Splicing variants of ADAR2 and ADAR2-mediated RNA editing in glioma (Review)

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Abstract. The roles of alternative splicing and RNA editing in gene regulation and transcriptome diversity are well documented. Adenosine deaminases acting on RNA (ADARs) are responsible for adenosine-to-inosine (A-to-I) editing and exemplify the complex association between RNA editing and alternative splicing. The self-editing activity of ADAR2, which acts on its own pre-mRNA, leads to its alternative splicing. Alternative splicing occurs independently at nine splicing sites on ADAR2 pre-mRNA, generating numerous alternative splicing variants with various catalytic activities. A-to-I RNA editing is important in a range of physiological processes in humans and is associated with several diseases, including amyotrophic lateral sclerosis, mood disorders, epilepsy and glioma. Reduced editing at the glutamine/arginine site of the AMPA receptor subunit GluA2 in glioma, without any alteration in ADAR2 expression, is a notable phenomenon. Several studies have tried to explain this alteration in the catalytic activity of ADAR2; however, the underlying mechanism remains unclear. The present review summarizes the relevant literature and shares experimental results concerning ADAR2 alternative splicing. In particular, the present review demonstrates that shifts in the relative abundance of the active and inactive splicing variants of ADAR2 may reduce the ADAR2 editing activity in glioma. Dominant expression of ADAR2 splicing variant with low enzyme activity causes reduced RNA editing of GluA2 subunit at the glutamine/arginine site in glioma.

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Abbreviations: A-to-I, adenosine-to-inosine; ADAR, adenosine deaminase acting on RNA; ASV, alternative splicing variant; dsRBD, dsRNA-binding domain

Key words: alternative splicing, splicing variant, ADAR2, RNA editing, glioma

Contents

1. Introduction

Carcinogenesis is a complex, multistage process. Advances in gene sequencing technology have demonstrated that certain DNA mutations and chromosomal abnormalities are important in tumorigenesis and tumor development. Aberrations in RNA, a central element in gene expression, are also vital in tumorigenesis, tumor development and malignant growth at the posttranscriptional and epigenetic level. Posttranscriptional modifications, including RNA editing and alternative splicing, render it possible to diversify the transcriptome, while restricting the size of the genome. Members of the adenosine deaminase acting on RNA (ADAR) family of enzymes, which catalyze adenosine-to-inosine (A-to-I) RNA editing, have been associated with alternative splicing in glioma (1,2). Levels of A-to-I RNA editing are reportedly reduced in glioma, and alternative splicing variants (ASVs) of ADAR2 are expressed at various levels (3). Notably, the expression of ADAR2 mRNA is unaltered in glioma, and the underlying mechanism of this phenomenon remains unclear. The present review discusses alternative splicing and RNA editing in glioma, specifically in terms of the ADAR2 spliced isoforms.

2. RNA editing and ADARs

RNA editing was first identified in trypanosomes by Benne *et al* (4), who concluded that alterations in nucleotide sequences occur during or following transcription of the frameshift gene by a RNA editing process. A subsequent study identified a RNA duplex unwinding activity when antisense RNA was injected into fertilized frog eggs (5). The unwinding activity was later demonstrated to arise from structural alterations in the RNA when adenosine (A) is converted

to inosine (I) (6). The following types of RNA editing have been identified (7): Uridine (U) insertion or deletion (8); cytosine (C) insertion and dinucleotide insertion (9); small nucleolar RNA-mediated nucleotide modification of ribosomal RNAs (10); transfer (t)RNA editing (11); C-to-U editing (12); and A-to-I editing (13). In mammals, A-to-I editing is the most common type of RNA editing causing genetic diversity (14). A-to-I RNA editing occurs at over one hundred million genomic sites, which are located in the majority of human genes (15). ADAR enzymes, responsible for the catalytic conversion of A to I (14), share a highly conserved catalytic deaminase domain (DM) at their C-terminal, which binds to double-stranded DNA (dsRNA) synergistically with the N-terminus dsRNA-binding domain (dsRBD) (16-18). In vertebrates, three members of the ADAR family (ADAR1, ADAR2 and ADAR3) have been identified (19). There are three dsRBDs for ADAR1, and two for ADAR2 and ADAR3 (18). ADAR1 and ADAR2 are ubiquitously expressed in humans and exhibit catalytic activity, whereas ADAR3 is expressed specifically in the brain and has no catalytic activity (14). ADAR3 has been revealed to competitively inhibit deaminase activities of other ADARs by binding to dsRNA (20). The adenosine deaminase reaction catalyzed by ADAR2 is site-specific (21), as demonstrated in studies of the GluA2 subunit of glutamate AMPA receptor (21-23), in which the conversion of a glutamine (Q) to an arginine (R) codon is exclusively mediated by ADAR2 (23). ADAR2 activity is essential for brain development and function (24), and >99.9% of RNA editing occurs at the Q/R site of GluA2 in the human central nervous system (25). In addition, RNA editing plays a role in controlling microRNA (miRNA) biogenesis (26). A recent study of the mouse brain revealed that reproducible alterations in the sequence and abundance of mature miRNAs are induced by ADAR2 (27). Furthermore, ADAR2-mediated editing is site-specific, as opposed to sequence-specific, and ADAR2 edits the coding and noncoding regions of mRNAs (28,29). Therefore, a single-base modification during the recoding process during editing may affect the coding potential of the RNA and its splicing.

3. Alternative splicing

Studies conducted in 1977 revealed that the coding regions in DNA sequences are not continuous, and the final tRNA or mRNA is a spliced product (30,31). In 1978, Gilbert presented 'Why genes in pieces?' (32), in which the terms 'intron' and 'exon' were first introduced. The research by Gilbert led to an additional study, which confirmed that alternative splicing occurs in eukaryotic cells (33). In 1994, the level of alternatively spliced human genes was estimated to be only 5% (34). However, by the 21st century, data from The International Genome Sequencing Consortium predicted that ≥50% of human genes are alternatively spliced (33,35,36). In 2008, Wang *et al* (37) and Pan *et al* (38) demonstrated that >90% of multi-exon genes are alternatively spliced, and the majority of these are specifically alternatively spliced tissue. Wang *et al* (37) also described various types of alternative splicing, including exon skipping, intron retention, alternative 5' splice sites, alternative 3' splice sites, mutually exclusive exons, mutually exclusive 5' untranslated regions (UTRs), mutually exclusive 3' UTRs and tandem UTRs. Alternative splicing results in the expression of diverse proteins and affects transcription factors, cell signaling, transmembrane proteins and secreted extracellular proteins (39). Consequently, the structural and functional alterations in these proteins and signaling pathways may be involved in carcinogenesis (39).

4. Alternative splicing of human ADAR2 mRNA

The human ADAR2 gene is located on the long arm of chromosome 21 (21q22.3) and spans ~153 kbp (40-42). A study by Slavov and Gardiner (42) revealed that the genomic structure of the human *ADAR2* gene consists of 15 exons. The structure of ADAR2 mRNA is illustrated in Fig. 1. Kawahara *et al* (43) followed the exon and intron identifiers reported by Slavov and Gardiner to divide ADAR2 mRNA into four regions based on alternative splicing sites: The first region includes exons 2-1, in which two ASVs occur; the second region includes exons 2-3, in which three ASVs occur; the third region includes exons 4-8, in which two ASVs occur; and the fourth region includes exons 9-10. So far, a total of nine splicing sites in ADAR2 mRNA have been confirmed (42-48). Alternative splicing at these sites occurs independently, resulting in dozens of ADAR2 spliced isoforms. This renders it challenging to analyze tissue-specific and developmental-stage-dependent properties of the ADAR2 ASVs *in vivo* (49). Studies have identified that alternative splicing occurs at exon 2 and exon 4-6, which are dsRBD and DM coding regions, and these affect the enzyme activity of ADAR2 (43,44,46). In addition, inclusion of exon 5a results in the generation of a protein, which has ~50% reduction in activity (44). The exception is the splicing variants, which have a distinctive truncated shorter C-terminal structure, and exhibit no editing activity, if splicing occurs at exon 9 (45). Agranat *et al* (48) described a splicing event that the 93 nucleotide sequence located in intron 7 was included as exon 7a. This also occurs outside ADAR2 function domain coding regions, but does not lead to a catalytic activity product. Alternative exon 7a is expressed tissue-specifically, with high levels in the skeletal muscle, heart and testis, and low levels in the brain (48). Alternative splicing sites in human ADAR2 mRNA are summarized in Table I, which is adapted from a previous study by the present authors (50).

5. Reduced RNA editing in glioma

Although extensive sequencing and analysis of the human genome have revealed a clear association between genes and cancer, numerous questions have also been raised. Currently, in the postgenomic era, the field of epigenetics has received much attention. Epigenetic alterations, including alternative splicing and ADAR-mediated A-to-I RNA editing, have been associated with several types of cancer, including breast cancer, neuroblastoma and hepatocellular carcinoma, and there is a particularly clear association with glioma (3,39,51).

Glioma is the most common type of tumor in the central nervous system, and is classified into four grades that reflect the degree of malignancy. Glioblastoma multiforme (grade IV) is the most aggressive type of glioma and is fatal (1). An association between RNA editing and glioma, particularly the pathogenesis of glioblastoma, was first identified by Maas *et al* (1), who demonstrated that there was

Table I. Summary of alternative splicing sites in human *ADAR2* mRNA.

Author, year	ASV, exon	Effect on ADAR2 transcript	Effect on ADAR2 protein	Effect on catalytic activity	(Ref.)
Slavov and Gardiner, 2002	-1-1	Inclusion of exon 1a	28-amino-acid N-terminal extension	Unknown	(42)
Kawahara *et al*, 2005	1-3	Skipping of exon 2	Generation of a 12-amino-acid protein	None	(43)
Kawahara *et al*, 2005	9-10	Inclusion of intron 9	Unknown	Unknown	(43)
Kawahara *et al*, 2005	9	Splices exon 9, 83 nt downstream from stop codon	Unknown	Unknown	(43)
Gerber *et al*, 1997	5-6	Inclusion of exon 5a	Insertion of AluJ cassette in the catalytic domain	Decreased	(44)
Lai *et al*, 1997	9	Truncates 3' end of the coding region	Replacement of 29 C-terminal residues with 2 amino acids	None	(45)
Rueter *et al*, 1999	1-2	Addition of 47 nt to 5' end of exon 2	Generation of a 9-kDa protein	Decreased	(46)
Maas and Gommans, 2009	-1-1	Inclusion of exon 0	49-amino-acid N-terminal extension	Unknown	(47)
Agranat *et al*, 2010	7-8	Inclusion of exon 7a	Nonsense-mediated mRNA decay	None	(48)

Adapted from Li *et al* (3). ADAR, adenosine deaminases acting on RNA; ASV, alternative splicing site; nt, nucleotide.

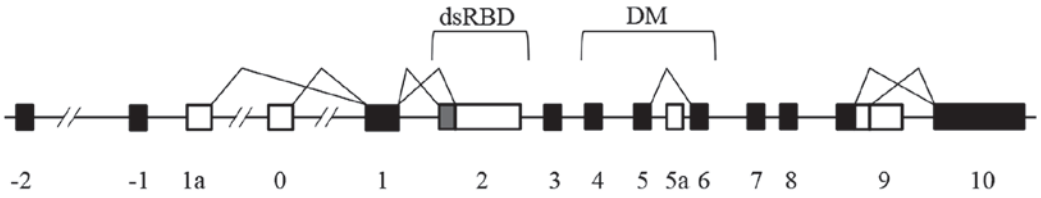

Figure 1. Exon-intron structure of human *ADAR2* gene. Boxes illustrate exons, while lines illustrate introns. Filled boxes indicates coding and open boxes indicate non-coding. Shaded part of exon 2 indicates the 47 nucleotide cassette, where self-editing occurs, leading to the formation of a splicing site. ADAR, adenosine deaminases acting on RNA; dsRBD, N-terminus dsRNA-binding domain; DM, deaminase domain.

reduced editing at the Q/R site of GluR-B, with no corresponding alteration in ADAR2 expression and no difference in the alternative splicing of ADAR2 mRNA in tumor and normal tissues. Cellular mechanisms that regulate ADAR2 catalytic activity are unknown, but may involve posttranslational modification or controlled subcellular localization of ADAR2 (1). Another study demonstrated that a reduction in editing levels was associated with the grade of malignancy in pediatric astrocytomas, which was attributed to altered ADAR2 catalytic activity (2). Furthermore, that study reported that ADAR2 overexpression inhibited cell proliferation and migration *in vitro*. In addition, alternative splicing within exon 2 of the ADAR1 pre-mRNA in high-grade tumors generated a 110-kDa protein, as opposed to the full-length 150-kDa protein. Overexpressed ASVs of ADAR1 are hypothesized to form heterodimers with ADAR2, disrupting the balance between ADAR1, ADAR2 and ADAR3, and competing for specific ADAR2 editing activity at the Q/R site of GluR-B (2). These findings suggest that the alternative splicing events in ADAR1 regulate ADAR2-mediated RNA editing.

A previous study demonstrated a significant loss of ADAR2 editing activity in newly diagnosed and recurrent pediatric high-grade astrocytoma (52). Notably, ADAR2 editing activity was substantially rescued in the only patient with prolonged survival, suggesting that ADAR2 activity/expression is a possible prognostic marker. Other results suggest attenuated A-to-I editing of miRNA-376a* promotes invasiveness of glioblastoma cells *in vitro* and orthotopic xenograft mouse models (53). In addition, a recent study demonstrated that ADAR2 editing activity inhibits glioblastoma growth by modulating the cell division cycle 14B/S-phase kinase-associated protein 2/p21/p27 axis (54).

6. ADAR2 ASVs regulate RNA editing in glioma

Reduced RNA editing without a significant alteration in ADAR2 expression has been widely documented (1-3,55); however, the underlying mechanism remains unclear. Due to the large number of ADAR2 ASVs, ADAR2 expression may not associate fully with its editing activity. This raises the

question of whether alternative splicing is another regulatory factor that may interfere with ADAR2-mediated RNA editing. However, the precise association between RNA editing and alternative splicing is complex and remains unclear.

Rueter *et al* (46) demonstrated that the addition of 47 nucleotides to the 5' end of exon 2 occurs subsequent to RNA editing within intron 1, which reduces ADAR2 activity *in vivo*. A previous study by the present authors revealed the expression levels of this ADAR2 ASV in human glioma tissues and glioma-derived U251 and BT325 cell lines (55); the ADAR2 ASV was expressed in 10% of low-grade astrocytomas, 16.7% of oligodendrogliomas, 12.5% of anaplastic astrocytomas and 25% of glioblastomas multiforme. In addition, the increased expression of this self-editing-induced ASV corresponded to the increasing malignancy of the glioma, and its expression appeared to be associated with the malignant features of glioma, as identified in the glioblastoma multiforme group of ADAR2 ASV^+ patients, who had more severe peritumoral brain edema, tumor invasion in more brain lobes and a shorter median survival time compared with ADAR2 ASV^- patients. Therefore, it appears that ADAR2 mRNA levels are not altered in glioma-derived cell lines or glioma tissues, the self-editing of the ADAR2 pre-mRNA generates an ADAR2 ASV in glioma-derived cell lines and glioma tissues, and the expression of this ADAR2 ASV may be associated with the malignancy of glioma.

Early studies by the present authors lead to a hypothesis that ADAR2 splicing isoforms may affect its enzyme activity; therefore leading to investigations concerning the association between splicing isoform expression and the clinical features of glioma. However, the identified percentage of ADAR2 ASVs (10-25%) was not enough to theoretically explain the reduced RNA editing of the GluA2 Q/R site. Hideyama and Kwak (56) demonstrated that expression of Q/R site-unedited GluA2 requires >50% reduction of ADAR2 activity; therefore, the present authors analyzed the differences in the alternative splicing patterns of ADAR2 in glioma U87, U251 and A172 cell lines and normal human astrocytes HA1800 cells (50). Quantitative polymerase chain reaction identified no significant differences in the ADAR2 pre-mRNA splicing patterns at exon 1a or 2 between the glioma-derived cell lines and normal human astrocytes. However, transcripts including exon 5a were predominantly expressed in the glioma-derived cell lines, and transcripts without exon 5a were relative to its expression in normal human astrocytes. Taken together, these findings indicate that alternative splicing in glioma cells causes an abnormal increase in the expression of exon 5a, leading to the active suppression of ADAR2 activity and a reduction in A-to-I RNA editing.

Therefore, A-to-I RNA editing is regulated by the pattern of ADAR2 alternative splicing in glioma. Collectively, *in vitro* results suggest an association between the increased expression of abnormal ADAR2 isoforms or specific ADAR2 ASVs and the malignant characteristics of astrocytoma. Further studies concerning human glioma tissues are required to support *in vitro* findings, using normal brain white matter as a control. The primary aim of such a study would be to determine whether the alterations in ADAR2 ASV expression may be used as a novel marker for the molecular classification of glioma and to monitor tumor progression in patients.

7. Conclusion

In summary, abnormal expression and activity of ADAR2 contributes to abnormal RNA editing. Additional study into specific ADAR2 ASVs is required to identify the specific RNA abnormalities that are associated with tumorigenesis and tumor development, and to determine the complex associations between ADAR-mediated A-to-I editing and the alternative splicing of pre-mRNA ADAR2 in specific diseases. Furthermore, additional study is required to establish the precise regulation of A-to-I editing by ADAR2 ASVs and how they contribute to glioma genesis and progression. Overall, future aims would be to identify novel ADAR2 target genes, investigate the rescue potential of ADAR2 editing activity and define the underlying rules of ADAR2 ASVs differential expression. Identification of subtle alterations in the transcriptome introduced by A-to-I RNA editing and ADAR2 isoforms with differential activity generated by alternative splicing may continue to lead to additional and notable findings.

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