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JSM Genetics & Genomics

Research Article

RNA Editing in Zebrafish *Grin1b* is Regulated by Age and Influenced by Gender

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Submitted: 02 March 2016

Accepted: 22 April 2016

Published: 26 April 2016

Keywords

- RNA editing
- Adenosine-to-inosine
- *Grin1b*
- Zebrafish
- Aging
- *D. rerio*

Abstract

We recently described RNA editing in two sites of the *Grin1b* gene transcript of *Danio rerio* embryos. Upon further investigation, we discovered the editing of one of these sites changes with age (embryo vs. adult), as well as differing between male and female adults. RNA editing of this target represents an opportunity for dissection of the structural control elements that assist zebrafish ADARs in this coordinate regulation. We suggest that RNA editing research and genome editing strategies may be reinforcing and complementary.

ABBREVIATIONS

D. rerio: Danio rerio; ADAR: Adenosine Deaminase that Acts on RNA; A-to-I: Adenosine-to-Inosine; BP: Base Pairs

INTRODUCTION

RNA editing generates diversity in the transcriptome through recoding of mRNA transcripts and transcript interactions with regulatory RNAs. The translated proteins from edited and unedited transcripts may be subtly or profoundly different in function. A-to-I RNA editing is conserved from *Drosophila* to vertebrates, and occurs via similar structurally-directed mechanisms [1,2]. The majority of known RNA editing targets are ion channels and synaptic docking components which are expressed in many tissues including the nervous system and heart.

Alterations in RNA editing have potential clinical significance and have been linked in humans to amyotrophic lateral sclerosis (Lou Gehrig's disease), depression, schizophrenia, and epilepsy. A growing body of evidence also supports their involvement in cancer progression.

Adenosine-to-Inosine (A-to-I) RNA editing occurs in the primary messenger RNA transcript, following transcription, usually before the completion of splicing in the nucleus. RNA editing is an enzymatic process directed by the formation of secondary structures in RNA targets, formed by RNA secondary structure formation; the RNA transcript is imperfectly self-complementary and structures are formed by the pairing of complementary bases. Many of these substrates are recognized by the class of A-to-I editing enzymes, called Adenosine Deaminases that Act on RNA (ADARs). These enzymes edit RNA but not DNA.

RNA editing is detectable as Adenosine/Guanosine mixed signals in the sequenced products of reverse transcription; subsequently, controls can be performed to distinguish editing from DNA polymorphism and polymerase errors. Specific adenosines are converted by the ADAR enzymes to inosines and the ribosomes in the cell recognize these inosines as guanosines. Many codons may be respecified by A to G changes in first, second, or third positions of the codon, and one amino acid codon thereby changed into another. The alterations of single amino acids in ion channels by genetic mutation or RNA editing can have profound effects on the kinetics of an action potential depending upon the amino acid substituted and the position of the amino acid in the final protein structure.

Non-recoding edits can permit, enhance, or prevent the binding of small regulatory RNAs such as miRNAs to target transcripts [3]. Edits in untranslated regions or most codon third positions may affect mRNA fate, functioning as a form of regulatory feedback, likely negative.

In the course of investigating the editing status of several predicted Adenosine-to-Inosine mRNA editing sites in zebrafish embryos, we verified editing in a site that was discovered serendipitously during sequencing (designated E6). *Grin1b* (*NMDAR1.2*) encodes an NMDA receptor; this gene is known to contribute to the process of synaptic plasticity, as does RNA editing of synaptic components in general. The E6 *grin1b* site does not result in recoding in the resultant grin1b protein product, and we currently interpret this to reflect an important contribution to regulation of the transcript itself or of protein synthesis such as the results of Li and colleagues [4]. Upon further analysis, we discovered that the editing of the E6 site is sensitive to age,

occurring at characteristic levels in the embryo and in the adult fish, as well as consistently different in adult males and in adult females. We propose that this region of *grin1b* contains elements that form secondary structures that direct or mediate temporal and gender-specific aspects of editing regulation.

MATERIALS AND METHODS

Staging of zebrafish*:* zebrafish (*Danio rerio* Tubingen strain) were collected as embryos at 48-72 hours, and as non-synchronously aging adults of mixed gender. Adult Danio were kept at a constant temperature of 25°C (77°F) and fed *ad libitum* once daily. Tilapia (*Oreochromis mossambicus*) were obtained as fingerlings from Aquaponics USA (Yucca Valley, California). Fish used for this study were anesthetized in accordance with university institutional animal care and use council policies. Whole embryos were used for these analyses while the anterior quarter of the adult fish was used for RNA preparation (approximately 100 mg of brain tissue/fish). Further investigations into editing in other tissues such as the heart are planned.

Three independent RT-PCRs were performed for each data point followed by restriction analyses of editing at site E6 with an enzyme, *BstNI*, sensitive to A/G changes in its recognition sequence [5]; error is presented as standard deviation. Earlier analyses determined that DNA polymorphism was not present as a potential confounder at the E6 site, as well as a site designated E5; recent SNP analysis suggests that E5 may be a rare polymorphism, but this would not account for the mixed peaks seen only in cDNA-based electropherograms, suggesting additional complexity at this site. For further technical details and interpretation of data see Pozo and Hoopengardner, 2012 [5].

Briefly, following RT-PCR, E6 specific bands were extracted from agarose gels and restriction digests were performed; band intensities were analyzed using the Kodak Gel Logic system and software and scaled with regard to band size.

RESULTS

Editing with regard to age

Our former studies established that the frequency of embryonic editing at site E6 was 21.36% with a standard deviation of 4.47 % [5].

Our current data support the frequency of adult editing at site E6 in males at 35.03% (standard deviation of 3.40%) and in females at 30.83% (standard deviation of 3.65%).

Based on prior work in Drosophila [6] and other organisms, we hypothesized that editing in Danio would increase from embryonic to adult stages. Although insects are a common model system for editing, the zebrafish, as a simple model vertebrate system, is well suited for the examination of editing in clinically relevant targets.

At site E6, the difference in editing is very significant (paired t-test, $p < 0.05$) compared to adults collectively ($p = 0.0051$), as well as significant compared to males alone ($p = 0.0136$) and to females ($p = 0.0467$), respectively.

Editing with regard to gender

Percent editing frequencies for editing site E6. RT-PCR samples were treated with the restriction enzyme *BstNI* for site E6; an average frequency was calculated. Site E6 is edited at a frequency of 35.03% in males and 30.83% in females.

At site E6, influences of gender on editing may be suggested (see Discussion), although the standard error in adult males and females indicate that editing values occur in both sexes across a similar range.

At site E6, the value of a paired t-test comparing adult males and females is 0.2188.

A non-synchronized aging population and a low sample size were used for initial forays into the regulation of these sites in the adult. We are unable to conclusively assert that these male/female values are significantly different or unique to each sex, but they are influenced by gender as opposite male and female trends across a similar range. The editing values for site E6 were frequently higher for males (Figure 1). The mean and median for the editing values of this site in each sex are distinct. The Figure 1 graph and (Figure 2) box plot highlight this. The values may also be influenced by fertility and the shared environment in which males and females were kept. We suggest that future experiments may clarify this regulation.

Species comparisons

We initially hypothesized that editing at the E6 site or in this region generally, were conserved in other teleosts, and selected tilapia for further analysis. Primers specific for the tilapia (*Oreochromis mossambicus*) *grin1b* ortholog in the F3-R3 region [5] were designed and used for RT-PCR. The resulting amplicon was submitted for sequencing, however, examination of tilapia *grin1b* transcripts in this region detected no conserved editing at the expected adenosine positions and no additional sites specific to *O. mossambicus*. Tilapia primers used were O.mossambicus. grin1b. F1681 AATCTGGCTGCCTTCCTGGTG and O.mossambicus. grin1b.R1991 AACAGCTCTCCCGTGGTCAC; the Danio grin1b primers used were previously detailed [5].

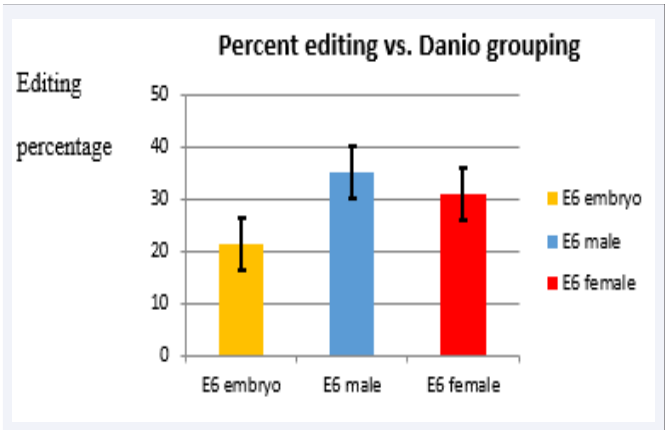

Figure 1 Editing percentages of Danio with regard to gender and age. Error bars represent standard deviation (Stdev). *E6 embryo- statistically significant compared to adult males, adult females, or adults collectively. $p<0.05$, Student's paired t test.

DISCUSSION

Editing regulation at the substrate level

Secondary structures predicted by mfold [7] at 25 degrees Celsius show small incremental differences in free energy with editing changes. Of the top predicted structures, the unedited 311 bp fragment folded yields a delta G (dG) of -119.41 kcal/ mol (Fig. 3 mfold pdf, modeled at 25 degrees C with mfold 2.3).

Purine variation at the E6 site yields slight changes in free energy dependent on adenosine or guanosine at this location; the dG values are -119.41 and -119.92 kcal/mol, respectively, with no stem changes in the E6 region.

We suggest that this small scale change confers a selective advantage, especially in the context of additional nearby sequence changes due to allelism or editing. Future covariation analyses may elucidate this model more fully (contrasting hairpin structures are shown in (Figure 4)).

In addition, these apparently subtle changes may favor codon alterations that influence translation rates, as suggested for aspects of bacterial translational pausing [4].

The effects of temperature and caloric reduction on aging extension or RNA editing in Danio are not completely understood, however, editing is responsive to temperature changes and is hypothesized to be adaptive in species that occupy environments that differ strongly in temperature [8]. For this reason, care was taken that fish be kept at a constant temperature of 25°C (77°F). A non-restrictive feeding regimen was also used to prevent potential confounders relating to caloric restriction. A 14 hour light/10 hour dark cycle (standard) was maintained to avoid the alteration of circadian rhythms and breeding.

COMPARISONS OF EDITING REGULATION

Timing of editing and sexual dimorphism

There is a general increase in editing in the E6 site with age; this change of editing from early to late life is seen in animal models [6,9] and our results are consistent with this trend. Indeed, increased editing in target sites might be used as a biomarker of maturation and the dysregulation of editing as a symptom of disease or neuropathy. The severity of human neuropathies generally increases with age [10]. The pathology of Alzheimer's disease is also subject to sexual dimorphism in humans [11]. Research suggests that periodic patient editing profiles would be a useful clinical tool.

Differences in editing between male and female fish at site E6 are affected by gender; more detailed analyses involving synchronized maturation would be required to assess this more completely. Since the fish were not synchronized in age, these variations may also be affected by temporal aging. Additional staging series would be required to prove this point; we refer to other papers on temporal control that follow this general methodology of collectively summing adults as a single stage of maturation [6,9].

A full editing series with respect to aging in Danio would be extensive. Studies of Danio lifespan revealed that the majority of a wild-type cohort lived for 42 months (3.5 years) , while the oldest survived for 66 months (greater than five years) [12].

Further analysis

To our knowledge, this is the first paper to provide initial data to support editing differences regulated with regard to gender in zebrafish. In addition, we further characterize a site in Danio that

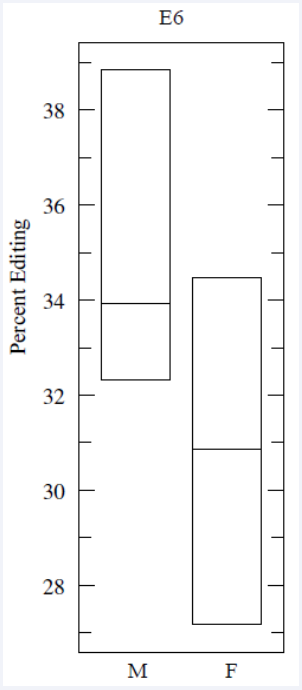

Figure 2 E6 adult male (M) and female (F) editing values.

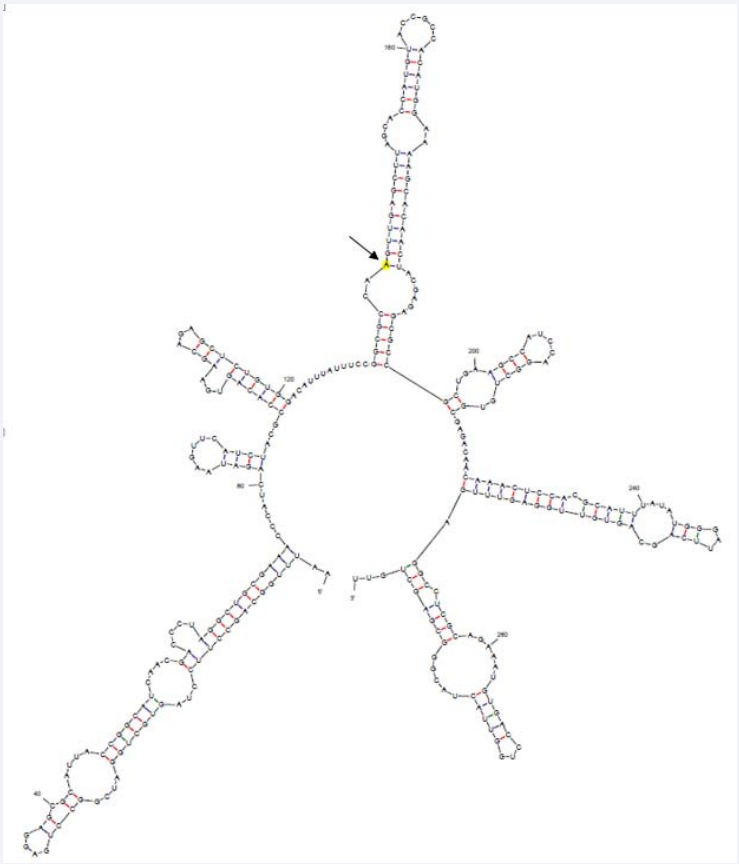

Figure 3 Unedited RNA structure; the E6 site (position 141) adenosine is indicated. Delta G value, -119.41 kcal/mol.

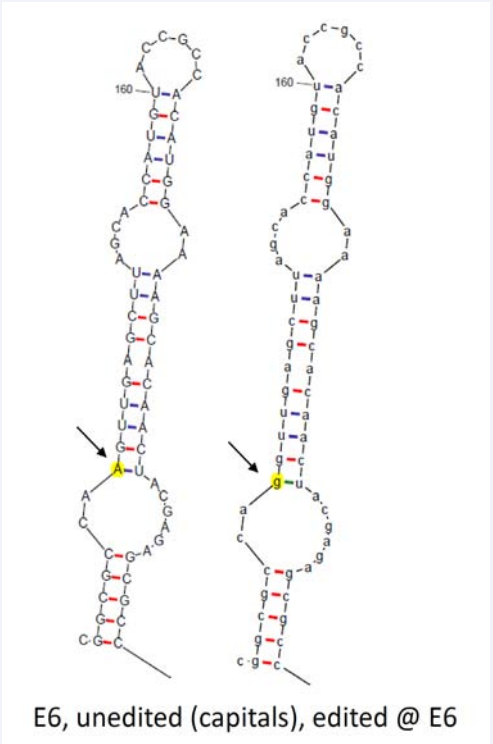

Figure 4 RNA structure comparisons; the E6 site is indicated, separately, in edited and unedited hairpins.

is responsive to both age and gender via predicted changes in free energy.

Further experiments may address these questions in cotransfection assays using modified *grin1b* constructs or in studies of Danio ADAR enzyme kinetics.

RNA editing research identifies nucleotides of RNA transcripts at which alteration may provide subtle or profound benefit to the organism without fixation in the genome; such sites provide a means for rapid adaptation. The fixation of these purine changes as encoded guanosines in the genome via genome editing strategies may provide important functional information and detail the necessity of purine flexibility in resultant transcripts.

ACKNOWLEDGMENTS

We wish to thank Dr. Betsy Dobbs-McAuliffe for the donation of zebrafish embryos and adults.

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Cite this article

Pozo P, Hoopengardner B (2016) RNA Editing in Zebrafish Grin1b is Regulated by Age and Influenced by Gender. JSM Genet Genomics 3(1): 1009.