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Inhibition of rabies virus replication by multiple artificial microRNAs

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ABSTRACT

The RNA interference (RNAi) technology has been recognized as a promising antiviral therapy for a few years. One of the potential limitations for applying this technology against wild type rabies virus is its high rate of genetic variation. Recently, an RNAi vector system that incorporated modified dsRNA within microRNA structure [or artificial miRNAs(amiRNAs)] has been described. This allowed expression of multiple amiRNAs of single or multiple targets from a single construct. In this study, we evaluated a benefit of using amiRNA vector against different rabies strains. We found that applying single targeting amiRNA against challenged rabies virus standard (CVS) rabies nucleocapsid (N) mRNA resulted in more than 90% reduction of viral genome in Neuro2A cells up to 72 h after infection. Multiple amiRNAs aiming at single or multiple NmRNA target(s) yielded comparable inhibitory results as with a single amiRNA against perfectly matched target. Although the level of each mature miRNA generated from multiple amiRNA construct was slightly reduced as assessed by stem-loop RT and real-time PCR techniques, its effectiveness remained unchanged even when an ineffective or scrambled amiRNA was also included in the transcript. Against highly pathogenic wild type virus, single amiRNA construct activity was reduced when mismatching with target sequence occurred at critical site whereas multiple targeting amiRNA construct remained highly effective. Our results suggest the benefit of using multiple targeting amiRNAs when confronting with wild type rabies virus, the sequence of which is not completely known.

1. Introduction

RNA interference (RNAi), a process by which double-stranded (ds) RNA directs sequence-specific degradation of perfectly complimentary RNA (Leonard and Schaffer, 2006), can be initiated in target cells by either applying exogenous synthetic double-stranded RNA(dsRNA) molecules or using plasmid/viral vector constructs containing short-hairpin RNA(shRNA). RNAi has been used successfully to inhibit a number of viral infections (Haasnoot et al., 2007), including positive-stranded RNA viruses, such as human immunodeficiency virus (Boden et al., 2003; Rossi, 2006), West Nile virus (Bai et al., 2005), and hepatitis C virus (Randall et al., 2003), and negative-stranded viruses, such as vesicular stomatitis virus (Bitko et al., 2005), and respiratory syncytial virus (Barik, 2004).

The challenges for developing effective RNAi-based therapy against viruses especially those of RNA origin are high rate of genetic variability and mutation and problem in delivering therapeutic into the target. This is particularly true in the case of strictly neurotropic virus, such as rabies (Hemachudha et al., 2002). Blood–brain barrier remains intact during the course of rabies virus (RV) infection (Laothamatas et al., 2003; Roy et al., 2007). Synthetic dsRNA cannot pass the blood–brain barrier effectively. Alternative method for the delivery of RNAi into the central nervous system (CNS) is required.

Recently, the polymerase-II-promoter-driven plasmid vector for expressing a pre-miRNA backbone that can produce artificial miRNA (amiRNA) has been developed (Chung et al., 2006; Stegmeier et al., 2005; Sun et al., 2006). Theoretically, this type of vector provides unique benefits in designing antiviral therapy. It has been demonstrated that expression of amiRNA is more effective and less toxic than regular shRNA vector (Boden et al., 2004; Li and Ding, 2006; Qu et al., 2007; McBride et al., 2008). This strategy also allows multiple miRNAs to be expressed coordinately from a single precursor RNA and processed into individual amiRNAs (Xia et al., 2006). The simultaneous use of amiRNAs against multiple targets may circumvent the possibility of viral resistance or escape caused by target mismatch with miRNA as previously reported in the case of human immunodeficiency virus (Liu et al., 2008). Once effective combinations can be provided, the cargo could be integrated into viral vectors, such as lentivirus, adenovirus or adeno-associated virus, for delivery into the CNS.

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In this study, RV was used as target. RV has a single non-segmented negative-strand RNA genome that comprises only five genes that encode viral proteins, namely, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L) (Warrell and Warrell, 2004). There are two modes of viral RNA synthesis, transcription and replication. Both require the association of the N, P and L proteins with the viral genomic RNA to form a ribonucleoprotein (RNP) complex. Transcription is initiated at the 3′ end of the genomic RNP and involves sequential production of a leader RNA and 5′ monocistronic mRNAs (N-P-M-G-L). The dissociation of the polymerase at each gene border results in a progressive reduction in the amount of transcript generated. In the replication mode, the polymerase complex initiates at the 3′ ends of both genomic and antigenomic RNPs and full-length RNPs are produced (Finke et al., 2003; Finke and Conzelmann, 2005).

We used a commercially available mi155 backbone vector to express amiRNA(s) designed against Challenge Virus Standard strain of RV nucleocapsid (N) transcripts, the most conserved region. We transfected the plasmid into Neuro2A cells, and infected the cells with different strains of RV. We tested each amiRNA separately and in different combinations. We also tested the application of amiRNAs after viral infection. Our results show that miRNA, either as single or multiple amiRNA(s), against single or multiple targets of rabies NmRNA, can inhibit RV replication in vitro. Nucleotide mismatch with the RV target at the critical region, such as in the RNA induced silencing complex (RISC) cleavage site, can affect the inhibitory activity. The use of multiple targeting amiRNAs, thus, may be useful under circumstance that pre-treated target sequence is not completely appreciated. The inclusion of only one effective amiRNA among multiple targeting ones yielded comparable result although the level of each miRNA was reduced.

2. Materials and methods

2.1. Cells and viruses

Neuro2A cells (ATCC cat no. CCL-131) were maintained in HyQ MEM/EBSS media (Hyclone) supplemented with 10% Fetal bovine serum, 2 mM L-glutamine, 1.5 g/l $NaHCO_3$, 1 Mm Sodium Pyruvate, 1× Non-Essential Amino Acids (NEAA), 100 U of penicillin/ml and 100 μg of streptomycin/ml at 37 °C, 5% CO_2. RV Challenge Virus Standard (CVS)-11, HEP-flury-GFP laboratory fixed strains and street virus were provided by Drs. Khawplod and Morimoto, Queen Saovabha Memorial Institute, Thai Red Cross Society. All virus stocks showed tissue culture infection dose$_{50}$ ($TCID_{50}$) of 10^{-5}. In all experiments, 10 or 100 $TCID_{50}$ were used. Street (or wild type) virus was an isolate originating from rabies infected Thai dog, obtained in the laboratory after primary isolation in cell culture. HEP Flurry and CVS strains were laboratory fixed viruses after continued cell-culture passages. Street virus and fixed strains differed in many aspects. Fixed strains had defined incubation period, lower virulence and ability to infect, ability to spread within the body as compared to street virus.

2.2. Plasmids

The RV gene targeting sequences were designed by using the miRNA design algorithm (http://rnaidesigner.invitrogen.com/rnaiexpress/). Complementary single-stranded DNA oligos encoding the pre-miRNA were synthesized, annealed, and cloned into pcDNA6.2-GW/EmGFP-miR (Invitrogen), a Pol II miR RNAi expression vector which contained specific miR-155 flanking sequences. The version of vectors without EmGFP, which were used in immunofluorescence studies, was created by digesting the vector with DraI. To generate a chain of miRNA in one primary transcript, the miRNA cassettes from the donor plasmid were excised with restriction enzymes, BamH1 and Xho1, and put in the backbone miRNA vector pre-cut with Bgl II and Xho1 (Fig. 2A).

2.3. Transfection with miRNA expression plasmid

Plasmid DNAs were mixed with lipofectamine 2000 (Invitrogen) at the ratio of 1:1 (1 μl of lipofectamine 2000 per 1 μg of DNA) in opti-MEM medium (Invitrogen). The complexes were then added to Neuro 2A culture (plated at 1×10^5 cells per well in 24-well plates on the previous day). Scramble miRNA plasmid was used as a control.

2.4. Viral challenges assay

For pre-exposure experiments, Neuro2A cells were grown in 24 wells plates overnight before transfected with miRNA plasmids. At 12 h after transfection, two different amounts (10 or 100 $TCID_{50}$) of stock virus ($TCID_{50}$ of 10^{-5}) were added to the cells in each well. The media was changed after 6 h of incubation at 37 °C. RNA was isolated 24, 48, and 72 h after the viral challenge. For post-exposure experiments, Neuro2A cells were infected with 10 $TCID_{50}$ of CVS virus 12 h before transfected with miRNA plasmid. Total RNAs were collected at 24 h after transfection.

2.5. RNA isolation

Total RNA from cells was isolated 24, 48, and 72 h after infection with the RNeasy mini kit (Qiagen). Culture supernatants were harvested after 48 h, and RNA from supernatant fluid was purified using QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions.

2.6. Quantification of viral genome and transcripts

2.6.1. RT

Reverse transcription was performed using M-MLV reverse transcriptase (Promega) with 4 μl of total RNA isolated from infected cells/ supernatants (20 ng/μl). Oligo dT was used as a RT primer for mRNA detection. RT primer used for detecting viral genome was 5′-AGAAGGATCGTGGAGCACCATACTCTCA-3′.

2.6.2. Real-time PCR

4 μl of cDNA were added to 10 μl of QuantiTect SYBR green PCR master mix (Qiagen), 0.5 μl of 20 μM forward primer, 0.5 μl of 20 μM reverse primer, 5 μl of RNase-free water. Real-time PCR was carried out in Rotorgene 6000 (Corbett). RNA levels were measured by SYBR green incorporation using the following thermal cycling profile: 95 °C for 1 min, followed by 40 cycles of amplification (95 °C for 15 s, 55 °C for 20 s, and 72 °C for 30 s). For N gene (CVS) the forward primer is 5′-CTGGCAGACGACGGAACC-3′ and the reverse primer is 5′-CATGATTCGAGTATAGACAGCC-3′. For N gene (HEP-Flury) the forward primer is 5′-CTGGCAGATGACGGAACT-3′, the reverse primer is 5′-CATGATTCGAGTATAGAC-AGCT. For the detection of viral genome, the following primers were used: forward primer 5′-AGAAGGATCGTGGAGCACCATACTCTCA -3′ and reverse primer TACCAGCCCTGAACAGTCTTCA-3′. The data shown was an average of three different samples.

2.7. Quantification of mature miRNA

2.7.1. Stem-loop RT-PCR

5 μl of sample RNA (2 ng/μl) were mixed with 7.0 μl of RT master mix from Taq man Micro RNA Reverse Transcription kit (ABI) (1.5 μl of 10× RT buffer, 0.15 μl of 100 mM dNTPs,

0.19 μl of 20 U/μl RNase Inhibitor, 1.0 μl of 50 U/μl Multiscribe RT enzyme, 4.16 μl of RNase-free water). 3 μl of specific loop-RT primer (N1: CGACTCATGCTGACGAATTTTGAGTCGCAAA CTTGA, N2: GCGACTCATGCTGACGAATTTTGAGTCGCGTCTCT 200 nM) were add to the mixture. RT reaction was kept at 16 °C for 30 min then at 42 °C for 30 min before it was terminated by incubating the mixture at 85 °C for 5 min.

5 μl of cDNA from the RT reaction were mixed with 4.0 μl of LightCycler Taqman Master Mix (Roche), 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reverse primer, 0.2 μl of 10 μM Ppobe, 10 μl of RNase-free water. The sequences of the PCR primers and probe were NP1 forward primer: CCGCCCTACATCATCCG, NP2 forward primer: CCCCTAAAGATGCATGTTCAG, reverse primer: GCGACTCATGCTGACGAA, NP1 probe 6FAM-TTTGAG+TCGCAAAC+T+TGATCC—BBQ, NP2 probe: 6FAM-TTTGAG+TCGCG+TC+TCTG—BBQ. The base LNA is mark + in front of its base. PCR was performed using the following thermal cycling profile: 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 5 s, 55 °C for 5 s, and 60 °C for 5 s). Signals were measured in LightCycler Version 1.5 (Roche).

2.7.2. Immunofluorescence

At 48 h post-infection, cells were fixed in 80% acetone and stained with FITC-labeled rabies virus N protein-specific antibody. Cells were visualized and the images digital captured on the Nikon eclipse TE2000 microscope using a 10× objective and appropriate filters.

3. Results

RV NmRNA was chosen as target for miRNA inhibition since RNP complex, the association of N, P, and L proteins with viral genomic RNA, are important for both viral transcription and replication (Finke and Conzelmann, 2005; Finke et al., 2003). Furthermore, N protein has been known to be the most conserved and the most abundant of all rabies viral proteins (Kissi et al., 1995). Previous studies have demonstrated that N protein is required for the protection of viral genome from cellular ribonuclease activity (Sokol et al., 1969; Yang et al., 1999). Three different regions of CVS rabies N mRNA were selected as targets in designing artificial miRNA (Fig. 1A). Their inhibitory actions on rabies viral replication were determined.

4. Inhibitory effect against RV replication by amiRNA against CVS-RV NmRNA could be demonstrated in both pre- and post-challenging models

The efficacy of 3 amiRNAs against different regions of RV CVS-N was evaluated in pre-challenging model by transfecting each plasmid into Neuro2A cells 12 h prior to challenge with CVS of RV. RNA was collected from cells or supernatant fluid 24, 48 and 72 h after infection for qRT-PCR analysis. In comparison with Neuro2A cell transfected with control plasmid, cells expressing either amiR(N1), amiR(N2), or amiR(N3) exhibited a marked reduction in N mRNA, but not GAPDH mRNA, at all designated time points after infection as measured by real-time PCR (data at 24 h as shown in Fig. 1B). Amount of RV N proteins were also reduced in all mi(N) transfected group compared to control as shown by immunofluorescence staining with anti-N antibodies (Fig. 1C). Measurement of viral genome in cells (data at 24 h post-infection as shown in Fig. 1D) and in culture supernate (data at 48 h post-infection as shown in Fig. 1E) demonstrated that all 3 of mi(N) were highly effective in reducing viral genome. These results indicated that the application of designed amiRNAs against NmRNA could efficiently inhibit viral replication. This was unlikely to be due to non-specific effect of the pol II amiRNA plasmid. Whereas these amiRNA-NmRNAs were highly effective in inhibiting RV replication, all other amiRNAs against different RV transcripts (2 anti-P, 3 anti-G, and 2 anti-negative-strand viral genome) using the same algorithm for selecting targets as our mi(N) with several control plasmids, had no significant inhibitory effect upon RV replication (data not shown). Nevertheless, these results did not indicate that amiRNAs against other transcripts than N, in particular P, had no inhibitory effect, as the P of RV is absolutely essential for virus replication. Only limited sequences of P were tested in this experiment. In post-challenging model, transfection of Neuro2A cells with anti-N amiRNA plasmids 12 h post-infection also resulted in a marked reduction of RV genome in cells 24 h later (Fig. 1F).

5. Multiple amiRNAs aiming single or multiple targets of CVS-NmRNA in one pre-miRNA transcript did not confer greater inhibition of RV replication than single amiRNA-NmRNA

The use of mir-155 based vector in our study offered expression of multiple amiRNAs in a single transcript. Using a cloning strategy described in Section 2 (Fig. 2A), plasmids containing different tandem copies (from 2 to 6) of amiR(N1) or amiR(N2) alone or in combination with other designed amiRNAs were created. In order to assess the effect of expressing multiple copies of amiRNAs in one pre-miRNA transcript, we used stem-loop RT and real-time PCR techniques to measure the level of each mature amiRNA separately (Chen et al., 2005). We found that levels of mature amiRNA(N2) expressed from a pre-miRNA transcript containing three copies, amiRC(N2 × 3), and six copies, amiRC(N2 × 6), of amiR(N2) cassettes were 180% and 270%, respectively as compared to a construct with a single amiRNA(N2) cassette (Fig. 2B). Correspondingly, combining one amiR(N2) with 2 and 5 copies of other amiRNAs [amiRC(N1 + N2 + N3) and amiRC(N1 × 4 + N2 + N3)] reduced the level of mature amiR(N2) produced from the vector to approximately 47% and 39% of the amiRNA(N2) vector, respectively (Fig. 2C). Combining 3 copies of amiRNA to the same target of NmRNA does not result to a statistically significant improvement of the inhibitory effect (Fig. 2D). Nevertheless, adding scrambled (or ineffective) amiRNA to the multiple amiRNA construct did not reduce the overall efficacy of the plasmid (Fig. 2E).

6. Designed amiRNA-NmRNA remained effective even when applied on to imperfectly matched target providing that mismatch was not in critical region

Due to the high degree of specificity, a single nucleotide mismatch between amiRNA and target viral RNA may reduce its effectiveness. Under the natural circumstance for therapeutic purpose, an already prepared, custom made amiRNA may have to be applied even before the complete viral sequence is appreciated. In order to simulate such event, we considered what would be the chance that single pre-designed amiRNA could form a perfect base pair with unknown street RV. Based on data of 414 nucleotide sequences of RV N gene from 237 samples of rabies infected dogs in Thailand during 1998–2002 (Denduangboripant et al., 2005), we were unable to find a stretch of 21-nucleotides that was perfectly conserved. Only one region of 33 nucleotides (base 227–259) that any chosen stretch of 21-nucleotides within could perfectly match to more than 95% of sequences input (Fig. 3). Further analysis revealed that within the region of 414 bases studied, there were 141 bases that showed significant degree of genetic variability (5–49%). These points of high variability rate are present in every 10–15 nucleotides across the sequences studied. Therefore, even

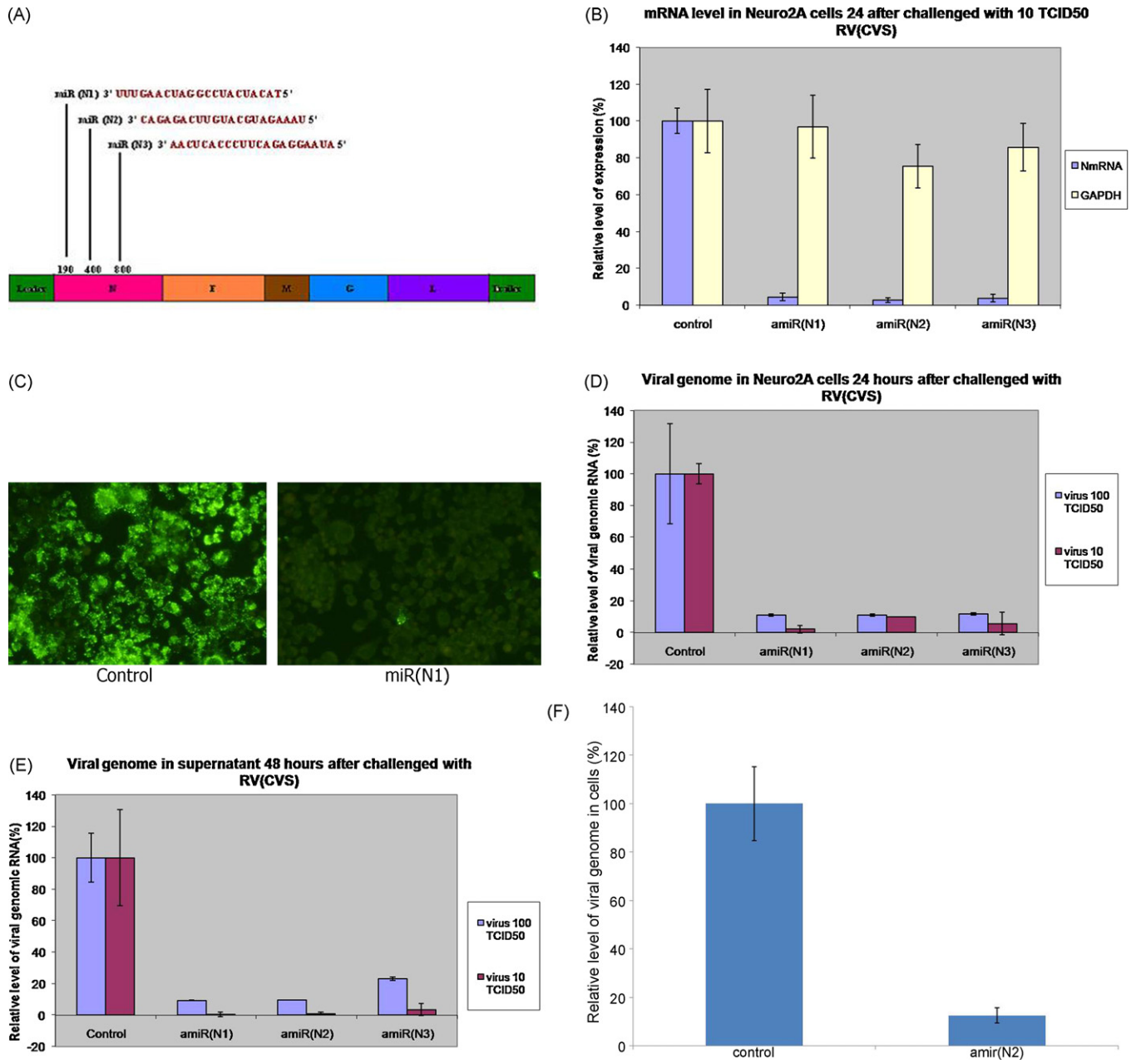

Fig. 1. Designing miRNA against rabies virus. (A) A schematic diagram depicting the location of the artificial miRNAs in association with viral mRNAs encoded within the RV genome. Total RNA was extracted from Neuro2A cells transfected with either amiR(N1), amiR(N2), amiR(N3) or control plasmid 24 h after challenge with 10 or 100 $TCID_{50}$ CVS RV. It was then analyzed for (B) level of mRNA of viral N mRNA and host cells GAPDH mRNA. (C) Neuro2A cells transfected with either amiR(N1) or control plasmid were fixed 48 h after the CVS RV infection and were subjected to direct-immunofluorescent staining with FITC-conjugated anti-N antibody. (D) Level of viral genome in cells 24 h after infection and (E) levels of viral genome in the culture supernatant 48 h after infection were measured by real-time PCR. (F) In post-exposure experiment, CVS RV were add to Neuro2A cells 12 h before transfection with amiRNA plasmid. Levels of viral genome in cells were measured 24 h after transfection by real-time PCR.

with a carefully selected sequence design, one to two nucleotides mismatch(es) could not be avoidable.

We next evaluated the effect of mismatch on inhibitory activity by applying our amiRNAs, which was designed for CVS RV NmRNA, against another laboratory RV strain, HEP-flury (Fig. 4A). Our results demonstrated that amiRNA designed against CVS strain could strongly inhibit HEP-flury RV replication as shown by immunofluorescence staining with anti-N antibody (Fig. 4B).

There are marked differences between strains of rabies virus in their ability to infect, spread within the body, and produce disease. Laboratory strains, such as CVS and HEP-flury, have low pathogenicity when inoculated peripherally in low doses. Wild type or street virus is far more dangerous than fixed strain in terms of causing disease in humans. We tested the effect of amiRNA designed against CVS NmRNA on street RV. We found that amiR(N1) and amiR(N2) which exhibited equal effectiveness in all previous tests, showed different behavior when encountering street RV target with single mismatch (Fig. 4C). The amiRNA(N1) construct, which contained a mismatch at the RISC cleavage site, showed a reduction in RV inhibition while amiRNA(N2) still remained effective. Therefore, mismatch(s) at some regions could be tolerable whereas mismatch at critical site could interfere with the single amiRNA activity. Comparing the activity between a single targeting and multiple targeting vector against target containing mismatches, the amiRC(N1 × 3) reduced viral genome to 36% of control, multiple targeting plasmid, amiRC(N1 + N2 + N3), was capable of reducing viral

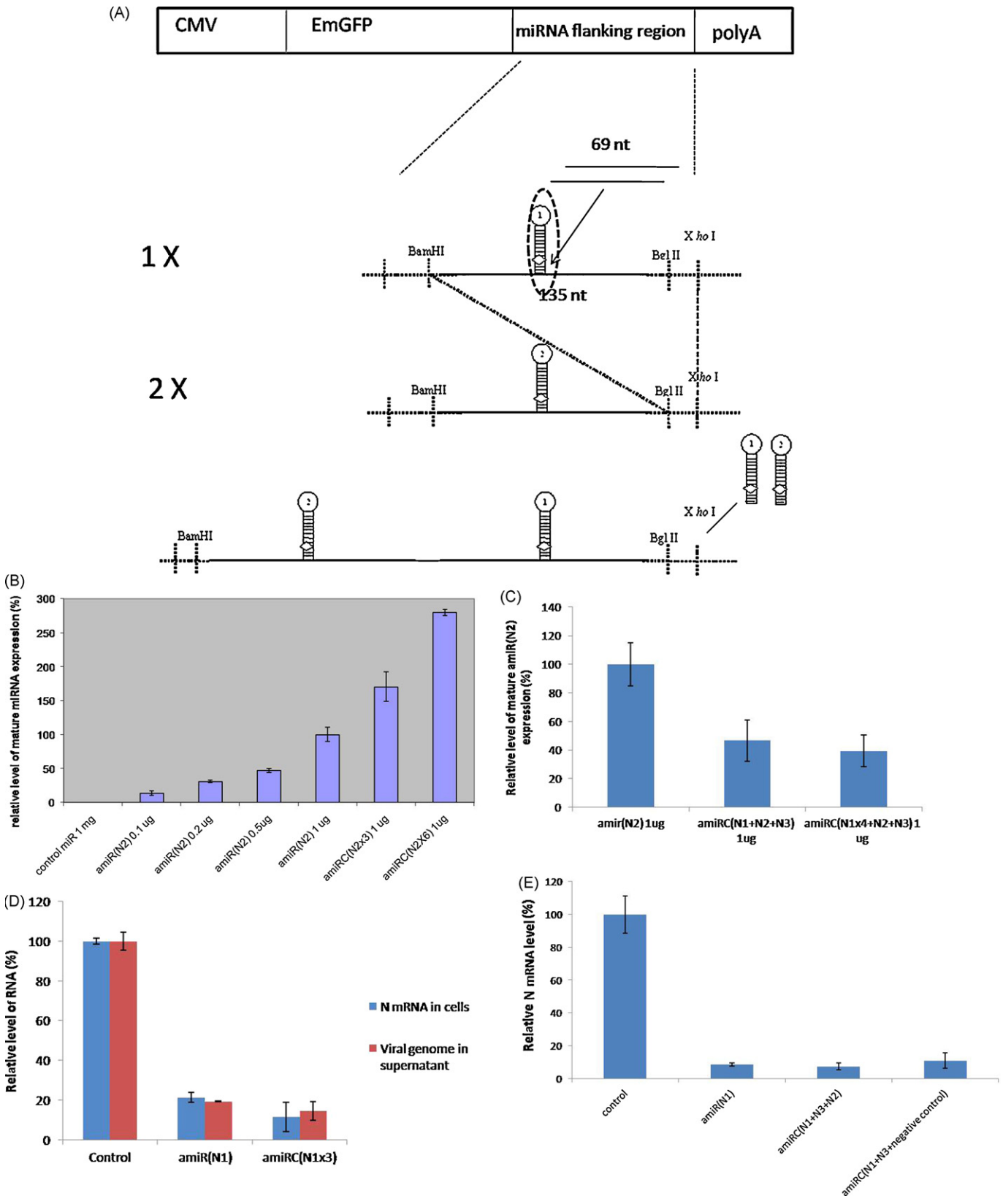

Fig. 2. Effect of chaining on the level of mature miRNA expression. (A) A schematic diagram shows cloning strategy for combining multiple miRNA cassettes into one pre-miRNA transcript. (B) Quantitative measurements of mature miR(N2) level in total RNA extracted from Neuro2A cells transfected with either 0.1, 0.2, 0.5 and 1 μg of amiR(N2) plasmid, 1 μg of plasmid containing a three copies of miR(N2) (miRC(N2 × 3)) or six copies of miR(N2) (miRC(N2 × 6)) using stem-loop RT real-time PCR technique. (C) Quantitative measurements of mature miR(N2) from Neuro2A cells transfected with 1 μg of amiR(N2) plasmid or plasmid containing one cassette of miR(N2) together with two or five other miRNA cassettes, respectively. (D) Real-time PCR analysis of levels of viral genome in cells transfected with control plasmid, amiR(N1) or vector containing 3 copies of miR(N1) (miRC(N1 × 3) 24 h after challenged with 10 $TCID_{50}$ CVS RV. (E) Level of viral N mRNA in cells transfected with control miR plasmid, miR(N1), plasmid containing a chain of miR(N1) together with miR(N2) and miR(N3) (miRC(N1 + N3 + N2)), and plasmid containing a chain of miR(N1) together with miR(N3) and negative control miRNA (miRC(N1 + N3 + negative control)), 24 h after challenged with 10 $TCID_{50}$ CVS RV.

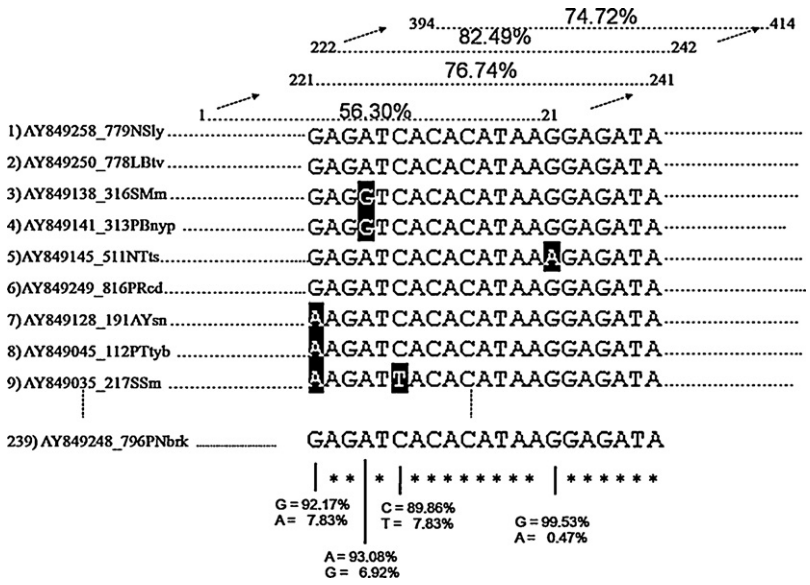

Fig. 3. Diagram shows genetic variability of street RV in Thailand and its effect on miRNA target selection.

genome to 6.8% of the control. Thus, at 24 h after infection, viral genome found in cells transfected with amiRC(N1 + N2 + N3) vector was only 18.8% of what observed in amiRC(N1 × 3) transfected cells (Fig. 4E).

7. Discussion

In this study, we demonstrated that plasmid-based amiRNA designed against RV N mRNA could strongly inhibit RV replication of both laboratory RV strains (CVS, HEP-Flurry) and the highly pathogenic street RV in neural cell line. Together with the results from recent studies which showed that short N cDNA could interfere with RV replication (Wunner et al., 2004), and that synthetic siRNA against laboratory strain RV NmRNA could reduce $TCID_{50}$ in BHK-21 cells (Brandao et al., 2007), supported that N mRNA could be a good target for intervention in order to inhibit RV replication.

It remained to be tested whether targets other than NmRNA could be RNAi targets. For example, in the case of VSV, negative-stranded RNA virus closely related to RV, it was shown that siRNAs against P mRNA was highly effective in reducing viral replication (Barik, 2004). In our experiment, only 2 sequences of P were tested, therefore, we cannot exclude the significance of P alone as an effective target for inhibition. It also remained to be investigated whether combining amiRNA-PmRNA to amiRNA-NmRNA can improve the inhibitory effect beyond what we observed with amiRNA-NmRA alone. siRNA against NmRNA of Nipah virus was more effective than siRNA against LmRNA (Mungall et al., 2008). This also suggested that transcript quantity might also play a role in siRNA efficacy.

It should be noted that although anti-N amiRNA worked in all experiments we tested, some fluctuations of each vector efficacy between experiments were observed. For example, viral N-mRNA and genome were reduced to 15–20% of control in some experiments (as compared to less than 10% as shown in Fig. 1D). This might be due to the limitation of our transient transfection system that there was a small, variable, percentage of cells that did not uptake plasmid. In most of the experiments, at least 80–90% of cells were transfected based on presence of strong EmGFP signals. It may also be possible that this percentage may be higher as faint signal cannot be counted with confidence. Our test may not be capable of comparing vector efficacy reliably among those that could reduce viral genome to less than 15% of control. The observation that more amiR(N1) did not give more inhibition could be due to the possibility that the assay might not be anymore in the linear range. Further, this might be the maximum of what miRNA can do. Regarding to whether the high inhibition rate was due to an excessive use of vector DNA, optimization of DNA and transfection reagents had been pre-determined ensuring that DNA amount was not excessive. The total amount of DNA used per well remained constant (1 μg) in all experiments. We added control plasmid DNA to 1 μg when we used less than 1 μg of test plasmid.

One of the potential advantages in using miRNA-based expression vector was the ability to express multiple amiRNAs from a single transcript as compared to only one in regular shRNA vector. It has been shown that two co-transfected shRNAs compete against each other for transport and for incorporation into the RISC resulted in a reduction in shRNA processing and its activity. Yet, when the same shRNAs were embedded in miR-30 backbone vectors, there was no significant change in their ability to down-regulate its target (Castanotto et al., 2007). The mir-155 based vector similar to what we used in this study has been previously shown to inhibit reporter construct more efficiently when multiple copies, up to 8, were expressed (Chung et al., 2006). Besides, the whole procedure in making multiple amiRNAs is simple and economical. However, our construct containing up to 6 copies of the same amiRNA-NmRNA did not significantly improve the efficacy beyond that containing one copy when tested against live CVS RV. Based on our quantitative assay for the level of mature amiRNA produced from each plasmid, we found that each addition of amiRNA cassette (up to 6) to the transcript would result to an increase of the total amount of mature amiRNA generated, but not in a linear dose dependency (less than three-fold increase in mature miRNA). While part of this was due to the differences in vector length between 1× and 6×, which contributed to 10% change in molar amount of DNA used in each transfection, it is possible that the amiRNA processing from each copy was also affected. The constructs containing transcripts with 3 and 6 amiRNA copies expressed each mature amiRNAs at the levels of 47%, and 39%, respectively, of those found with construct containing transcript with a single amiRNA copy. There have been reports using different miR-based vectors in constructing multiple amiRNAs and different effect in miRNA processing. Report on using vector expressing 5 amiRNAs designed against HIV from polycistronic mir-17–92 cluster, each of which

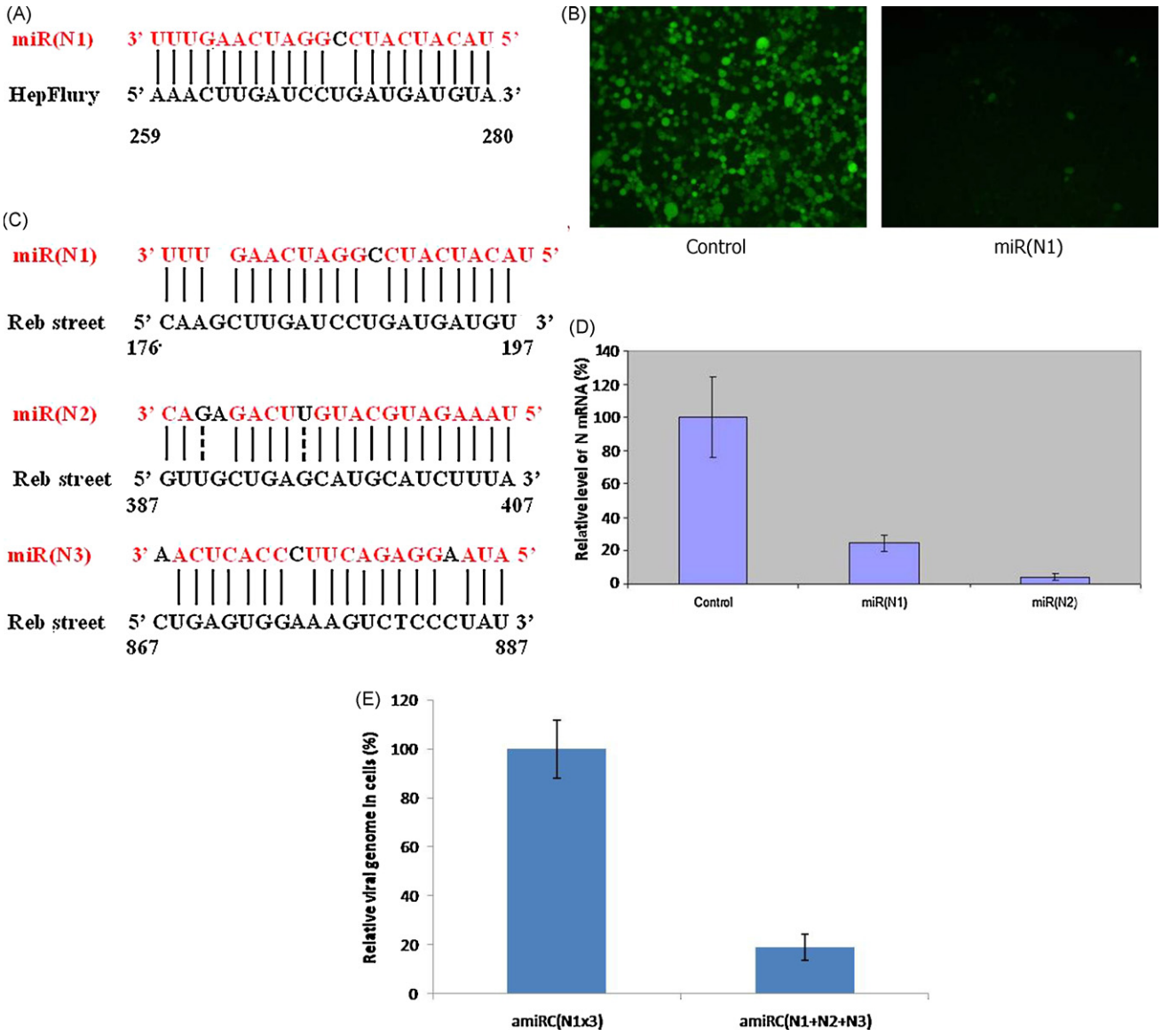

Fig. 4. Effect of miRNA designed against CVS strain on HEP-flurry and street RV strain. (A) A schematic diagram depicting the location of the artificial miRNAs in association with HEP-flurry N mRNA. (B) Neuro2A cells transfected with miR(N1) or control plasmid were fixed 48 h after being infected with HEP-flurry-GFP virus. (C) A schematic diagram depicting the location of the artificial miRNAs (miR(N1), miR(N2), and miR(N3)) in association with street rabies N mRNA. (D) Real-time PCR analysis for viral genome from Neuro2A cells transfected with miR(N1), miR(N2), or control miRNA plasmid 24 h after being infected with 10 $TCID_{50}$ street RV. (E) Real-time PCR demonstrated the inhibitory effect of amiRC(N1 + N2 + N3) compared to amiRC(N1 × 3) on amount of street virus genome detected in cells 24 h after infection.

was incorporated into different amiRNA backbones, showed that some amiRNA processing could even be increased as compared to vector expressing single miRNA (Liu et al., 2008). In contrast, in a miR-30 based system, there was a report that expressing 2 tandem copies of amiRNA in a single transcription unit was less effective for RNAi activity than a single copy of the same amiRNA (Zhou et al., 2005). Recent reports using miR-106 cluster demonstrated that maintenance of the native flanking primary miRNA (pri-miRNA) sequences and key structural features of the native miRNAs are critical for efficient siRNA processing (Aagaard et al., 2008). Alternative approaches next to shRNA and miRNA-like constructs have been described. These included long-hairpin RNAs(lhRNA) and modified hairpin RNA(mhRNAs) (Haasnoot et al., 2007). Both systems also have a potential advantage in the generation of multiple siRNAs to enable simultaneous targeting of different sites. Efficient inhibition by lhRNAs has been reported for virus such as HIV-1 and HBV (Konstantinova et al., 2006; Liu et al., 2007; Weinberg et al., 2007). Long mhRNAs of 50–100 bp was shown to effectively suppress replication of HBV without activating the interferon pathway (Akashi et al., 2005).

Although using construct expressing multiple amiRNA against different regions of N with perfect match did not produce significantly different results from construct expressing only one effective miRNA, adding ineffective miRNA to the chain did not significantly reduce overall inhibitory effect of other miRNA. This would be applicable for use in case that virus is known but without complete information on virus sequence. Our results showed that small degree of imperfect base pair can be tolerated. However, in the case of acute lethal infection such as rabies, which is prevalent in canine endemic countries, mismatch at critical site with significant reduction of efficacy as shown in our study may be avoided by using multiple amiRNAs. This has been demonstrated previously in HIV model to prevent viral escape in such chronic infection (Rossi et al., 2007; ter Brake et al., 2008). Database showed that the highest possible mutation rate site within the region we studied (130/141) were limited to two possible bases per location. Thus, chaining 2

artificial miRNA against the same target sequences with a single nucleotide difference will allow more regions to be available for si/miRNA target selection, while still in keeping of a high probability of forming a perfect match with street virus. While our data seems promising, it remains to be seen whether this level of reduction can be maintained over a longer period of time and how this strategy work in vivo model. Prevention of viral escape would also be an important issues, it can be examined by passaging the virus on cells expressing multiple amiRNAs.

Taken together, our data may support the use of vector containing multiple miRNAs against different targets in RV. It should be at least equally effective while providing safety where one or two pre-designed miRNA in the chain does not work properly.

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