Abstract
Esophageal squamous cell carcinoma (ESCC), the major histologic form of esophageal cancer, is a heterogeneous tumor displaying a complex variety of genetic and epigenetic changes. Aberrant RNA editing of adenosine-to-ino sine (A-to-I), as it is catalyzed by adenosine deaminases acting on RNA (ADAR), represents a common posttranscriptional modification in certain human diseases. In this study, we investigated the status and role of ADARs and altered A-to-I RNA editing in ESCC tumorigenesis. Among the three ADAR enzymes expressed in human cells, only ADAR1 was overexpressed in primary ESCC tumors. ADAR1 overexpression was due to gene amplification. Patients with ESCC with tumoral overexpression of ADAR1 displayed a poor prognosis. In vitro and in vivo functional assays established that ADAR1 functions as an oncogene during ESCC progression. Differential expression of ADAR1 resulted in altered gene-specific editing activities, as reflected by hyperediting of FLNB and AZIN1 messages in primary ESCC. Notably, the edited form of AZIN1 conferred a gain-of-function phenotype associated with aggressive tumor behavior. Our findings reveal that altered gene-specific A-to-I editing events mediated by ADAR1 drive the development of ESCC, with potential implications in diagnosis, prognosis, and treatment of this disease. Cancer Res; 1–12. ©2013 AACR.

Introduction
Esophageal squamous cell carcinoma (ESCC), the leading cause of cancer death worldwide, is a heterogeneous tumor displaying a complex variety of genetic and epigenetic changes. ESCC is characterized by its remarkable geographic distribution and high-risk areas include Northern China, especially in Henan province, Northern Iran, and South Africa (1). Despite the recent advances in treatment, the benefit of all kind of treatment is not satisfactory. The prognosis of patients with ESCC is still poor and the 5-year overall survival rate is ranging from 20% to 30% (2). Therefore, it is very important to search for biologic markers, which can diagnose cancer as early as possible, estimate reaction to chemotherapy or radiotherapy in those patients with ESCC, and predict overall survival rate of patients undergoing treatment.

In human cancers, aberrant posttranscriptional modifications such as alternative splicing and RNA editing may lead to tumor-specific transcriptome diversity. The best-characterized type of RNA editing found in mammals converts cytosine (C) to uracil (U) and adenosine (A) to inosine (I). In humans, the most common type of RNA editing is conversion of A to I, which is catalyzed by the double-stranded RNA (dsRNA)-specific ADAR (adenosine deaminases acting on RNA) family of protein (3). As inosine preferentially base pairs with cytidine, inosines are interpreted as guanosines by the translation and splicing machineries. Thus, the ADARs may recode transcripts, which results in a proteome that is divergent from the genome (4–7) and therefore, modulates the protein sequence and function of gene products. Because of the diverse impacts of RNA editing on gene expression and function, it is possible that the misregulation of A-to-I RNA editing may play a role in tumorigenesis by either inactivating a tumor suppressor or activating genes that promote tumor progression.

The ADAR gene family includes three members, ADAR1 (also known as ADAR), ADAR2 (ADARB1), and ADAR3 (ADARB2). ADAR1 and ADAR2 are expressed in most tissues; ADAR3 is exclusively detected in brain tissue (8). There are two isoforms of ADAR1, a full-length ADAR1 p150 and an N-terminally
truncated ADAR1 p110 (9). The p150 isoform is produced from an IFN-inducible promoter and the p110 form is constitutively expressed, which is initiated from a downstream methionine as the result of the skipping of the exon containing the upstream methionine. Because the p150 isoform of ADAR1 can be induced by IFNs and found in cytoplasm, possibly it exerts its functions to target viruses that replicate in the cytoplasm. However, the ADAR1 p110 isoform exerts its A-to-I modification in the nuclear pre-mRNA. Our recent study has reported that the recoding RNA editing of a gene AZIN1 (antizyme inhibitor 1) is specifically catalyzed by ADAR1, and the hyper-editing pattern of AZIN1 gene predisposes to human hepatocellular carcinoma (10). Until now, the roles of RNA editing enzyme ADARs and the edited transcripts of target genes in the development of ESCC have not been studied. Here, we demonstrated that the RNA editing enzyme ADAR1, but not ADAR2 and ADAR3, was significantly overexpressed in primary ESCC tumor compared with their matched nontumor specimens. Moreover, the role of ADAR1 and the altered gene-specific editing pattern were further investigated in clinical specimen, cell models, and mice.

Materials and Methods

Cell lines
Six Japanese ESCC cell lines (KYSE140, KYSE410, KYSE180, KYSE30, KYSE510, and KYSE520) were obtained from DSMZ, the German Resource Centre for Biological Material (11). A Chinese ESCC cell line HKESC1 was kindly provided by professor Srivastava (Department of Pathology, The University of Hong Kong, Hong Kong, China), and two Chinese ESCC cell lines EC18 and EC109 was kindly provided by professor Tsao (Department of Anatomy, The University of Hong Kong, Hong Kong, China), and two Chinese ESCC cell lines EC18 and EC109 was kindly provided by professor Tsao (Department of Anatomy, The University of Hong Kong, Hong Kong, China). All nine ESCC cell lines were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL). All cell lines used in this study were regularly authenticated by morphologic observation and tested for absence of mycoplasma contamination (MycopAlert, Lonza Rockland). The cells were incubated at 37°C in a humidified chamber containing 5% CO₂.

Clinical samples

**Cohort 1.** A total of 69 paired primary ESCC tumor tissues and their matched nontumorous tissues that were surgically removed, snap-frozen in liquid nitrogen (for protein, RNA, and DNA extraction) were obtained from Linzhou Cancer Hospital (Henan, China) between 2010 and 2011.

**Cohort 2.** A total of 180 paired primary ESCC tumor tissues and their matched nontumor tissues that were surgically removed and embedded in a paraffin block [for tissue microarray (TMA) construction] were obtained from Linzhou Cancer Hospital along with clinicopathological summaries between 2001 and 2005.

None of these patients received preoperative chemotherapy and radiotherapy. All clinical samples used in this study were approved by the committees for ethical review of research involving human subjects at Zhengzhou University (Zhengzhou, China), The University of Hong Kong, and National University of Singapore (Singapore).

Analysis of RNA editing

Direct sequencing was performed on 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 (12), PCR products were subcloned into the T-easy vector (Promega), and approximately 50 individual plasmids were sequenced for each sample. For each sample, three independent real-time PCR (RT-PCR) reactions were performed.

FISH

The detailed procedure for normal karyotype preparation was described in "Supplementary Materials and Methods." A BAC clone at 1q21.3 containing the ADAR1 gene (RP11-61L14) was labeled with SpectrumRed (Vysis). The chromosome 1 centromere probe was labeled with SpectrumGreen (Vysis). FISH reaction was performed according to the method described previously (13).

cDNA synthesis and quantitative real-time PCR

As described previously (10), the total RNA was isolated and the equal amounts of cDNA were synthesized using the Advantage RT-for-PCR kit (Clontech) and used for quantitative PCR (qPCR) analysis. The sequences of primers are included in "Supplementary Materials and Methods."

Cell proliferation assay

Cells were seeded in 96-well plate at a density of 0.5 to 1 × 10⁵ cells per well. The cell growth rate was measured using Cell Proliferation XTT Kit (Roche Diagnostic) according to the manufacturer’s instruction. Three independent experiments done in triplicate were performed.

Focus formation and colony formation in soft agar

Briefly, 1 × 10⁵ cells were seeded in a 6-well plate. After culture for 12 days, surviving colonies (>50 cells per colony) were counted and stained with crystal violet (Sigma-Aldrich). Triplicate independent experiments were performed and the data were expressed as the mean ± SD of triplicate wells within the same experiment. For soft agar assay, 2 × 10³ cells in 0.4% low-melting agarose (Lonza Rockland) were placed on the top of the bottom layer of 0.6% low-melting agarose in a 6-well plate. After 2 to 4 weeks, surviving colonies (>80 cells/colonies) were counted.

Cell migration assay

The Transwell cell migration assay was performed using Bio-coat cell migration chambers (BD Biosciences) containing polyethylene terephthalate membranes (PET) of 8 μm pore size according to the manufacturer’s instructions. Briefly, 1 to 2 × 10⁵ cells in FBS-free RPMI were added. RPMI supplemented with 10% FBS was added to the bottom chamber as a chemoattractant. After 24 to 36 hours, the number of cells that had migrated through the filter pores was stained with crystal violet (Sigma-Aldrich), counted, and imaged using SPOT imaging software (Nikon).
Matrigel invasion assay

We performed invasion assays using 24-well BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s instructions. Briefly, 1 to 2 × 10^5 cells in FBS-free RPMI were added to the top chamber, and 10% FBS in RPMI was added to the bottom chamber as a chemoattractant. After 36 to 48 hours of incubation, cells that invaded the Matrigel were fixed and stained with crystal violet (Sigma-Aldrich). The number of cells was counted and imaged using SPOT imaging software (Nikon).

In vivo tumorigenicity assay

We subcutaneously injected approximately 2 × 10^6 of EC109-CTL or EC109-AR1 cells into the left or right flank of 4- to 5-week-old male severe combined immunodeficient (SCID) mice (n = 5), respectively. To compare the tumorigenic abilities of the wild-type or edited AZIN1 gene, we subcutaneously injected approximately 2 × 10^6 of EC109-LacZ, EC109-wt/AZI, or EC109-edt/AZI cells into the right flank of SCID mice (n = 6). We monitored tumor formation in the SCID mice over a 4-week period and calculated the tumor volume weekly by the formula V (volume) = 0.5 × L (length) × W (width) × L. All animal experiments were approved by and performed in accordance with the Institutional Animal Care and Use Committees of National University of Singapore.

Antibodies and Western blot analysis

Mouse anti-ADAR1, anti-AZIN1, and anti-β-actin antibodies were purchased from Abcam. The mouse anti-ADAR2 and anti-GAPDH antibodies were purchased from Sigma-Aldrich and Santa Cruz Biotechnology. Protein lysates were quantified and resolved on a SDS-PAGE gel, transferred onto a polyvinylidene difluoride membrane (Millipore), and immunoblotted with a primary antibody, followed by incubation with a secondary antibody. The blots were visualized by enhanced chemiluminescence (GE Healthcare).

Immunohistochemical staining

The TMA blocks were sectioned (5 mm thick) for immunohistochemical (IHC) staining. Briefly, sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. For antigen retrieval, the slides were immersed in 10 mmol/L citrate buffer (pH 6.0) and boiled for 15 minutes in a microwave oven. Nonspecific binding was blocked with 5% normal goat serum for 10 minutes. The slides were incubated in a 1:100 dilution of anti-ADAR1 (Abcam) at 4°C overnight in a humidified chamber. The slides were then sequentially incubated with biotinylated goat anti-mouse immunoglobulin G (IgG; 1:100 dilution, Santa Cruz Biotechnology) for 30 minutes at room temperature and streptavidin-peroxidase conjugate for 30 minutes at room temperature. Isotope-matched human IgG was used in each case as a negative control. Finally, the 3,3'-diaminobenzidine (DAB) Substrate Kit (Dako Ltd.) was used for color development followed by Mayer’s hematoxylin counterstaining. On the basis of staining intensities, the ADAR1 immunoreactivity was scored as negative (0; total absence of staining), weak expression (1; faint staining in <50% of cells), moderate expression (2; moderate staining in >25% to <75%, or strong staining in <25% of tumor cells), and strong expression (3; moderate staining in >75%, or strong staining in >25% of tumor cells).

Statistical analysis

Unless otherwise indicated, the data are presented as the mean ± SD of three independent experiments. The SPSS statistical package for Windows (version 16; SPSS) was used to perform the data analyses. The ADARI or ADAR2 expression levels in any two groups of clinical samples (e.g., ESCC tumors and matched nontumor tissues) were compared using a Wilcoxon signed rank test. Kaplan–Meier plots and log-rank tests were used for overall survival analysis. For the TMA analysis, which was based on the scores of IHC staining, ADAR1 expression levels in the primary ESCC tissues and their matched nontumor tissues were compared using the Wilcoxon signed rank test. A paired Student t test was used to compare the editing levels as well as the total mRNA levels of FLNB or AZIN1 in ESCC and their matched nontumor specimens of patients. Spearman correlation coefficients were used to evaluate the positive correlation between the expression of ADAR1 and the editing level of AZIN1 or FLNB in clinical samples. An unpaired two-tailed Student t test was used to compare the number of colonies, the number of migrative and invasive cells, and tumor volume between any two preselected groups. P < 0.05 was considered to be statistically significant.

Results

RNA editing enzyme ADAR1 is significantly upregulated in primary ESCCs and its clinical implications

A-to-I RNA editing is a posttranscriptional modification in stem-loop structures within precursor mRNA, which is catalyzed by dsRNA-specific ADAR enzymes (14). To investigate the expression profiles of the three RNA editing enzymes in ESCC specimens, we examined the expression levels of ADAR1, ADAR2, and ADAR3 in 69 pairs of primary ESCC tumor and their corresponding nontumor tissues that were obtained from Linzhou Cancer Hospital (cohort 1). As detected by the qRT-PCR, only ADAR1 was significantly upregulated in ESCC samples (P = 0.0045) and approximately 48 of 69 (69.57%) ESCC specimens demonstrated the higher expression of ADAR1 than their matched nontumor specimens (Fig. 1A, left). There was no significant difference in ADAR2 expression between primary ESCCs and their matched nontumor specimens (P = 0.1465; Fig. 1A, right). In addition, ADAR3 was undetectable in all of the samples (data not shown). To confirm our findings, Western blot analysis of ADAR1 and ADAR2 expression was performed in the paired ESCC and nontumor specimens of 35 randomly selected ESCC cases from the cohort 1. Consistently, the upregulation of ADAR1 protein (particularly the p110 isoform) in tumors was observed in 19 of 35 (54.29%) ESCCs, and there was no obvious difference in ADAR2 protein expression between ESCC and their matched nontumor specimens (Fig. 1B).

To investigate the clinical implication of ADAR1 during the development of ESCC, we constructed a panel of TMAs consisting of 180 matched pairs of primary ESCC and nontumor
specimens that were collected from Linzhou Cancer Hospital (cohort 2). Informative results of IHC staining were observed in 136 matched pairs of ESCC and nontumor specimens. Non-informative samples included lost samples, inappropriately stained samples, and samples with too few cells; such were not used as valid data. By performing IHC staining, we observed the

![Figure 1. ADAR1 is significantly overexpressed in primary ESCC samples and its clinical implication.](image)

A. Box plots represent the relative ADAR1 (left) and ADAR2 (right) expression levels in 69 matched pairs of ESCC and nontumor specimens in cohort 1. The data are presented as box plots with the median (horizontal line), 25% to 75% (box), and 5% to 95% (error bar) percentiles for each group. B. Western blot analyses of ADAR1 and ADAR2 expression levels in six paired ESCC and nontumor specimens in cohort 1. GAPDH was used as a loading control. C. Example of the ADAR1 expression level detected in a matched pair of primary ESCC and nontumor tissue in cohort 2. The boxed regions are magnified and displayed in the bottom panels. Scale bar: 200 μm. D. Kaplan–Meier plots for the overall survival rate of patients with (n = 90, red line) or without (n = 46, blue line) the tumoral overexpression of ADAR1. E. FISH analysis of the ADAR1 gene (red signal) and the control chromosome 1 centromere probe (green signal) specifically hybridized to the chromosome 1 (indicated by white arrow) of normal human karyotype (left). A representative example of ADAR1 gene amplification (red signals) in a matched pair of primary ESCC and nontumor tissue (middle and right panels). Scale bar, 500 μm.
A-to-I Editing Mediated by ADARs in ESCC

differential nuclear expression of ADAR1 between the primary ESCC tumor and their matched nontumor tissues (Fig. 1C). A detailed analysis of the IHC data revealed that the ADAR1 was overexpressed in 66.18% (90/136) of the informative ESCC tumor and their matched nontumor tissues (Fig. 1C). The ADAR1 was specimen-specific silenced by the introduction of two specific short hairpin RNAs (shRNA) against ADAR1 gene (Fig. 3A). Compared with cells transduced with the control scramble shRNA (510-CTL), cells transfected with two shRNAs targeting ADAR1 (510-shAR1 #5 and 510-shAR1 #7) were found to be less tumorigenic, as manifested by the decreased cell growth rates, the decreased migrative and invasive capabilities compared with 180-CT and EC109-CTL cells, respectively (Fig. 2E). Xenograft studies in mice demonstrated that the growth rate of tumors derived from EC109-AR1 cells was markedly higher than those derived from EC109-CTL cells (Fig. 2F).

Table 1. Cox proportional hazard regression analyses for overall survival

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age</td>
<td>1.160 (0.716–1.878)</td>
<td>0.547</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Gender</td>
<td>1.364 (0.857–2.170)</td>
<td>0.190</td>
<td>—</td>
<td>—</td>
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<tr>
<td>LN metastasis</td>
<td>1.141 (0.601–2.164)</td>
<td>0.688</td>
<td>0.749 (0.577–0.971)</td>
<td>0.029*</td>
</tr>
<tr>
<td>Differentiation</td>
<td>0.759 (0.587–0.982)</td>
<td>0.036*</td>
<td>8.534 (1.949–37.358)</td>
<td>0.004*</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>10.227 (2.506–41.733)</td>
<td>0.001*</td>
<td>1.599 (0.731–3.501)</td>
<td>0.240</td>
</tr>
<tr>
<td>Tumor invasion</td>
<td>2.995 (1.435–6.248)</td>
<td>0.003*</td>
<td>0.513 (0.321–0.822)</td>
<td>0.005*</td>
</tr>
<tr>
<td>ADAR1 overexpression</td>
<td>0.577 (0.364–0.916)</td>
<td>0.020*</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
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Abbreviation: CI, confidence interval.
\*Statistical significance (P < 0.05) is shown in bold.
Figure 2. ADAR1 functions as an oncogene during ESCC progression. A, Western blot analyses showing expression of ADAR1 and ADAR2 proteins in the indicated cell lines. β-actin was the loading control. B, cell growth rates of the indicated cell lines were compared by XTT assays. The results are expressed as the mean ± SD of triplicate wells within the same experiment (*, *P < 0.05; **, *P < 0.01; ***, *P < 0.001, unpaired, two-tailed Student t test). C, quantification of foci formation induced by the indicated stable cell lines. Triplicate independent experiments were performed and the data were expressed as the mean ± SD of triplicate wells within the same experiment (*, *P < 0.05; ***, *P < 0.001, unpaired, two-tailed Student t test). Scale bar, 0.5 cm. D, quantification of colonies (formed in soft agar) that were induced by the indicated cell lines. (Continued on the following page.)
FLNB: spearman $r = 0.75, P = 0.025$; Fig. 4A and Supplementary Fig. S2). In clinical samples, the editing levels of AZIN1 and FLNB in ESCC tumors were significantly higher than those in nontumor specimens (AZIN1: $P < 0.0001$; FLNB: $P < 0.0001$, paired Student t test; Fig. 4B), suggesting that the hyperediting patterns of AZIN1 and FLNB might play a role in ESCC progression. We also determined the total mRNA levels of AZIN1 and FLNB in the same clinical cases as described above. The mRNA levels of AZIN1 and FLNB were significantly increased in ESCC tumors than their matched nontumor specimens (AZIN1: $P = 0.043$; FLNB: $P = 0.023$, paired Student t test; Supplementary Fig. S3), indicating that the hyperediting phenotypes of AZIN1 and FLNB in tumors would not be affected by the low endogenous transcript levels. Furthermore, we defined the fold-change of ADAR1 in ESCC cases with the ratio of the relative quantification values of tumors to that of the corresponding nontumor specimens. In addition, the fold-change of AZIN1 or FLNB editing level in ESCC cases was characterized by the ratio of the editing frequency of tumors to that of their matched nontumor specimens. As expected, there was a positive correlation between the editing level of AZIN1 or FLNB and the expression level of ADAR1 in ESCC cases (AZIN1: Spearman $r = 0.3689, P = 0.0018$; FLNB: Spearman $r = 0.4186, P = 0.0003$; Fig. 4C). To provide direct evidence that ADAR1 regulates AZIN1 and FLNB editing, the introduction of the ADAR1 p110 expression constructs into EC109 and KYSE180

Figure 3. Silencing ADAR1 by RNAi inhibits its tumorigenicity. A, Western blot analyses showing expression of ADAR1 and ADAR2 proteins in the indicated cell lines. $\beta$-actin was the loading control. B, cell growth rates of the indicated cell lines were compared by XTT assays. The results are expressed as the mean ± SD of triplicate wells within the same experiment ($^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$, $^{***}$, $P < 0.001$, unpaired, two-tailed Student t test). C, quantification of foci formation induced by the indicated stable cell lines. Triplicate independent experiments were performed and the data were expressed as described above ($^{*}$, $P < 0.01$; $^{**}$, $P < 0.001$, unpaired, two-tailed Student t test). Scale bar, 0.5 cm. D, quantification of cells from the indicated cells that migrated through the PET-membrane or invaded through the Matrigel-coated membrane ($^{*}$, $P < 0.05$; $^{**}$, $P < 0.001$, unpaired, two-tailed Student t test). Scale bar, 100 μm.
cell lines led to the increased editing frequencies of AZIN1 and FLNB transcripts as well as the expression level of ADAR1 in nine ESCC cell lines. B, the AZIN1 (left) and FLNB (right) editing levels in 69 paired ESCC and matched nontumor specimens in cohort 1 (paired Student t test). C, correlation between the expression level of ADAR1 and the editing level of AZIN1 (left) or FLNB (right) in 69 paired ESCC and matched nontumor specimens in cohort 1. D and E, sequence chromatograms of the AZIN1 and FLNB transcripts in EC109 (D) or KYSE180 cells (E) that transiently transduced with an ADAR1 p110 lentivirus (+ADAR1 p110) or LacZ control lentivirus (+control). The percentages of edited AZIN1 and FLNB transcripts were detected as described in the Materials and Methods section. An arrow indicates the editing position. F, sequence chromatograms of the AZIN1 and FLNB transcripts in KYSE510 cells (E) that transiently transfected with two shRNAs against ADAR1 gene (shAR1 #5 and shAR1 #7) or the control shRNA (control). An arrow indicates the editing position.

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The functional alteration of AZIN1 transcript as a result of the A-to-I RNA editing during ESCC progression

As a specific RNA editing target of ADAR1 protein, AZIN1 gene encodes a protein that undergoes an amino acid substitution from serine (Ser) to glycine (Gly) at residue 367 (10). In this study, the functional difference between the wild-type and the edited AZIN1 protein was further studied. For this purpose, we introduced V5-tagged wild-type AZIN1 (wt/AZI) or edited AZIN1 (edt/AZI) expression constructs into two ESCC cell lines (KYSE180 and EC109; Supplementary Fig. S4A). Cells transduced with the edt/AZI lentivirus (180-edt/AZI and EC109-edt/AZI) with 100% of their AZIN1 transcripts edited (Fig. 5A) had accelerated growth rates and higher frequencies of focus and colony formation in soft agar than cells transduced with the wt/AZI (180-wt/AZI and EC109-wt/AZI) or control LacZ lentivirus (180-LacZ...
Figure 5. The edited AZIN1 confers more aggressive tumorigenic phenotypes during ESCC progression. A, sequence chromatograms of the AZIN1 transcript in the indicated cell lines. An arrow indicates the editing position. B, cell growth rates of the indicated cell lines were compared by XTT assays. The results are expressed as described above (\( P < 0.05; \), \( P < 0.001; \)). C, quantification of foci formation induced by the indicated stable cell lines. Triplicate independent experiments were performed and the data were expressed as described above (\( P < 0.01; \), \( P < 0.001; \)). Scale bar, 0.5 cm. D, quantification of colonies (formed in soft agar) that were induced by the indicated cell lines. Triplicate independent experiments were performed and the data were expressed as described above (\( P < 0.05; \), \( P < 0.01; \)). Scale bar, 200 μm. E, quantification of cells from the indicated cells that migrated through the PET-membrane or invaded through the Matrigel-coated membrane. (\( P < 0.05; \), \( P < 0.01; \), \( P < 0.001; \), unpaired, two-tailed Student t test). Scale bar, 200 μm. F, growth curves of tumors derived from the indicated cell lines over a period of 4 weeks. Data are presented as the mean ± SD (\( n = 6; \), \( P < 0.05; \), \( P < 0.001; \), unpaired, two-tailed Student t test).
ADARs, the enzymes required for the conversion of A-to-I in dsRNA, were first noticed as cellular RNA unwinding activity (19, 20), as they lead to destabilization of RNA duplexes by introducing I-U mismatch. ADARs exhibit strict tissue-specific and environment-dependent expression patterns (21, 22). There are three orthologs: ADAR1 and ADAR2 occur in most animals, whereas ADAR3 is vertebrate and brain specific. The ADAR1 p150 isoform is presumably responsible for the A-to-I editing of viral RNAs produced by viruses (14, 23), but not of the nuclear pre-mRNAs (24). It has been reported that all the three editing enzymes ADAR1, ADAR2, and ADAR3 were downregulated in brain tumors. Overexpression of ADAR1 and ADAR2 in the U87 glioblastoma multiforme cell line resulted in a decreased proliferation rate, suggesting that the reduced A-to-I editing in brain tumors is involved in the pathogenesis of cancer (18). On the contrary, ADAR1 or/and ADAR2 were also found to be upregulated in tumor tissues, such as prostate cancer and breast cancer (25). In this study, we investigated the expression profiles of all three RNA editing enzymes in ESCC clinical samples. ADAR1 and ADAR2 were abundantly expressed in ESCC samples, but ADAR3 was undetectable in all samples. Because of the genomic amplification of the ADARI gene, which is mapped to chromosome 1q21, ADAR1 was the only RNA editing enzyme found to be significantly upregulated in primary ESCC tumors compared with their matched nontumor specimens in two individual cohorts (cohort 1 and 2). Clinically, the tumoral overexpression of ADAR1 was correlated with the shorter overall survival time of patients with ESCC, which could be used independently for the prediction of a poor prognosis. Consistently, our functional assays have indicated that ADAR1 could induce tumorigenic phenotypes in cell models and animals.

ADARs can change the structure of RNA by changing an Adenosine Uracil (AU) base pair to an Inosine Uracil (IU) mismatch. Conceivably, ADARs could affect any biologic process that is associated with the sequence- or structure-specific interaction of RNA (14). So far, ADARs have been shown to alter the protein coding, create or delete splice sites, and modulate transcript stability. The A-to-I editing can be very specific, leading to deamination of select adenosine residues, or it can be almost random and lead to nonselective conversion of many adenosines. For long dsRNA (<100 bp) within 3′ untranslated region (3′-UTR), many adenosine residues are edited promiscuously, leading to approximately 50% of adenosines being converted to inosines. However, in terms of A-to-I editing within protein-coding sequences, it is highly selective, and an imperfect fold-back dsRNA structure is formed between the exon sequence surrounding the editing site(s) and a downstream intronic complementary sequence termed editing-site complementary sequence (8). Until now, only a few recoding RNA editing events (e.g., Q/R site editing in the glutamate receptor) have been verified. The editing events within 3′UTRs can affect transcript stability via affecting microRNA targeting or the nuclear retention of transcripts (26–29). However, the editing targets within coding region will cause amino acid change and affect protein function rather than protein level, suggesting that the recoding editing events are of high potentials to play a role in tumorigenesis by either inactivating a tumor suppressor or activating genes that promote tumor progression. As reported previously, two recoding editing events at codon 2269 (Met → Val) of the FLNB gene and codon 367 (Ser → Gly) of the AZIN1 gene are catalyzed by ADAR1 protein (10, 12). In this study, we proved that ADAR1 was responsible for the editing of FLNB and AZIN1 in ESCC cells and clinical samples, suggesting that as a result of the differential expression of ADAR1 in ESCC tumors, the editing alterations in the specific genes may be responsible for the ADAR1-induced malignant phenotypes during ESCC progression. Therefore, we selected one of the recoding editing targets, AZIN1, for further study. Intriguingly, we found the editing frequency of AZIN1 was significantly increased in primary ESCC tumors compared with their matched nontumor specimen, suggesting there is a hyperediting phenotype of the AZIN1 editing event during ESCC progression. AZIN1, termed antizyme inhibitor 1, blocks the effects of antizyme on ornithine decarboxylase (ODC). This protein has substantial similarity to ODC itself but has no ODC activity (30). It binds to antizyme more tightly than ODC and thus releases ODC from the antizyme–ODC complex (30, 31). Although the physiologic importance of AZIN1 is not yet fully understood, a strong case can be made for it to be included as a component of the polyamine pathway. Downregulation of antizyme inhibitor in lung cancer cells reduced ODC levels and led to growth inhibition (32). Also, it has been reported that overexpression of AZIN1 in NIH3T3 fibroblasts provides growth advantage through neutralization of antizyme functions (33). In the present study, we demonstrated that although the wild-type AZIN1 could promote the tumorigenicity, the edited AZIN1 conferred “gain-of-function” phenotypes that were manifested by more aggressive behaviors during ESCC progression, which has also been found in human hepatocellular carcinoma as reported recently (10). All these findings suggest that the ADAR1-induced editing alteration may play a pivotal role in human solid tumors and the precise regulation of the expression level of ADARs is essential for accurate editing and the altered expression of ADARs could be at the origin of cell transformation.
The characterization of editing events is a necessary step toward fully understanding the function and regulation of transcriptome, and investigating the connection between RNA editing and cancer progression is only the initial step in this research. The existence of RNA editing, as opposed to hard-wired genomic mutation, indicates that it can be spatiotemporally controlled. Indeed, there is a great excitement that we could develop potential therapeutic approaches for targeting this epigenetic process. Here, we propose two feasible approaches: (i) modulating the expression of RNA editing enzyme ADARs by overexpressing or silencing ADARs and (ii) reinstating a specific hyperedited or hype-edited transcript (34). In this study, we reported that one of the RNA editing enzyme ADAR1 was overexpressed in ESCC tumors, which can be used as an independent factor for prognosis prediction. As a result of the tumor overexpression of ADAR1, the edited AZIN1 transcript is more abundant in tumors, leading to “gain-of-function” phenotypes during ESCC progression. Therefore, we speculate that monitoring the expression level of ADAR1 or the editing activities of key editing targets represent a useful biomarker for the detection of disorders in ESCC and our study may also provide the a key biologic process for rationale drug development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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