Global regulation of alternative splicing by adenosine deaminase acting on RNA (ADAR)


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Regulation of AS by ADAR

Outline

1. Introduction

2. Materials

3. Results
   - Collection of editing sites
   - A-to-I editing sites rarely fall within the canonical splicing motifs
   - In silico assay suggesting A-to-I RNA editing modifies SREs
   - ADAR has prominent influence on global splicing pattern
   - Splicing pattern changes by ADAR KD $\Leftrightarrow$ enrichment of A-to-I editing?
   - ADAR KD shows significant changes in splicing and RNA processing genes

4. Summary
A major mechanism for gene regulation and transcriptome diversity.

Yet the extent of the phenomenon, the regulation and specificity of the splicing machinery are only partially understood.
ADAR enzymes

- **ADAR**: Adenosine Deaminase Acting on RNA.
- Adenosine-to-inosine (A-to-I) RNA editing of pre-mRNA by ADAR enzymes, which bind **double strand** RNAs.
  - ADAR (ADAR1): two distinct prevalent isoforms: p110 & p150.
  - ADARB1 (ADAR2).
  - ADARB2 (ADAR3).
  - ADAR1 knockout: mice die in the embryonic stage.
  - ADAR2 knockout: mice suffer from seizures and die at an early age.
- **Cytosine** is base-paired by reverse transcriptase with **inosine** during cDNA synthesis.
A-to-I RNA editing and mRNA splicing are indeed coordinated in specific genes.

This coordination may be governed by the RNA Pol II carboxy-terminal domain (CTD) [Laurencikiene et al. EMBO report 2006; Ryman et al., RNA 2007].

- CTD helps ensure that editing precedes splicing of the GluR-B transcript.

Efficient exonic RNA editing often depends on intronic editing complementary sequences (ECS) for duplex formation.

Examples for interrelations between editing and splicing are evident early in evolution [Jin et al., BMC Evol. Biol. 2007; Agrawal & Stormo, RNA 2005].
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An editing site strengthens an enhancer signal within the exon [Agrawal & Stormo, *RNA* 2005].

Exonization of intronic sequences:
- RNA editing in the NARF gene in human might lead to exonization of an intronic sequence and the birth of the eighth exon of this gene [Lev-Maor et al., *Genome Biol.* 2007].

Editing at the branch site of PTPN6 gene in AML patients was found to impair splicing of the intron, with a probable role in leukemogenesis [Beghini et al., *Hum. Mol. Genet.* 2000].
Contribution of this paper

To analyze how ADAR **globally** affects alternative mRNA splicing.

- A systematic approach;
- High-throughput expression analysis (exon-specific microarray) & sequencing of transcript data sets (ESTs/mRNA);
- Massively parallel sequencing (MPS; NGS).
A-to-I RNA editing rarely targets canonical splicing motifs.
- Yet it was found to affect splicing regulatory elements (SREs) within exons.

Cassette exons were found to be significantly enriched with A-to-I editing sites compared with constitutive exons.
RNA-seq & exon-specific microarray revealed that ADAR knockdown in HepG2 & K562 cell lines leads to **global changes** in gene expression.

- Hundreds of genes change their splicing patterns.
- This cannot be explained by putative editing sites alone.

Genes showing significant changes in their splicing pattern are frequently involved in RNA processing & splicing activity.

Direct A-to-I RNA editing is **NOT** likely to be the primary mechanism for ADAR-mediated regulation of AS.

- The regulation is suggested to be achieved by modulating trans-acting factors.
Brief summary of the results (contd.)

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Genomic data

- **hg19/GRCh37** (UCSC ftp site): DNA reference for detection of editing sites.
- **RefSeq**:
  - To define genes and exon–intron boundaries.
  - To determine 5’-SS and 3’-ss consensus motifs.
- **dbSNP 132** (from UCSC table browser): SNP sites.
- Repeat data: taken from **RepeatMasker**.
Transcript data

- EST & mRNA data and their alignments to hg19:
  - EST: 249,717 reads; mRNA: 7,510,566 reads.
  - Taken from UCSC table browser.

- 454 Life Science RNA-seq data: SRA003647/SRP000614.
  - Including:
    - HBRR (Human Brain Reference RNA);
    - UHRR: Universal Human Reference RNA.
  - Alignment against hg19 using BLAT.

- RNA-Seq data (short reads):
  - U87MG cell line:
    - 2 x control + 2 x ADAR KD.
    - Downloaded (Bahn et al. Genome Res. 2012).
  - HepG2 & K562 cell line
    - Illumina GAIIx (76bp; paired-end; alignment: using TopHat).
Microarray experiment setting

- Using an exon-specific microarray (Affymetrix exon 1.0 st).
  - A probe set for each annotated exon.
  - Feasible to compare expression levels of individual exons and thus to evaluate AS.
- For each cell line, control samples and ADAR KD samples were tested.
A-to-I RNA editing sites detection and collection

- Comparison of mRNA/EST/RNA-seq reads and the reference genome (preliminary SNV sites).

Then process the following filters:

- Quality assurance:
  1. The SNV site must be distal (20 bases) from sequence ends.
  2. Exclude known SNP sites.
  3. Enough support:
     - ≥ 5% of all aligned reads that cover the site;
     - ≥ 2 ESTs or ≥ 1 mRNA.

- Others specific to ADAR enzymes:
  1. Clustering: [...] A-to-G ... A-to-G ... A-to-G ... A-to-G ...] ← 32 bases.
  2. Double strand: \[12 \text{ bp} \rightarrow \text{A-to-G} \rightarrow 12 \text{ bp} \rightarrow 80\% \text{ complementary to } 1000 \text{ bp} \rightarrow \text{A-to-G} \rightarrow 1000 \text{ bp} \rightarrow ...

- Including previous published (putative) editing sites (e.g., DARNED).
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- Including previous published (putative) editing sites (e.g., DARNED).
Verification of editing sites from RNA-seq analysis

To verify editing sites predicted based on mRNA/EST, using:

- SAMtools `pileup` and `varScan` [Koboldt et al. *Bioinformatics* 2009] on the RNA-seq alignment and scanning for A-to-G or T-to-C.
- Only sites in both replicates were considered.
Sites passed all filters:

- 33,687 A-to-G sites (42%; total: 80,127 sites).
- 20,283 A-to-G sites in 3,630 genes (the rest: poorly annotated regions?)
  - 156 in CDS;
  - 14,958 in intronic regions;
  - 513 in non-coding exonic regions;
  - 1,115 in non-coding introns;
  - 3,452 in 3’UTR;
  - 89 in 5’UTR;
Category of editing sites predicted based on mRNA/EST (contd.)
Reduction in editing level following ADAR KD based on RNA-seq

**Figure A**
- Graph showing the G/(A+G) levels in HepG2 and K562 cells.
- Control (black bars) and KD (white bars) conditions.
- Significant difference indicated by stars.

**Figure B**
- Graph showing the G/(A+G) levels in HepG2 and K562 cells.
- Control (black bars) and KD (white bars) conditions.
- Significant difference indicated by stars.
Results

Collection of editing sites

Reduction in editing level following ADAR KD (contd.)

- Adenosines not known to be edited (in edited genes).
Constitutive exons vs. cassette exons

- Categorize the editing sites in exons →
  1. constitutive;
  2. cassette;
  3. other (e.g., retained introns, alternative 5’ ss and 3’ ss).
Constitutive exons vs. cassette exons (contd.)

- Enriched in cassette exons over constitutive exons.

**TABLE 1.** A-to-I editing in constitutive and cassette exons

<table>
<thead>
<tr>
<th>Exon type</th>
<th>No. of A-to-I sites&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Exon lengths (bases)</th>
<th>A-to-I per base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutive</td>
<td>363</td>
<td>22,119,390</td>
<td>$1.64 \times 10^{-5}$</td>
</tr>
<tr>
<td>Cassette</td>
<td>114</td>
<td>2,380,193</td>
<td>$4.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>Other</td>
<td>56</td>
<td>1,171,293</td>
<td>$4.8 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Overall, editing sites are enriched in cassette exons when normalized to the combined exon lengths ($\chi^2$ P-value < $10^{-10}$). Similar results were found using different editing sites sets (see Supplement 1, Supplemental Table S4).

<sup>a</sup>Editing sites were taken from DARNED (Kiran and Baranov 2010).
Constitutive exons vs. cassette exons (contd.)

- Mismatches in exons (mRNA/EST).
- Mismatches at 150-base intronic regions flanking exons (mRNA/EST).
Constitutive exons vs. cassette exons (contd.)

- A possible explanation:
  - Cassette and their flanking introns contain more *Alu* repeats.

- Nevertheless, counting only non-*Alu* editing sites:
  - cassette vs. constitutive:
    - $1.52 \times 10^{-5}$ sites/base vs. $1.29 \times 10^{-5}$ sites/base. (not significant)
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Fundamental sequence motifs required for canonical splicing

1: 3’ splice site (5’ ss);
2: poly-pyrimidine-tract (PPT);
3: branch point
   - branch site (BS) consensus motif: yUnAy);
4: 5’ splice site (5’ ss).
Results

A-to-I editing sites rarely fall within the canonical splicing motifs

Editing sites rarely fall within the primary consensus sites of canonical splicing

- For editing sites within 5’ ss or 3’ ss consensus motifs:
  - Only 3 and 2 are within 5’ ss and 3’ ss resp (mRNA/EST).
  - Only 24 are within 5’ ss or 3’ ss for ALL data.

- For editing sites overlapping the branch-site consensus motif yUnAy:
  - None.
Splicing regulatory elements (Data preparation)

- Experimentally proved SREs:
  - Akerman et al. [Genome Biol. 2009].
  - Piva et al. [Bioinformatics 2009].

- Computational verification:
**Results**

*In silico* assay suggesting A-to-I RNA editing modifies SREs

**Created/abolished SREs**

- Splicing factor proteins (e.g., hnRNPs and SR-proteins) bind to SREs in mRNA and regulate the type and the level of intron inclusion/exclusion.

  - The approach: Searching for potential SREs in exons that have ≥ 1 putative editing site.
    - **Control:** randomly select adenosines in the exon and then change them to guanosines.
      - Count the cases where the changes create or abolish SREs.
    - Repeat the process for 1,000 times.

  - The same procedure was applied for SREs located within the 150-base intronic regions flanking the exons.
Regulation of AS by ADAR

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In silico assay suggesting A-to-I RNA editing modifies SREs

Note: Only 244 editing sites within 193 exons of 188 genes, while AS analysis of RNA-seq and microarray data revealed thousands of AS regions.
Microarray analysis

- Tools:
  - **Partek** genomic suite (for microarray data).
  - AS ANOVA (for splicing analysis) & express ANOVA (for total gene expression level).

- Only genes without a large change in expression (fold-change $\leq 1.5$) were considered in the AS analysis.

- Lists of AS genes were extracted using four different significant thresholds (AS ANOVA $P$-values of 0.001, 0.01, 0.015, 0.025).

- Under the independence assumption:

  $N_{exp} = \left( \frac{N_{HepG2}}{N} \right) \times \left( \frac{N_{K562}}{N} \right) \times N.$

  - $N$: the total number of genes represented on the array.
  - $N_{HepG2}$: the number of detected genes in HepG2.
  - $N_{K562}$: the number of detected genes in K562.
Regulation of AS by ADAR

Results

ADAR has prominent influence on global splicing pattern

Microarray analysis (contd.)
Results

ADAR has prominent influence on global splicing pattern

Differential expression & alternative splicing analysis of RNA-seq

- Both are done using AltAnalyze.
  

- Differential expression analysis:
  
  - Count the reads for each constitutive exons in Ensembl genes (V.62) and use AltAnalyze quintile normalization.
  - Use fold-change ratios of 1.4, 2, and 2.5.
  - # DE genes in both cell lines (as they did in the microarray analysis).
Regulation of AS by ADAR

Results

ADAR has prominent influence on global splicing pattern

A simple example of quintitle normalization

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
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<td>3</td>
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<tr>
<td>B</td>
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<td>1</td>
<td>4</td>
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<td>4</td>
<td>6</td>
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<tr>
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<td>2</td>
<td>8</td>
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<td>iv</td>
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</table>

\[ A \quad 5 \quad 4 \quad 3 \quad \text{becomes} \quad A \quad 2 \quad 1 \quad 3 \]
\[ B \quad 2 \quad 1 \quad 4 \quad \text{becomes} \quad B \quad 3 \quad 2 \quad 4 \]
\[ C \quad 3 \quad 4 \quad 6 \quad \text{becomes} \quad C \quad 4 \quad 4 \quad 6 \]
\[ D \quad 4 \quad 2 \quad 8 \quad \text{becomes} \quad D \quad 5 \quad 4 \quad 8 \]

\[ A (2 \quad 1 \quad 3)/3 = 2.00 = \text{rank} \quad i \]
\[ B (3 \quad 2 \quad 4)/3 = 3.00 = \text{rank} \quad ii \]
\[ C (4 \quad 4 \quad 6)/3 = 4.67 = \text{rank} \quad iii \]
\[ D (5 \quad 4 \quad 8)/3 = 5.67 = \text{rank} \quad iv \]

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<td>4.67</td>
<td>2.00</td>
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Regulation of AS by ADAR

Results

ADAR has prominent influence on global splicing pattern

B

Differential expression analysis

- Overlap expression
- Expected overlap expression

Intersection

Union
Differential expression & alternative splicing analysis of RNA-seq (contd.)

Alternative splicing analysis:

- Base on alignment and junctions obtained by TopHat.
- Using AltAnalyze
  - Splice-index (SI).
    - Expression of a given exon relative to the total gene expression.
  - Analysis of splicing by isoform reciprocity (ASPIRE) [Ule et al. *Nature Genetics* 2005].
    - Using reads mapped to junctions.

Only exons detected as AS by both methods and whose genes were NOT found to be differentially expressed (|fold-change| ≤ 2) were considered as AS exons.

- Genes with ≥ 1 AS exon were considered as AS genes.
Results

ADAR has prominent influence on global splicing pattern
Are genes that significantly change their splicing pattern in ADAR KD cells enriched with A-to-I RNA editing sites?
### TABLE 2. Editing in AS genes detected by RNA-seq

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Splicing pattern</th>
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<th>Genomic size (bases)</th>
<th>A-to-I per base</th>
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<tr>
<td>HepG2</td>
<td>Changed</td>
<td>12,043</td>
<td>338,829,808</td>
<td>$3.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>HepG2</td>
<td>Unchanged</td>
<td>15,973</td>
<td>731,790,993</td>
<td>$2.18 \times 10^{-5}$</td>
</tr>
<tr>
<td>K562</td>
<td>Changed</td>
<td>12,971</td>
<td>343,111,651</td>
<td>$3.78 \times 10^{-5}$</td>
</tr>
<tr>
<td>K562</td>
<td>Unchanged</td>
<td>14,897</td>
<td>679,627,158</td>
<td>$2.19 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Editing sites are enriched in AS genes ($\chi^2$ P-value $< 10^{-10}$ for both HepG2 and K562).

<sup>a</sup>Editing sites were taken from DARNED (Kiran and Baranov 2010).
It is still difficult to determine if editing mediates the AS regulation, or whether other ADAR-dependent events are responsible for this effect on AS.

Based on the RNA-seq experiments, only 17 verified editing sites in the vicinity (1,000 bases flanking) of the detected AS cassette exons are detected.

Direct editing is not the sole explanation for AS.
ADAR KD shows significant changes in splicing and RNA processing genes

**Significant changes in RNA processing and splicing machinery genes?**

| TABLE 3. Detected AS genes are enriched with RNA processing and splicing functions |
|-----------------------------------------------|------------------|------------------|------------------|------------------|
| ID   | Term                        | HepG2 No. of AS genes | FDR          | K562 No. of AS genes | FDR          | U87MG No. of AS genes | FDR          |
| 6396 | RNA processing              | 229               | 1.2 x 10^{-17} | 246               | 2.8 x 10^{-19} | 130               | 5.4 x 10^{-9} |
| 16071|mRNA metabolic process      | 153               | 2.9 x 10^{-10} | 168               | 1.0 x 10^{-12} | 95                | 7.8 x 10^{-9} |
| 3723 | RNA binding                 | 290               | 6.0 x 10^{-20} | 305               | 2.1 x 10^{-20} | 152               | 8.8 x 10^{-7} |
| 8380 | RNA splicing                | 123               | 2.0 x 10^{-9}  | 128               | 8.4 x 10^{-9}  | 80                | 2.5 x 10^{-8} |
| 398  | Nuclear mRNA splicing, via spliceosome | 71               | 5.6 x 10^{-6}  | 71                | 1.8 x 10^{-4}  | 43                | 0.002          |

All three RNA-seq data sets (HepG2, K562, and U87MG) show significant enrichment for these functions.
ADAR-dependent AS in *HNRNPR* based on U87MG RNA seq [Bahn et al. *Genome Res.* 2012].
Results

ADAR KD shows significant changes in splicing and RNA processing genes
The KD sample contains more reads supporting inclusion of the second exon than reads supporting its exclusion ($P$-value: 0.0075).
Summary of the results

- A-to-I RNA editing rarely targets canonical splicing motifs, yet it affects SREs within exons.

- Cassette exons were found to be significantly enriched with A-to-I editing sites compared with constitutive exons.

- ADAR knockdown in HepG2 & K562 cell lines leads to global changes in gene expression and hundreds of genes changing their splicing pattern, yet this cannot be explained by putative editing sites alone.

- Genes showing significant changes in their splicing pattern are frequently involved in RNA processing & splicing activity.

- The primary mechanism for ADAR-mediated regulation of AS is suggested to be achieved by modulating trans-acting factors.
Thank you.