

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

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**Kusuma School of Biological Sciences**

**INDIAN INSTITUTE OF TECHNOLOGY, DELHI**

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

by

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Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to the



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# CERTIFICATE

This is to certify that the thesis titled “Mapping of RNA G-quadruplexes in mirtrons and understanding their biological role”, being submitted by Ms. Uzma Salim in the Indian Institute of Technology, Delhi for the award of the degree of “Doctor of Philosophy” is a record of the bonafide research carried out by him, which has been prepared under my supervision and guidance in conformity with rules and regulation of the Indian Institute of Technology Delhi, India. The results prescribed in it have not been submitted in part or full to any other University or Institute for the award of any Degree/Diploma.

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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

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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.

## सार

मिट्रॉन गैर-विहित माइक्रोआरएनए के प्रमुख वर्ग का प्रतिनिधित्व करते हैं जो ड्रोसा-स्वतंत्र, स्प्लिसिंग-निर्भर मार्ग के माध्यम से इंट्रॉन से प्राप्त होते हैं। हेयरपिन युक्त इंट्रॉन के अनियमित स्प्लिसिंग से डिसर/एगो-मध्यस्थ विहित माइक्रोआरएनए बायोजेनेसिस पर प्रतिकूल प्रभाव पड़ सकता है। अकशेरुकी जीवों में, टर्मिनल यूरेडाइल ट्रांसफ़ेरेस (TUTases) द्वारा मिट्रॉन हेयरपिन की 3' टेलिंग मिट्रॉन बायोजेनेसिस को दबा देती है। हालाँकि, कशेरुकियों में मिट्रॉन बायोजेनेसिस को विनियमित करने वाले तंत्रों को अभी भी ठीक से समझा नहीं गया है। जी-क्वाड्रप्लेक्स डीएनए और आरएनए दोनों में जी-समृद्ध अनुक्रमों द्वारा निर्मित न्यूक्लिक एसिड द्वितीयक संरचनाएँ हैं, और वे महत्वपूर्ण स्प्लिसिंग विनियामक के रूप में उभरे हैं। हमारे अध्ययन में पाया गया कि मानव मिट्रॉन विशेष रूप से 5' भुजा में आरएनए जी-क्वाड्रप्लेक्स से समृद्ध होते हैं। यह संवर्धन मिट्रॉन बायोजेनेसिस में आरएनए जी-क्वाड्रप्लेक्स की संभावित भूमिका का सुझाव देता है। सावधानीपूर्वक विश्लेषण से पता चला कि पौधे और अकशेरुकी मिट्रॉन की 5' भुजाएँ यूरेसिल (यू) से समृद्ध हैं, जबकि कशेरुकी मिट्रॉन की 5' भुजाएँ ग्वानिन (जी) से समृद्ध हैं। न्यूक्लियोटाइड संवर्धन में यह बदलाव मिट्रॉन बायोजेनेसिस में एक विकासवादी अनुकूलन का संकेत दे सकता है। आगे के विश्लेषण से पता चला कि अधिकांश स्तनधारी मिट्रॉन में एक आरएनए जी-क्वाड्रप्लेक्स होता है, जो पौधों या अकशेरुकी मिट्रॉन में नहीं देखा गया है। दिलचस्प बात यह है कि स्तनधारी मिट्रॉन में लगभग सभी आरएनए जी-क्वाड्रप्लेक्स 5' भुजा में स्थित थे। मानव मिट्रॉन में पूर्वानुमानित आरजी4 इन विट्रो में एक जी-क्वाड्रप्लेक्स संरचना बनाते हैं, और 5' भुजा में जी-क्वाड्रप्लेक्स गठन मिट्रॉन के छांटने के लिए ड्रोसा-स्वतंत्र स्प्लिसिंग की सुविधा प्रदान करता है। 5' भुजा में आरएनए जी-क्वाड्रप्लेक्स का विघटन द्रोषा नॉकआउट कोशिकाओं में स्प्लिसिंग और परिपक्वता को बाधित करता है, जबकि आरएनए जी-क्वाड्रप्लेक्स-मोटिफ के बाहर उत्परिवर्तन मिट्रॉन बायोजेनेसिस को प्रभावित नहीं करते हैं। इस प्रकार, हमारे निष्कर्ष संकेत देते हैं कि 5' भुजा में आरएनए जी-क्वाड्रप्लेक्स स्तनधारी मिट्रॉन के विकासवादी परिदृश्य में महत्वपूर्ण विनियामक तत्व हैं। इस ज्ञान को मानव-संक्रमित हर्पीज वायरस तक विस्तारित करने से पता चला कि हर्पीज सिम्प्लेक्स वायरस (HSV) के 50% से अधिक प्री-माइक्रोआरएनए में आरएनए जी-क्वाड्रप्लेक्स होता है। आगे के विश्लेषण ने hsv1-mir-H27 और इसके एनालॉग hsv2-mir-H20 को संभावित मिट्रॉन के रूप में पहचाना। द्रोषा नॉकआउट कोशिकाओं में hsv1-mir-H27 की अभिव्यक्ति ने प्रदर्शित किया कि प्री-माइक्रोआरएनए को ड्रोसा-स्वतंत्र तरीके से संसाधित किया जा सकता है, जिससे पुष्टि होती है कि यह वायरस-एनकोडेड माइक्रोआरएनए एक मिट्रॉन है। hsv1-mir-H27 के लिए पूर्वानुमानित

स्लिस साइट्स मिरट्रॉन के सफल स्लिसिंग की ओर ले जाती हैं। hsv1-mir-H27 प्री-माइक्रोआरएनए में आरएनए जी-क्वाड्रप्लेक्स इन विट्रो में एक जी-क्वाड्रप्लेक्स संरचना बनाता है, और मानव मिरट्रॉन की तरह, 5' आर्म में आरएनए जी-क्वाड्रप्लेक्स गठन ड्रोशा-स्वतंत्र स्लिसिंग की सुविधा देता है। 5' आर्म में आरएनए-जी-क्वाड्रप्लेक्स का क्रमिक विघटन द्रोशा नॉकआउट कोशिकाओं में स्लिसिंग को रोकता है, जबकि आरएनए-जी-क्वाड्रप्लेक्स-मोटिफ के बाहर उत्परिवर्तन मिरट्रॉन बायोजेनेसिस को प्रभावित नहीं करते हैं। यह दर्शाता है कि प्री-माइक्रोआरएनए की 5' आर्म में आरएनए जी-क्वाड्रप्लेक्स गठन इसके स्लिसिंग में महत्वपूर्ण रूप से योगदान देता है। इस प्रकार, Hsv1-mir-H27 पहला मानव-संक्रमित वायरल मिरट्रॉन है जिसकी पहचान की गई है। इस कार्य ने NFE2L1 को hsv1-miR-H27 के एक वास्तविक लक्ष्य के रूप में प्रकट किया। ICP0 लोकस से उत्पन्न, hsv1-miR-H27 अभिव्यक्ति बढ़ी हुई कोशिकीय एपोटोसिस से जुड़ी है, कोशिका मृत्यु पथों को संशोधित करने में इसकी संभावित भूमिका को दर्शाता है। यह प्रो-एपोटोटिक कैस्पेज़ 3/7 गतिविधि को बढ़ाता है, जो hsv1-miR-H27-NFE2L1 अक्ष के माध्यम से HSV1-प्रेरित अपोटोसिस में भागीदारी का सुझाव देता है। इस प्रकार, hsv1-mir-H27 ICP0-प्रेरित सेलुलर अपोटोसिस के लिए जिम्मेदार संभावित अज्ञात गैर-कोडिंग कारक हो सकता है। हालाँकि, संक्रमण मॉडल की तुलना में hsv1-miR-H27 द्वारा प्रेरित अपोटोसिस न्यूनतम है, जिसका अर्थ है कि अतिरिक्त वायरल प्रोटीन या तनाव कारक इस प्रतिक्रिया को बढ़ा सकते हैं। इनमें ऑक्सीडेटिव तनाव, संक्रमण से प्रोटीओटॉक्सिक तनाव या कोशिका मृत्यु को बढ़ाने के लिए NFE2L1-PSMB6 मार्ग को प्रभावित करने वाले अन्य वायरस-व्युत्पन्न तत्व शामिल हो सकते हैं। अपोटोसिस प्रतिक्रिया में इन एम्पलीफायरों की भूमिका को पूरी तरह से समझने के लिए आगे की जांच की आवश्यकता है।

यह शोध स्तनधारियों और वायरस में मिट्रॉन जैवजनन को समझने में एक महत्वपूर्ण कदम है। यह छोटे आरएनए जीव विज्ञान में आरएनए-जी-क्वाड्रप्लेक्स की अतिरिक्त भूमिकाओं को प्रकट करता है और मेजबान कोशिका प्रक्रियाओं के वायरल हेरफेर में hsv1-miR-H27 के लिए संभावित रूप से महत्वपूर्ण भूमिका का सुझाव देता है। ये निहितार्थ आरएनए जीव विज्ञान में हमारे निष्कर्षों के महत्व को रेखांकित करते हैं और मिट्रॉन की भूमिका, उनके प्रसंस्करण और जैवजनन, और सेलुलर प्रक्रियाओं को विनियमित करने में उनके डाउनस्ट्रीम प्रभावों में अनुसंधान के कई रोमांचक मार्गों का मार्ग प्रशस्त करते हैं।

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## List of Abbreviations

|               |                                                                                                                  |
|---------------|------------------------------------------------------------------------------------------------------------------|
| AFP           | Alpha fetoprotein                                                                                                |
| AKT           | Protein Kinase B                                                                                                 |
| AREs          | AU rich elements                                                                                                 |
| ASSP          | Alternative Splice Site Prediction                                                                               |
| ATF6          | Activating Transcription Factor 6                                                                                |
| ATRX          | Alpha thalassemia/mental retardation syndrome X linked                                                           |
| B-DNA         | B form DNA                                                                                                       |
| BLV           | Bovine Leukemia Virus                                                                                            |
| BMVC          | Benzimidazole Methyl Viologen Conjugate                                                                          |
| BRACO-19      | N,N'- (9- (4- (Dimethylamino)phenylamino)acridine-3,6-diyl)bis (3-(pyrrolidin-1-yl)propanamide) trihydrochloride |
| bZIP          | Basic Leucine Zipper                                                                                             |
| CaMKII        | Calcium/Calmodulin dependent Protein Kinase II                                                                   |
| CASP8 mediate | Caspase 8 mediated                                                                                               |
| CD            | Circular Dichroism                                                                                               |
| CDKN2A        | Cyclin Dependent Kinase Inhibitor 2A                                                                             |
| cDNA          | Complementary DNA                                                                                                |
| CMV           | Cytomegalovirus                                                                                                  |
| CNC-bZIP      | Cap 'n' Collar basic Leucine Zipper                                                                              |

## Abbreviations

|       |                                       |
|-------|---------------------------------------|
| CpG   | Cytosine phosphate Guanine            |
| cRNA  | Complementary RNA                     |
| DBR1  | Debranching Enzyme Homolog 1          |
| DDX41 | DEAD-Box Helicase 41                  |
| DEGs  | Differentially Expressed Genes        |
| DNA   | Deoxyribonucleic Acid                 |
| DSC   | Differential Scanning Calorimetry     |
| EBV   | Epstein Barr Virus                    |
| EcoRI | Escherichia coli Restriction Enzyme I |
| EDTA  | Ethylenediaminetetraacetic Acid       |
| FACS  | Fluorescence Activated Cell Sorting   |
| FAK   | Focal Adhesion Kinase                 |
| FBS   | Fetal Bovine Serum                    |
| FDR   | False Discovery Rate                  |
| GFP   | Green Fluorescent Protein             |
| GPI   | Glycosylphosphatidylinositol          |
| HSV-1 | Herpes Simplex Virus Type 1           |
| ICP0  | Infected Cell Protein 0               |
| ICP34 | Infected Cell Protein 34.5            |

## Abbreviations

|          |                                                         |
|----------|---------------------------------------------------------|
| ICP4     | Infected Cell Protein 4                                 |
| ICP6     | Infected Cell Protein 6                                 |
| IFN      | Interferon                                              |
| IL-6     | Interleukin 6                                           |
| LATs     | Latency Associated Transcripts                          |
| MDV-1    | Marek's Disease Virus Type 1                            |
| mRNA     | Messenger RNA                                           |
| MTA2     | Metastasis Associated 1 Family Member 2                 |
| MUC1     | Mucin 1                                                 |
| NaCl     | Sodium Chloride                                         |
| NFE2L1   | Nuclear Factor Erythroid 2 Like 1                       |
| NMR      | Nuclear Magnetic Resonance                              |
| OSM      | Oncostatin M                                            |
| PBS      | Phosphate Buffered Saline                               |
| PCR      | Polymerase Chain Reaction                               |
| PDS      | Pyridostatin                                            |
| PI3K     | Phosphoinositide 3 kinase                               |
| PI3K-Akt | Phosphoinositide 3 kinase Protein Kinase B pathway      |
| PIGT     | Phosphatidylinositol Glycan Anchor Biosynthesis Class T |

## Abbreviations

|        |                                                                                |
|--------|--------------------------------------------------------------------------------|
| PTEN   | Phosphatase and Tensin Homolog                                                 |
| qPCR   | Quantitative Polymerase Chain Reaction                                         |
| RISC   | RNA Induced Silencing Complex                                                  |
| RLU    | Relative Light Units                                                           |
| RNA    | Ribonucleic Acid                                                               |
| RNAi   | RNA Interference                                                               |
| SNP    | Single Nucleotide Polymorphism                                                 |
| SPR    | Surface Plasmon Resonance                                                      |
| tRNA   | Transfer RNA                                                                   |
| TUTase | Terminal Uridylyltransferase                                                   |
| UTR    | Untranslated Region                                                            |
| UV     | Ultraviolet                                                                    |
| VEGFA  | Vascular Endothelial Growth Factor A                                           |
| XhoI   | Xanthomonas holcicola Restriction Enzyme I                                     |
| YWHAE  | Tyrosine 3 Monooxygenase/Tryptophan 5 Monooxygenase Activation Protein Epsilon |
| Z-DNA  | Z form DNA                                                                     |