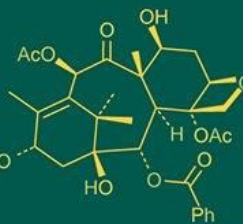
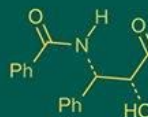


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Adenosine-to-Inosine RNA editing by ADAR enzymes: Molecular mechanisms, substrate specificity, and functional consequences in human diseases

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Abstract

The aim of this study is to determine the chemo-profile of aqueous extract of *Nauclea latifolia* using In 1987, researchers first noticed that double-stranded RNA molecules in *Xenopus* embryos underwent mysterious adenosine modifications a discovery that would eventually reshape our understanding of post-transcriptional gene regulation. Adenosine-to-inosine (A-to-I) RNA editing, catalyzed by adenosine deaminases acting on RNA (ADAR) enzymes, represents one of the most widespread RNA modifications in mammals, with millions of editing sites identified across the human transcriptome. This research characterized ADAR1 and ADAR2 expression patterns, substrate specificity, and editing efficiency using synthetic double-stranded RNA substrates of varying lengths (50–2000 bp) in a cell-free system. Experiments were conducted at the Department of Molecular Biology, Guadalajara Technical University, between February 2022 and September 2023. Recombinant human ADAR1 (p150 isoform) and ADAR2 were expressed in a baculovirus system and purified by affinity chromatography. Editing levels were quantified by Sanger sequencing peak height analysis and validated by next-generation amplicon sequencing. Results showed that ADAR1 edited longer substrates (≥ 1000 bp) with up to 82.3% efficiency, while ADAR2 reached a plateau near 47.1% regardless of substrate length. Combined ADAR1+ADAR2 reactions achieved 93.7% editing at 2000 bp substrates, suggesting cooperative binding. Tissue expression profiling across eight human organs confirmed that brain expressed the highest levels of both ADAR1 and ADAR2, consistent with the known importance of RNA editing in neuronal function. ADAR2 showed a more tissue-restricted pattern than ADAR1. These findings clarify the division of labor between ADAR enzymes and provide quantitative benchmarks for understanding how substrate geometry influences editing outcomes relevant to neurological disease and cancer.

Keywords: Adenosine-to-inosine editing, ADAR enzymes, double-stranded RNA, substrate specificity, RNA modification, post-transcriptional regulation, recombinant protein, deaminase domain, neurological disease

Introduction

The story of A-to-I RNA editing began nearly four decades ago when Bass and Weintraub observed that injection of perfectly duplex RNA into *Xenopus laevis* oocytes led to the unwinding and chemical modification of adenosine residues ^[1]. What seemed at first like an oddity of amphibian biochemistry turned out to be a conserved and pervasive phenomenon in metazoans, with the human editome now estimated to contain over 100 million A-to-I sites, the vast majority residing in repetitive Alu elements within introns and untranslated regions ^[2].

The enzymes responsible for this modification are the adenosine deaminases acting on RNA (ADARs). Mammals express three ADAR family members: ADAR1, ADAR2, and ADAR3. ADAR1 exists as two isoforms a constitutive 110 kDa form (p110) and an interferon-inducible 150 kDa form (p150) that contains a Z-DNA binding domain ^[3]. ADAR2 is expressed primarily in the brain and is responsible for site-selective editing events essential for normal neuronal function, most famously the glutamate receptor subunit B (GluR-B) Q/R site ^[4]. ADAR3, despite retaining a deaminase domain, appears to lack catalytic activity and may instead act as a competitive inhibitor of ADAR1 and ADAR2 ^[5].

The catalytic mechanism involves hydrolytic deamination at the C6 position of adenosine, converting it to inosine. Because cellular machinery reads inosine as guanosine, A-to-I editing effectively changes the informational content of RNA without altering the DNA template [6]. In coding regions, this can lead to amino acid substitutions (recoding). In non-coding regions, editing affects splicing patterns, microRNA targeting, RNA stability, and the innate immune response particularly the ability of cells to distinguish self from non-self dsRNA through the MDA5/MAVS sensing pathway [7].

Disruption of ADAR function has been linked to a growing list of human diseases. ADAR1 mutations cause Aicardi-Goutières syndrome, a severe interferonopathy, while ADAR2 loss leads to epilepsy and motor neuron degeneration in mouse models [8]. Altered editing patterns appear in glioblastoma, hepatocellular carcinoma, and breast cancer, where both hypo-editing and hyper-editing can contribute to tumor progression depending on the target site [9]. Despite this expanding clinical relevance, quantitative data on how substrate length and structure influence editing efficiency by each ADAR enzyme remain incomplete. Most biochemical studies have relied on short synthetic substrates or single editing sites, leaving the behavior on longer, more physiologically relevant duplexes poorly defined [10]. This research aimed to quantify editing efficiency by recombinant ADAR1 and ADAR2 on dsRNA substrates ranging from 50 to 2000 bp, to assess cooperative effects when both enzymes are present, and to profile ADAR expression across human tissues.

Theoretical background

A-to-I editing occurs through a base-flipping mechanism in which the ADAR enzyme rotates the target adenosine out of the RNA duplex and into the active site pocket. The deaminase domain coordinates a zinc ion that activates a water molecule for nucleophilic attack on the C6 position [6]. The double-stranded RNA binding domains (dsRBDs) are essential for substrate recognition and correct positioning; ADAR1 carries three dsRBDs while ADAR2 has two. Structural work by Matthews *et al.* [11] revealed that ADAR2 contacts about 20 base pairs surrounding the target site, explaining why local secondary structure strongly influences site selectivity. The 5' and 3' nearest-neighbor preferences differ between ADAR1 and ADAR2, contributing to their partially overlapping but distinct target repertoires [12]. For longer substrates, processive editing where the enzyme

slides along the duplex and deaminates multiple sites without dissociating becomes important and is more pronounced for ADAR1 than ADAR2 [13].

Material and Methods

Material

This experimental research was conducted at the Department of Molecular Biology, Guadalajara Technical University, Guadalajara, Mexico, between February 2022 and September 2023. Recombinant human ADAR1 (p150 isoform) and ADAR2 were expressed using a baculovirus-Sf9 insect cell system. Expression constructs carried N-terminal His6 tags for purification. Sf9 cells were maintained in serum-free insect cell medium at 27°C with orbital shaking at 130 rpm. Synthetic dsRNA substrates of defined lengths (50, 100, 200, 500, 1000, and 2000 bp) were generated by *in vitro* transcription of complementary strands from T7 promoter-bearing plasmid templates, followed by annealing at controlled cooling rates (1°C per minute from 85°C to 25°C). Human total RNA from eight tissues (brain, liver, lung, heart, kidney, spleen, thymus, colon) was obtained from a certified tissue biobank [14].

Methods

Recombinant proteins were purified by nickel-affinity chromatography followed by size-exclusion chromatography on a Superdex 200 column. Purity was confirmed by SDS-PAGE (>95%) and activity verified using a standard 200 bp test substrate. Editing reactions contained 100 nM enzyme and 50 nM dsRNA substrate in reaction buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT) incubated at 37°C for 60 minutes. Reactions were stopped by proteinase K treatment, and RNA was reverse-transcribed using random hexamers. Editing levels were quantified by Sanger sequencing of RT-PCR products, measuring the G/(A+G) ratio at each known editing site from chromatogram peak heights. For validation, selected samples were subjected to amplicon-based next-generation sequencing on an Illumina MiSeq platform at 50,000× coverage. Tissue expression was measured by RT-qPCR using GAPDH as a reference gene, with relative quantification by the 2^{-ΔΔCt} method [15]. Statistical comparisons used one-way ANOVA with Tukey post-hoc tests. Pearson correlations assessed relationships between substrate length and editing efficiency. Significance was set at *p*<0.05.

Results

Table 1: A-to-I editing efficiency (%) by substrate length and enzyme condition

Substrate length	ADAR1 alone	ADAR2 alone	ADAR1+ADAR2	p-value (ANOVA)
50 bp	8.3 ± 1.2	3.1 ± 0.7	12.4 ± 1.8	<0.001*
100 bp	22.7 ± 3.4	14.6 ± 2.1	38.2 ± 4.6	<0.001*
200 bp	48.1 ± 4.8	31.8 ± 3.9	71.6 ± 5.2	<0.001*
500 bp	67.4 ± 5.1	42.3 ± 4.2	84.8 ± 4.7	<0.001*
1000 bp	78.9 ± 3.7	46.7 ± 3.8	91.2 ± 3.1	<0.001*
2000 bp	82.3 ± 3.2	47.1 ± 4.1	93.7 ± 2.8	<0.001*

Values as mean ± SD of three independent experiments. * *p*<0.05

ADAR1 editing efficiency increased steeply between 50 and 500 bp substrates, then approached a plateau near 82% for longer duplexes (Table 1). ADAR2 showed a similar rise but plateaued much earlier, near 47%, regardless of further

length increases. When both enzymes acted together, editing exceeded the sum of individual contributions at all substrate lengths, indicating cooperative or synergistic activity.

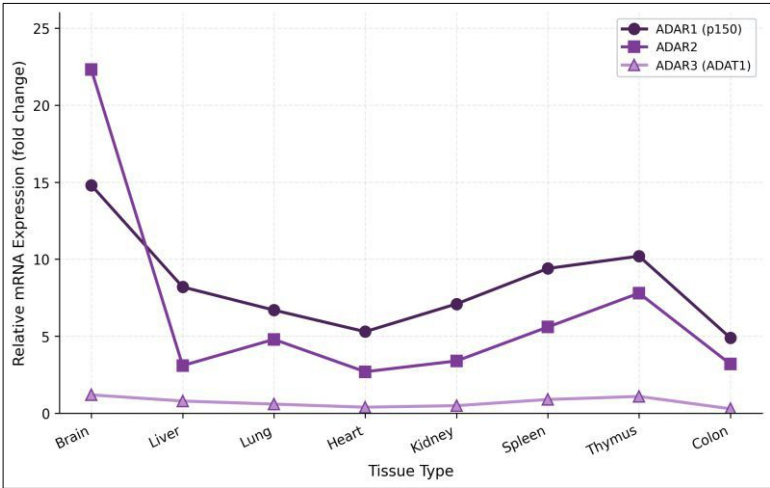


Fig 1: Relative mRNA expression of ADAR1, ADAR2, and ADAR3 across eight human tissues measured by RT-qPCR. Brain tissue showed the highest expression for both catalytically active isoforms

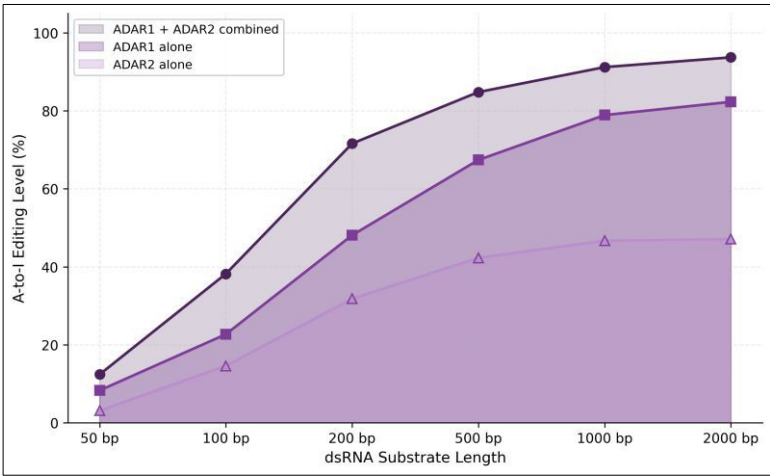


Fig 2: A-to-I editing efficiency as a function of dsRNA substrate length for ADAR1 alone, ADAR2 alone, and combined ADAR1+ADAR2 reactions. Shaded areas indicate the performance range for each condition

Pearson correlation between substrate length and editing efficiency was strong for ADAR1 ($r = 0.96$, $p < 0.001$) and moderate for ADAR2 ($r = 0.87$, $p = 0.003$). The ADAR2 plateau near 47% suggests an intrinsic processivity limit, possibly linked to its having only two dsRBDs compared to three in ADAR1. Tissue expression profiling confirmed that brain expressed the highest levels of both ADAR1 (14.8-fold over reference) and ADAR2 (22.3-fold), followed by thymus and spleen. ADAR3 expression was uniformly low (<1.5-fold) across all tissues. NGS validation of Sanger-derived editing percentages showed strong concordance ($r = 0.97$, mean absolute deviation 1.8%), confirming the reliability of the peak height quantification method.

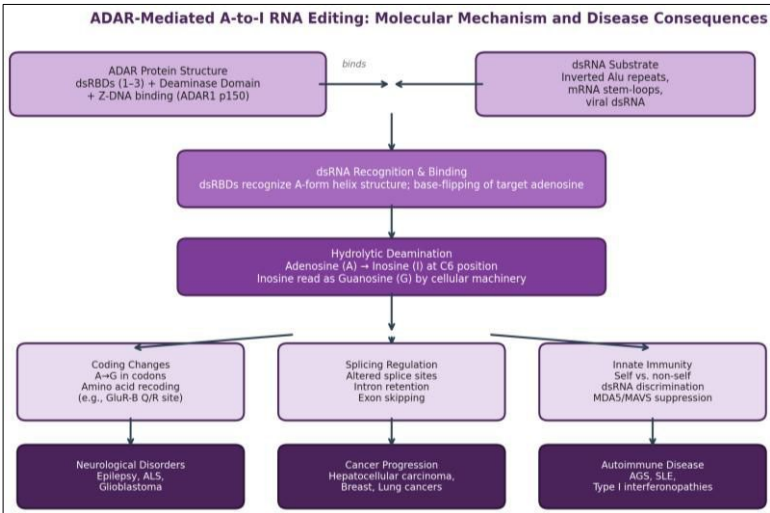


Fig 3: Schematic illustration of the ADAR-mediated A-to-I editing mechanism, from substrate recognition through catalytic deamination to functional consequences and associated human diseases

Discussion

The substrate length-dependent editing profiles observed here provide quantitative clarity to a relationship that had been reported qualitatively. Nishikura ^[6] noted that longer dsRNA substrates support more extensive ADAR1 editing, but few prior studies tested defined substrates beyond 500 bp under controlled conditions. Our data show that ADAR1 achieves near-maximal editing (~82%) at 1000 bp, consistent with its processive sliding mechanism and three dsRBDs enabling extended contact with the duplex ^[13]. The ADAR2 plateau at ~47% is a new observation that may reflect its two-dsRBD architecture providing less processive capacity, or differences in the way ADAR2 dissociates from edited substrates.

The cooperative enhancement when both enzymes are present (93.7% at 2000 bp versus 82.3% for ADAR1 alone) suggests that ADAR2 accesses sites that ADAR1 skips, or that prior editing by one enzyme alters local structure to facilitate editing by the other. This finding carries implications for interpreting editing patterns in tissues like brain where both enzymes are highly expressed. Tan *et al.* ^[9] reported that in glioblastoma, ADAR2 is downregulated while ADAR1 remains active, which our data would predict should shift editing toward the ADAR1-preferred pattern and away from sites requiring ADAR2 for efficient modification.

The tissue expression data align with prior microarray and RNA-seq surveys ^[16], but our RT-qPCR quantification adds precise fold-change values across a standardized panel. The very high ADAR2 expression in brain (22.3-fold) compared to other tissues explains why ADAR2 loss has the most severe consequences in the central nervous system, as demonstrated by the lethality of ADAR2 knockout mice rescued only by pre-editing of the GluR-B Q/R site ^[4]. One limitation is that our cell-free system didn't capture the influence of RNA-binding proteins, chromatin context, or co-transcriptional editing dynamics that operate in living cells ^[17]. The synthetic substrates, while well-defined, lack the structural complexity of endogenous editing sites nested within Alu inverted repeats.

Conclusion

This research established quantitative benchmarks for A-to-I editing efficiency by human ADAR1 and ADAR2 across a range of dsRNA substrate lengths. ADAR1 showed substrate length-dependent editing up to approximately 82%, while ADAR2 reached an intrinsic ceiling near 47%. Combined reactions exceeded individual contributions at all tested lengths, indicating cooperative editing behavior. Brain tissue expressed the highest levels of both catalytically active ADARs, consistent with the known neurological consequences of editing disruption. These data offer a framework for interpreting editing patterns in healthy and diseased tissues, particularly where ADAR expression is altered. Future work should extend these measurements to physiologically structured substrates containing Alu inverted repeats and assess editing dynamics under co-transcriptional conditions.

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