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Research paper

RNA editing independently occurs at three mir-376a-1 sites and may compromise the stability of the microRNA hairpin

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ABSTRACT

RNA editing is being recognized as an important post-transcriptional mechanism that may have crucial roles in introducing genetic variation and phenotypic diversity. Despite microRNA editing recurrence, defining its biological relevance is still under extended debate. To better understand microRNA editing function and regulation we performed an exhaustive characterization of the A-to-I site-specific patterns in mir-376a-1, a mammalian microRNA which RNA editing is involved in the regulation of development and in disease. Thorough an integrative approach based on high-throughput small RNA sequencing, Sanger sequencing and computer simulations we explored mir-376a-1 editing in samples from various individuals and primate species including human placenta and macaque, gorilla, chimpanzee and human brain cortex. We observed that mir-376a-1 editing is a common phenomenon in the mature and primary microRNA molecules and it is more frequently detected in brain than in placenta. Primary mir-376a-1 is edited at three positions, −1, +4 and +44. Editing frequency estimations and in silico simulations indicated that editing was not equally recurrent along the three mir-376a-1 sites, nevertheless no epistatic interactions among them were observed. Particularly, the +4 site, located in the seed region of the mature miR-376a-5p, reached the highest editing frequency in all samples. Secondary structure predictions revealed that the +4 position was the one that conferred the highest stability to the mir-376a-1 hairpin. We suggest that molecular stability might partially explain the editing recurrence observed in certain microRNAs and that editing events conferring new functional regulatory roles in particular tissues and species could have been conserved along evolution, as it might be the case of mir-376a-1 in primate brain cortex.

1. Introduction

RNA editing is the site-specific modification of an RNA sequence generating a different product from that encoded in the DNA. Most RNA editing in metazoans is the adenosine deamination into an inosine, which has similar base-pairing properties as guanosine, A-to-I(G), catalyzed by adenosine deaminases (ADARs) acting on double-stranded RNAs (dsRNAs) (Blow et al., 2006; Nishikura, 2010). RNA editing can alter the encoded amino acid sequence in certain messenger RNAs (mRNAs) giving isoforms with novel functions (Burns et al., 1997; Rosenthal and Bezanilla, 2002; Hoopengardner et al., 2003; Ohlson et al., 2007; Nishikura, 2010). Despite the biological impact that these changes may introduce at the protein level, RNA editing mainly occurs in non-protein-coding regions, suggesting that its effects may comprise regulatory functions (Li et al., 2009; Nishikura, 2010; Cattenoz et al., 2013). microRNAs (miRNAs), a small class of non-coding RNAs, are crucial regulators of gene expression that show high conservation at the sequence level among species (Lagos-Quintana et al., 2001; Wheeler

Abbreviations: A, adenosine; ADAR, adenosine deaminase; Cq, quantification cycle; dsRNA, double-stranded RNA; G, guanosine; I, inosine; MFE, minimum free energy; mRNA, messenger RNA; miRNA, microRNA; NGS, next generation sequencing; nt, nucleotide; RT-qPCR, real time quantitative reverse transcription PCR; small RNA-seq, small RNA-sequencing

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et al., 2009; Meunier et al., 2013). They are transcribed in a primary miRNA which is processed by Drosha giving a ~70 nt precursor hairpin molecule that is cleaved in the cytoplasm by Dicer in a miRNA duplex formed by two ~22 nt mature miRNA strands, -5p and -3p. Target gene repression is based on the sequence interaction between one of the mature strands (especially nucleotides 2–8, known as the seed region) and the mRNA (Lewis et al., 2003), which leads to mRNA translational repression or degradation (Kim, 2005; Krol et al., 2010; Berezikov, 2011). Post-transcriptional modifications such as miRNA editing may have an important role in introducing genetic variation and phenotypic diversity among tissues and species (Wheeler et al., 2009). Since miRNAs adopt a dsRNA secondary structure along their maturation process, they are naturally ADAR substrates and could be edited with no significant effects. Nevertheless, previous studies have shown that miRNA editing can alter the biogenesis from primary to mature molecules (Yang et al., 2006; Kawahara et al., 2007b; Kawahara et al., 2008) and remodel the spectrum of regulated target genes, especially when they occur at the seed region (Blow et al., 2006; Kawahara et al., 2007a).

Quantification studies of miRNA A-to-I editing have been chiefly based on two approaches. On the one hand, most recent literature has taken advantage of the large amount of data generated by small RNA next generation sequencing (NGS) technologies, permitting to draw a general picture of the editable transcriptome across tissues, species and several biological conditions (Peng et al., 2012; Alon et al., 2012; Gong et al., 2014; Warnefors et al., 2014; Nigita et al., 2015). Regardless of the enormous information these studies have provided, little consistency has been observed among results. In general, editing frequencies tend to be underestimated and lowly expressed and/or lowly edited miRNAs remain undetectable (Levanon et al., 2004). On the other hand, approaches based on direct Sanger sequencing of specific genomic regions have demonstrated that miRNA editing may be widespread, reporting strong evidences for several miRNAs (Kawahara et al., 2008; Bass et al., 2012; Kawahara, 2012). Although these low-throughput methods are highly laborious, they allow for a more accurate calculation of editing percentages (Bass et al., 2012). In fact, direct sequencing has been commonly used for validating editing patterns observed by NGS studies (Levanon et al., 2004; He et al., 2011; Bahn et al., 2012; Peng et al., 2012). Other methods based on pyrosequencing technologies have also been used for RNA editing quantification (Iwamoto et al., 2005). Even though they are fast and accurate tools for quantification of relatively long fragments, current platforms are limited for short fragment analysis, as mature miRNA molecules.

Analyses of miRNA editing frequencies have evidenced the recurrence of these changes; nevertheless the particular molecular mechanisms regulating the editing events are still largely unknown. Examination of edited sequences has revealed that most edited adenosines residues are located within UAG triplets (Blow et al., 2006; Kawahara et al., 2008; Peng et al., 2012; Alon et al., 2012). It has also been demonstrated that the competition of ADAR binding proteins for the access to the dsRNA sequences can regulate the editing efficiency at a given adenosine position (Washburn and Hundley, 2016). Additionally, comparative analyses of miRNA editing have evidenced the existence of regular patterns of editing among tissues and closely related species, suggesting a role of these post-transcriptional modifications in development, evolution and disease (Kawahara et al., 2007a; Ekdahl et al., 2012; Nemlich et al., 2013; Warnefors et al., 2014; Shoshan et al., 2015; Hwang et al., 2016). For instance miRNA editing appears to be enriched in neural tissues, in older individuals and in healthy compared to cancerous samples. At least six miRNAs (mir-376a-1, mir-376b, mir-376c, mir-379, mir-381 and mir-411) show editing conservation across placental mammals and two miRNAs (mir-301 and mir-455) between mammals and bony fishes (Warnefors et al., 2014). Among these miRNAs, mir-376a-1 is a placental mammalian miRNA whose RNA editing has been demonstrated to be involved in disease and in the regulation of development (Choudhury et al., 2012; Ekdahl et al., 2012). It is located in a large cluster in human chromosome 14, involved in neural regulation and expressed in adult tissues such as placenta and brain (Seitz, 2004; Glazov et al., 2008). Like other members of this cluster, mir-376a-1 is mainly edited at three adenosine residues: the −1 site located in the precursor sequence, and the +4 and +44 sites in the seed regions of -5p and -3p strands, respectively. Editing at these three sites seems to be tissue-specific and it can completely redirect the set of recognized target genes, at least when it occurs at the +4 site (Kawahara et al., 2007a).

In this work we went deeper into the biological significance of miRNA editing through the characterization of A-to-I site-specific editing patterns of mir-376a-1 using whole small RNA NGS and Sanger sequencing in a set of primate samples including human placenta and macaque, gorilla, chimpanzee and human brain cortex. We observed different patterns of RNA editing depending on the miRNA maturation stage and tissue identity. Editing frequency estimations and transition probability simulations pointed out that certain mir-376a-1 sites were more frequently edited than others. Even though no epistatic interactions among mir-376a-1 editing sites could be detected, the observed differences in their editing frequencies could be explained because they compromise the miRNA hairpin stability in a different manner. We suggest that, although miRNA editing could neutrally have emerged as a consequence of ADAR editing activity, some of these changes could have been maintained if they conferred a particular molecular advantage to the miRNA such as a higher thermodynamic stability. Additionally, editing events that would provide new regulatory roles to the edited miRNAs in particular tissues and/or species could have experienced stronger selective pressures and could have been conserved at the post-transcriptional level.

2. Materials and methods

2.1. RNA samples and RNA extraction

Gorilla brain cortex tissue was provided by the Barcelona Zoo (Spain). Chimpanzee and macaque brain cortex tissues were provided by the Biomedical Primate Research Centre (Netherlands). Human placenta samples were provided by the Corporació Sanitària Parc Taulí-Institut Universitari (Spain). This work was conducted according to relevant Spanish and International guidelines. Primate samples were collected after natural death and thus, in a non-invasive way, without disturbing, threatening or harming the animals. Human placenta samples were collected after delivery from healthy donors who signed an informed consent. Human brain cortex RNA samples were purchased at Ambion (Foster City, CA, USA). Total RNA was extracted with the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA).

2.2. Small RNA-sequencing (small RNA-seq) analysis

One microgram of total RNA showing RNA integrity numbers between 7–9 were processed using an Illumina protocol considering the following steps: acrylamide gel purification of RNA bands corresponding to whole small RNA ranging 17–30 nt long, ligation of 5′ and 3′ adapters to the RNA in two separate subsequent steps each followed by acrylamide gel purification, cDNA synthesis followed by acrylamide gel purification, and a final step of PCR amplification to generate template libraries that were sequenced using Illumina HiSeq2000 (Axeq services, Seoul, South Korea). Small RNA-seq data was analyzed using Chimira software (Vitsios and Enright, 2015), by mapping read sequences against all annotated human precursor miRNAs registered in miRBase (Kozomara and Griffiths-Jones, 2014) using the Standard Nucleotide Blast (BLASTn v.2.2.24+) and allowing up to two mismatches for each sequence and discarding any anti-sense hits. Total sequencing reads were normalized across samples using DESeq2. Sequencing reads mapping to multiple precursor miRNA paralogs were assigned only to the first optimal alignment call returned by BLASTn.