease in nucleoprotein RNA content in the presence of chimeric construct encoding shRNA and ribozyme while approximately 35% and 45% decrease in RNA level was observed with shRNA and ribozymes respectively (Fig. 3).

Discussion and Conclusion:

Several respiratory and other viruses have been targeted till date by RNA interference technology. Human metapneumovirus is highly prone to mutation and therefore, use of one antiviral approach targeting a single region of viral genome may result in rapid generation of mutant resistant strains as described earlier in case of HIV (Boden et al., 2003; Das et al., 2004). Several investigators have earlier demonstrated the efficacy of mono-specific siRNAs and ribozymes capable of inhibiting influenza virus and hMPV gene expression (Tompkins et al., 2004; Ge et al., 2003; McCown et al., 2003; Lazarev et al., 1999; Nitschinsk et al., 2018) and bispecific siRNA construct targeting the genes of HIV (Anderson et al., 2003). In the present study, we have shown that the novel siRNA-Rz chimeric construct targeted to hMPV nucleoprotein RNA interferes with the gene expression very efficiently and to achieve it, we

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used specific oligo sequence (linker) to link shRNA and ribozyme in the chimeric construct. As described in our previous work (Kumar et al., 2010), the linker could efficiently be cleaved under cytoplasmic condition and therefore, separation of shRNA and ribozymes could have happened inside the cells transfected with chimeric construct allowing the shRNA and ribzoymes to act independently and synergistically. Further, we examined the modulation of target gene expression using constructs expressing individual shRNA and ribozymes to assess that both shRNA and ribozymes were actively and independently inhibiting the N gene expression. A synergistic action of shRNA and ribozymes in the chimeric construct could significantly downregulate the expression of N gene of hMPV. Although significant inhibition of N gene expression was achieved with our chimeric construct, it needs to be evaluated with different subtypes of hMPV with a range of challenge doses.

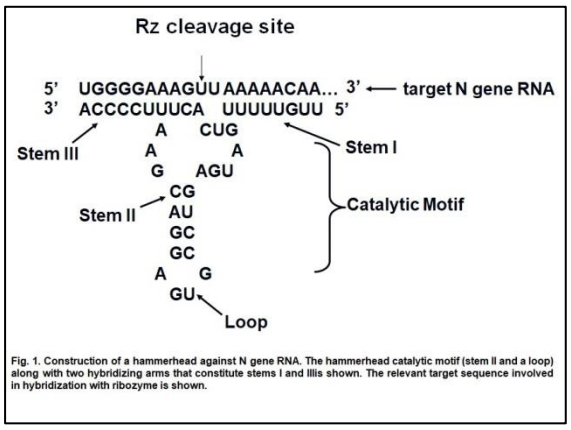

Fig. 1. Construction of a hammerhead against N gene RNA. The hammerhead catalytic motif (stem II and a loop) along with two hybridizing arms that constitute stems I and IIIis shown. The relevant target sequence involved in hybridization with ribozyme is shown.

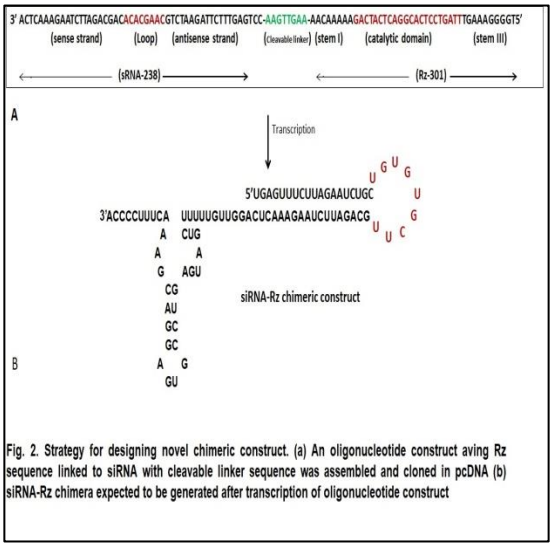

Fig. 2. Strategy for designing novel chimeric construct. (a) An oligonucleotide construct aving Rz sequence linked to siRNA with cleavable linker sequence was assembled and cloned in pcDNA (b) siRNA-Rz chimera expected to be generated after transcription of oligonucleotide construct

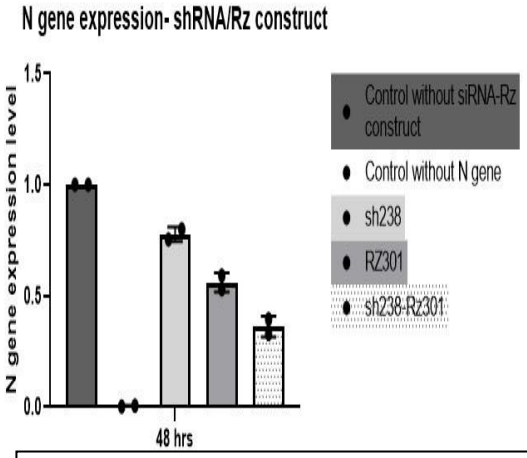

Fig 3. Real time RT-PCR shows the modulation of N gene expression in the presence of shRNA-Rz construct, siRNA-238 and Rz-301. Real time RT-PCR was performed using SYBR Green in duplicate and the level of N gene RNA was compared by calculating ΔΔCt value. The level of N gene RNA in cells transfected with only N gene clone was taken as the reference whose value was taken as "1" and compared the RNA level in all other experiments with the reference value