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Submission date: 27-Mar-2023 01:13AM (UTC-0700)
Submission ID: 2047854696
File name: A1_2_2009 Koraka et al_j.virusres.2009.02.016_Q1.pdf (537.42K)
Word count: 3682
Character count: 18872

Virus Research 142 (2009) 213–216

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

RNA secondary structures in the proximal 3′UTR of Indonesian Dengue 1 virus strains

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 19 February 2009
Accepted 25 February 2009
Available online 13 March 2009

Keywords:
Flaviviruses
Dengue viruses
3′UTR
Secondary structure

ABSTRACT

The characteristics of DENV-1 viruses, isolated during the 2001–2002 outbreak in Indonesia were studied. The secondary structure of the 3′UTR of different DENV-1 strains derived from Indonesian patients was compared with the 3′UTR of previously described DENV-1 sequences. The complete 3′UTR of DENV-1 was sequenced from 13 patients suffering from the severe form of dengue virus infection (dengue hemorrhagic fever). Prediction of RNA secondary structure of the 3′UTR revealed some previously unidentified conserved structures in the proximal region of the 3′UTR, the role of which in viral replication is still unknown. In addition our data suggest that some structural elements previously described in the distal part of the 3′UTR are partly dependent on the proximal part of the UTR. Our data support the existence of previously unidentified conserved secondary structures in the proximal part of the 3′UTR and their roles need to be further investigated.

Dengue viruses belong to the genus *Flavivirus* of the family *Flaviviridae*. They can be further subdivided into four antigenically distinct serotypes (DENV-1–4). With an estimated 100 million infections and 200 000 deaths per year, mainly among children (Anon., 2004), dengue is the most prevalent mosquito borne virus and among the most important public health problems world-wide.

Flaviviruses are single stranded positive sense RNA viruses coding three structural and seven non-structural proteins. The genome of dengue viruses is approximately 11 kb and contains a single open reading frame, which is flanked by 5′ and 3′ untranslated regions (UTRs). The length of the UTRs varies among different dengue serotypes (89–101 nt and 432–466 nt for 5′ and 3′UTR, respectively). The exact function of the UTRs has not been completely elucidated; however several studies have suggested that both the 5′ and 3′UTRs of flaviviruses are important for virus replication, translation and virulence (Alvarez et al., 2005; Mandl et al., 1998; Men et al., 1996; Proutski et al., 1997, 1999; Zeng et al., 1998). The 3′UTR of dengue viruses consists of a variable region of approximately 100 nt proximal to the stop codon of the NS5 gene and a distal region of approximately 300 nt. The secondary structure of the distal part of the 3′UTR of dengue viruses and other flaviviruses has been studied extensively and is shown to contain conserved structural elements among all flaviviruses, although some differences have been observed between mosquito and tick-borne flaviviruses (Hahn et al., 1987; Proutski et al., 1997; Rauscher et al., 1997). Those structures are believed to play an important role in the synthesis of minus strand RNA and consequently in virus replication. The 3′distal part of the UTR forms a long stable hairpin (Hahn et al., 1987; Proutski et al., 1997; Rauscher et al., 1997; Zeng et al., 1998), which stabilizes viral genome and enhance initiation of translation. The proximal part of the 3′UTR shows a high degree of sequence variation, however, the structural elements of this region have not been extensively described.

In this study we characterized the secondary structure of the 3′UTR of DENV-1 strains isolated from Indonesian patients and compared them with the structure of the 3′UTR of DENV-1 strains available in GenBank (accession numbers available from the authors upon request). To this end, we used the Genetic Algorithm (GA) incorporated in the STAR program, which is also able to predict pseudoknots. The validity of the predictions obtained by this program has been described elsewhere where experimental data confirmed *in silico* predictions (Gultyaev et al., 1995; van Batenburg et al., 1995). Our computations were executed with default settings of population size 10 and standard growth increments. During validation of our predictions the same strains were modeled with a different algorithm (mFOLD available from

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doi:10.1016/j.virusres.2009.02.016

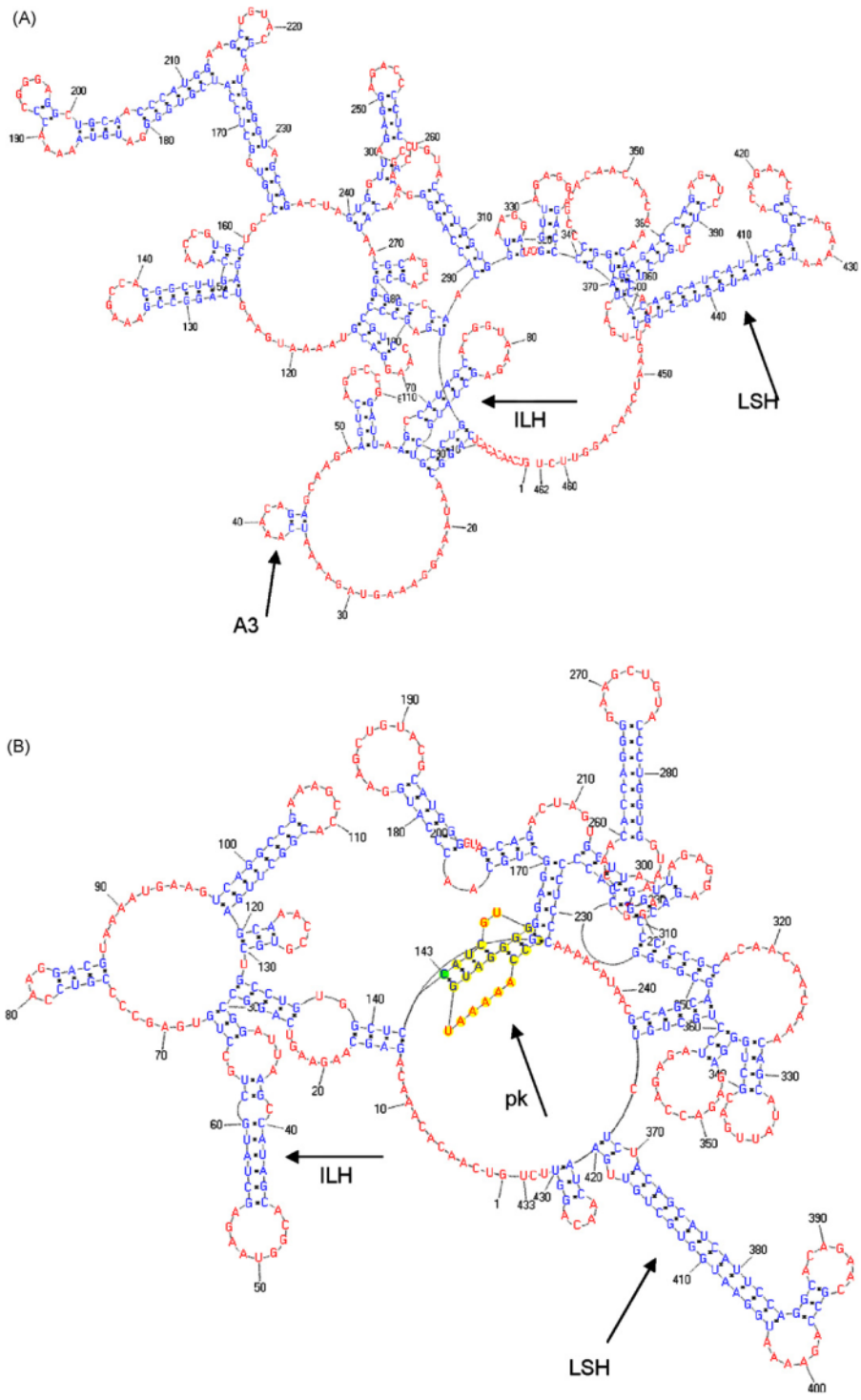

Fig. 1. Effect of the proximal DENV-1 3′UTR to the formation of structural elements in the distal part. The Abidjan strain is depicted in this figure: (A) wild type sequence; (B) 29-nt deletion introduced *in silico*. Only the conserved structural elements ILH (identified in this study) and the long stable hairpin (LSH, reference 9) are shown with arrows. The formation of a pseudoknot (pk) is highlighted and shown with an arrow (B).

the internet at: http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) with similar results (not shown). In order to obtain more accurate predictions using short sequences, we initially identified the structural elements of the proximal part (first 100 nt of the 3′UTR) using 37 DENV-1 strains. In order to simulate the natural folding of the RNA, in a second step analysis we modeled the structure of the complete 3′UTR of the same 37 DENV-1 strains.

Two regions, A and B of structural elements were identified. First, we identified the structural elements formed in Region A (immediately downstream of the stop codon) of the DENV-1 3′UTR. This region shows the highest degree of sequence variability and consequently structural variability. There was only one stem loop (structure A3, Fig. 1A) that was considered semi-conserved among DENV-1 strains since it was present in 31 DENV-1 strains (Supplementary Table 1). Region B was defined as a structurally conserved region, when studied on the population or individual level and could be displayed as an internal loop hairpin (ILH) (Fig. 1) (Proutski et al., 1997). However, in some instances the internal loop was lost so that the structural element of Region B was displayed as a relatively long hairpin. Although the structure of this region was conserved among all but one DENV-1 strains that we tested, the exact position of the structural element (i.e. ILH or hairpin) varied slightly among the different strains.

In a second step analysis we predicted the secondary structure of the complete 3′UTR of the 37 strains of DENV-1. Conserved structures in the distal part of the 3′UTR as described by Proutski et al. were present in all our predictions (data not shown), confirming the validity of our models. In addition we observed that some strains formed pseudoknots in the region upstream the long stable hairpin as has been predicted by Olsthoorn and Bol (2001). The ILH structure remained the consensus structural element of region B, formed by the nucleotides 66–91 (Fig. 1; Supplementary Table 1). However, some of the strains that we analyzed displayed a hairpin instead of an ILH. A DENV-1 strain from Fujian was the only strain that did not posses the ILH or the hairpin in Region B. Instead the nucleotides of this region base-paired with nucleotides in the distal part of the UTR. This finding suggests that Region B is structurally conserved among DENV-1 strains and provides strong evidence of its existence in vivo.

Next, we simulated the effect of short and long deletions that have been observed in some DENV-1 strains (Nukui et al., 2006 and in this study) on the maintenance of the different structural elements. This would indicate the relative importance of such structural elements in a particular genetic background (individual level) but also identify structures conserved among DENV-1 variants (population level). Towards that end, deletions were introduced *in silico* in all DENV-1 strains used in this study. The results of these predictions are summarized in Supplementary Table 1. The conserved structures A3 and B were not influenced when deletions were introduced, although the nucleotides involved in the formation of those structures varied between strains. Interestingly, the Fujian strain, which lacked formation of ILH or hairpin in Region B in the wild type, acquired this after introduction of both the short or long deletions. The structural element A3 remained semi-conserved, when the complete 3′UTR was modeled after introducing the deletions, whereas region B remained conserved among all DENV-1 strains although fewer strains displayed a hairpin, instead the ILH was formed (Supplementary Table 1). An interesting observation after modeling the complete 3′UTR was the influence that the deletions had in the formation of pseudoknots (Supplementary Table 1; Fig. 1). Some DENV-1 strains lost their pseudoknot after introduction of either short or long deletion and some other strains formed a pseudoknot, suggesting that the proximal part may influence the formation of some structural elements in the distal part of the 3′UTR (Supplementary Table 1).

Next we looked for the presence of the conserved structures A3 and B in the variable region of 3′UTR of DENV-2, -3 and -4 strains that have been deposited in the GenBank (accession numbers available from the authors upon request). Although the length of the variable region varied among the different serotypes according to the total length of the 3′UTR, region A and B was formed also in the DENV-2 and -3 strains that we analyzed. Similar to DENV-1, the conserved structure A3 was also found in the majority of DENV-2 and -3 strains, either as a short hairpin or a stem loop, involving the consensus sequence AGGC. This structure was also found in the DENV-4 strains that were studied albeit in lower frequency compared with DENV-1, -2 and -3. In region B the ILH was conserved among DENV-2 and -3. For DENV-4 only 12 strains were available in the GenBank by the time of data analysis. DENV-4 had different characteristics of the 3′UTR compared to the other serotypes. The 3′UTR of DENV-4 is shorter than the other three dengue virus serotypes, having an approximately 20 nt shorter variable region. ILH was conserved in 6 out of 12 DENV-4 strains studied whereas 3 strains were predicted to form a hairpin instead. The remaining 3 strains studied were predicted to form a pseudoknot in the B region. This tertiary structure was not predicted to occur in the variable regions of DENV-1, -2 and -3.

The RNA secondary structure of viral genomes and in particular of the untranslated regions is believed to play central roles in the viral life cycle, in particular in initiation or enhancement of translation and replication. The secondary structure of flavivirus 3′UTR's has been extensively described and the structural elements of the 3′distal part are well identified (Hahn et al., 1987; Proutski et al., 1997; Rauscher et al., 1997; Thurner et al., 2004). A considerable amount of experimental data confirms the importance of this region in flaviviruses life cycle (Alvarez et al., 2005; Chiu et al., 2005; Mandl et al., 1998; Men et al., 1996; Proutski et al., 1997). In our study we observed the same structures in several DENV-1 strains, confirming their conservation. However, little is known about the proximal part of the 3′UTR (Proutski et al., 1997), which lie immediately after the stop codon of the ORF. It bears considerable sequence variability and its structure and importance have not been well characterized. There was no specific pattern for the presence or absence of the structural elements identified in Region A (except A3 which was considered semi-conserved among DENV-1 strains). Those elements were formed in an interdependent manner, favoring the formation of A3 and ILH in Region B. Secondly, we identified the conserved structural elements, immediately upstream of the 3′distal part (Region B). Those elements (ILH or hairpin) were conserved among 36 of the 37 DENV-1 strains modeled (Supplementary Table 1). The presence of a structural element in itself does not indicate any functional significance in terms of biological properties of the virus. If a certain element is present in all viral strains or is maintained by means of compensatory mutations, it is assumed to exist. Such a conserved element might be indispensable for virus replication or might influence other biological properties such as virulence. In contrast, non-conserved elements are dispensable for virus replication and are likely to influence other biological properties. To assess the relevance of the conserved structures A3 and B, we modeled the complete 3′UTR of DENV-2, -3 and -4. If these structures are important one would expect them to be conserved among all DENV serotypes. Our predictions confirm the existence of these structures in the majority of the strains analyzed. Although there is no evidence of co-variation in region A, we cannot exclude that this region may influence parameters associated with virus virulence.

The presence of pseudoknots in RNA structures is considered to be important for stabilization of the RNA. Alternatively, pseudoknots could also affect replication rate of viruses (Bodaghi et al., 2000). Previously, pseudoknots have been observed in flavivirus 3′UTR and in particular in DENV either in naturally existing strains

of all DENV serotypes (Olsthoorn and Bol, 2001) or in truncated 3′distal sequences of DENV-3 (Proutski et al., 1997; Shi et al., 1996). In this study some DENV-1 strains formed a pseudoknot with nucleotides 178–193. Neither Thurner et al. nor Proutski et al. reported pseudoknots in naturally occurring DENV-1 strains (Proutski et al., 1997; Thurner et al., 2004). The difference with the study of Thurner et al. is the algorithm used for prediction of secondary structures. Although Proutski et al., used the same GA algorithm to predict secondary structures as in this study, they used a consensus sequence for each of the flaviviruses that they described in their study. We modeled 37 different strains of the same DENV serotype and the pseudoknots were observed in six of them. The positive predictive potential of GA was shown by Proutski et al. They were able to predict pseudoknots using the GA by modeling a truncated 3′UTR of DENV-3 for which the formation of the pseudoknot was experimentally supported (Shi et al., 1996). In addition, experimental data support the existence of pseudoknot in a DENV-4 strain (Romero et al., 2006), a structure that was also predicted to exist in three of the strains that we studied (see above). However, the existence and functional importance of pseudoknots in the DENV-1 3′UTR remains to be determined.

The 17-nt and 29-nt deletions identified by Nukui et al. (2006) were of particular interest. Those strains must have been circulating in SE Asia since at least 2001, suggesting that DENV-1 viruses bearing these deletions are viable and able to circulate in a relatively wide geographic region. However their biological importance is not clear. Tajima et al. introduced a similar deletion in a molecular clone of a different DENV-1 strain and showed that this deletion was dispensable for replication of the mutant clone (Tajima et al., 2006). Men et al. also showed that deletion of the proximal part of the 3′UTR in a DENV-4 molecular clone did not influence the replication of the mutant clone (Men et al., 1996). In contrast, deletions of part of the 3′UTR of other flaviviruses, Kunjin virus or TBE virus have been reported to influence replication of the virus (Khromykh and Westaway, 1997; Mandl et al., 1998).

We have seen that the influence of the deletions on the RNA secondary structure seemed to be strain dependent and that might also explain the observations of Tajima et al. Indeed in our modeling system, the structure of the proximal part of the 3′UTR of the strain that Tajima et al. used to prepare the molecular clone (referred as strain "Japan" in Supplementary Table) did not change after introduction of either the small or the long deletion. On the other hand, some other strains of DENV-1 had remarkable changes in the stem and loop formation of Region A after introduction of the deletions (Supplementary Table). Of particular note was the effect of the deletions in the structure of the distal part of the UTR especially in pseudoknot formation since their presence or absence in a particular strain was influenced by the introduction of the deletions. These findings suggest that distal and proximal regions of the 3′UTR of DENV-1 strains are in some extent dependent and the significance of these findings as well as the effects in viral life cycle warrants further investigation. Studies are ongoing to investigate the role of these deletions on replication efficiency and tropism in vitro using DENV-1 strains for which deletions are predicted to have an effect on the conserved structures.

Acknowledgements

The authors would like to thank all patients and their legal guardians as well as the nurses and doctors, participating in the observational study in Dr. Kariadi Hospital, Semarang, Indonesia. This study was partly founded by the Royal Dutch Academy of Arts and Science (K.N.A.W.).

The authors would also like to thank Dr. R.A.M. Fouchier and Dr. S. Noppornpanth for helpful discussions and critically reviewing the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.02.016.

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