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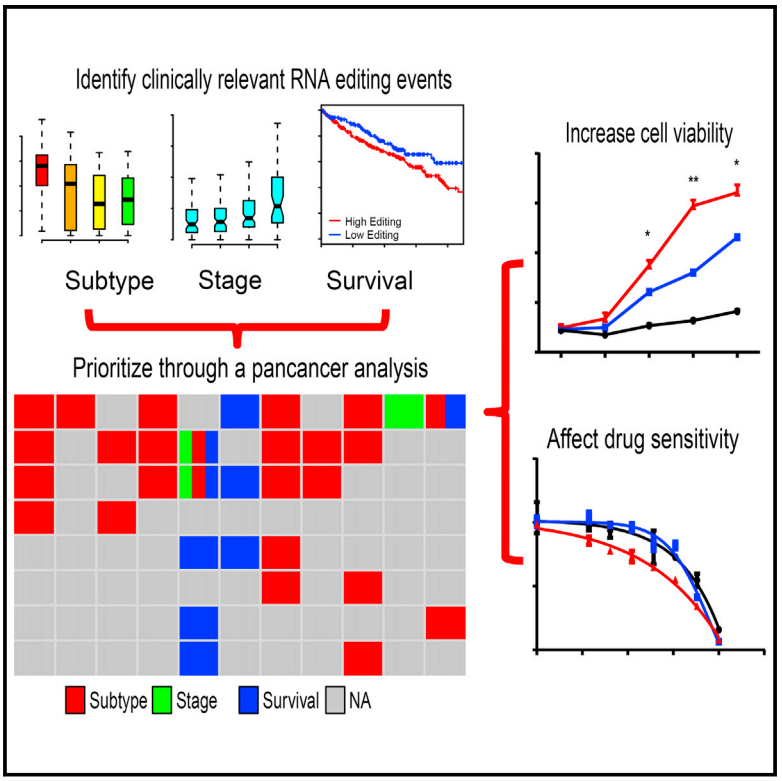
The Genomic Landscape and Clinical Relevance of A-to-I RNA Editing in Human Cancers

Graphical Abstract



Highlights

- A systematic analysis of genome-wide RNA editing events across tumor types was done
- A considerable number of clinically relevant RNA editing events are revealed
- The functional effects of cross-tumor nonsynonymous RNA editing events are shown
- Evidence that nonsynonymous RNA editing may affect drug sensitivity is provided

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In Brief

Han et al. characterize global A-to-I RNA editing profiles across 17 cancer types and experimentally demonstrate the effects of several cross-tumor nonsynonymous RNA editing events on cell viability and drug sensitivity.

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The Genomic Landscape and Clinical Relevance of A-to-I RNA Editing in Human Cancers

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SUMMARY

Adenosine-to-inosine (A-to-I) RNA editing is a widespread post-transcriptional mechanism, but its genomic landscape and clinical relevance in cancer have not been investigated systematically. We characterized the global A-to-I RNA editing profiles of 6,236 patient samples of 17 cancer types from The Cancer Genome Atlas and revealed a striking diversity of altered RNA-editing patterns in tumors relative to normal tissues. We identified an appreciable number of clinically relevant editing events, many of which are in noncoding regions. We experimentally demonstrated the effects of several cross-tumor nonsynonymous RNA editing events on cell viability and provide the evidence that RNA editing could selectively affect drug sensitivity. These results highlight RNA editing as an exciting theme for investigating cancer mechanisms, biomarkers, and treatments.

INTRODUCTION

RNA editing is a widespread post-transcriptional mechanism that confers specific and reproducible nucleotide changes in selected RNA transcripts (Bass, 2002; Keegan et al., 2001). As for functional consequences, RNA editing events can result in missense codon changes (Maas and Rich, 2000), modulation of alternative splicing (Rueter et al., 1999), or modification of regulatory RNAs (Kawahara et al., 2007; Tomaselli et al., 2015) and their binding sites (Liang and Landweber, 2007). In humans, the most common type of RNA editing is adenosine to inosine (A to I) (Piskol et al., 2013), which is catalyzed by ADAR enzymes (Bass et al., 1997). Despite some issues in earlier attempts, recently several groups have developed computational methods for accurately detecting A-to-I RNA editing from next-generation sequencing data on a large scale (Bahn et al., 2012; Peng et al., 2012; Ramaswami et al., 2012, 2013). As a result, more than 1 million A-to-I RNA editing sites have been confidently detected in the human genome (Bazak et al., 2014; Ramaswami et al., 2013). However, the vast majority of

Significance

ADAR-mediated A-to-I RNA editing represents a widespread, phylogenetically conserved, post-transcriptional mechanism to engender genomic diversity by reproducibly changing RNA sequences without a concomitant change in DNA sequences. The role of RNA editing in human cancer is only beginning to emerge from early studies of individual candidates in a few cancer types. Our systematic analysis of RNA editing across 17 cancer types demonstrates an appreciable number of RNA editing events associated with clinical characteristics of tumors and patient outcomes, some of which show functional effects on cell viability and drug sensitivity. Thus, aberrant RNA editing provides an underexplored mechanism to reproducibly alter protein or regulatory RNA sequences that could act as drivers and represent potential biomarkers or therapeutic targets in cancer.

Table 1. Summary of TCGA RNA-seq Data Used in This Study

Cancer Type Name (TCGA Code)	No. of Normal Samples	No. of Tumor Samples	Sequence Strategy	Read Length	Average Mappable Reads	No. of Informative Editing Sites
Colorectal cancer (CRC)	0	228	single end	76	21,793,066	8,493
Uterine corpus endometrioid carcinoma (UCEC)	4	316	single end	76	25,324,332	14,217
Glioblastoma multiforme (GBM)	0	154	paired end	76	106,403,279	37,934
Lung adenocarcinoma (LUAD)	58	488	paired end	48	133,297,582	54,362
Liver hepatocellular carcinoma (LIHC)	50	200	paired end	48	139,117,210	23,540
Bladder urothelial carcinoma (BLCA)	19	252	paired end	48	144,059,158	39,270
Kidney renal papillary cell carcinoma (KIRP)	30	198	paired end	48	146,793,890	36,686
Prostate adenocarcinoma (PRAD)	52	374	paired end	48	147,246,105	43,078
Brain lower grade glioma (LGG)	0	486	paired end	48	149,851,835	51,806
Head and neck squamous cell carcinoma (HNSC)	42	426	paired end	48	157,436,457	35,510
Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC)	3	196	paired end	48	161,207,521	32,797
Breast invasive carcinoma (BRCA)	105	837	paired end	50	161,673,379	76,555
Kidney renal clear cell carcinoma (KIRC)	67	448	paired end	50	166,049,114	63,717
Stomach adenocarcinoma (STAD)	33	285	paired end	75	169,720,033	26,389
Lung squamous cell carcinoma (LUSC)	17	220	paired end	50	171,002,267	36,822
Thyroid carcinoma (THCA)	59	498	paired end	48	171,399,819	52,701
Kidney chromophobe (KICH)	25	66	paired end	48	174,113,816	22,317

these sites are in noncoding and repetitive element regions of the genome and have unknown functional relevance. Therefore, the research focus on RNA editing has moved from the identification of novel sites to characterization of the mechanisms by which they mediate their functions and their consequences on cellular function.

To date, a critical role of A-to-I RNA editing in human cancer has been reported for only individual examples. In prostate cancer, A-to-I RNA editing in the androgen receptor impairs the protein's ability to interact with androgenic or anti-androgenic ligands (Martinez et al., 2008); in liver cancer, the edited form of *AZIN1* has a stronger affinity for antizyme and induces cytoplasmic-to-nuclear translocation of *AZIN1*, and a low editing level is sufficient to confer more aggressive tumor behavior (Chen et al., 2013); in colorectal cancer (CRC), A-to-I RNA editing in *RHOQ* promotes the invasion potential (Han et al., 2014); and in glioblastoma, *ADAR2*-mediated RNA editing in *CDC14B* modulates the Skp2/p21/p27 pathway and plays a critical role in the pathogenesis of this disease (Galeano et al., 2013). Despite these intriguing findings, the global pattern of A-to-I RNA editing in human cancer genomes has not been systematically characterized, and the functional importance and clinical relevance of RNA editing in cancer remain largely unknown. Here, we aimed to address these questions through a systematic analysis of A-to-I RNA editing events using RNA-sequencing data from The Cancer Genome Atlas (TCGA) project (Weinstein et al., 2013).

RESULTS

Overview of A-to-I RNA Editing Patterns across Major Cancer Types

To perform a comprehensive, high-quality analysis of A-to-I RNA editing in cancer genomes, we developed a computational pipeline based on ~1.4 million high-confidence RNA editing sites annotated in the Rigorously Annotated Database of A-to-I RNA Editing (RADAR) (Ramaswami and Li, 2014) (Figure S1A). The RNA editing sites in RADAR were collected from recent transcriptome-wide RNA editing identification studies and underwent extensive manual curation. We further applied a series of filters to remove the potential contamination of SNPs or somatic mutations (Experimental Procedures). Thus, this RNA editing data set represents a reliable and global candidate set to start with. From TCGA RNA sequencing (RNA-seq) data, we assessed the RNA editing signals at these candidate sites in 6,236 samples of 17 cancer types or related normal tissues (Table 1 and Figure 1A). For each cancer type, we detected a large number of RNA editing candidate sites with editing signals, but many of them were sufficiently covered only in a limited sample set. Therefore, we defined "informative" RNA editing sites as those sites with detected signals and coverage $\geq 10\times$ in ≥ 30 tumor samples (and related normal samples) for a cancer type (Experimental Procedures) and focused on these sites in subsequent analyses to ensure adequate statistical power. The number of

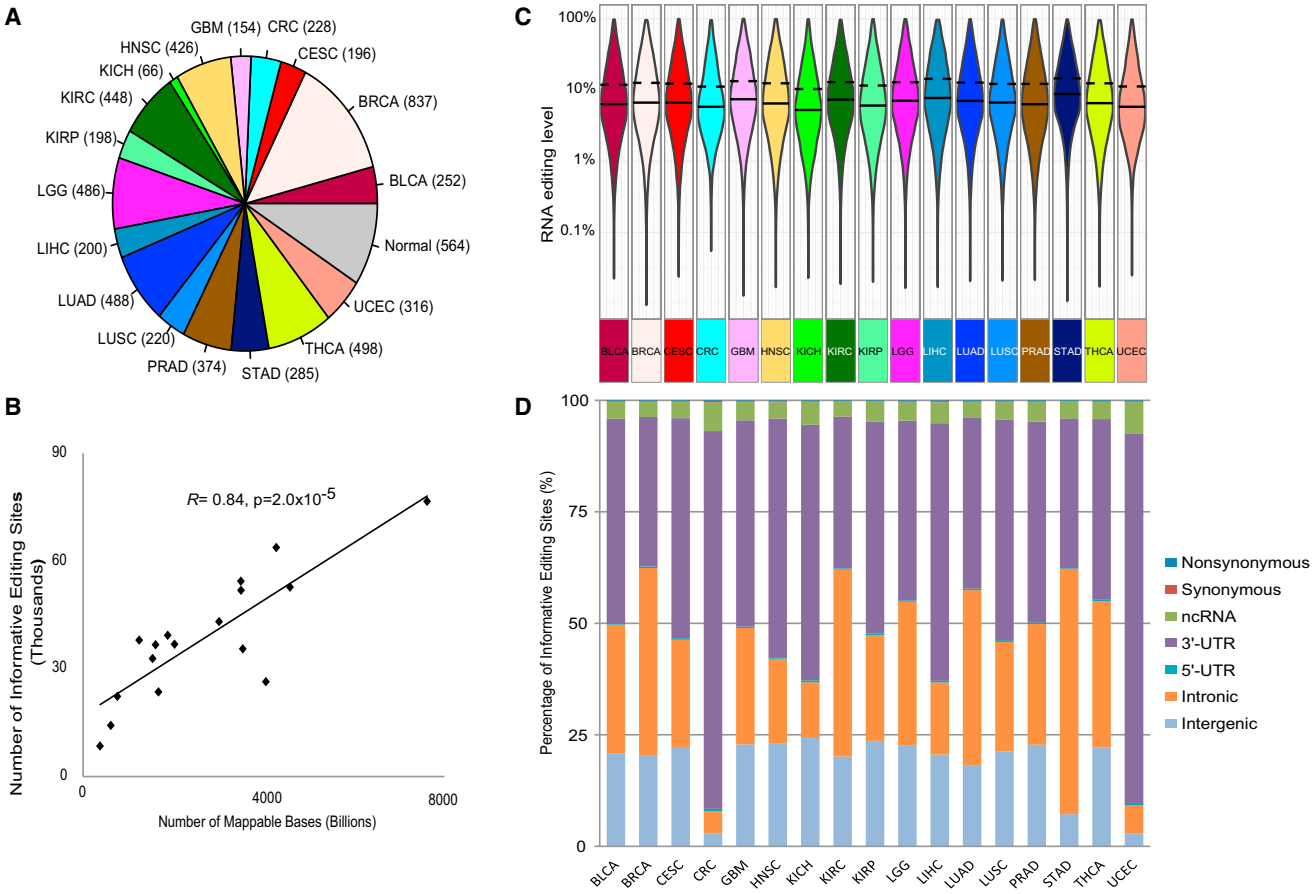


Figure 1. Overview of A-to-I RNA Editing Patterns in Human Cancer

(A) Numbers of TCGA tumor and normal samples analyzed in this study.

(B) Correlation between the number of total mappable RNA-seq bases and the number of informative RNA editing sites across different cancer types.

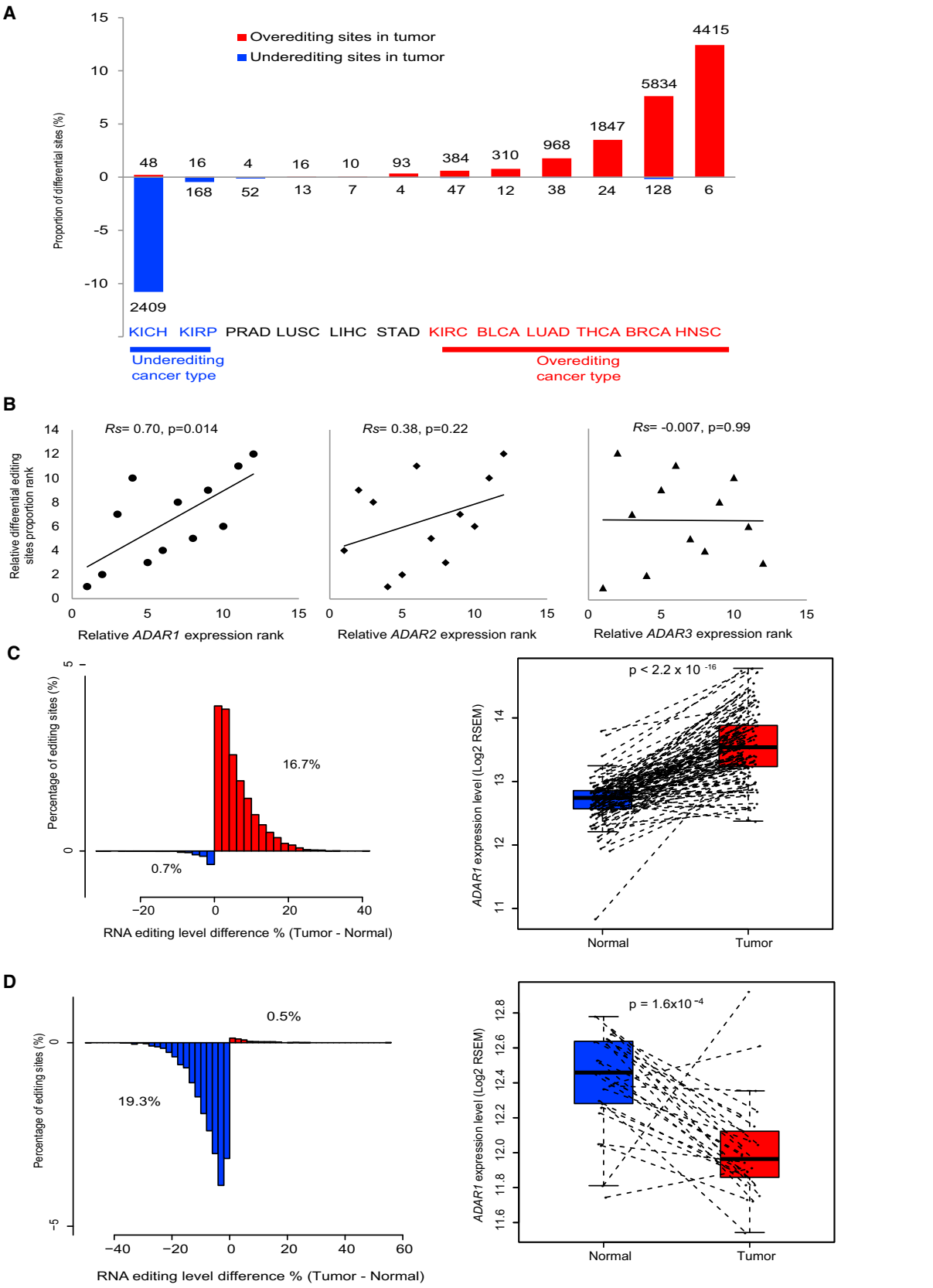
(C) The editing-level distributions at informative editing sites in different cancer types. Dashed and solid lines denote average and median for each cancer type, respectively.

(D) The distributions of informative RNA editing sites in different types of RNA regions.

See Table 1 for definitions of abbreviations of cancer types. See also Figure S1.

informative RNA editing sites per cancer type ranged from 8,493 in CRC to 76,555 in breast invasive carcinoma (BRCA) (Figure 1B). This large variation among cancer types is mainly because (1) the number of tumor samples per cancer type varied markedly (from 66 in kidney chromophobe [KICH] to 837 in BRCA; Table 1) and (2) the number of mappable reads per sample varied greatly among cancer types because of different sequencing strategies (from 22 million in CRC to 174 million in KICH; Table 1). Indeed, across the 17 cancer types, the number of informative editing sites showed a strong linear correlation with the total number of mappable bases or the total number of mappable reads (Figure 1B, Pearson's correlation [R] = 0.84, p = 2.0 × 10^{-5}, Spearman's correlation [Rs] = 0.75, p = 7.4 × 10^{-4}; Figure S1B, R = 0.89, p = 2.0 × 10^{-6}, Rs = 0.82, p = 7.0 × 10^{-5}). These results also indicate that the informative editing sites we identified show no significant bias toward one or a few well-studied cancer types.

We first quantified the editing levels at the informative RNA editing sites (defined as the proportion of edited reads among the total mapped reads at a given site in a TCGA BAM file). As a quality control, we randomly selected a few samples, remapped the raw RNA-seq reads using the previously established mapping pipeline that can accurately detect both *Alu* and non-*Alu* RNA editing events (Ramaswami et al., 2012, 2013), and obtained very consistent RNA-editing levels (Rs > 0.93). Thus, for the informative edited sites surveyed, the RNA-seq mapping procedures used had little effect on the quantification of RNA-editing levels. Figure 1C shows the overall editing-level distributions at informative RNA editing sites in different cancer types (Figure S1C shows the distributions in normal tissues). We next examined the distribution of these editing sites in different types of transcribed regions (Figure 1D). Across different cancer types, most of the informative RNA editing sites were in 3′ UTRs and intronic regions, as observed previously in mouse tissues (Gu et al., 2012), but the editing sites in coding regions were relatively limited (Figure S1D shows the numbers of nonsynonymous and synonymous RNA editing sites in different cancer types). Furthermore, we did not detect any correlation between RNA-editing level and the local GC content (Figure S1E). Because our analysis was based on RNA-seq data, the observed genomic



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distribution of informative RNA editing sites could be affected by the coverage bias of the mRNA-seq platform. However, because of the large number of candidate editing sites examined, we still obtained sufficient sampling power to survey RNA editing activities in different transcribed regions.

Diversity of RNA Editing Patterns in Tumors Relative to Normal Samples

The global RNA editing differences between cancer samples and related normal tissues remain largely uncharacterized, and previous studies have suggested that this is a complex topic (Fumagalli et al., 2015; Jiang et al., 2013; Maas et al., 2001; Nemlich et al., 2013; Qin et al., 2014; Tomaselli et al., 2015). For example, an earlier study found reduced editing in brain tumors (Paz et al., 2007), while a recent study suggested a mixture of gene-specific hyper- and hypo-editing activities in liver cancer (Chan et al., 2014). However, these studies were either based on a small set of RNA editing sites or were limited to single cancer types. To obtain a comprehensive view of RNA editing patterns in tumor samples, we focused on 12 tumor types with available RNA-seq data for matched normal tissues from the same patients (Figure 2A). For each cancer type, we identified RNA editing sites with significantly differential editing activity between matched tumor and normal samples (paired Wilcoxon test, false discovery rate [FDR] < 0.05, and mean editing-level difference among comparison groups [Diff] $\geq$ 5%). Although with this criterion, the editing levels at most sites remained similar, we observed a great diversity of "altered" RNA editing patterns across these cancer types: significant numbers of RNA editing sites showed over-editing patterns in head and neck squamous cell carcinoma, BRCA, thyroid carcinoma, and lung adenocarcinoma tumors, while significant numbers of editing sites showed under-editing patterns in KICH and kidney renal papillary cell carcinoma tumors.

To identify the molecular determinants underlying these patterns, we performed two complementary analyses, one focusing on the general pattern across cancer types and the other on the editing abundance within each cancer type. We first analyzed the correlations of ADAR expression with the "net" proportion of over-editing RNA sites (defined as the percentage of over-editing sites minus the percentage of under-editing sites) and found that the proportion was highly correlated with the relative *ADAR1* mRNA expression level (defined as the fold change relative to normal samples) (Rs = 0.70, p = 0.014; Figure 2B) but not with that of *ADAR2* (Rs = 0.38, p = 0.22; Figure 2B) or *ADAR3* (Rs = −0.007, p = 0.99; Figure 2B). Figures 2C and 2D show the detailed RNA editing change patterns in two representative cancer types. In BRCA, 12,770 (16.7%) informative RNA editing sites showed significant over-editing in the tumor samples compared with matched normal samples, whereas only 553 (1.2%) showed significant under-editing in tumor samples (paired Wilcoxon test, FDR < 0.05; Figure 2C, left). In contrast, in KICH, only 110 informative RNA editing sites (0.5%) showed over-editing in the tumor samples, whereas 4,318 (19.3%) showed under-editing in tumor samples (paired Wilcoxon test, FDR < 0.05; Figure 2D, left). Indeed, *ADAR1* mRNA expression was much higher in BRCA than in matched normal samples (fold change = 1.81, paired Wilcoxon test, $p < 2.2 \times 10^{-16}$; Figure 2C, right), while *ADAR1* was significantly under-expressed in KICH (fold change = 0.76, paired Wilcoxon test, $p = 1.6 \times 10^{-4}$; Figure 2D, right). We further performed sample-based analysis within each cancer type and found that the number of informative sites with editing signals showed the strongest correlation with the *ADAR1* mRNA expression among the three ADAR enzymes (Table S1). These results suggest that the global altered RNA editing patterns in tumors are more likely to be affected by *ADAR1* than the other two editing enzymes. However, because the mRNA expression of ADAR may not directly reflect enzyme editing activity (Wahlstedt et al., 2009), and the dimer formation and the interactions among the ADAR enzymes could be important for editing activity (Chen et al., 2000; Chilibeck et al., 2006; Cho et al., 2003), further efforts are required to elucidate the relative contributions of the three ADAR enzymes to the observed RNA editing patterns.

An Appreciable Level of Clinically Relevant RNA Editing Sites in Various Cancer Types

Given the large number of A-to-I RNA editing events observed across tumor types and distinct editing patterns at some sites between tumor and normal tissues, a fundamental question is what fraction of RNA editing events in tumors are functionally tumorigenic or clinically valuable. To address this question, we focused on the RNA editing sites showing correlations with tumor subtype, clinical stage, and patient survival. Clinical stage and patient survival are well-established clinical variables, while tumor subtype often facilitates clinical decisions. In a sense, they all characterize intertumoral heterogeneity among the same disease. Thus, we referred to RNA editing sites showing nonrandom editing patterns with regard to these biologically and clinically meaningful parameters as "clinically relevant editing sites." Specifically, we identified such sites within each cancer type using three complementary computational analyses (Figure 3A): (1) differential analysis of RNA editing level among established tumor subtypes (FDR < 0.01, Diff $\geq$ 5%), which identified 2,660 RNA editing sites in total; (2) differential analysis of RNA editing level among tumor stages (FDR < 0.05, Diff $\geq$ 5%), which identified 684 RNA editing sites in total; and (3) correlation analysis of RNA editing level with patient overall survival (FDR < 0.05,

Figure 2. Comparison of the Overall A-to-I RNA Editing Patterns between Paired Tumor and Normal Samples

(A) Numbers of over-editing sites (red) and under-editing sites (blue) across different cancer types.

(B) The correlation between the "net" proportion of over-editing sites (defined as the percentage of over-editing sites [red] minus the percentage of under-editing sites [blue]) and the relative mRNA expression of *ADAR1* (left), *ADAR2* (middle), and *ADAR3* (right) (fold change relative to normal tissues). To robustly detect a meaningful relation, the rank-based Spearman correlations were used and plotted.

(C) Distribution of editing-level difference in BRCA relative to matched normal breast tissue samples (left) and the mRNA expression level of *ADAR1* (right) (red in tumor and blue in normal).

(D) Distribution of editing level difference in KICH samples relative to matched normal kidney samples.

In (C) and (D), the paired Wilcoxon test was used to assess the difference between paired tumor and normal samples. The boxes show the median ± 1 quartile, with whiskers extending to the most extreme data point within 1.5 interquartile range from the box boundaries. See Table 1 for definitions of abbreviations of cancer types. See also Table S1.

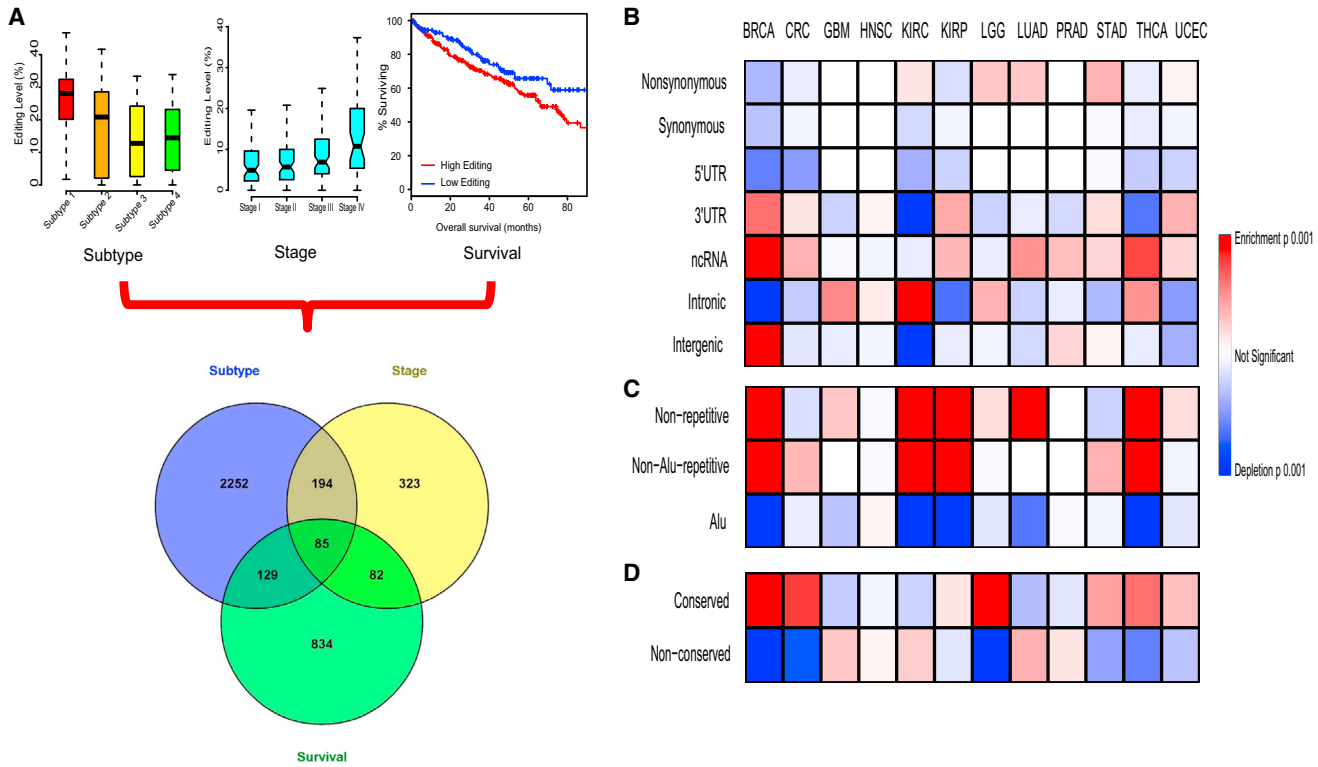


Figure 3. Identification and Patterns of Clinically Relevant RNA Editing Sites

(A) Overview of clinically relevant RNA editing sites identified by three complementary computational analyses: differential analysis among tumor subtypes, differential analysis among tumor stages, and correlation analysis with patient overall survivals. An explicative cartoon is shown for illustration purposes.

(B–D) Statistical significance for the enrichment or depletion patterns of clinically relevant RNA editing sites through coverage-dependent permutation tests across 12 tumor types for different types of RNA regions: gene annotation (B), non-repetitive (C), non-*Alu* repetitive and *Alu* elements, and evolutionary conservation (D). ncRNA, non-coding RNA.

See Table 1 for definitions of abbreviations of cancer types. See also Table S2.

Diff $\geq$ 5%), which identified 1,130 RNA editing sites in total. Among the 17 cancer types, 12 cancers contained such clinically relevant sites, ranging from 4 in prostate adenocarcinoma to 2,059 in BRCA (Table S2). To rule out the potential confounding effect of tumor purity, we repeated the analysis using ABSOLUTE-based (Carter et al., 2012) tumor purity as a covariate. For the 9 cancer types with available tumor purity data, we found that 97.9% of the clinically relevant sites originally identified still remain significant (Table S2). We also calculated the correlation of ADAR expression levels with tumor purity and found no strong correlation (Table S2). Therefore, tumor purity appeared to have little effect on our results.

In order to investigate the distributions of clinically relevant editing sites in different types of RNA regions, we classified the RNA editing sites from three parallel perspectives: gene annotation, sequence repetitive elements, and evolutionary conservation. Because the power to detect clinically relevant editing sites in our analysis was affected by sample size and quality of clinical data (e.g., the follow-up time) in a given cancer type, we examined the distribution patterns for each cancer type separately. Furthermore, given the potential effects of coverage bias in different RNA regions due to gene expression or the mRNA-seq platform, instead of directly comparing the proportions of clinically relevant RNA editing sites among different RNA regions, we performed a coverage-dependent permutation test to assess the enrichment and depletion patterns (Experimental Procedures). In terms of gene annotation, we found that clinically relevant RNA editing sites tend to be in noncoding RNAs as well as in nonsynonymous and intronic regions in some cancer types (Figure 3B). In terms of sequence repetitive elements, clinically relevant sites show consistent depletion patterns in *Alu* elements (Figure 3C). In terms of evolutionary conservation, clinically relevant sites tend to be conserved among humans, chimpanzees, and macaques (Figure 3D). Together, these analyses based on different types of RNA-region classification help understand which factors affect the overall distributions of clinically relevant RNA editing sites.

"Driver" Functional Effects of Clinically Relevant Nonsynonymous RNA Editing Events

Because clinically relevant RNA editing events at nonsynonymous sites could directly result in amino acid changes, we focused on these RNA editing sites and assessed their functional effects experimentally. To boost the discovery power, we performed the above analyses for nonsynonymous RNA editing sites with a relaxed FDR cutoff and identified 35 RNA editing sites with potential clinical relevance (FDR < 0.2, Diff $\geq$ 5%; Table S3). Interestingly, 8 of these RNA editing events (22.9%)

showed clinically relevant patterns in more than one cancer type (Figures 4A and S2). This pan-cancer analysis suggests that some A-to-I nonsynonymous RNA editing may be a "master" driver event and play a critical functional role in different tumor contexts. We focused on four top editing candidate sites (S367G in *AZIN1*, I164V in *COPA*, I635V in *COG3*, and R764G in *GRIA2*) for further investigation (Figures 4A and S2B). The functional effects of RNA editing at the residue S367G in *AZIN1* (identified in eight cancer types by our analysis; Figures 4B and S2C) have been characterized in liver cancer (Chen et al., 2013). Differential editing activity at I164V in *COPA* (identified in seven cancer types; Figures 4C and S2D) between tumor and normal samples has been reported in liver cancer (Chan et al., 2014) but has not been functionally characterized. I635V at *COG3* (identified in six cancer types Figures 4D and S2E) was only reported in a recent RNA-editing methodology study (Ramaswami et al., 2012). *GRIA2* (also known as *GluR-B*) contains two known RNA editing sites: the Q607R editing in the second transmembrane domain is well studied (Herb et al., 1996; Higuchi et al., 1993) but has insufficient coverage in our data set; the role of R764G (identified in two cancer types; Figure 4E) has not been functionally characterized in cancer. We confirmed the occurrence of these RNA editing events in an independent set of breast tumor samples using an orthogonal Sequenom approach (Figures 5A and S3A).

Given the availability of high-quality antibodies, we assessed the functional effects of the editing events in *AZIN1*, *GRIA2*, and *COG3* using various functional assays. To examine the effects on cell proliferation or survival, we performed cell viability assays (upon overexpression) in MCF10A cells, a normal human breast epithelial cell line. Given similar levels of wild-type (WT) and edited proteins (Figure S3B), the edited *AZIN1* (*AZIN1*^{S367G}), *GRIA2* (*GRIA2*^{R764G}), and *COG3* (*COG3*^{I635V}) significantly increased cell survival relative to the WT gene (t test, $p < 0.05$; Figure 5B; see Experimental Procedures). We obtained similar results of cell viability assays on the basis of cell counting (Figure S3C). Because these RNA editing events show cross-tumor clinical relevance, we further examined their effects in a different lineage. We performed similar viability assays in Ba/F3 cells, which is a murine leukemia cell line and an established drug screening platform for subsequent investigation (Cheung et al., 2014; Liang et al., 2012), and observed the same patterns (t test, $p < 0.05$; Figure 5C). To examine the effects on cell survival, we assessed levels of active caspase-3 in MCF10A and found no significant changes (Figure S3D). These results were confirmed by a cell death detection ELISA kit (data not shown). To examine the effects on cell migration, we performed wound healing assays in MFC10A and observed no substantial effects (Figure S3E).

Therapeutic Liability of Clinically Relevant Nonsynonymous RNA Editing Sites

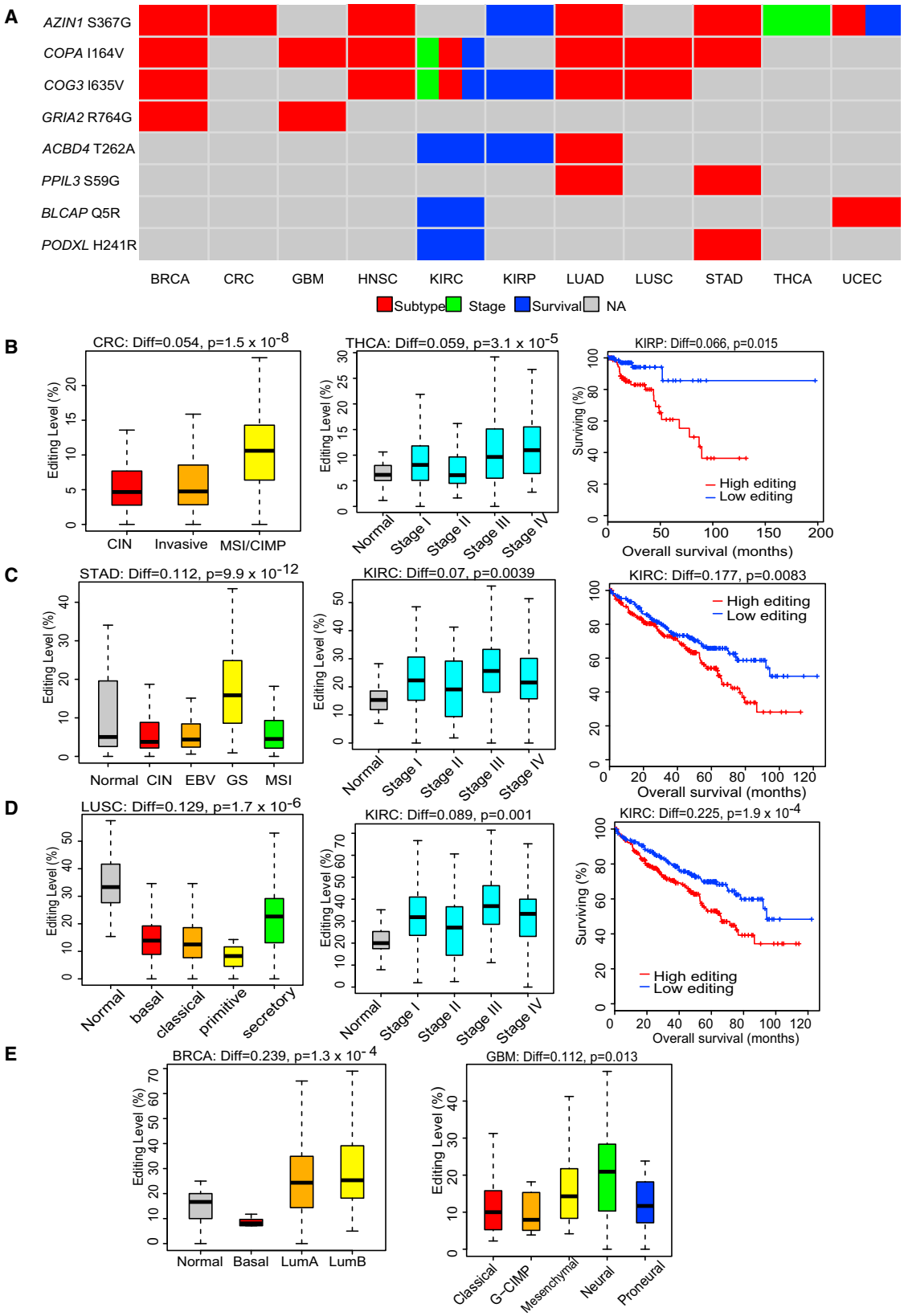
A critical question about RNA editing is whether some RNA editing could affect the response of cancer therapies. This question has significant clinical implications but has never been investigated. Given their confirmed "driver" behaviors in Ba/F3 (Figure 5C), we focused on the RNA editing in *AZIN1*, *GRIA2*, and *COG3* and examined whether these events alter drug sensitivity using a high-throughput Ba/F3 differential cytotoxicity screen (Cheung et al., 2014; Quayle et al., 2012). Ba/F3 cells depend on interleukin-3 (IL-3) for proliferation but readily become IL-3 independent in the presence of an oncogene or oncogenic event (Liang et al., 2012). We screened 145 compounds targeting major signaling pathways in Ba/F3 addicted to these RNA editing events (in the absence of IL-3) and performed a "counterscreen" with the same Ba/F3 cells cultured with exogenous IL-3 to control for the cytotoxic activity of the compounds. In addition, we used a spontaneously transformed Ba/F3 cell line (originally transfected with *PIK3R1* but not expressing significant levels of *PIK3R1*) as negative controls. Strikingly, compared with the WT genes, the edited genes selectively affected the sensitivity of Ba/F3 cells to several targeted therapeutics, including *AZIN1*^{S367G} for the IGF-1R inhibitor BMS536924, *GRIA2*^{R764G} for MEK inhibitors CI1040 and PD0325901, and *COG3*^{I635V} for MEK inhibitors CI1040, PD0325901, and trametinib (Figure 6A shows representative examples).

Furthermore, we examined the editing levels of the 35 clinically relevant nonsynonymous RNA editing sites (Table S3) in cell lines from the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012) and examined their correlations with the sensitivity data (half maximal inhibitory concentration [IC_{50}]) of 24 drugs available at the CCLE portal. Interestingly, we found that the editing levels of 16 RNA editing sites were significantly correlated with drug sensitivity (FDR < 0.1; Figure 6B). Furthermore, across RNA editing sites, the drug clustering analysis showed meaningful patterns: three chemotherapy agents, paclitaxel, irinotecan, and topotecan, were clustered together and their sensitivities were associated with the editing in *AZIN1* and other sites; erlotinib was in the same cluster as the HER2 agent lapatinib; two RAF inhibitors, PLX4720 and RAF265, were adjacent to each other; and two MEK inhibitors, AZD6244 and PD0325901, were tightly correlated. These results suggest that the effects of RNA editing on drug response are not limited to the cases we examined.

DISCUSSION

The advent of next-generation sequencing data has drawn widespread attention to the analysis of RNA editing (Li et al., 2011; Peng et al., 2012; Piskol et al., 2013; Ramaswami et al., 2012, 2013); however, these studies have mainly focused on RNA editing events in normal tissues. More recently, a functional role for RNA editing in tumorigenesis has begun to emerge, but related studies have been limited to individual examples. The present study represents a systematic investigation of the global pattern and clinical relevance of A-to-I RNA editing across a broad range of cancer types and normal tissues.

The number of A-to-I RNA editing sites in humans is huge, but most sites exhibit editing at very low levels (Bazak et al., 2014), leading to a great challenge in detecting editing sites in a comprehensive manner. To ensure high-quality analysis, we started with the high-confidence RNA editing sites reported in previous studies rather than calling novel editing sites without prior knowledge. We focused on RNA editing events with detected editing signals in multiple TCGA samples and further filtered those with potential mutational signals at the DNA level. Although "false" RNA-editing sites due to SNPs or mutations might not be completely removed, such noise in our data should be very rare. Because of the large number of RNA editing



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candidates identified in normal tissues in the RADAR database, we obtained sufficient numbers of RNA editing sites to assess the global patterns of A-to-I RNA editing. Furthermore, the strong linear correlation between informative editing sites per cancer type and the total number of mapped reads (or bases) across cancer types indicates that our RNA editing sets are not biased toward well-studied cancer types.

The rich TCGA data set allowed researchers to address some important questions about RNA editing on a large scale (Paz-Yaacov et al., 2015). We revealed a diversity of altered RNA editing events in tumor samples relative to normal tissues, which correlates best with the *ADAR1* expression level globally. Note that this observation does not rule out the important role of *ADAR2*-mediated editing events in specific cancer types, as demonstrated in previous studies (Cenci et al., 2008; Galeano et al., 2013; Maas et al., 2001; Tomaselli et al., 2015). On the basis of the correlations of RNA editing levels with tumor subtype, stage, or survival, we detected an appreciable number of RNA editing sites with potential clinical relevance (~3.5% of the total informative RNA editing sites examined). These editing sites show marked editing difference for distinct patient groups within a cancer type, and they may represent promising biomarker candidates for further assessment. An alternative way to infer clinically relevance could be based on levels of edited transcripts. However, unlike the editing level, which is a parameter independent from the expression level of the edited gene, the levels of edited transcripts are linked to the gene expression level itself. Indeed, we observed that for a large proportion of RNA editing sites with clinical correlations based on the level of edited transcripts, their gene expression levels also showed corresponding correlations, suggesting the potential confounding effects of gene expression on detecting clinical relevance. Therefore, we focused on the editing-level-based clinically relevant sites in this study. Our data sets (both raw data and clinically relevant sites) have been made publically available through Synapse (Omberg et al., 2013) and thus provide a valuable resource for systematically dissecting the clinical utility of RNA editing.

Importantly, we experimentally investigated the functional effects of several nonsynonymous RNA editing events with potential clinical relevance across multiple tumor types, including the well-studied editing site in *AZIN1* and the other two previously functionally uncharacterized RNA editing sites in *COG3* and *GRIA2*. Moreover, our study provides the evidence that a specific RNA editing event could selectively affect therapeutic responses. We demonstrated that the RNA editing event in *COG3* and *GRIA2* increased sensitivity to some targeted agents, whereas the editing in *AZIN1* engendered decreased sensitivity. Mutations in cancer genes can increase or decrease sensitivity to the same therapeutic agent on the basis of where they are located in the targeted pathway. For example, mutations in the *EGFR* increase sensitivity to drugs targeting the EGFR. However, mutations in *KRAS*, which is clearly a driver, can result in resistance to EGFR inhibitors. Furthermore, if the editing is a neomorph, it could either increase or decrease to the sensitivity to a specific drug. Thus, some RNA editing events may be functionally equivalent to "driver" mutations, making a notable contribution to tumor initiation and growth as well as playing a critical role in response to cancer therapy. Together, our findings highlight RNA editing as an exciting theme for investigating cancer mechanisms, identifying biomarkers, and developing therapeutic targets. Further efforts should be made to characterize the function of other clinically relevant RNA editing events (especially those in noncoding regions), to elucidate the interactions of these editing events with other types of molecular aberrations, and to investigate their utility in clinical practice.

EXPERIMENTAL PROCEDURES

Characterization of A-to-I RNA Editing Profiles

We downloaded RNA-seq BAM files of 5,672 patient tumor samples across 17 TCGA cancer types and their related 564 non-tumor tissue samples (if available) from the University of California, Santa Cruz, Cancer Genomics Hub (CGHub; https://cghub.ucsc.edu). We also downloaded 740 BAM files of CCLE cell lines from CGHub. The detailed read mapping procedure (BAM generation) was previously described in TCGA marker papers (Brennan et al., 2013; Cancer Genome Atlas Research Network, 2008, 2012, 2013; Cancer Genome Atlas Network, 2012a, 2012b; Kandoth et al., 2013).

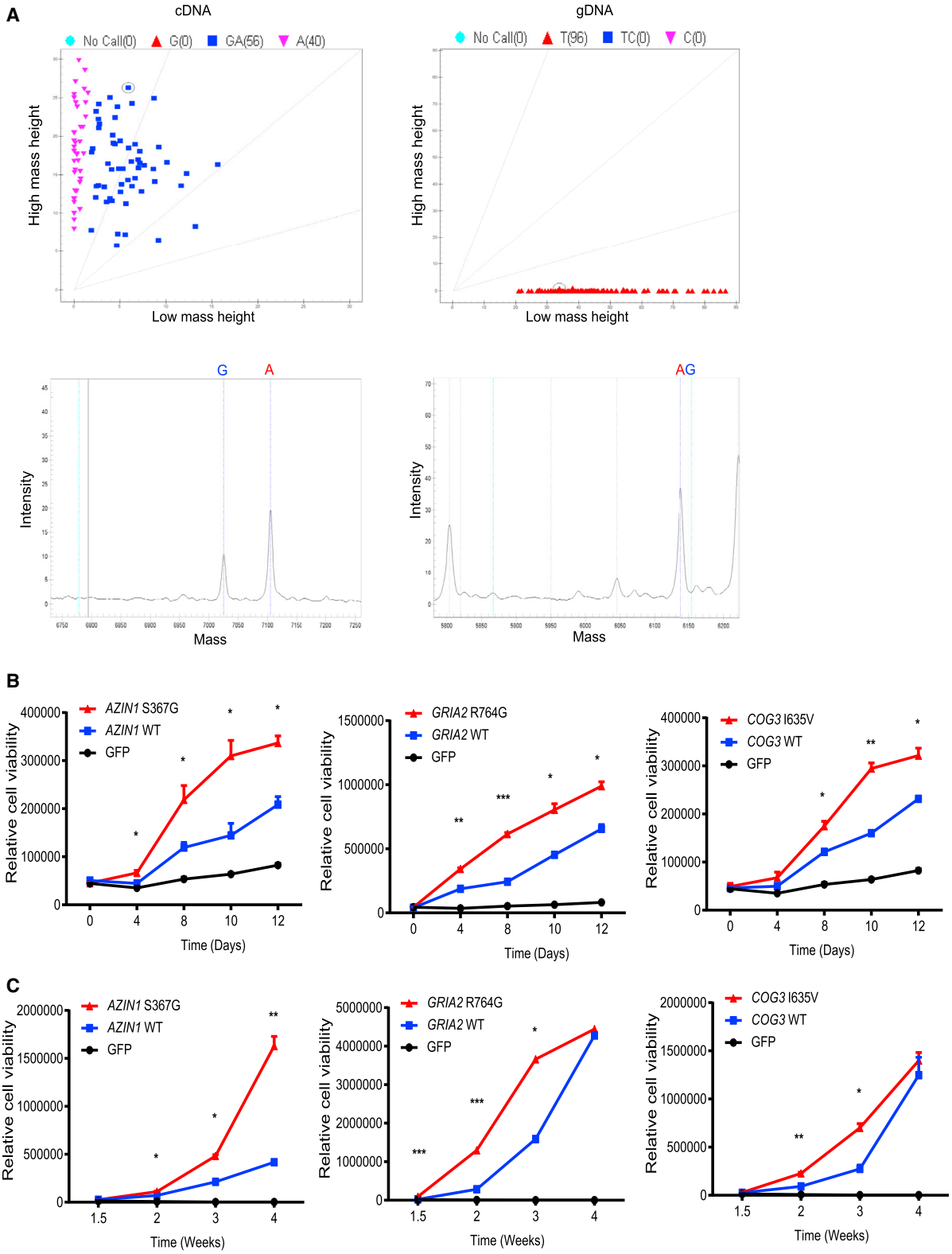
We obtained a comprehensive collection of ~1.4 million A-to-I RNA editing sites from RADAR (http://rnaedit.com) (Ramaswami and Li, 2014). Note that these RNA editing sites were directly called from RNA-seq data from normal tissues and tumor samples, not from the comparison of editing profiles upon ADAR perturbation. We re-annotated them by ANNOVAR (Wang et al., 2010) and then filtered ~4,000 sites annotated in dbSNP (version 137), COSMIC, and TCGA somatic mutations. On the basis of the RNA-seq reads mapped to the human reference genome (hg19), the editing level at a specific site in a given sample was calculated as the number of edited reads divided by the total number of reads (Ramaswami et al., 2013), and only the nucleotides with base quality $\geq$20 were used. Those editing sites with at least three edited reads in at least 3 samples per tissue type were considered to be detected RNA editing sites. To ensure adequate statistical power, we further identified the informative RNA editing sites among the detected RNA editing sites by requiring at least 30 samples (including normal samples if available) with coverage $\geq$ 10 in a tissue/tumor type. Thus, given a cancer type, the tumor samples and their related normal samples had the same set of informative RNA editing sites in our analysis. To further rule out the possibility of potential contamination due to undetected SNPs or somatic mutations, we obtained whole-genome sequencing data from International Cancer Genome Consortium and whole-exome sequencing data from TCGA for the cancer types we surveyed and assessed if there were some potential mutational signals at informative RNA editing sites. We found potential mutational signals at only 310 sites out of 112,572 across the 17 cancer types (0.28%) and excluded them from our analysis.

Figure 4. Clinical Relevance of Nonsynonymous A-to-I RNA Editing Sites

(A) The clinical relevance of eight nonsynonymous RNA editing sites identified in multiple cancer types. For each cancer type, the gray box indicates not significant, the red box indicates the significant differential editing among tumor subtypes (FDR < 0.2, Diff $\geq$ 5%), the green box indicates the significant differential editing among stages (FDR < 0.2, Diff $\geq$ 5%), the blue box indicates the association with the overall survival (FDR < 0.2, Diff $\geq$ 5%).

(B–E) Representative plots showing clinical relevance of nonsynonymous RNA editing events in *AZIN1*^{S367G} (CRC subtype: CIN, chromosomal instability; MSI, microsatellite instability) (B), *COPA*^{I164V} (stomach adenocarcinoma [STAD] subtype: CIN, chromosomal instability; EBV, Epstein-Barr virus positive; GS, genomically stable; MSI, microsatellite instability) (C), *COG3*^{I635V} (D), and *GRIA2*^{R764G} (E). The boxes show the median ± 1 quartile, with whiskers extending to the most extreme data point within 1.5 interquartile range from the box boundaries. G-CIMP, glioma CpG island methylation phenotype.

See Table 1 for definitions of abbreviations of cancer types. See also Figure S2 and Table S3.



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Comparisons of RNA Editing Patterns between Cancer and Normal Samples

For the comparison between tumor and normal samples, we required the informative RNA editing sites with at least five pairs of tumor and normal samples with coverage $\geq$ 10. If sufficient matched normal samples in which a site had adequate coverage were not available, the site was excluded from our analysis. We used the Wilcoxon test to detect RNA editing sites with differential editing between tumor and normal samples and defined significantly differential editing sites as FDR < 0.05 and Diff $\geq$ 5%. TCGA mRNA expression data were obtained from Synapse: syn300013 (Omberg et al., 2013). We used the paired Student's t test to detect differentially expressed ADAR enzymes between normal and tumor samples.

Identification of Clinically Relevant RNA Editing Sites

We obtained clinical information, including tumor subtypes, disease stage, and patient overall survival time, from TCGA marker papers or the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). We used the Wilcoxon test or Kruskal-Wallis nonparametric ANOVA to detect RNA editing sites with differential editing among different tumor subtypes and considered FDR < 0.01 to be statistically significant. We used the Wilcoxon test or Kruskal-Wallis nonparametric ANOVA to detect RNA editing sites with differential editing among different tumor stages and considered FDR < 0.05 to be statistically significant. We used the univariate Cox test to examine whether the RNA editing level was significantly correlated with patient survival and considered FDR < 0.05 to be statistically significant. We chose different FDR cutoffs on the basis of the signal abundance in each analysis. Groups with fewer than five samples were excluded from the analysis. We required a Diff $\geq$ 5% for at least two groups, thereby ensuring a sufficient biological difference. The gene-annotation-based RNA type of an RNA editing site was annotated by ANNOVAR, and the sequence repetitive status and evolutionary conservation status (i.e., the conservation among humans, chimpanzees, and macaques) were annotated as in RADAR (Ramaswami and Li, 2014). To test the effect of tumor purity, we obtained the tumor purity data on the basis of ABSOLUTE from Synapse: syn1710466 (Carter et al., 2012) and repeated the analysis with the tumor purity as a covariate in the ANOVA. We repeated the above analysis for the nonsynonymous RNA editing sites only and considered FDR < 0.2 to indicate statistical significance. We then ranked the nonsynonymous RNA editing sites on the basis of the number of cancer types with detected significance.

To assess if clinically relevant RNA editing sites are enriched in some RNA regions, we performed a coverage-dependent permutation test. First, for each cancer type, we classified all the informative RNA editing sites into ten coverage groups (each with the same number of editing sites) on the basis of the median coverage of a given RNA editing site across all sufficiently covered samples. Second, given the numbers of clinically relevant sites observed in each group, we randomly selected the same number of RNA editing sites as "pseudo clinically relevant sites," so that the whole pseudo set would have the same coverage distribution as the true clinically relevant sites. We then counted the frequencies of pseudo clinically relevant sites for each type of RNA region. We repeated this process 1,000 times, and on the basis of the obtained distributions of these permutations, we assessed the statistical significance of the enrichment of the clinically relevant sites relative to the random expectation (defined as the frequency of permutations with the number of pseudo clinically relevant sites no fewer than the observed true clinically relevant sites). We did this analysis for each cancer type separately.

Sequenom Validation

Four selected RNA editing sites, *AZIN1*^{S367G}, *COPA*^{I164V}, *COG3*^{I635V}, and *GRIA2*^{R764G}, were validated on in-house breast cancer samples by Sequenom MassARRAY at the MD Anderson Sequenome Core Facility, as previously described (Liang et al., 2012).

Generation of Stable BaF3 and MCF10A Cell Lines

The mutant open reading frames corresponding to the RNA editing sites in *AZIN1*, *GRIA2* (the mutation was introduced at the R764G site only and the codon at Q607R remained as WT CAG), and *COG3* were made by site-directed mutagenesis and confirmed by Sanger sequencing. Virus were produced by transfecting human embryonic kidney 293PA (HEK293PA) cells with the GFP control vectors or pHAGE-V5-puromycin expression vectors (carrying *AZIN1*-WT, *AZIN1*-S367G, *GRIA2*-WT, *GRIA2*-R764G, *COG3*-WT, or *COG3*-I635V), and the Lentiviral Packaging Mix (psPAX2 and pMD2.G). BaF3 cells were transduced by the virus and were added RPMI 1640 medium/5% FBS in the low IL-3 (0.0001 ng/ml) and put back into the incubator for 4 weeks, followed by selection with puromycin (0.6 μg/ml) and IL-3 withdrawal. Stable Ba/F3 cells were maintained in medium without IL-3. MCF10A cells were transduced by the virus, followed by selection with puromycin (0.6 μg/ml). Stable MCF10A cells were maintained in completed Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) full medium with 5% horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 10 μg/ml insulin (Sigma), 100 ng/ml Cholera Toxin (Sigma), and 0.5 mg/ml hydrocortisone. After 7 days of antibiotic selection, expression of the constructs was verified by western blots.

Cell Extract Preparation and Western Blotting

Whole-cell lysates for western blotting were extracted with RIPA (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease, and phosphatase inhibitor cocktail). Cell lysates (20 ug) were loaded onto 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane, and protein expression was depicted with an enhanced chemiluminescence western blot detection kit (Amersham Biosciences). Antibodies used were AZIN1, antizyme inhibitor 1Polyclonal antibody (Proteintech), GRIA2, AMPA receptor (GluR 2) (E1L8U) Rabbit mAb (Cell Signaling Technology), COG3 polyclonal antibody (Proteintech), V5 Tag Mouse Monoclonal Antibody (Life Technologies), and ERK2 (Santa Cruz Biotechnology).

BaF3 and MCF10A Cell Viability Assay

BaF3 cells were transduced by the virus and resuspended in BaF3 low IL-3 medium (0.0001 ng/ml). Then the cells were transferred to a 96-well plate, and the assays were performed at weeks 1.5, 2, 3, and 4. Stable MCF10A cell lines were seeded into 96-well plates, and the assays were performed at days 0, 4, 8, 10, and 12. CellTiter-Glo (Promega) was added to access cell viability according to the manufacturer's instructions. The cell viability measurement was also performed on the basis of cell counting after trypsin digestion. The significance of differences was analyzed with Student's t test, and $p < 0.05$ was considered statistically significant.

Apoptosis Assay

Cells (1.5×10^4) were seeded into six-well plates for 24 hr before incubation with MCF10A full medium, MEBM added BPE (Lonza), or DMEM without glucose and L-glutamine for another 24 hr. Apoptosis-induced DNA fragmentation was measured using the Cell Death Detection ELISA Kit (Roche Applied Science) according to the manufacturer's instructions. Apoptosis-induced the active form of caspase-3 was tested using the PE Rabbit Anti-Active Caspase-3 (BD Biosciences) according to the manufacturer's instructions. The BD Canto II analyzer was used to read active caspase-3 in the PE channel.

Wound Healing Assay

MCF10A cells (3.5×10^4) were seeded into 96-well ImageLock plates for 24 hr in DMEM/F12 medium included with 1% horse serum, 4 ng/ml EGF, 2 μg/ml

Figure 5. Sequenom Validation and Functional Effects of Nonsynonymous RNA Editing Sites on Cell Viability

(A) Sequenom validation of *AZIN1*^{S367G}. (Top) Results of a group of samples at cDNA and genomic DNA (gDNA), respectively, where each blue symbol represents the AG genotype of a sample. (Bottom) Results of an individual sample in cDNA and gDNA, respectively, where there are one "A" peak and one "G" peak in cDNA but only one "A" peak in gDNA.

(B) The effects of *AZIN1*^{S367G}, *GRIA2*^{R764G}, and *COG3*^{I635V} in MCF10A cell viability assays.

(C) The effects of *AZIN1*^{S367G}, *GRIA2*^{R764G}, and *COG3*^{I635V} in BaF3 cell viability assays.

Two-sided t test was used to assess the difference. Error bars denote ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. See also Figure S3.

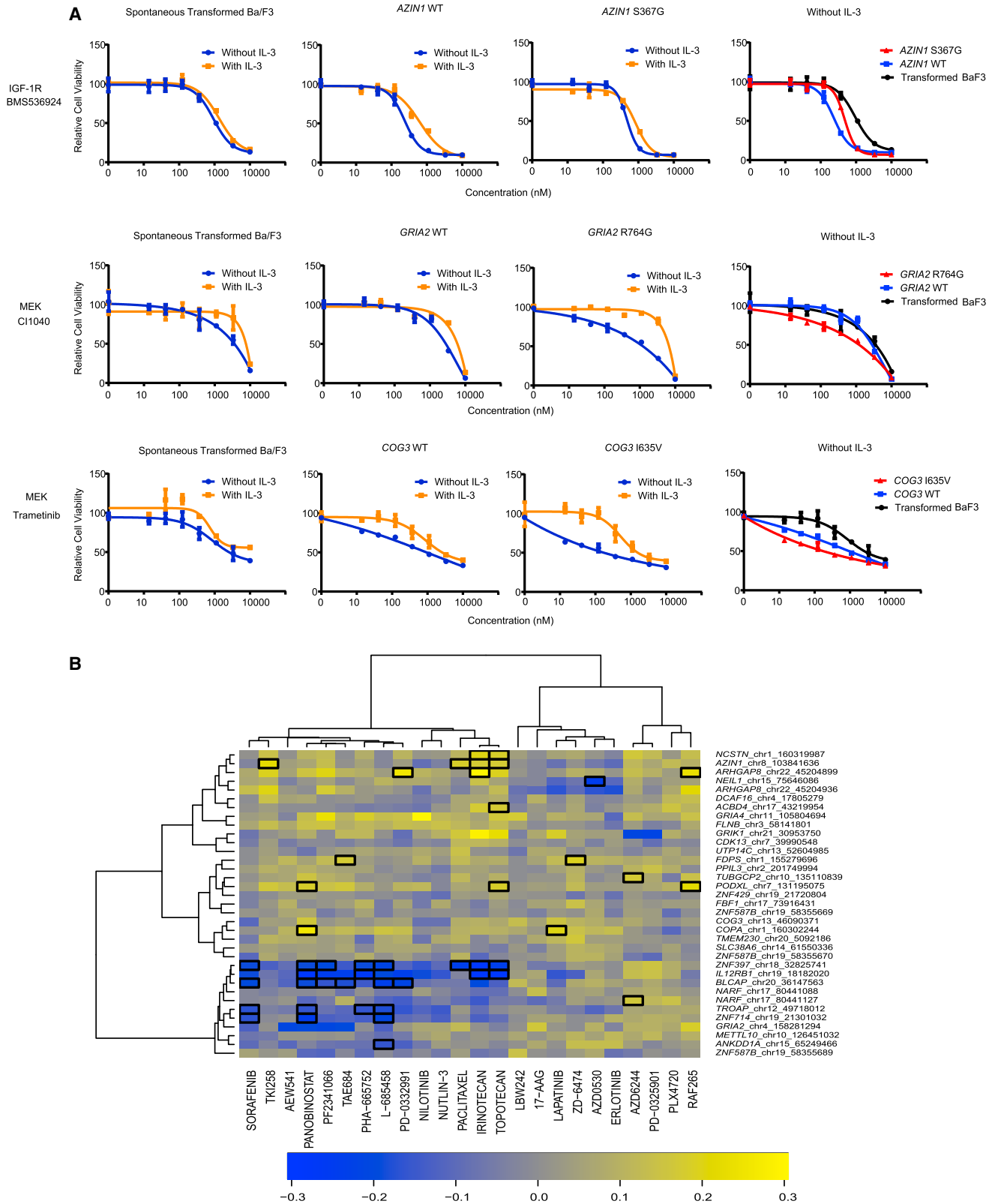

Figure 6. Effects of Nonsynonymous RNA Editing Sites on Drug Sensitivity

(A) Spontaneously transformed Ba/F3 cells (negative control), Ba/F3 cells stably expressing *AZIN1* and *AZIN1*^{S367G}, *GRIA2* and *GRIA2*^{R764G}, and *COG3* and *COG3*^{I635V} were screened against the drug library with or without IL-3 for 72 hr. Dose-response curves are shown for the IGF-1R inhibitor BMS536924, the MEK

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insulin, 20 ng/ml Cholera Toxin, and 0.1 mg/ml hydrocortisone. Automated 96-well cell migration (scratch wound) on IncuCyte was analyzed using the IncuCyte Cell Migration Kit (Essen BioScience), which comprises a 96-pin wound-making tool (WoundMaker), a Cell Migration Analysis software module, and a starter batch of 96-well ImageLock plates.

Ba/F3 Drug Screening Assay

The IL-3-dependent Ba/F3 parental cell line was maintained in RPMI 1640 medium containing 5% FBS and 5 ng/ml IL-3. The spontaneously transformed Ba/F3 cell line was maintained in RPMI 1640 medium containing 5% FBS without IL-3. Stable Ba/F3 cell lines expressing the WT and edited genes were obtained and maintained by selection of puromycin (0.6 μg/ml) and IL-3 withdrawal. The 145-compound library was purchased from the John S. Dunn Gulf Coast Consortium for Chemical Genomics. These compounds were dissolved in DMSO as 10 mM stock solutions. The day before treatment, cells (1×10^4) were seeded in 96-well plates in medium with or without IL-3. Eight serial dilutions of each compound were prepared in media, and final drug concentrations ranged from 0–10 μM. Cells were treated with DMSO or drug compounds in the presence or absence of IL-3 for 72 hr. Cell viability was determined using PrestoBlue (Promega) for mitochondrial dehydrogenase activity. Drug screening was repeated independently to ensure the reproducibility of the results.

To comprehensively assess the effects of RNA editing sites on drug sensitivity, we downloaded the drug screening data from CCLE (http://www.broadinstitute.org/ccle/home) and calculated the correlations between the RNA editing level and IC_{50}.

ACCESSION NUMBERS

The accession number for the RNA editing data reported in this paper is Synapse: syn2374375.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.08.013.

AUTHOR CONTRIBUTIONS

H.L. conceived of and supervised the project. L.H., G.B.M., and H.L. designed and performed the research. L.D., R.Z., Y. Yang, Y. Yuan, Jun Li, J.R., Z.J., S.W., and J.B.L. contributed to the data analysis. S.Y., X.X., Jie Li, H.M.W., A.K.E., N.N., R.M., Y.H. L., L.W.C, K.J.J., Y.L., and S.L.K. performed the experiments. L.H., X.X., G.B.M., and H.L. wrote the manuscript, with input from all other authors.

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inhibitors CI1040 and trametinib. The drugs were dissolved in DMSO, and only DMSO was added at the drug concentration of zero as a control. At each drug dose, the relative cell viability (measured on the basis of three independent replicates) was obtained by normalizing the absolute cell viability to the DMSO control to remove the baseline difference between Ba/F3 cells with and without IL-3. Error bars denote ± SD.
(B) A heatmap showing the correlations of the RNA editing levels of 35 clinically relevant nonsynonymous sites with the IC_{50} values of 24 clinical drugs across CCLE cell lines. The highlighted boxes indicate significant correlations at FDR < 0.1.

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