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ADAR-Mediated RNA Editing Predicts Progression and Prognosis of Gastric Cancer

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BACKGROUD & AIMS: Gastric cancer (GC) is the third leading cause of global cancer mortality. Adenosine-to-inosine RNA editing is a recently described novel epigenetic mechanism involving sequence alterations at the RNA but not DNA level, primarily mediated by ADAR (adenosine deaminase that act on RNA) enzymes. Emerging evidence suggests a role for RNA editing and ADARs in cancer, however, the relationship between RNA editing and GC development and progression remains unknown. **METHODS:** In this study, we leveraged on the next-generation sequencing transcriptomics to demarcate the GC RNA editing landscape and the role of ADARs in this deadly malignancy. **RESULTS:** Relative to normal gastric tissues, almost all GCs displayed a clear RNA misediting phenotype with ADAR1/2 dysregulation arising from the genomic gain and loss of the *ADAR1* and *ADAR2* gene in primary GCs, respectively. Clinically, patients with GCs exhibiting ADAR1/2 imbalance demonstrated extremely poor prognoses in multiple independent cohorts. Functionally, we demonstrate in vitro and in vivo that ADAR-mediated RNA misediting is closely associated with GC pathogenesis, with ADAR1 and ADAR2 playing reciprocal oncogenic and tumor suppressive roles through their catalytic deaminase domains, respectively. Using an exemplary target gene *PODXL* (podocalyxin-like), we demonstrate that the ADAR2-regulated recoding editing at codon 241 (His to Arg) confers a loss-of-function phenotype that neutralizes the tumorigenic ability of the unedited *PODXL*. **CONCLUSIONS:** Our study highlights a major role for RNA editing in GC disease and progression, an observation potentially missed by previous next-generation sequencing analyses of GC focused on DNA alterations alone. Our findings also suggest new GC therapeutic opportunities through ADAR1 enzymatic inhibition or the potential restoration of ADAR2 activity.

Keywords: Transcriptome; Editome; RNA Editing; ADARs.

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Gastric adenocarcinoma (gastric cancer [GC]) is a deadly malignancy highly prevalent in Asia and the third leading cause of global cancer mortality.[1,2] A heterogeneous disease arising from the complex interplay of host factors and environmental risk agents, such as *Helicobacter pylori*, GCs have been shown to exhibit a wide spectrum of molecular aberrations.[3] At the DNA level, we and others have shown that GCs can exhibit distinct patterns of chromosomal amplifications and deletions involving oncogenes and tumor-suppressor genes (*ERRB2*, *FGFR2*, and *RB1*), gene fusions (eg *CD44-SLC1A2*, *CLDN18-ARHGAP26*), microsatellite instability, and somatic mutations in genes, such as

Abbreviations used in this paper: ADAR, adenosine deaminase that act on RNA; A-to-G, adenosine to guanosine; A-to-I, adenosine to inosine; CDS, coding sequences; CM, catalytic mutant; DR, down-regulation; edt, edited; EV, empty vector; GC, gastric cancer; mRNA, messenger RNA; NT, non-tumor; OE, overexpression; OS, overall survival; PCR, polymerase chain reaction; PODXL, podocalyxin-like; Ras, Rat sarcoma; SG, Singaporean; TCGA, The Cancer Genome Atlas; UTR, untranslational region; VAF, variation frequency; wt, wild-type.

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TP53, *ARID1A*, and *RhoA*.[3–10] Clinically, however, few of these molecular alterations have significantly impacted the treatment of GC patients to date, with the exceptions of traztuzumab treatment in *ERBB2*-positive GC and ramucirumab (an anti-angiogenic therapy) in advanced GC.[11,12] As such, there remains a compelling need to identify novel molecular targets and oncogenic processes operative in GC, which might be exploited for therapy.

Besides DNA sequence alterations, epigenetic alterations are also emerging as major players in GC pathogenesis, involving DNA methylation, histone modifications (acetylation, methylation), and expression of GC-associated microRNAs or long non-coding RNAs.[13–15] Pertinent to this study, RNA editing is another recently described epigenetic mechanism in which sequence alterations are introduced into the transcripts of expressed RNAs, while leaving the underlying DNA sequence intact and unmodified. The most frequent type of RNA editing in humans involves adenosine to inosine (A-to-I) editing, and is primarily mediated by ADAR (adenosine deaminase that act on RNA) enzymes. Molecularly, A-to-I editing has been shown to increase both transcript and proteome diversities, as inosine residues are recognized as guanosine by the general cellular machinery. Previous studies have shown that RNA editing can contribute to transcriptomic and phenotypic diversity, through protein recoding, alternative splicing, altered microRNA regulation, and changes in transcript localization, expression, and degradation.[16,17] Genome-wide studies have also suggested that RNA editing is pervasive, with >85% of RNAs likely to be edited in noncoding and/or coding sequences.[18,19]

Recently, our group has demonstrated important roles for ADAR-mediated RNA editing in human cancer, including liver and esophageal malignancies.[20–22] To date, however, the role of ADARs and the landscape of RNA edited targets (the "editome") in GC remains unknown. We hypothesized that since prior sequence alteration studies of GC have largely focused on genetic variation at the DNA level,[3,7] important oncogenic contributions from RNA editing ("RNA mutations") might have been missed. In this study, we addressed this knowledge gap by leveraging high-throughput transcriptome sequencing (RNA-Seq) of primary GCs and cell lines to dissect the relationship between RNA editing and GC progression and prognosis. We found evidence of widespread RNA misediting and ADAR deregulation occurring in GCs relative to normal gastric tissues, and a significant correlation between ADAR deregulation and GC patient survival. Using an exemplary target gene *PODXL* (podocalyxin-like), we discovered an ADAR2-regulated recoding RNA editing event causing an amino acid substitution from histidine (His) to arginine (Arg) at codon 241 of PODXL, conferred a loss-of-function phenotype that neutralizes the tumorigenic ability of the unedited *PODXL*. These results highlight RNA editing as an important pathogenic mechanism in gastric carcinogenesis, and ADAR enzymes as potential GC therapeutic targets.

Materials and Methods

The detailed Materials and Methods can be found in the Supplementary Materials.

Targeted RNA Editing Analysis

Direct sequencing was performed on polymerase chain reaction (PCR) products, and the editing frequency was calculated using software ImageJ (http://rsb.info.nih.gov/ij/). The reliability of this method was further verified by cloning of individual sequences as described previously.[20] PCR products were subcloned into the T-easy vector (Promega, Madison, WI), and approximately 50 individual plasmids were sequenced for each sample. For each sample, 2–3 independent reverse transcription PCR reactions were performed.

Statistical Analyses

Unless otherwise indicated, the data are presented as mean ± SD of 3 independent experiments. The SPSS statistical package for Windows (version 16; SPSS Inc, Chicago, IL) was used to perform the data analyses. The *ADAR1* or *ADAR2* expression levels in any 2 groups of samples were compared using the Mann-Whitney U test. The editing levels of editing sites between 2 preselected groups were compared using the Mann-Whitney U test. An unpaired 2-tailed Student *t* test was used to compare the number of invaded cells, foci, and tumor volume between any 2 preselected groups. A *P* value <.05 was considered to be statistically significant.

Results

Global Identification of Adenosine-to-Inosine/Guanosine (A-to-I or A-to-G) RNA Editing Sites in Gastric Cancer by RNA-Seq

We performed high-throughput RNA-Seq of 14 matched pairs of gastric tumors and non-tumor (NT) gastric samples, generating a mean of 170.3-million reads that could be uniquely aligned to the human genome (hg19). The aligned reads provided substantial coverage (a mean of 68.1%) for the vast majority of human messenger RNA (mRNA) transcripts annotated in the UCSC genome browser (Supplementary Table 1). In order to perform a comprehensive high-quality analysis, we applied a 2-phase method for analyzing RNA editing sites in GC genomes. Relative to the number of RNA variants distributed in different genomic regions (coding sequences [CDS], untranslational regions [UTRs], introns, upstream/downstream, intergenic and pseudo/noncoding RNAs) and detected in one single sample, there was a drastic drop in those present in multiple samples (ranging from 2 to 10) (Supplementary Figure 1*A*). In particular, concomitant with a decrease in the number of RNA variants in CDS, a 5-fold increase in the proportion of A-to-G variants was observed, among all types of RNA variants present in ≥6 samples compared with those only detected in one sample (Supplementary Figure 1*B*). This may help to improve the lower technical validation rate of A-to-I (G) sites in CDS (<40%) than that in 3′ UTRs (90%–95%), as reported previously.[20] In addition, approximately 96.2% (5076 of 5276) of A-to-I(G) RNA editing sites detected in ≥6 samples by our RNA-Seq were annotated in the Rigorously Annotated Database of A-to-I RNA Editing.[23] To avoid false-positive results due to the DNA contamination, 4 matched pairs of tumors and NT samples (2000639, 2000721, 2000986, and 980417) were selected for whole

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