Targeting RNA editing of antizyme inhibitor 1: A potential oligonucleotide-based antisense therapy for cancer

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INTRODUCTION
RNA editing is a widespread co- or post-transcriptional modification process that introduces changes in RNA sequences encoded by the genome, contributing to “RNA mutations.” Editing of adenosine to inosine (A to I) in double-stranded RNA (dsRNA), catalyzed by the adenosine deaminase acting on RNA (ADAR) family of enzymes, is the most common type of RNA editing in mammals.1 In vertebrates, a family of 3 ADAR proteins, ADAR1, ADAR2, and ADAR3, has been characterized.2 ADAR1 and ADAR2 (ADARs) catalyze all currently known A-to-I editing events. In contrast, ADAR3 has no documented deaminase activity. Inosine essentially mimics guanosine (G); therefore, ADAR proteins introduce a virtual A-to-G substitution in transcripts. Such changes can lead to specific amino acid substitutions,3–5 alternative splicing,6,7 microRNA-mediated gene silencing,8,9,10 or changes in transcript localization and stability.11,12,14

Aberrant editing on specific transcripts and their association with cancer progression have been discovered in many cancer types.15–17 The protein-recoding type of RNA editing contributes to tumorigen-esis mainly through enhancing the activity of oncogenes or reducing the activity of tumor suppressors.18–21

AZIN1 (antizyme inhibitor 1) is one of the best-studied ADAR1 targets in cancer. Editing of AZIN1 results in a serine (S) to glycine (G) substitution at residue 367, and the edited form AZIN1S367G is more stable and has a stronger affinity...
to antizyme (AZ) than AZIN1\(^{\text{wild-type}}\). AZ binds and degrades proteins associated with cell growth and proliferation such as ornithine decarboxylase (ODC) and cyclin D1 (CCND1). AZIN1\(^{\text{S607G}}\) inhibits AZ-mediated degradation of ODC and CCND1 by competing with AZIN1\(^{\text{wild-type}}\) for binding to AZ, thereby facilitating entry into cell cycle and possessing much stronger tumorigenic capabilities than AZIN1\(^{\text{wild-type}}\). Importantly, RNA editing level of AZIN1 is significantly higher in different cancer types such as hepatocellular carcinoma (HCC),\(^5\) esophageal squamous cell carcinoma (ESCC),\(^6\) nonsmall-cell lung cancer (NSCLC),\(^6\) and colorectal cancer (CRC)\(^5\) compared with corresponding non-cancerous tissues. Elevated editing frequency of AZIN1 has also emerged as a prognostic factor for overall survival and disease-free survival and an independent risk factor for lymph node and distant metastasis.\(^5\) All these findings strongly suggest that suppressing AZIN1 editing may contribute to the development of novel RNA therapies for cancer treatment.

Unfortunately, RNA therapeutics targeting cancer-driven or associated RNA editing events have not yet been identified or introduced into the clinic. In this study, we employed chemically modified antisense oligonucleotides (ASOs) to sensitively and specifically inhibit AZIN1 editing. ASOs can be defined as single- or double-stranded oligonucleotides, \(\sim 15-25\) nucleotides (nt) in length, that can bind to RNA through Watson-Crick base pairing. In an effort to increase cellular uptake, stability, target binding affinity, and specificity while reducing nuclease cleavage and immune response from TLRs (Toll-like receptors), backbone modifications (e.g., phosphodiester, phosphorothioate [PS]), and sugar ring modifications such as 2'-O-Me (2'-O-methyl), 2'-O-MOE (2'-methoxyethyl), and alkynucleic acid (LNA) have been widely used for ASOs. To confer site-specific RNA editing inhibition, ASOs must be able to enter the cell nucleus and sequester or block ADAR proteins from binding to target RNAs. They must also not trigger RNase H or RNA interference (RNAi) response against RNA targets. To date, ASOs containing 2'-O-Me or 2'-O-MOE in combination with the PS backbone modification, or highly modified ASOs including phosphorodiamidate morpholino oligomer (PMO) and peptide nucleic acid (PNA), have been utilized to modulate splicing and inhibit translation.\(^22\) However, for RNA editing inhibition, very few studies showed that morpholino ASO and 2'-O-Me/LNA-modified ASOs were capable of inhibiting editing of certain targets with low or modest potency, such as the Q/R site of GluA2\(^{23}\) (glutamate ionotropic receptor AMPA type subunit 2), HTR2C\(^{26}\) (5-hydroxytryptamine receptor 2C), and NEIL1\(^{26}\) (Nei like DNA glycosylase 1).

In this study, we applied a minigene system to uncover the editing site complementary sequence (ECS) of AZIN1 that forms dsRNA with the editing region at exon 11, which allows ADAR1 protein to bind and catalyze the deaminase reaction. Based on this finding, we designed and synthesized PNAs, 2'-O-Me alone, or 2'-O-Me/PS-modified ASOs that target either the editing region or the ECS and evaluated their potential to inhibit AZIN1 editing and cancer cell viability in vitro and in vivo. We found that complete 2'-O-Me sugar ring modification in combination with partial PS backbone modification may be an optimal chemistry for RNA editing inhibition, and ASOs that target the ECS, but not the editing region, could effectively abolish AZIN1 editing without affecting splicing and translation. 2'-O-Me/PS-modified ASO3.2, which targets the ECS, specifically inhibits the viability of cancer cell lines as well as cells derived from HCC patient-derived xenografts (PDXs) in vitro and tumour incidence and growth in xenograft models. Altogether, our study develops an ASO-based RNA editing inhibitor and provides an attractive approach for targeting cancer-associated RNA editing substrates. The delivery of therapeutically effective, chemically stabilized ASOs into human liver and central nervous system (CNS) has been achieved recently,\(^27\) suggesting that developing an ASO-based RNA editing inhibitor of AZIN1 may hold great promise for the treatment of cancer, particularly liver cancer.

RESULTS

An 8-nt sequence at 3’ end of exon 12 is the core ECS and indispensable for AZIN1 editing

Uncovering the ECS of AZIN1 transcript would help to decipher the precise dsRNA structure, which is essential for AZIN1 editing. To this end, AZIN1 minigene constructs were generated by inserting fragments of different length covering the edited exon 11 and flanking exons and introns into either pRK7 or pcDNA3.1 vector (Figure 1A). HTR2C, which is a well-characterized editing target with its dsRNA structure well delineated in many studies,\(^28-30\) was used to generate HTR2C minigene as a positive control. Upon co-transfection of the HTR2C minigene and ADAR1 expression construct, \(\sim 75.8\%\) of endogenous AZIN1 was edited, indicating a successful ADAR1 overexpression (Figure 1B, left; Figure S1). Moreover, three known editing sites were detected in exogenous HTR2C transcripts (Figure 1B, right), supporting the feasibility of using the pRK7 minigene system in this study. Among all AZIN1 minigenes, only AZIN1 transcripts transcribed from the minigene containing fragment A (FA), which lacks a 90-bp sequence at the 3’ end of exon 12, was unable to be edited (Figures 1A and 1C). This observation could be confirmed by using pcdNA3.1-based minigenes, ruling out the possibility of artifacts of the pRK7 minigene system (Figure 1D). These findings suggested that the ECS is most likely to be at the 3’ end of exon 12.

To precisely locate the ECS, RNA sequence corresponding to fragment E (FE) was used for secondary structure prediction by RNAfold.\(^31\) Of note, the 3’ end of exon 12 forms dsRNA with the edited sequence (Figure 1E). FE minigene was utilized to generate 3 additional minigenes by deleting a 29-bp sequence at the 3’ end of exon 12 (FE-1) or introducing an 8-bp internal deletion (FE-2) or point mutations (FE-3) into the potential ECS (Figure 2A). As predicted by RNAfold, both deletion and mutations dramatically alter the dsRNA structure (Figure 2B). Using the same strategy described above, we observed that transcripts transcribed from FE-1, -2, and -3 were not edited upon ADAR1 overexpression (Figure 2C; Figure S2). Further, in vitro RNA editing analysis showed that AZIN1 transcripts transcribed from FB or FE, but not FA, FE-2, and FE-3, could be edited in the presence of purified ADAR1 protein (Figures 2D). All these data supported that an 8-nt sequence (5’-
Figure 1. The 3’ end sequence of exon 12 is required for AZIN1 editing

(A) Schematic diagram of AZIN1 minigene constructs generated by inserting five different fragments (FA, FB, FC, FD, or FE) covering the edited exon 11 and flanking exons (exons 10 and 12) and introns (introns 9, 10, 11, and 12) into either pRK7 or pcDNA3.1 vector. Red arrow indicates relative position of the editing site. (B and C) Sequencing chromatograms illustrate editing of endogenous AZIN1 (B, left) and exogenous HTR2C (B, right) or AZIN1 (C) transcripts transcribed from pRK7-based minigene constructs in HEK293T cells co-transfected with the indicated pRK7 minigenes and empty vector (EV) or ADAR1 expression construct (ADAR1). (D) Sequencing chromatograms illustrate editing of endogenous and exogenous AZIN1 transcripts in HEK293T cells co-transfected with pcDNA3.1-based minigenes and EV or ADAR1. (E) Predicted dsRNA structure of AZIN1 by RNAfold. An 8-nt sequence indicated by black arrows is the potential core ECS. Editing site is indicated by red arrowhead. Base pair probabilities are shown by a color spectrum. (B–D) Percentage of editing is calculated as area of “G” peak over the total area of “A” and “G” peaks. *, no editing detected. Black arrow indicates the position of editing site.
GCUUUUCC-3') at the 3' end of exon 12 is the core ECS and indispensable for dsRNA formation and AZIN1 editing.

**Identifying ASOs with pronounced in vitro editing inhibitory effects**

Based on the elucidation of the AZIN1 dsRNA, 7 entirely 2'-O-Me-modified ASOs (ASO1, ASO5, ASO6, and ASO7 target the editing region, and ASO2, ASO3, and ASO4 target the ECS region), an antisense PNA (ASP1), and 2 dsRNA-binding PNAs (DSP1 and DSP2) were designed and synthesized (Figure 3A; Table S1). As negative controls, 4 ASOs were designed, including ASO-ctl (6 point mutations were introduced into ASO2), ASO-ctl1, ASO-ctl2, and ASO-ctl3 (share the same sequence with ASO3 except carrying one, double, and triple point mutations, respectively) (Figure 3A). The AZIN1
Figure 3. Screening of effective ASOs that can bind to AZIN1 duplex and inhibit AZIN1 editing in vitro
(A) Illustration of the design of ASOs. A short RNA duplex containing the editing region consisting of partial exon 11 with the edited adenosine (highlighted in red) and partial exon 12 containing the ECS region (the 8-nt core ECS is highlighted in green) is used for designing ASOs that target either the editing or ECS region. Point mutations introduced into each control ASO are highlighted in purple. Sequences of each oligo and their characteristics are listed in Table S1. (B) REMSA was performed to examine the binding of each ASO (2.5 μM) to 32P-labeled AZIN1 RNA duplexes (86 nt in length). The sequence and predicted secondary structure of the duplex probe are listed in Table S3, Figure 3A, and Figure S3A. Vehicle control (VC), no ASO added. (C) Binding of ASO1, 3, 5, or 7 to the duplexes described in (B) was detected by REMSA, at different

(legend continued on next page)
RNA duplex was designed with the use of RNAfold to cover both the editing region and the ECS region (Figure S3A). Binding of each oligo to the AZIN1 RNA duplex was examined by RNA electrophoretic mobility shift assay (REMSA) (Figure 3B; Figure S3B). We observed a strong band shift in the presence of ASO1, ASO3, ASO5, and ASO7, whereas very weak or no shift was detected upon the addition of ASO2, ASO4, ASO6, all 3 PNA, and 4 control ASOs (Figure 3B). Further, with increasing amounts of ASO1, 3, 5, or 7 added, a dose-dependent increase in the amount of shifted duplex probe was detected, confirming the binding capability of these ASOs to AZIN1 with sub-micromolar affinity (Figure S3). Because PNA are shorter than ASOs, further testing using a shortened AZIN1 probe showed that AS1 (12-mer) remains incapable of binding to the AZIN1 duplex, whereas DSP1 and DSP2 could bind through PNA-dsRNA triplex formation with a modest binding affinity (Figure S3B, micromolar).

Next, to examine whether the binding of each oligo to the AZIN1 duplex is sufficient to inhibit AZIN1 editing, ASO1, 3, 5, and 7 and control ASOs were used in in vitro editing assays. With the addition of ASO3, AZIN1 editing was completely abolished; compared to ASO3, ASO1 was slightly less effective but repressed editing from 75.7% to 2.4%; and ASO5 and ASO7 demonstrated minor and no inhibition, respectively (Figure 3D). All control ASOs except ASO-ctl1 were unable to inhibit AZIN1 editing in vitro. As seen in Figure S4, a weak binding of ASO-ctl1 to the AZIN1 duplex probe was observed when we added 10-fold more ASO-ctl1. We therefore excluded ASO-ctl1 from the following experiments. Surprisingly, even though ASO5 could directly target the editing site therefore excluded ASO-ctl1 from the following experiments. Surprised, even though ASO5 could directly target the editing site, we screened the basal editing level of AZIN1 among 15 cancer cell lines (9 HCC, 3 ESCC, and 3 NSCLC). AZIN1 editing was only detected in an ESCC line, KYSE510, and a NSCLC line, H358 (Figure S5A). Unexpectedly, all 7 ASOs that target the editing region (ASO1, 1.1, 1.2, 1.3, 5, 6, and 7) led to skipping of exon 11 in KYSE510 and H358 cells (Figure 4B; Figures S5B and S5C), possibly due to the existence of splicing factor binding sites at the editing region predicted by SpliceAid 2.2 (Figure 4C). Notably, 3 ECS-targeting ASOs (ASO3.1, 3.2, and 3.3), but not the control ASOs, inhibited AZIN1 editing, without causing major changes in the splicing and expression of AZIN1 (Figures 4D–4F). It is likely that additional PS modification in 2’-O-Me-modified ASO3 increases the chemical stability, resulting in improved editing inhibition efficacies of ASO3.1, 3.2, and 3.3 in cells.

To ensure that the inhibitory effects of ASO3.1 and ASO3.2 on AZIN1 editing are indeed due to their binding to AZIN1 transcript in vivo, we confirmed that ASO could enter the nucleus (Figure 4G), as premRNA editing takes place in the nucleus. By performing RNA immunoprecipitation (RIP) followed by real-time quantitative PCR (qPCR) in HEK293T cells overexpressed with Flag-tagged ADAR1 (Flag-ADAR1) or Flag empty vector control (Flag-EV), we observed that the treatment of ASO3.1 or 3.2 significantly reduced the binding of ADAR1 to AZIN1 transcripts (Figure 4H). Of note, even though the sequence and modification patterns of ASO-ctl, ASO-ctl2, and ASO-ctl3 are not identical, all of them were unable to bind to AZIN1 and inhibit its editing (Figures 3B and 4F).

Altogether, our findings suggested that the editing region at exon 11 of AZIN1 pre-mRNA is non-targetable, whereas ASOs targeting the ECS could specifically and effectively inhibit ADAR1’s binding to AZIN1, leading to suppression of AZIN1 editing in cancer cells.

**ASO3.2 specifically inhibits cancer cell viability and proliferation**

We next studied whether ASO3.1 and ASO3.2 specifically inhibit cancer cell viability through repressing AZIN1 editing. To this end, in addition to KYSE510 and H358, an AZIN1 editing null ESCC cell line, KYSE180, was also included in the study. We observed that concentrations as indicated. (D) *In vitro* RNA editing analysis of AZIN1 transcripts transcribed from the FE minigene, after incubation with purified ADAR1 protein and 200 nM of the indicated ASO. Left: sequencing chromatograms illustrate editing of *in vitro* transcribed AZIN1 transcripts in the indicated samples. Percentage of editing is calculated as area of “G” peak over the total area of “A” and “G” peaks. Black arrow indicates the position of editing site. *, no editing detected. Right: data are presented in the bar chart as the mean ± SD of technical triplicates from a representative experiment of 3 independent experiments. The value shown on the top of each bar is the mean value. n.d., not detectable.
both ASO3.1 and ASO3.2 inhibited cell viability of KYSE510 and H358 with low half-maximal inhibitory concentration (IC_{50}) values (ASO3.1: K510 45.8 nM and H358 37.6 nM; ASO3.2: K510 62.3 nM and H358 48.4 nM), whereas they demonstrated much less effect on cell viability of KYSE180 (ASO3.1: 272 nM; ASO3.2: 723 nM) (Figure 5A). Of note, KYSE180 demonstrated ~2.6-fold lower sensitivity to ASO3.2 than ASO3.1, implying that ASO3.2 may confer a higher specificity to repress editing and cancer cell viability than ASO3.1 (Figure 5A). After confirming that there was no obvious difference in the effect on cancer cell viability among ASO-ctl, ASO-ctl2, and ASO-ctl3 (Figure S6), we chose ASO-ctl as the control ASO for the following experiments. To further confirm the specific inhibitory effect of ASO3.2, 3 cell lines were used for cell viability and foci formation assays. We found that ASO3.2 only inhibited cell viability of KYSE510 and H358, but not KYSE180 (Figures 5B and 5C). In addition to cancer cells, we also did not observe any obvious effect on the viability of healthy human hepatocytes, which are free of AZIN1 editing (Figure 5B; Figure S5A).

As reported by us previously, AZIN1^{S367G} could enhance G1/S transition and increase the protein expression of oncoproteins ODC and CCND1.\(^5\) Cell cycle analysis showed that upon treatment with ASO3.2, KYSE510 and H358 cells, but not KYSE180 cells, demonstrated an increase in the percentage of sub-G1 phase (apoptotic cells). Because ~20% of ASO3.2-treated KYSE510 and H358 cells entered apoptosis from G1 or other phase of the cell cycle, we did not observe a clear attenuation of G1/S transition compared to cells treated with ASO-ctl or ASO3 (Figures 5D and 5E). However, a reduction in CCND1 and ODC protein expression was detected in ASO3.2-treated KYSE510 but not KYSE180 cells (Figure S7), suggesting that ASO3.2 could specifically inhibit AZIN1 editing in cancer cells, leading to reduced cell viability and proliferation.

**ASO3.2 effectively inhibits tumor incidence and growth in vivo**

We next investigated the effect of ASO3.2 on tumor incidence and growth. KYSE510 cells were pre-treated with ASO-ctl or ASO3.2, followed by subcutaneous injection into dorsal flanks of mice. Tumor incidence rate of mice injected with ASO3.2-pre-treated cells was lower than the control group (Figure 6A), and tumors derived from ASO-ctl-pre-treated cells grew significantly faster than tumors originated from ASO3.2-pre-treated cells (Figure 6B). Suppression of AZIN1 editing was also confirmed in xenograft tumors derived from ASO3.2-pre-treated cells at end point (Figure 6C).

We further investigated the impact of ASO3.2 on tumor growth by intratumoral injection. Extracellular vesicles (EVs) are small membrane vesicles released from different types of cells and increasingly being recognized as natural RNA carriers and novel drug delivery vehicles.\(^33,34\) In this study, ASO3.2 or ASO-ctl was loaded into EVs derived from human red blood cells (RBCs) (ASO-RBCEVs), an ideal source of EVs with promising properties for RNA drug delivery.\(^35\) To test the cellular uptake of ASO-RBCEVs, Cy5-labeled ASO3.2 was loaded into RBCEVs that were labeled with carboxyfluorescein succinimidyl ester (CFSE), which fluoresces only in the presence of esterase when they are either loaded into RBCEVs or internalized into cells.\(^36\) Upon treatment with Cy5-ASO loaded into CFSE-RBCEVs, 100% of KYSE510 cells were Cy5 and CFSE double positive, indicating a very high cellular uptake of ASO-RBCEVs (Figure 6D). Next, mice were injected with untreated KYSE510 cells subcutaneously for tumor development, followed by administration of ASO-RBCEVs or naked (unloaded) ASO intratumorally every 4 days (Figure 6E). Notably, intratumoral injection of ASO3.2-RBCEVs significantly inhibited tumor growth, whereas no obvious difference was observed in tumor growth between mice treated with naked ASO-ctl and ASO3.2 (Figure 6F). Altogether, ASO3.2 could effectively inhibit tumor incidence and growth in vivo.

**ASO3.2 specifically inhibits cell viability of cells derived from HCC patient-derived xenografts**

To evaluate whether ASO3.2 could be a promising RNA therapeutics for cancer treatment, we went on to examine the effect of ASO3.2 on HCC PDX-derived cells. We examined the editing level of AZIN1 in several PDX cells and identified PDX1 and PDX11 as AZIN1 editing-positive and -negative PDX lines, respectively (Figure 6G). As seen in
Figure 6H, PDX1 demonstrated significantly reduced cell viability upon ASO3.2 treatment, whereas no obvious changes in cell viability were observed in PDX11 cells.

DISCUSSION
As reported by us and others in the past decade, dysregulated A-to-I editing is implicated in the pathogenesis of various cancers, such as breast cancer, glioma, multiple myeloma (MM), chronic myeloid leukemia, HCC, CRC, gastric cancer, and ESCC. Transcripts aberrantly edited by ADARs in cancer tissues such as AZIN1, GLI1 (glioma-associated oncogene 1), and DHFR (dihydrofolate reductase) remarkably contribute to cancer progression and metastasis. Unlike DNA editing, genetic information manipulated by RNA editing is reversible and tunable. Since ADAR1 has multiple functions that are critical for normal development such as hematopoiesis and organ development, simply modulating the expression of ADARs may cause considerable off-target effects. An alternative strategy may be to disrupt ADAR enzymes to specific editing sites at target transcripts.

We were the first to report that ADAR1-mediated editing at codon 367 of AZIN1 produces a much more aggressive form, AZIN1S367G, than the wild-type AZIN1, and the increased level of AZIN1S367G predisposes to HCC and ESCC. Later on, several groups reported that increased AZIN1 editing is implicated in other types of cancer such as NSCLC and CRC. All these findings suggest that targeting AZIN1 editing holds great promise for cancer treatment. To achieve the goal of repressing cancer-associated AZIN1 editing in cells and tissues, ASOs were chosen, due to the facts that (1) they are smallest (~15–25-mer) among all RNA-based therapeutics; (2) they have high ability to enter cells through liposome formulation or conjugation with cell-penetrating moieties; and (3) they can be modified to have adequate stability and target binding affinity as well as less immunogenicity.

Like splice-switching and translation-inhibiting ASOs, these editing-inhibiting ASOs must bind to target pre-mRNA transcripts in the nucleus, without eliciting RNase H activation or RNAi. The 2’-O-methyl group that substitutes the hydrogen at the 2’ of RNA (2’-O-Me-RNA) exists in physiological conditions. This modification increases both nuclease resistance and target binding affinity (by 0.9°C modification), while it decreases native immune reactions. To target A-to-I RNA editing, it is very important to identify the ECS and understand the secondary structure of the target RNA (i.e., which nucleotide is in a base pair, hairpin loop, or bulge). This information will be very helpful to designing ASOs targeting either the editing region or the ECS, without disturbing the potential binding of RNA-binding proteins (RBP)s (e.g., splicing factors). In addition, a properly designed ASO-based editing inhibitor is expected to invade the secondary structure to prevent editing. In this study, we uncover that the 3’ end sequence of AZIN1 exon 12 forms dsRNA with the editing region, allowing us to design and synthesize multiple 2’-O-Me/PS-modified ASOs and PNAs that target either the editing region or the ECS. We observed that (1) ASO1 or ASO3 could either substantially inhibit or completely abolish AZIN1 editing in vitro, respectively, (2) ASO2, 4, and 6 were incapable of binding to AZIN1 duplex, and (3) although ASO7 could bind to AZIN1, it still failed to inhibit editing in vitro. All these observations suggest that the 5’ upstream sequence of the editing site and the 3’ downstream sequence of the ECS may favor the binding of ASO to AZIN1 and the consequent inhibition of ADAR1 binding. This information may be useful for future understanding of ADAR1 substrate binding and deamination. However, whether ASO1 and ASO3 inhibit AZIN1 editing by a strand-invasion mechanism remains for our investigation.

Moreover, it has been reported that PNAs incorporating modified nucleobase such as thio-pseudouracil (L) and guanidine-modified 5-methyl cytosine (Q) can selectively bind to dsRNAs over single-stranded RNAs and dsDNAs in a sequence-specific manner. Besides, PNAs have a neutral peptide-like backbone, are chemically stable and resistant to nucleases, and offer enhanced specificity of RNA sequence and structure recognition. In this study, we also tested the inhibitory effect of PNAs on AZIN1 editing. As ASO4 (20-mer) was unable to bind to AZIN1 duplex, it is not surprising that the antisense PNA ASP1 (12-mer) was also incapable of binding to AZIN1. Although dsRNA-binding PNAs DSP1 and DSP2 could bind to AZIN1 with modest affinity, they failed to inhibit editing at nanomolar concentrations, possibly attributed to the insufficient blockage of ADAR1-AZIN1 dsRNA interaction by DSP1 (10-mer) or DSP2 (8-mer) due to their rather short length. All these observations imply that a relatively long and chemically stable ASO (e.g., complete 2’-O-Me sugar ring modification in combination with partial PS backbone modification) may be an optimal chemistry for RNA editing inhibition. However, it is interesting to test whether additional modifications (e.g., LNA) of 2’-O-Me or 2’-O-Me/PS-modified ASOs would further improve the editing inhibitory potency in vitro and in vivo.
Figure 6. ASO3.2 specifically inhibits tumor incidence and growth in vivo
(A) Left: schema of subcutaneous injection of ASO-pretreated KYSE510 cells into mice. ASO3.2- or ASO-ctl-pre-treated cells were injected into right or left dorsal flank of mice, respectively. Right: cumulative tumor incidence curves of mice injected with the indicated pre-treated cells (100 nM ASO3.2 or ASO-ctl for 48 h), estimated by the Kaplan-Meier method. (B) Representative tumors derived from pre-treated KYSE510 cells as described above 6 weeks after subcutaneous injection (n = 6 mice per group). Growth curves of tumors derived from the indicated cells over a period of 6 weeks. Data are presented as the mean ± SD. (C) Sequencing chromatograms illustrate editing of AZIN1 in 2 representative pairs of xenograft tumors. (D) FACS analysis of Cy5 versus CFSE fluorescence in KYSE510 cells treated with 50 nM unlabeled or Cy5-labeled ASO-ctl/ASO3.2 that were loaded into the CFSE-labeled RBCEVs. Percentages of Cy5/CFSE double-positive cells (Q2) are highlighted in red. (E) Schema of RBCEV-based ASO delivery to mice bearing cancer xenografts. (F) Left: representative tumors derived from KYSE510 cells after receiving i.t. injection of ASO3.2-RBCEVs or ASO-ctl-RBCEVs every 4 days (n = 6 mice per group). For each injection, a total of 1 μg of ASO was loaded into 50 μg of RBCEVs and resuspended in 20 μL of PBS. Right: representative tumors after receiving multiple i.t. injections of naked (unloaded) ASO3.2 or ASO-ctl. The same experimental procedures were conducted as described on left. Growth curves of each group of tumors over a period of 7 weeks. Data are presented as the mean ± SD. Black arrow indicates each injection. (G) Sequencing chromatograms illustrate editing of AZIN1 in PDX1 and PDX11 cells. (H) Cell viability of PDX1 or PDX11 cells was measured by CTG assays after treatment with ASO3.2 or ASO-ctl by Lipofectamine transfection for 48 h. Data are presented as the mean ± SD of 4 replicates from a representative experiment of 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 determined by unpaired, two-tailed Student’s t test.
editing region led to a substantial exon 11 skipping, probably due to the blockage of splicing regulators (e.g., SRSF1, SRSF3, SRSF6) to the editing region, suggesting that the editing region is not targetable. Moreover, even though 2′-O-Me-modified ASO3 that targets the ECS could completely abolish AZIN1 editing \textit{in vitro}, only ASO3.1 and ASO3.2, which have further complete and partial PS modification respectively, could effectively abolish \textit{AZIN1} editing in cancer cells. This could be due to the advantages of PS modification such as strong resistance to endo- and exonuclease digestion, increased serum stability, and reduced renal clearance. It is also known that PS modification may cause non-specific binding to plasma proteins and other nucleotide sequences. This may explain why the fully PS-modified ASO3.1 demonstrated less specificity of inhibition of \textit{AZIN1} editing and tumor cell viability than the partially PS-modified ASO3.2. We further confirmed that ASO3.2 specifically inhibited cell viability of \textit{AZIN1}\textsuperscript{5367G}-expressing cancer cells and cells derived from HCC PDXs but not \textit{AZIN1}\textsuperscript{5367G-null} cancer cells, PDX lines, and normal hepatocytes. Further, in the pre-treated xenograft tumor model, ASO3.2 inhibited tumor incidence and growth. This observation was also supported by the intratumoral injection model in which we delivered ASO3.2 into tumor cells with an RBCEV-based delivery approach and found that intratumoral injection of ASO3.2 that was loaded into RBCEVs, but not naked (unloaded) ASO3.2, significantly suppressed tumor growth.

Until now, 4 ASOs have been clinically approved, all of which are chemically modified. Two recently FDA-approved switching-splint ASOs, eteplisen and nusinersen, entered the clinic in 2016/2017, raising the enthusiasm in the field for developing ASO therapeutics for treating diseases. In this study, we show that ASO-mediated inhibition of \textit{AZIN1} editing effectively suppresses tumor incidence and growth, suggesting that many cancer patients, particularly those demonstrating high editing level of \textit{AZIN1} in tumors, may benefit from this \textit{AZIN1}-targeting, ASO-based therapeutics.

MATERIALS AND METHODS

Cell lines

All cell lines were maintained in RPMI-1640 medium (Biowest) supplemented with 10% fetal bovine serum (FBS) (Biowest). All cell lines used in this study were regularly authenticated by morphological observation and tested for mycoplasma contamination. PDX lines were cultured in DMEM-F12 (Biowest) and supplemented with 1:50 B27 supplement without vitamin A (Thermo Fisher), 1:100 insulin-transferrin-selenium supplement (Gibco), 1 mM EDTA (Ambion), Triton X-100 (Sigma-Aldrich), and 10 mM nicotinamide (Sigma-Aldrich), 10 mM recombinant human (Leu15)-gastrin I (Sigma-Aldrich), 25 ng/mL recombinant human hepatocyte growth factor (HGF) (Abcam), 50 ng/mL recombinant human epidermal growth factor (EGF) (Abcam), 50 ng/mL recombinant human basic fibroblast growth factor (bFGF) (Abcam), 5 μg/mL heparin (Sigma-Aldrich), and 10 ng/mL recombinant human FGF-10 (Abcam). All cancer cell lines and PDX lines were incubated at 37°C in a humidified incubator containing 5% CO₂.

Normal human hepatocytes

HepaCur human hepatocytes are isolated from the perfused livers of humanized FRG KO mice. The freshly isolated hepatocytes are guaranteed to be ≥ 95% human and have a viability of ≥ 70%. Isolated hepatocytes were cultured in HypoThermosol FRS (BioLife Solutions, catalog # 101373), which is an optimized hypothermic preservation medium. Prior to plating, HypoThermosol FRS was changed to HMM (HepaCur Maintenance Medium, catalog # HMM500), according to the manufacturer’s protocol. Subsequently, 1 × 10⁶ cells were plated into each well of a 96-well plate and treated with ASOs on the same day. The cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

RNA extraction, cDNA synthesis, quantitative PCR, and Sanger sequencing

Total RNA was extracted with the RNasy Mini Kit (QIAGEN), according to the manufacturer’s protocol. cDNA synthesis was conducted with the Advantage Reverse Transcription Kit (Clontech Laboratories), following the manufacturer’s protocol. Real-time qPCR was performed with GoTaq DNA polymerase (Promega) on the QuantStudio 5 Real-Time PCR System (Applied Biosystems). The relative expression of \textit{AZIN1} or \textit{ADAR1} (defined as “relative expression”) is given as 2⁻ΔCT (ΔCT = CT(AZIN1/ADAR1) – C(T(β-actin)) and normalized to the relative expression that was detected in the corresponding control cells, which was defined as 1.0. Semiquantitative PCR was done with the FastStart Taq Kit (Roche), following the manufacturer’s protocol. Purified PCR amplions were identified by Sanger sequencing. ImageJ was used to calculate the percentage of A-to-I(G) editing. The percentage of editing is calculated as the area of “A” peak over the total area of “A” and “G” peaks. Sequences of primers are listed in Table S2.

Generation of minigene constructs

To clone \textit{AZIN1} sequences for the pRK7 or pcDNA3.1 minigene construction, placental DNA (Sigma-Aldrich) was used for PCR using PrimeSTAR Max DNA Polymerase (Takara), following the manufacturer’s protocol. The KAPA HiFi HotStart PCR Kit (KAPA Biosystems) was utilized to introduce internal deletion or point mutations. Sequences of primers used for cloning are listed in Table S2.

\textbf{In vitro RNA editing assay}

First, forced overexpression of Flag-tagged ADAR1 protein was performed by transfecting Flag-ADAR1 plasmid into HEK293T cells. Cells were harvested 48 h after transfection and lysed in lysis buffer containing 50 mM Tris-Cl pH 7.5 (Ambion), 150 mM NaCl (Ambion), 1 mM EDTA (Ambion), Triton X-100 (Sigma-Aldrich), and 1× cComplete EDTA-free Protease Inhibitor Cocktail (Roche). Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) were used for immunoprecipitation of cell lysate to obtain Flag-ADAR1 proteins. 100 μg/mL Flag elution buffer was obtained by dissolving 3× FLAG peptide (Sigma) in Tris-buffered saline (TBS) containing 50 mM Tris-Cl pH 7.4 and 150 mM NaCl. Flag elution buffer was used to elute the proteins from the magnetic beads. Eluant was stored at −80°C until further use. \textit{In vitro} transcription of the minigene constructs was done with the RiboMAX Large Scale RNA Production System-SP6.
Sequences of probes are listed in Table S3.

To test the editing inhibitory effect of ASOs in vitro, ASOs were incubated with in vitro transcribed RNA transcripts prior to the addition of purified ADAR1 protein. PCR amplification was then conducted, and purified PCR products were sent for Sanger sequencing. Sequences of primers used for in vitro editing analysis are listed in Table S2.

RNA electrophoretic mobility shift assay

**Binding of each oligo to the AZIN1 RNA duplex probe**

The 86-nt AZIN1 RNA duplex probe was transcribed in vitro with RibobMax Large Scale RNA Production System-T7 (Promega), following the manufacturer’s protocol. Next, 50 pmol of the RNA probe was incubated at 37°C for 30 min with rSAP (shrimp alkaline phosphatase) (NEB) to dephosphorylate the RNA. EDTA (0.8 μL, 250 mM) was added and incubated at 65°C for 20 min to heat inactivate rSAP. The mixture was then incubated with 1 μL of 100 mM MgCl2 with T4 PNK (polynucleotide kinase) (NEB) and ATP, [γ-32P] (PerkinElmer) at 37°C for 30 min. The mixture was then heated to 95°C to denature the duplex and was left to anneal slowly to room temperature, after which 80 μL of distilled water was added and transferred to an Illustra MicroSpin G-25 Column (GE Healthcare) for purification. LightShift Chemiluminescent RNA EMSA Kit (Thermo Scientific) 10× REMSA Binding Buffer (100 mM HEPES pH 7.3, 200 mM KCl, 10 mM MgCl2, 10 mM DTT) was used to incubate the samples. Samples were mixed with 1 μL of RNA duplex (with a final concentration of 25 nM) and the respective oligonucleotides and incubated for 30 min. TBE (Tris-borate EDTA) gel was pre-run before samples were added. The gel was dried and then exposed to BioMax Light Film (Carestream, Sigma-Aldrich) and developed.

**Binding of each PNA to the truncated AZIN1 RNA duplex probe**

Both strands (ECS-s and ES-s) were added together and slowly cooled from 95°C to room temperature to form the truncated RNA duplex before annealing of the PNAs at 40°C for 10 min. Both steps were carried out in an incubation buffer of 200 mM NaCl, 0.5 mM EDTA, and 20 mM HEPES, pH 7.5. After annealing of the PNA, the samples were allowed to cool to room temperature before incubation at 4°C overnight. The gel was run at constant voltage of 250 V for 5 h in a running buffer of 1× TBE, pH 8.3. The gel was then stained in ethidium bromide for 30 min before it was imaged with the Typhoon Trio Variable Mode Imager.

Sequences of probes are listed in Table S3.

**ASO treatment**

All ASOs were purchased from Integrated DNA Technologies (IDT). PNAs (ASP1 and DSP1 and DSP2) were synthesized and purified according to the protocol reported previously.19 Cells were seeded the day before treatment to achieve 50% confluence on the day of treatment. Cells were then treated (transfected) with ASOs that were diluted in Opti-MEM to the desired concentration by Lipofectamine 2000. The subsequent analysis was conducted 48 h after the treatment with ASO. Three independent experiments were carried out, each with three technical replicates conducted.

**RNA immunoprecipitation**

HEK293 cells were either untreated or treated with ASO-ctl or ASO3.2 for 6 h, followed by transfection with 4 μg of Flag empty vector (Flag-EV) or Flag-tagged ADAR1 (Flag-ADAR1). At 48 h after transfection, cells were collected and lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with complete protease inhibitor (Roche) and SUPERase In (Invitrogen). Lysate was then incubated with anti-FLAG M2 magnetic beads (Sigma) overnight at 4°C with rotation, followed by washing 6 times with 1× TBS buffer (50 mM Tris and 150 mM NaCl). A total of 10% of beads was used for protein elution, and the rest was used for RNA extraction using the RNeasy miniprep kit (QIAGEN). Extracted RNA was reverse transcribed with the Advantage RT-for-PCR Kit (Clontech) with oligo dT, and subsequently qPCR was performed. %Input = 2^-ΔCt × 100; ΔCt = CtREP – [Ctinput – dilution factor]. Fold enrichment was calculated by normalizing the %Input (each sample) to %Input (Flag-EV).”

**Cell viability assay**

The CellTiter-Glo Luminescent Cell Viability (CTG) Assay (Promega) was used to measure cell viability after cells were treated with ASOs. Cells were seeded and treated in 96-well clear flat-bottom plates (Corning) for 2 days prior to the addition of the CTG assay reagent. A total of 100 μL of cell lysate was transferred to a 96-well white flat-bottom plate (Corning). The GloMax Discover Microplate Reader (Promega) was used to read the intensity of luminescence.

**Western blot analysis**

Protein lysates were prepared with Radioimmunoprecipitation assay (RIPA) buffer (Sigma) supplemented with 1× cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and quantified by Bradford assay (Bio-Rad). Protein lysates were then separated by 8%–10% SDS-PAGE, followed by incubation with primary antibodies (1:1,000 dilution) overnight at 4°C and incubation with secondary antibodies (1:10,000 dilution) at room temperature for 1 h. Primary antibodies used are anti-ADAR1 (Abcam, ab88574), anti-AZIN1 (Proteintech, 11548-1-AP), anti-β-actin (1:10,000; Santa Cruz Biotechnology, sc-47778), anti-Flag-horseradish peroxidase (1:10,000; Sigma-Aldrich, A8592), anti-ODC (Proteintech, 28728-1-AP), and anti-CCND1 (Proteintech, 60186-1- Ig).

**Foci formation assay**

For foci formation assay, cells were seeded to obtain 50% confluency prior to the treatment of ASO. Cells were stained with crystal violet (Sigma-Aldrich) 48 h after treatment.
Cell cycle analysis by PI staining and fluorescence-activated cell sorting

Cells were treated with ASOs for 48 h prior to the cell cycle analysis. After the treatment, cells were fixed with 70% ethanol in −20°C overnight. After washing with phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl), cells were resuspended in 1 mL of staining solution containing 50 μg/mL propidium iodide (PI) (Invitrogen) and 0.5 μL of 10 mg/mL RNase (Thermo Scientific) and incubated for 1 h at 37°C. Stained cells were analyzed on the LSRII (BD Biosciences), and the results were analyzed on FACSDiva Software (BD Biosciences).

Loading of ASO into RBCEVs

Blood samples were obtained from healthy donors by Hong Kong Red Cross, and EVs were produced from RBCs according to our established protocol.35 ASOs were loaded into RBCEVs at a ratio of 1 to 50 with ExoFect Transfection Reagent (System Biosciences) according to the manufacturer’s protocol. RBCEVs were washed twice with PBS thrice for 5 min each. The coverslips were mounted with methanol for 10 min at room temperature. Fixed cells were stained with PI (Invitrogen) and 0.5 μL of 10 mg/mL RNase (Thermo Scientific) and incubated for 1 h at 37°C. Stained cells were analyzed on the LSRII (BD Biosciences), and the results were analyzed on FACSDiva Software (BD Biosciences).

Labeling RBCEVs with CFSE

A total of 200 μg of ASO-loaded RBCEVs was incubated with 400 μL of 10 μM CFSE at 37°C for 2 h. A total of 0.5 mL of CFSE-labeled RBCEVs was loaded onto a prepacked qEV-original size exclusion column (Izon Science, Christchurch, New Zealand) and eluted with PBS in 40 fractions (0.5 mL/fraction). Fractions 7 to 11 were combined and centrifuged at 21,000 × g for 30 min at 4°C. The supernatant was removed, and the RBCEV pellet was washed twice with PBS, resuspended, and quantified with a NanoDrop spectrophotometer (Thermo Fisher).

Fluorescence imaging

Cells were cultured on coverslips for 24 h and treated with 100 nM unlabeled or 6-carboxyfluorescein (6-FAM)-labeled ASO3.2. At 48 h after treatment, cells were washed with PBS before fixation with methanol for 10 min at room temperature. Fixed cells were washed with PBS thrice for 5 min each. The coverslips were mounted onto slides with SlowFade Gold AntiFade Mountant with DAPI (Thermo Fisher Scientific) and viewed under a Zeiss Axio Imager M2 microscope.

Cellular uptake of ASOs by flow cytometry analysis

KYSE510 cells were treated with either Cy5-labeled or unlabeled ASO-ctl/ASO3.2 that were loaded into CFSE-labeled RBCEVs (at a ratio of 1 to 50). After 48 h, cells were trypsinized, washed, and resuspended in the fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.5% FBS). Flow cytometry of cells was performed with LSRII (BD Biosciences) and analyzed on FACSDiva Software (BD Biosciences). The cells were initially gated based on FSC-A and SSC-A to exclude the debris and dead cells (low FSC-A). The cells were further gated based on FSC-width versus FSC-height, to exclude doublets and aggregates. Subsequently, the CFSE-positive cells were gated in fluorescein isothiocyanate (FITC). Cy5-positive cells were gated with Cy5.

In vivo tumorigenicity assays

Pre-treatment model

KYSE510 cells were pre-treated with 100 nM ASO3.2 and ASO-ctl using Lipofectamine 2000 (Invitrogen) for 48 h, followed by the subcutaneous injection of 4 × 10⁶ pre-treated cells into left and right dorsal flanks of 4- to 6-week-old NOD scid gamma (NSG) mice (n = 6 mice per group). Tumor growth was monitored by measuring tumor length (L) and width (W) at indicated time points. Tumor volume was calculated by the formula V = 0.5 × L × W². All animal experiments were approved by and performed in accordance with the Institutional Animal Care and Use Committees of National University of Singapore (NUS, Singapore).

Intratumoral injection model

A total of 2 × 10⁶ KYSE510 cells were injected subcutaneously to the right and left flanks of 4- to 6-week-old NSG mice for tumor development. When tumors were visible (~1 mm in diameter), mice were divided into two groups (6 mice per group) for multiple intratumoral (i.t.) injection of ASO-loaded RBCEVs (Group 1: RBCEVs-based delivery) or naked ASO (Group 2: naked ASO) every 4 days for 7 weeks. For each injection of ASO-loaded RBCEVs per tumor, a total of 1 μg of ASO was loaded into 50 μg of RBCEVs and resuspended in 20 μL of PBS. For each injection of naked ASO per tumor, a total of 13.5 μg of ASOs (ASO-ctl or ASO3.2) was dissolved in 20 μL of PBS. Tumor growth was monitored by measuring tumor length (L) and width (W) at indicated time points. Tumor volume was calculated by the formula V = 0.5 × L × W². All animal experiments were approved by and performed in accordance with the Institutional Animal Care and Use Committees of National University of Singapore (NUS, Singapore).

Statistical analysis

Unpaired, two-tailed Student’s t test was used for statistical analysis of changes in cell viability and tumor growth rate between the control and treatment groups. For all figures: *p < 0.05; **p < 0.01; ***p < 0.001.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.05.008.

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AUTHOR CONTRIBUTIONS
L.C. conceived and supervised the study. L.C. and D.J.T.T. designed and performed the experiments. J.H., Y.S., T.H.M.C., S.J.T., W.L.G., L.C. and D.J.T.T. designed materials. L.C. and D.J.T.T. wrote the manuscript. M.T.N.L., and S.J.T. provided insightful suggestions and experimental materials. L.C. and D.J.T.T. wrote the manuscript.

DECLARATION OF INTERESTS
L.C., D.J.T.T., and G.C. are inventors in a patent application under the Patent Cooperation Treaty (PCT) (PCT/SG2020/050380) that is related to the work that is described in this manuscript. The other authors declare no competing interests.

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