Mapping of RNA G-quadruplexes in mirtrons and understanding their biological role

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Abstract

Mirtrons represent the predominant class of non-canonical miRNAs derived from introns through a Drosha-independent, splicing-dependent pathway. Unregulated splicing of introns containing hairpins can adversely impact Dicer/Ago-mediated canonical miRNA biogenesis. Among invertebrates, the 3' tailing of mirtron hairpins by terminal uridyl transferases (TUTases) suppresses mirtron biogenesis. However, the mechanisms regulating mirtron biogenesis in vertebrates remain poorly understood. G-quadruplexes are nucleic acid secondary structures formed by G-rich sequences in both DNA and RNA, and they have emerged as significant splicing regulators. Our study found that human mirtrons are enriched with rG4s specifically in the 5' arm. This enrichment suggests a potential role for rG4s in mirtron biogenesis. Careful analysis revealed that while the 5' arms of plant and invertebrate mirtrons are enriched with uracils (Us), the 5' arms of vertebrate mirtrons are enriched with guanines (Gs). This shift in nucleotide enrichment could indicate an evolutionary adaptation in mirtron biogenesis. Further analysis revealed that most mammalian mirtrons contain an RNA G-quadruplex (rG4), a feature not observed in plants or invertebrate mirtrons. Interestingly, almost all rG4s in mammalian mirtrons were located in the 5' arm. Predicted rG4s in human mirtrons form a G-quadruplex structure *in vitro*, and rG4 formation in the 5' arm facilitates Drosha-independent splicing for the excision of mirtrons. Disruption of rG4s in the 5' arm inhibits splicing and maturation in DROSHA knockout cells, whereas mutations outside the rG4-motif do not impact mirtron biogenesis. Thus, our findings indicate that rG4s in the 5' arm are critical regulatory elements in the evolutionary landscape of mammalian mirtrons. Extending this knowledge to human-infecting herpesviruses revealed that more than 50% of Herpes Simplex Virus (HSV) pre-miRNAs contain an rG4. Further analysis identified hsv1-mir-H27 and its analog hsv2-mir-H20 as putative mirtrons. Expression of hsv1-mir-H27 in DROSHA knockout cells demonstrated that the pre-miRNA could be processed in a Drosha-independent manner, confirming that this virus-encoded miRNA is a mirtron. Predicted splice sites for hsv1-mir-H27 lead to successful splicing of the mirtron. The rG4 in hsv1-mir-H27 pre-miRNA forms a G-quadruplex structure *in vitro*, and like human mirtrons, rG4 formation in the 5' arm facilitates Drosha-independent splicing. The sequential disruption of rG4s in the 5' arm inhibits splicing in DROSHA knockout cells, while mutations outside the rG4-motif do not affect mirtron biogenesis. This indicates that rG4 formation in the 5' arm of pre-miRNA significantly contributes to its splicing. Hsv1-mir-H27 is,

thus, the first human-infecting viral mirtron to be identified. This work revealed *NFE2L1* as a bona fide target of hsv1-miR-H27. Originating from the ICP0 locus, hsv1-miR-H27 expression is associated with increased cellular apoptosis, indicating its potential role in modulating cell death pathways. It enhances pro-apoptotic caspase 3/7 activity, suggesting involvement in HSV1-induced apoptosis through the hsv1-miR-H27-*NFE2L1* axis. Thus, hsv1-mir-H27 can be the potential unidentified non-coding factor responsible for ICP0-induced cellular apoptosis. However, the apoptosis induced by hsv1-miR-H27 is minimal compared to infection models, implying that additional viral proteins or stress factors may amplify this response. These could include oxidative stress, proteotoxic stress from infection, or other virus-derived elements affecting the *NFE2L1-PSMB6* pathway to enhance cell death. Further investigation is needed to fully understand the role of these amplifiers in the apoptosis response.

This research is a significant step in understanding mirtron biogenesis in mammals and viruses. It reveals additional roles for rG4s in small RNA biology and suggests a potentially crucial role for hsv1-miR-H27 in the viral manipulation of host cell processes. These implications underscore the importance of our findings in RNA biology and pave the way for numerous exciting avenues of research into the role of mirtrons, their processing and biogenesis, and their downstream effects in regulating cellular processes.