Targeting ADAR1 with a Novel Small-Molecule for the Treatment of Prostate Cancer

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Abstract

Despite initial response to androgen signaling therapy, most prostate cancer (PCa) patients eventually relapse and remain incurable. ADAR1-mediated A-to-G editing plays oncogenic roles in various tumors. However, the specific function of ADAR1 and the global RNA edited targets governing PCa progression remain underexplored. Here, we demonstrate that highly expressed ADAR1 as a crucial oncogenic target in PCa, and develop a novel small-molecule ADAR1 inhibitor ZYS-1 with significant anti-tumor efficacy and favorable safety profile. Either depletion or pharmacological inhibition of ADAR1 dramatically suppress PCa growth, inhibit metastasis, and potentiate immune response. We further reveal that the translation of MTDH is repressed by ADAR1 in an editing-dependent manner, which drives cell proliferation and invasion in PCa. Collectively, these results shed light on ADAR1 as a novel druggable target in PCa therapy and highlight the widespread applicability of ADAR1 inhibitors for a broad spectrum of malignancies.

Significance

ADAR1-mediated A-to-G editing is essential for PCa initiation and progression. Targeting ADAR1 with drugs is a promising therapeutic strategy. ZYS-1, an inhibitor of ADAR1, with strong anti-tumor efficacy and favorable safety in PCa models, and holds broad therapeutic effects against a wide panel of cancers.

Introduction

Prostate cancer (PCa) is the most frequently occurring malignancy and the second leading cause of cancer-related mortality among men in western countries (1–3), with an estimated over 1 million new cases and nearly 500,000 deaths expected to occur globally in 2020 (2). Primary PCa patients who are diagnosed with early precursor lesions are known as prostatic intraepithelial neoplasia (PIN), and treatments for these organ-confined PCa include surgery and brachytherapy (4, 5). PCa tumor growth is mainly dependent upon sustaining activated androgen receptor (AR) signaling. The backbone of PCa treatments includes androgen-deprivation therapy (ADT) and AR antagonists by blocking androgen production and/or function (6, 7). Despite the initial response, most patients eventually relapse to a therapy-resistant stage, which leads to metastatic castration-resistant prostate cancer (mCRPC) that remains incurable at the current time (7, 8). In addition to the sustained role of AR, there has been an urgent need to identify specific and novel drug targets. As such, the global understanding of the intricate mechanisms involved in these oncogenic processes is of critical importance for the development of novel therapeutic strategies (9–11).

Gene mutations are important in cancer onset and aggressiveness as well as in the generation of tumor heterogeneity. Besides alterations at the DNA level, RNA-based abnormalities have been emerging as important players in cancer pathogenesis (12, 13). RNA editing is a common form of epitranscriptomic mechanism that modifies the sequence of RNA transcripts without a concomitant change in its genomic blueprints (12, 14). High-throughput genome-wide studies suggest that editing occurs in more than 85%

of human RNA noncoding and/or coding regions (15). As for physiological consequences, RNA editing events may lead to expanding diversity in gene products and gene regulatory mechanisms by non-synonymous amino acid mutations, alternative RNA splicing, or modification of targeted RNAs (16, 17), thereby altering their stability, translational efficiency, and degradation (18). In humans, deamination of adenosine-to-inosine (A-to-I) within double-stranded RNA (dsRNA), mediated by adenosine deaminases acting on RNA (ADAR) family enzymes, is considered the most frequent type of RNA editing that impacts the benefit of cancer progress (15, 19, 20). Since inosine (I) is recognized as guanosine (G) by the translational machinery, this process may result in recoding transcripts and thus plays a vital role in cancer initiation and aggressiveness (21, 22). Three *ADAR* genes are encoded in mammalian cells, ADAR1 and ADAR2 are the two catalytically active enzymes, whereas ADAR3 is enzymatically inactive (23, 24).

The first cloned and discovered A-to-I dsRNA deaminase was ADAR1, which resides at chromosome 1q21, a region that is frequently amplified in cancer. There are two isoforms of ADAR1, the constitutively expressed N-truncated-p110 is mainly localized to the nucleus, where the interferon (IFN)-inducible full length-p150 is mainly cytoplasmic (25, 26). Increasing pieces of evidence have revealed a close association between majority RNA-editing events and elevated ADAR1 expression in various malignancies, thereby promoting tumorigenesis, therapeutic resistance and poor survival (27, 28). Crucially, several articles have reported higher RNA editing levels in prostate cancer (29–31). *In silico* analyses demonstrated that AR transcripts within more RNA editing events in PCa cell lines, meanwhile these cells also expressed high levels of ADAR1 (32). In addition, a recent study has suggested that elevated ADAR1 expression causes aberrant editing of the dominant-negative oncogene prostate cancer antigen 3 (PCA3) to the pathogenesis of PCa, whereby higher A-to-I editing of lncRNA PCA3 could upregulate expression of itself, and then downregulates the antisense tumor suppressor gene prune homolog 2 (PRUNE2) level, thereby, promoting PCa cell growth and metastasis (33). Despite PCA3 is a novel target of ADAR1, the significance of ADAR1 and the landscape of RNA-edited targets in PCa remain unanswered. Therefore, further identification of these aberrant targeted transcripts and investigation of the regulatory mechanisms can better enhance our understanding of PCa carcinogenesis.

It has been reported that loss of ADAR1 compromises tumor growth and metastasis in many cancer types (27). More importantly, emerging studies have shown that inhibition of ADAR1 in tumors not only potentiates the efficacy of epigenetic therapy, but also sensitizes tumors to immunotherapy and overcomes resistance to immune checkpoint blockade, which is beneficial for cancer therapy (34–36). Taken together, these studies suggest that ADAR1 maybe a new potential PCa therapeutic target, and exploitation of innovative targeted therapeutics against ADAR1 is warranted. Although to date, 8-azaadenosine (8-aza) has been considered as a potential ADAR1 inhibitor (37), there was not yet strong evidence suggesting its direct binding and inhibition with ADAR1 protein, and simultaneously, the action mode and therapeutic activity have not been adequately evaluated. Thus, there is still an urgent need to discover druggable inhibitors against ADAR1 to treat PCa and other cancers.

Herein, we carried out detailed investigation into RNA editing in PCa and the first identification of landscape of A-to-G RNA edited targets through systematic assays. Our results demonstrated that ADAR1 but not ADAR2 is abundantly expressed in prostate cancer compared with adjacent non-tumor tissues and associated with poor clinical outcomes. Furthermore, ADAR1 depletion inhibited PCa cells growth and metastasis *in vitro*, whereas overexpression had the opposite effects. Most importantly, loss of ADAR1 increased abundance of IFN- γ and CD8⁺ T cells infiltration *in vivo*. In addition, we identified metadherin (MTDH) as a crucial edited target of ADAR1 in PCa and provided a new insight that this RNA epitranscriptomic modification regulates MTDH protein levels through the translation modulation mechanism, which relied on the catalytic function of ADAR1. Rescue results showed that ADAR1/MTDH axis mediates tumor initiation and aggressiveness, which indicated the existence of a new pro-tumorigenesis pathway in PCa. To this end, using a series of structure-based virtual screening and validation assays, we further developed a novel small-molecule ADAR1 inhibitor, ZYS-1, which directly bind to ADAR1 and inhibit its deaminase activity with an IC₅₀ of 0.866 μ M. Consistent with ADAR1 knockdown, ZYS-1 not only strongly inhibited PCa cells proliferation and metastasis *in vitro*, but also dramatically suppressed tumor growth in mice with favorable safety profiles, and enhanced anti-tumor immunity, hopefully holding synergetic efficacy combining with immunotherapy. Collectively, our study suggests profound therapeutic effects and novel mechanism insights of targeting ADAR1 in PCa therapy, highlighting ADAR1 as a novel druggable target with broad potential for cancer therapy.

Results

Highly expressed ADAR1 is a favorable prognostic marker in prostate cancer

Since the involvement and role of ADAR1 in prostate cancer have not been dissected systematically, we first queried whether the expression of ADAR1 is high and associated with A-to-G editing level in PCa patients. We collected three pairs of matched tumor and normal tissues and performed RNA-sequencing to acquire their global RNA editing profile. There were 1949 genes with A-to-G editing frequency difference > 0 (Tumor vs Normal group, labeled in red), and 1543 genes with difference < 0 (labeled in blue) (Fig. 1A; Supplementary Table S4). ADAR1 expression level was significantly higher in tumor (Fig. 1I; Supplementary Fig. S1D), while ADAR2 expression was low compared to ADAR1 (data not shown) and was in a promiscuous pattern between tumor and normal tissues (Supplementary Fig. S1F). We next selected 16 patients from a previous reported TCGA PCa patients cohort (n = 31; ref. 30) according to their A-to-G editing level higher in tumor other than normal tissue (Supplementary Fig. S1A). This increase appears to be caused by higher *ADAR1* expression in tumor rather than *ADAR2* (Supplementary Fig. S1B and S1C). Higher *ADAR1* expression in PCa was again found in another TCGA cohort (n = 51; Fig. 1B). Interestingly, aged, high-risk and high Gleason grade patients had a higher *ADAR1* expression signature, which indicated worse clinical prognosis in patients with higher *ADAR1* expression (Fig. 1C-E). Notably, these patients' relapse-free survival (RFS) was significantly worse than those with low expression (Fig. 1F). Consistently, a tissue microarray from 38 PCa patients staining with ADAR1

antibody showed that ADAR1 protein intensity score in tumor was significantly ($P = 0.004$) higher than in adjacent normal tissue (Fig. 1G and H), and ADAR1 intensity score was apparently stronger in high Gleason grade patients (Fig. 1G, bottom). Furthermore, five prostate cancer cell lines were all detected to have a high level of ADAR1 protein (Fig. 1J; Supplementary S1E). Previous study has indicated loss of ADAR1 regulates tumor microenvironment (TME) and enhances immune inflammation through a MDA5/MAVS-dependent manner, as evidenced by increase of immune cells and decrease of myeloid-derived suppressor cells (MDSCs) (36). We used TIMER2.0 web tool to analyze correlation of ADAR1 and tumor immune infiltrating profile, and confirmed negative correlation with CD4⁺ T cells, CD8⁺ cells, M1 macrophages, and NK cells, and positive correlation with cancer-associated fibroblast (CAFs), endothelial cells and inhibitory immune cells M2 macrophage (Supplementary Fig. S1G). Collectively, these evidences suggest ADAR1 is aberrantly overexpressed in PCa tumors and associated with poor clinical outcomes.

Depletion of ADAR1 inhibits tumor cells growth in vitro and in vivo

To assess the effect of depletion of ADAR1 on PCa cells, we generated stable ADAR1-knockdown cells (shADAR1) and negative control cells (shNC) in DU-145 and VCaP cells using lentivirus-packaged short-hairpin RNA (shRNA). ADAR1 protein level was greatly decreased in knockdown cells relative to control without affecting ADAR2 (Fig. 2A; Supplementary Fig. S2A and S2B). A marked decrease of A-to-G editing level upon ADAR1 loss was observed in a known ADAR1 editing target *GLI1* (Fig. 2B; ref. 38). Consistent with previous studies in other tumors (13, 39–41), depletion of ADAR1 significantly inhibited PCa cell proliferation, migration, and invasion *in vitro* (Fig. 2C and I; Supplementary Fig. S2C). Via flow cytometry, we observed ADAR1 knockdown results in cell cycle arrest at G0/G1 phase and increased apoptosis (Fig. 2G and H). Next, to determine whether ADAR1 knockdown compromises tumor growth *in vivo*, we inoculated subcutaneously DU-145 shADAR1 and shNC cells into mice. Surprisingly, only one mouse grew little tumor while others (5/6) did not grow visible tumors, suggesting ADAR1 loss did abrogate tumor growth (Fig. 2J-L; Supplementary Fig. S2D). Next, we overexpressed empty vector (EV), Flag-tagged ADAR1-wild type (ADAR1-WT), and catalytically inactive ADAR1 E912A mutant (ADAR1-Mut) in DU-145 cell (Fig. 2D). A large increase in editing level of *GLI1* was found in ADAR1-WT cells relative to EV while ADAR1-Mut did not (Fig. 2E). Cell proliferation assay showed a higher growth rate of WT cells and comparable proliferative profile between Mut and EV cells, which demonstrated ADAR1's pro-proliferation ability is deaminase activity-dependent (Fig. 2F). These findings clearly implicate that loss of ADAR1 inhibits PCa tumor cell growth *in vitro* and *in vivo* via cell cycle arrest and apoptosis.

Depletion of ADAR1 significantly modulates transcriptional activity involved in tumor survival

To comprehensively understand the signaling modulation of ADAR1, we performed whole transcriptome sequencing (RNA-seq) on DU-145 shADAR1 and shNC cells with good concordance between two

biological replicates (Supplementary Fig. S3A). After ADAR1 knockdown, 139 and 48 genes were upregulated and downregulated, respectively, such as *ADAR*, *CDK6*, and *CDH1*, as shown in volcano plot and heat map (Fig. 3A; Supplementary Fig. S3B). In accordance with cell proliferation arrest phenotype observed in ADAR1 loss, gene set enrichment analysis (GSEA) using the Hallmarks gene set from the Molecular Signatures Database (MSigDB) showed substantial suppression of cell proliferation and cell-cycle related pathways, such as MYC targets, G2M checkpoint, and E2F targets (Fig. 3B). In addition, ADAR1 loss increased gene enrichment in P53 and apoptosis pathway, though not significant (Fig. 3D). It is worth noting that cell adhesion molecules (CAMs) pathway in KEGG (Kyoto Encyclopedia of Genes and Genomes) was dramatically upregulated (Fig. 3C), which may be attributed to suppressed epithelial-mesenchymal transition (EMT) signaling as evidenced by increased expression of epithelial marker (E-cadherin) and decreased expression of mesenchymal markers (N-cadherin) (Fig. 3F). We further confirmed reduced expression of cell proliferation and cell cycle-regulated protein CDK6 and c-MYC, and increased P53 protein and cleaved PARP upon ADAR1 KD (Fig. 3E and F). As an RNA editing enzyme, ADAR1 is responsible for avoiding hyper-activated IFN-I responses induced by endogenous dsRNA. Consistent with this, IFN- α response, IFN- γ response, and TNF α signaling via NF- κ B pathways were found to be positively enriched in shADAR1 group, which could be attributed to elevated expression of IFN-stimulated genes (ISGs) (Fig. 3C). We then used enzyme-linked immunosorbent assay (ELISA) to detect IFN- γ content in peripheral blood in shADAR1 and shNC xenograft mice since shADAR1 mice barely grew tumors. As expected, IFN- γ in PB of shADAR1 mice showed an approximate 2-fold increase relative to shNC mice (Fig. 3G). To assess immune infiltration, we performed flow cytometry to determine the abundance of cytotoxicity CD8⁺ T cells. In line with above bioinformatics results (Supplementary Fig. S1G), CD8⁺ T cells in shADAR1 mice spleen were significantly more than shNC (Fig. 3H; Supplementary Fig. S3C). All these results show the critical roles of ADAR1 in regulating signaling involved in tumor cell proliferation, apoptosis, immune response, and metastasis.

Characterize MTDH as a novel target of ADAR1 governing PCa carcinogenesis and metastasis

Subsequently, to investigate the mechanism of signaling modulation of ADAR1, we identified the direct targets of ADAR1 in PCa through multi-omics sequencing. In our RNA-seq data, a large decline of A-to-G editing events occurred in shADAR1 cells (Supplementary Fig. S4A), and these editing sites were mainly enriched in intron and 3' UTR (Supplementary Fig. S4B). To acquire ADAR1 bound transcripts, we performed RNA-immunoprecipitation sequencing (RIP-seq) using an ADAR1 antibody (Supplementary Fig. S5C). In all bound approximate 2000 RNA peaks, introns and exons account for 49.72% and 27.34% of peak region, respectively (Supplementary Fig. S4D). In addition, based on our RNA-seq data, we screened A-to-G editing frequency difference of more than 0.1 between shNC and shADAR1 group (shNC - shADAR1 > 0.1) to acquire 487 genes (Supplementary Fig. S4C). Subsequently, according to 487 editing genes, all bound 1993 peaks, and total genes (expression count > 200), we obtained 114 potential targets of ADAR1 (listed in Supplementary Table S3), as shown in the Venn diagram (Supplementary Fig. S4E).

GO and KEGG analysis showed that these 114 genes were mainly enriched in processes such as translation, viral process, cell cycle, ribosome, and apoptosis (Supplementary Fig. S5A and S5B). Based on their functions in literature, we further selected 28 genes and validated their bindings with ADAR1 using qPCR assay (Supplementary Fig. S4F). We found numbers of targets were positively accumulated in ADAR1 relative to IgG, such as *MTDH*, *EIF2AK2*, and *IFNAR1* (Fig. 4A; Supplementary Fig. S5D). Sanger sequencing was performed to validate editing sites indicated in RNA-seq data. *RBBP4*, *EIF2AK2*, *DFFA*, *IFNAR1*, and *MTDH* were confirmed with a decline of editing levels upon ADAR1 loss (Fig. 4B; Supplementary Fig. S5E).

To investigate the crucial targets governing tumor survival in PCa, we firstly examined their correlations with ADAR1. Bioinformatics analysis data suggested that *ADAR1* and *MTDH* have a strong positive correlation in PCa (Supplementary Fig. S6D). Our RNA-seq data also showed a large decline editing levels of *MTDH* in normal PCa tissues compared to tumors (Supplementary Table S4). Previous studies demonstrated that MTDH is an oncogenic protein associated with tumors growth and metastasis via modulation of cell cycle arrest, apoptosis, and EMT signaling, furthermore, MTDH loss also inducing cytotoxic T cell infiltration in colorectal and lung cancers (42, 43). These phenotypes are consistent with ADAR1 loss in PCa. Therefore, we speculated that MTDH might be the downstream target of ADAR1 in promoting PCa cell growth and metastasis. Firstly, RIP-qPCR and Sanger sequencing assay had confirmed that *MTDH* is a direct editing target of ADAR1 (Fig. 4A and B). Then we decided to elucidate the mechanism through which ADAR1 interacts and regulates *MTDH*. We examined expression of *MTDH* in RNA-seq data which did not show any difference between shNC and shADAR1 groups and confirmed using qPCR (Fig. 4C). However, the protein level of MTDH showed a pronounced reduction upon ADAR1 knockdown (Fig. 4C). Then, we overexpressed ADAR1-WT and -Mutant in shADAR1 cells, and found that wild-type ADAR1 could completely rescue MTDH protein level and cell viability while mutant-type could not even partially, demonstrating maintenance of MTDH protein is dependent on ADAR1's deaminase activity, which is also essential for cancer cells survival (Fig. 4D and E). Next, we decided to dissect why MTDH would dramatically reduce upon ADAR1 knockdown. Unexpectedly, knockdown of ADAR1 did not alter *MTDH* mRNA stability after transcription was inhibited by actinomycin D treatment (Fig. 4F). Therefore, we questioned whether ADAR1 would affect accumulation of *MTDH* mRNA in ribosome machinery. We conducted ribosome immunoprecipitation assay using anti-RPL22 (constituent of 60S ribosome protein) antibody, and results showed that *MTDH* mRNA enrichment significantly decreases in ribosome machinery upon ADAR1 knockdown (Fig. 4G and H). We speculated less degree of editing in 3' UTR of *MTDH* mRNA resulted in reduced ribosome occupancy. ADAR1 editing of endogenous RNA plays a role in preventing PKR activation, which allows PKR-eIF2 α axis downregulating translation during IFN response (44). Moreover, we observed PKR and eIF2 α activation following ADAR1 knockdown in DU-145 cell line, consistent with previous studies (Fig. 4I; ref. 44–46). This may be one reason for reduction of MTDH translation upon ADAR1 knockdown. We then overexpressed MTDH in ADAR1 knockdown cells and found MTDH could rescue compromised proliferation and invasion phenotype induced by ADAR1 loss (Fig. 4J-L), which demonstrated that suppressed proliferation and invasion upon ADAR1 loss in PCs is MTDH-dependent.

Finally, we were interested in clinical significance of MTDH in prostate cancer. Above mentioned three pairs of PCa tissues were subjected to Sanger sequencing and WB analysis to evaluate editing level and protein level of MTDH, respectively. We observed elevated editing level of MTDH in tumors relative to normal tissues (Supplementary Fig. S6A). Expression of MTDH was also significantly higher in tumors in both protein and mRNA levels (Supplementary Fig. S6B and S6C). Collectively, these findings demonstrate MTDH as a novel and critical downstream target of ADAR1 is responsible for cell proliferation and invasion, and may be a clinical biomarker for prostate cancer.

Discover a novel and potent small molecule inhibitor of ADAR1

Though others have reported 8-aza as a potential inhibitor of ADAR1, there has not yet been strong evidence suggesting its direct binding and inhibition with ADAR1 protein. 8-aza has been reported to slightly inhibit ADAR2 deaminase activity with an IC_{50} of 15 mM, and has only obscure evidence to show ADAR1 inhibition (37, 47).

To discover potential ADAR1 inhibitors, a structure-based virtual screening was conducted by LibDock and CDOCKER of Discovery Studio 2021 (Fig. 5A). According to a recent study of ADAR1 protein structure (48), homology modeling of ADAR1 was applied to this procedure and the pocket was defined as a catalytic pocket. Through two rounds of screening of SPECS and in-house libraries, we obtained the hit compound 8-bromoadenosine (8-Br-Ado, Fig. 5A), which has moderate inhibitory activity against ADAR1 with $IC_{50} > 20 \mu M$ (Supplementary Fig. S7B), and moderate cytotoxicity against DU-145 cell with an IC_{50} of 41.83 μM (Supplementary Fig. S7C). Binding mode showed 8-Br-Ado formed favorable hydrogen bonds with crucial residues in ADAR1 (Supplementary Fig. S7A).

Taking 8-Br-Ado as the lead compound, we designed and synthesized a series of derivatives for further structural optimization. A newly synthesized compound ZYS-1 (Fig. 5A) showed strong inhibitory activity against ADAR1 deaminase with an IC_{50} of 0.866 μM (Fig. 5D) and profound anti-proliferation activities against multiple PCa cell lines with IC_{50} within submicromolar magnitude, though, with several-folds higher IC_{50} values against RWPE1 (Fig. 5C; Supplementary Fig. S7D). Docking analysis suggested that in addition to hydrogen bonds with crucial residues, some extra weak interactions were formed between ZYS-1 and ADAR1 catalytic pocket, possibly helping better binding affinity (Fig. 5B). To detect direct binding of ZYS-1 to ADAR1 protein, we performed microscale thermophoresis assay (MST) and confirmed their interaction with a K_D value of $7.24 \pm 2.88 \mu M$ (Fig. 5F). Moreover, cellular thermal shift assay (CETSA) validated that ZYS-1 interacted with ADAR1 protein in cellular conditions (Fig. 5E). Next, we assessed the direct inhibitory function of ZYS-1 on ADAR1 in PCa cells. A comparable cell proliferation profile was observed in shADAR1 and 50 nM treated cells (Fig. 5G). We simultaneously treated shNC and shADAR1 cells with or without ZYS-1, and the difference of proliferation ratio (treated/untreated) started to show after 24 h and was more aggravated after 48 h (Fig. 5H). Furthermore, ADAR1 loss resulted in a about 2-fold increase of IC_{50} value of ZYS-1 in DU-145 cell (Supplementary Fig. S7E), meanwhile, forced expression of ADAR1 significantly attenuated growth inhibition by ZYS-1

(Supplementary Fig. S7F). As expected, ZYS-1 dose-dependently decreased editing level of *GLI1* in DU-145 and VCaP cells (Fig. 5I). This dose-dependent decrease was also confirmed using RNA-editing site specific quantitative PCR (RESSqPCR) (Fig. 5J; ref. 49). Both results demonstrate ZYS-1 inhibits catalytic activity of ADAR1 in cells. Taken together, these results indicate ZYS-1 directly binds to ADAR1 and inhibits its catalytic function.

ZYS-1 strongly suppresses PCa cell growth and invasion via ADAR1 inhibition

Consistent with the effect of ADAR1 knockdown, ZYS-1 not only inhibited cell proliferation (Fig. 5C and G), but also inhibited cell colony formation ability, and induced cell cycle arrest at G0/G1 phase and substantial apoptosis in a dose-dependent manner (Fig. 6A-C; Supplementary Fig. S8A). In addition, ZYS-1 significantly impaired DU-145 cell migration and invasion ability (Fig. 6D). Interestingly, we further found that ZYS-1 could reduce ADAR1 protein levels in DU-145 and VCaP cells, while did not alter *ADAR1* mRNA levels (Fig. 6E). These data suggest that ADAR1 protein degradation induced by ZYS-1 is in a post-transcriptional manner. However, ADAR1 degradation induced by ZYS-1 was independent of proteasome degradation (Supplementary Fig. S8C and S8D). Precise mechanism needs further investigation. Moreover, selectivity over ADAR2 was noteworthy as ZYS-1 did not change ADAR2 protein level (Supplementary Fig. S8B). Furthermore, ZYS-1 recapitulated signaling modulation of ADAR1 KD on E-cadherin, N-cadherin, P53, cleaved PARP, CDK6, and c-MYC protein in DU-145 and VCaP cells (Fig. 6F). We then evaluated effect of ADAR1 inhibition by ZYS-1 on MTDH. As expected, ZYS-1 dose-dependently reduced MTDH protein level but not changed its mRNA level, possibly via activation of PKR following targeting ADAR1 (Fig. 6G; Supplementary Fig. S8F). Furthermore, in line with ADAR1 KD, *MTDH* editing level was decreased following ZYS-1 treatment (Fig. 6H). *IFNAR1*, another potential target of ADAR1, was also observed a decrease in editing level (Supplementary Fig. S8G). Notably, forced expression of MTDH moderately rescued cell viability inhibited by ZYS-1 (Fig. 6I). Collectively, these results demonstrate that ZYS-1 suppresses tumor cell survival and invasion through ADAR1 inhibition.

ZYS-1 displays significant anti-tumor efficacy and favorable safety profile in vivo

As ZYS-1 exhibits a well anti-tumor effect *in vitro*, we then assessed the therapeutic effects of ZYS-1 *in vivo* using a DU-145-derived xenotransplantation mice model. Intraperitoneal administration of low dose (20 mg/kg/day) and high dose (40 mg/kg/day) of ZYS-1 significantly suppressed tumor growth relative to vehicle group in a dose-dependent manner, with tumor growth inhibition (TGI) rate achieving 66.68% and 81.83%, respectively (Fig. 7A-C). Especially, tumor growth almost stagnated after treatment for 20 days. Meanwhile, no differences in body weight or behavior (data not shown) were observed between dosing and control group (Supplementary Fig. S10A). Also, H&E staining of key organs of dosed mice did not exhibit apparent necrosis or inflammation (Supplementary Fig. S10B). Both results prove ZYS-1 is well tolerated and safe *in vivo* even after continuous administration. Next, H&E, Ki-67, and tunel staining

were performed to evaluate tumor proliferation and apoptosis. Necrosis area of dosing group tumor was markedly larger as shown in H&E staining (Fig. 7D). Proliferation level of tumor cells was lower after ZYS-1 treatment as indicated by Ki-67 staining (Fig. 7D and E). TUNEL staining was utilized to determine apoptosis and suggested ZYS-1 induced tumor cell apoptosis in a dose-dependent manner (Fig. 7D and F). We also performed immunohistochemical staining of ADAR1 and showed ZYS-1 decreased ADAR1 protein level dose-dependently in tumor, recapitulating the effects *in vitro* (Fig. 7D and G). Also, apoptosis-, cell cycle-, and EMT-associated protein level changes had a comparable tendency in tumors upon ZYS-1 treatment (Supplementary Fig. S10D). Subsequently, we assessed the acute toxicity of ZYS-1 in mice by treatment with various doses of ZYS-1 via intraperitoneal or oral administration. Lethal dose 50% (LD₅₀) of i.p. and p.o. was 208 and 687 mg/kg, respectively. Body and key organ weights and their H&E staining analysis were not found aberrant between dosed and control groups (Supplementary Fig. S9A and S9B). These evidences show that ZYS-1 has strong anti-tumor efficacy and is relatively safe *in vivo*.

Previous studies have demonstrated that loss of ADAR1 in tumor promotes IFN release and efficacy of immunotherapy such as PD-1 blockade (36). This sensitivity of tumor to immunotherapy is mediated by IFN release induced by ADAR1 loss. We thus determined the concentration of IFN- γ in tumor of xenograft mice. ELISA and WB analysis both validated increased human IFN- γ release in tumor in a dose-dependent manner (Fig. 7H; Supplementary Fig. S10D). Consistent with the increase of CD8⁺ T cells upon ADAR1 loss, we found that ZYS-1 treatment significantly elevated proportion of CD8⁺ T cells in tumor and spleen (Fig. 7I-K). In addition, immunosuppressive myeloid-derived suppressor cells (MDSCs) CD11b⁺Gr1⁺ were decreased in tumors after ZYS-1 treatment (Supplementary Fig. S10C). These results together suggest ZYS-1 not only exerts anti-proliferation and pro-apoptosis effects on tumor, but also ameliorate TME and enhance anti-tumor immunity by induction of IFN- γ release, CD8⁺ T cell infiltration, and decrease of MDSCs. Taken together, targeting ADAR1 is a promising therapy for prostate cancer and may synergize with immunotherapy such as PD-1 blockade deserving further studies.

ZYS-1 shows broad anti-tumor effects against various solid tumors and hematological malignancies

Previous studies have identified ADAR1's oncogenic roles in many tumor types, such as gastric cancer, lung cancer, glioblastoma, liver cancer, breast cancer, multiple myeloma, melanoma, pancreatic cancer, and leukemia (13, 36, 37, 39–41, 46, 50, 51). We thus chose several cancer cell lines to test anti-proliferation effect of ZYS-1 as ADAR1 level in these tumors was markedly higher than in non-tumor tissues in TCGA patients. ZYS-1 pronouncedly suppressed cell viability of all these cancer cell lines with low IC₅₀ values (< 1 μ M; Supplementary Fig. S11A-S11F). In addition, ZYS-1 exhibited excellent anti-proliferation activity towards primary AML cells from patients (IC₅₀ < 200 nM; Supplementary Fig. S11G). Particularly, ZYS-1 also potentiated ATRA-induced myeloid differentiation in these primary AML cells in a dose-dependent manner, possibly through an unknown mechanism by which ADAR1 inhibition on AML

myeloid differentiation (Supplementary Fig. S11H). Collectively, these results suggest targeting ADAR1 with small molecule inhibitor ZYS-1 has broad therapeutic potential against a wide range of tumor types.

Discussion

Prostate cancer (PCa) remains a leading cause of cancer death in men (52). However, it will inevitably progress into incurable metastatic CRPC in most patients following ADT and AR antagonist therapy (53). Therefore, identifying novel and efficacious drug targets is urgently needed to expand therapeutic options for lethal PCa. ADAR1-mediated deamination of adenosine-to-inosine (A-to-I) within double-stranded RNA (dsRNA) is the most prevalent form of RNA editing that modifies the sequence of RNA transcripts without changing its genomic blueprints, and its aberration has revealed a close association with autoimmune disease Aicardi-Goutieres Syndrome (AGS), neurodegenerative disease Autism and many kinds of cancer (14, 54). Although previous study has reported that PCA3/PRUNE2 axis is mediated by ADAR1 dependent RNA editing effects on tumor growth in AR-dependent prostate cancer cell lines (33), little is known about the role of ADAR1 in AR-negative prostate cancer, and the landscape of A-to-G editing targets in PCa remains underexplored. In the present study, we not only demonstrated that ADAR1-mediated A-to-G editing is a crucial carcinogenesis event in PCa, but also developed a novel small-molecule ADAR1 inhibitor, ZYS-1, that exhibits profound anti-proliferation activities against multiple cancer cells, not limited to PCa. Our data showed that ADAR1 is highly expressed in prostate cancer and associated with higher A-to-G editing levels. ADAR1 depletion and pharmacological inhibition dramatically suppressed PCa cells growth and metastasis, induced apoptosis, reduced the tumor burden and ameliorated immune response *in vivo*. We further identified oncogenic protein MTDH as a novel crucial edited target of ADAR1 driving tumor proliferation and invasion in PCa, whose translation was repressed by ADAR1 in an editing dependent manner. These data establish the critical regulatory role of ADAR1 in PCa initiation and progression, thereby expanding remedies for lethal PCa treatment.

Several studies have analyzed RNA editing profile in cancer from high-throughput whole transcriptome RNA-seq data in public databases, showing that A-to-G editing levels are higher in most tumors rather than in the matched normal tissues (28–31), including prostate cancer. Recently, Chan and colleagues reported that five types of cancers have hyperediting patterns in the mesenchymal (M) phenotypes rather than in the epithelial (E) phenotypes, including prostate cancer (55). However, the precise molecular mechanism through which ADAR1-mediated A-to-G editing leading to prostate carcinogenesis and metastasis was not fully explored in these studies. Here combined with the TCGA public data and the RNA-seq data of three pairs of matched PCa and normal tissues, we demonstrate that a higher A-to-G editing level in PCa appears to be caused by higher expression of *ADAR1* rather than *ADAR2*.

Subsequently, we identified 114 potential targets of ADAR1 through multi-omics analysis, which were mainly enriched in processes such as translation, viral process, cell cycle, ribosome and apoptosis. Based on their functions in literature, we further experimentally validated their binding with ADAR1. Importantly, we identified MTDH as a crucial edited target of ADAR1 in PCa. MTDH as an oncogenic protein has been implicated in various cancers, such as breast cancer, prostate cancer, hepatocellular carcinoma, colorectal cancer, gastric cancer, and lung cancer, which drove tumor development and drug resistance

via modulation of AKT/PI3K, NF- κ B, MAPK, and Wnt/ β -catenin signaling (42, 43, 56). Previous studies showed higher A-to-G editing levels of MTDH in breast cancer and hepatocellular carcinoma (57, 58), however, the connection of ADAR1 and MTDH has not been reported in PCa. In this study, we found elevated A-to-G editing level and higher expression level of MTDH in the three PCa specimens rather than normal tissues, and loss of ADAR1 reduced MTDH protein level, but not altered mRNA stability and transcript abundance. Rescue results showed that ADAR1/MTDH axis is regulated in an editing-dependent manner and mediates PCa cells proliferation and invasion. Indeed, we have shown that ADAR1 editing in 3' UTR of *MTDH* affects the accumulation of *MTDH* mRNA in ribosome machinery, and ADAR1 knockdown resulted in upregulation of p-PKR and p-eIF2 α , which trigger comprehensive translation shutdown (44). Rather, other studies suggested that ADAR1 regulates translation efficiency by affecting transcript spatial distribution and ribosome stalling (59, 60). More precise mechanisms towards MTDH post-transcriptional regulation still need to be illuminated future.

In addition, several recent articles reported loss of ADAR1 in tumors not only potentiates the efficacy of epigenetic therapy in colorectal cancer, but also confers susceptibility to the immunotherapy and overcomes resistance to immune checkpoint blockade in melanoma (35, 36). Therefore, development of small-molecule drugs against ADAR1 is of therapeutic significance. However, hitherto, due to the lack of crystal structural information for the deaminase domain of ADAR1, no drug has been successfully discovered against ADAR1. Here, using the homology model created in a recent study (48), by structure-based virtual screening, we obtained the hit compound 8-Br-Ado which moderately inhibited the deaminase activity of ADAR1 and cell proliferation. Next, through further structural optimization and subsequent validation assays, we developed a novel small-molecule ADAR1 inhibitor, ZYS-1, which could bind to ADAR1 and inhibited its deaminase activity with an IC₅₀ of 0.866 μ M. Recapitulating ADAR1 knockdown effect, ZYS-1 dramatically suppressed PCa cells growth and metastasis, induced apoptosis, reduced tumor burden and ameliorated immune response with a favorable safety profile *in vivo*, furthermore, modulating the certain signaling targets, e.g., *CDK6*, *MYC*, *CDH1*, *CDH2*, *TP53*, and *EIF2AK2*. Importantly, ZYS-1 impaired the A-to-G editing levels of the novel target *MTDH* and *IFNAR1*, and reduced MTDH protein level but not changed its mRNA level. Rescue assays showed that ZYS-1's contribution to anti-tumor activity in PCa was through inhibition of ADAR1/MTDH axis.

We first speculated ADAR1 protein degradation induced by ZYS-1 is in a post-transcriptional manner as ZYS-1 did not alter ADAR1 mRNA levels. A previous study has indicated that IFN signaling activation would induce ADAR1 degradation in an ubiquitination-dependent manner (61). We thus assumed that ZYS-1-induced ADAR1 degradation is ubiquitination/proteasome-dependent. At first, we used cycloheximide (CHX) to inhibit new protein synthesis. However, ZYS-1 only slightly induced ADAR1 protein degradation after 6 and 12 hours treatment (Supplementary Fig. S8C). Next, we used a proteasome inhibitor MG132 to inhibit proteasome-mediated protein degradation, however, MG132 treatment did not rescue the dramatic loss of ADAR1 upon ZYS-1 inhibition, suggesting independent of proteasome degradation (Supplementary Fig. S8D). Coincidentally, overexpressed m6A methyltransferase METTL3/14 has been suggested to regulate N⁶-methyladenosine (m6A) on *ADAR1* mRNA in

glioblastoma. The m6A binding protein YTHDF1 was demonstrated to recognize m6A modification on ADAR1 mRNAs and promote its translation, as evidenced by ADAR1 protein reduction without affecting its mRNA level upon YTHDF1 knockdown (62). Therefore, we postulated ADAR1 degradation upon ZYS-1 treatment maybe through an m6A-YTHDF1 dependent mechanism as ZYS-1 led to a significant reduction of YTHDF1 protein level in PCa cells (Supplementary Fig. S8E). Though this may be one reason for ADAR1 protein reduction upon ZYS-1 treatment, the precise mechanism still needs further exploration.

Finally, due to the lack of crystal structural information for the deaminase domain of ADAR1, we performed *in silico* docking to elucidate the binding mode between ZYS-1 and ADAR1 homology model. Docking analysis suggested that some crucial hydrogen bonds interactions (V894, K1003, V1004 and G1009) formed between ADAR1 and 8-Br-Ado or ZYS-1, which are located closely with catalytic metal-binding sites (C966, H988) and deaminase domain (E912, F972, D973, K996). However, in the interaction between ZYS-1 and ADAR1, halogens such as 2-F and 8-Cl formed some weak σ -hole interactions with C893, L1031 or T1033, similar to hydrogen bonds, which play an important role in the process of protein and ligand recognition and binding. This may be the reason why compound ZYS-1 exhibits a higher affinity to ADAR1 compared to 8-Br-Ado. Further study to obtain the crystal structure of ADAR1 has been undertaken in our collaborated laboratory. Expectantly, the co-crystal structure would elucidate the binding mode and more precisely guide to optimize ZYS-1 to enhance the inhibitory activity, pharmacokinetic profiles, safety properties or anti-tumor efficacy.

In summary, our study carries out a model in which the disturbing of ADAR1-mediated A-to-G editing in MTDH triggers its translational shutdown, modulating tumor cells proliferation and invasion in prostate cancer. Encouragingly, we discover and develop a novel small-molecule ADAR1 inhibitor ZYS-1 with strong anti-tumor efficacy and favorable safety profile. Meanwhile, we highlight the widespread applicability of ADAR1 inhibitors in various cancers. All these findings may represent a new therapeutic strategy for prostate cancer and more malignancies by targeting epitranscriptomic RNA editing with single agent or combination with other therapies.

Methods

Clinical samples

Three pairs of matched prostate tumor, normal prostate tissue, and fresh bone marrow (BM) blood of AML patients were obtained from Huai'an First People's Hospital with patients' informed consent. Fresh tissues were divided into several parts for protein and RNA isolation. Mononuclear cells (MNCs) were isolated from BM blood samples using Ficoll Paque Plus (GE Healthcare, 17-1440-02-1) for further studies. All samples used in this study were approved by medical ethics committee of Huai'an First People's Hospital, and China Pharmaceutic University. Clinicopathologic information of each patient was listed in Supplementary Table S1.

Cell Culture

All cell lines used in this study were cultured in recommended culture medium at 37°C in 5% CO₂. Sources and culture mediums of these cell lines were detailedly listed in Supplementary Table S1. All cell lines were detected no mycoplasma contamination annually and had valid STR profile. MNCs isolated from BM blood of AML patients were cultured in IMDM medium supplemented with 20% FBS, 10 ng/mL human cytokines IL-3, IL-6, SCF, TPO, and FLT3 ligand (all from Sino Biological).

Bioinformatics data mining and analysis

Expression data and clinical data of 497 PRAD patients in the Cancer Genome Atlas (TCGA) database were downloaded from the cBioPortal platform (<https://www.cbioportal.org>). Additionally, we downloaded expression data of 51 normal prostate tissue samples from the Genomic Data Commons (GDC, <https://portal.gdc.cancer.gov/>). To explore the prognostic value of *ADAR1*, we assigned PRAD patients into three subgroup, ADAR1-high (top 10%), ADAR1-intermediate, and ADAR1-low (bottom 10%), based on the expression levels of *ADAR1*. All statistical analyses were performed using R software (version 3.6.3).

Synthesis of ZYS-1

To a solution of 2-fluoroadenosine (5.70 g, 20 mmol) and pyridine (3.22 mL, 40 mmol) in acetonitrile (80 mL) was added drop-wise SOCl₂ (7.25 mL, 100 mmol) at 0 °C. The reaction mixture was continuously stirred for 4 h, and then subsequently warmed to room temperature for overnight. The resulting suspension was concentrated in vacuo. To the reaction mixture was added methanol (120 mL), water (12 mL), and NH₄OH (24 mL), followed by stirring for 0.5 h at r.t. The reaction mixture was concentrated and the solid was precipitated in the water phase, and the filter cake was obtained by filtration. The filter cake was re-dissolved in a small amount of methanol at 60 °C, and dichloromethane was added dropwise. The solid was precipitated by cooling, filtered and washed with cold methanol to obtain the product ZYS-1 as a white solid (5.45 g, 90%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.34 (s, 1H), 7.92 (s, 2H), 5.84 (d, *J* = 5.6 Hz, 1H), 5.58 (s, 2H), 4.67 (t, *J* = 5.4 Hz, 1H), 4.26–4.16 (m, 1H), 4.10 (q, *J* = 5.4, 5.0 Hz, 1H), 3.99–3.81 (m, 2H). ¹³C NMR (101 MHz, DMSO) δ 158.61 (d, *J* = 206.4 Hz), 157.70 (d, *J* = 19.3 Hz), 150.75 (d, *J* = 19.7 Hz), 140.01, 117.58, 87.40, 83.74, 72.69, 71.17, 44.76.

Cell Proliferation Assays

Cell proliferations were evaluated using the Cell Counting Kit-8 (Share-bio, SB-CCK8). Briefly, 5000–20000 cells/well were seeded into 96-well plate and treated with several diluted concentrations of test compounds or DMSO as control in triplicates. After treatment for 72 h, 10 μL of CCK-8 solution was added into each well, and the absorbance at 450 nm was measured using a microplate reader (Bio-Tek SynergyH1) upon incubation for 4 h at 37°C. Data were fitted in nonlinear regression and IC₅₀ values were calculated by GraphPad Prism 8.0. For cell growth assays, cell viabilities were determined at 0, 24, 48, 72, and 96 h.

Western Blotting

Western blotting assay was performed similarly as described previously (63). Cells were lysed in RIPA lysis buffer (Thermo Fisher Scientific, 89901) at 4°C, and the lysate supernatant were added with protease inhibitor (Roche, 04693132001) and phosphatase inhibitor (Roche, 04906845001). Protein concentration of lysates was quantified using a BCA protein assay kit (Thermo Fisher Scientific, 23227). Equal amounts of protein were loaded onto 8–12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane, which was blocked with 0.5% skim milk in Tris-buffered saline supplemented with 0.5% Tween 20 (TBS-T) and then incubated with indicated primary antibodies. Following washing in TBS-T, membranes were incubated with horseradish peroxidase-conjugated (HRP) secondary antibody for 1–2 h. The blots were visualized by a chemiluminescent detection system (Tanon, Shanghai, China).

Cell migration and invasion assays

Each transwell (Falcon, 353097) was seeded with 5×10^4 cells which have been starved in FBS-free medium for 12 h. Then, cells were allowed to migrate or invade to the bottom chamber for at least 24 h. Cells in the bottom of the chamber were fixed in 4% paraformaldehyde (PFA) for 10 min and stained with crystal violet for 10 min. For invasion assay, upper chambers were precoated with matrigel (Corning, 356234) and incubated for 12 h at 37°C. Images were taken using an inverted microscope (Nikon) and cell counts were quantified using ImageJ software.

Colony formation assay

500–1000 cells/well were plated to 24-well plate and allowed for growth for 10–14 days, during which medium in wells were replaced every three days. 4% PFA was used to fix colony for 10 minutes, followed by staining with crystal violet for 10 minutes. Images were taken using an inverted microscope and colony numbers were quantified using ImageJ software.

Lentivirus transfection

Lentivirus particles of short-hairpin RNA against *ADAR1* (pGV248-sh*ADAR1*) and its scrambled control (pGV112-shNC) were constructed and purchased from Genechem Co. Ltd. (Shanghai, China). For lentivirus transfection, $1-10 \times 10^5$ cells were seeded onto 6-well plate and allowed for adhesion for 24 h, followed by transfection with above lentivirus in the presence of HitransG P (Genechem, REVG005). After 16–24 h, medium containing virus was removed. Transfected cells were allowed for growth for 3–5 days, during which GFP fluorescence was observed every day, followed by treatment with 2 µg/mL puromycin for 24 h to select positive infected cells. All transfected cells were validated by qRT-PCR and western blotting, and maintained in medium containing 1 µg/mL puromycin. The lentiviral particles were used at 20 multiplicity of infection (MOI). The target sequences of the sh*ADAR1* and shNC were as follows: shNC: 5'-TTCTCCGAACGTGTCACGT-3'; sh*ADAR1* 1#: 5'-GATACTACACCCATCCATT-3'; sh*ADAR1* 2#: 5'-AGGGTATGTTGACTTTGAA-3'.

Transient overexpression of MTDH, ADAR1 WT and ADAR1 Mut

All plasmids including pGV141-EV, pGV141-*ADAR1* WT, pGV141-*ADAR1* Mut, and pGV141-*MTDH* were constructed and purchased from Genechem Co. Ltd. (Shanghai, China). Briefly, 3×10^5 DU-145 cells were seeded onto 6-well plate and allowed for adhesion to 70% confluence. Each well was added 200 μ L transfection complex in RPMI1640 medium containing 1 μ g plasmid and 1 μ L X-tremeGENE HP DNA Transfection Reagent (Roche, 06366236001). After 24–48 h transfection, expressions were validated by western blotting. The primers used for amplifying *ADAR1* and *MTDH* cDNA by PCR were listed in Supplementary Table S2.

Cellular Thermal Shift Assay (CETSA)

CETSA was performed following the protocol by Huang et al (64). Cells were harvested and lysed in PBS containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM DTT after freeze-thawed three times using liquid nitrogen. Upon centrifugation, supernatant were added ZYS-1 or DMSO and incubated at 25°C. Subsequently, the supernatant were divided equally and denatured at various temperatures for 5 min. These samples were analyzed by SDS-PAGE and western blot. Relative protein band intensity was analyzed by Image J software and the apparent aggregation temperature (T_{agg}) was fitted and calculated by GraphPad Prism 8.0 software.

RNA-seq and Data Analysis

Cells were harvested and total RNA was extracted using Beyozol Total RNA Extraction Reagent (Beyotime, R0011) following the instruction of the manufacturer. Total mRNA was enriched by Obligo(dT) beads, then fragmented into short fragments and reversely transcribed into cDNA with random primers. After the second-strand cDNA was synthesized, cDNA fragments were purified, end repaired, added poly(A), and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, amplified by PCR, and sequenced using Illumina HiSeq2500 by Genedenovo Biotechnology Co., Ltd (Guangzhou, China). Raw reads were cleaned to remove adapters or low quality reads and rRNA mapped reads. Clean reads were mapped to Homo sapiens genome. Gene expressions were quantified by FPKM (fragment per kilobase of transcript per million mapped reads) value and differential expression analysis was performed by DESeq2 software between two different groups with false discovery rate (FDR) below 0.05 and absolute fold change ≥ 2 considered differentially expressed genes. Gene Ontology (GO), Gene Set Enrichment Analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG), and hallmark gene sets in Molecular Signatures Database (MSigDB) were used to analyze the enriched pathways. Bioinformatic analysis was performed using Omicsmart platform (<https://www.omicsmart.com>).

Cell Cycle and Apoptosis Assays

For cell cycle analysis, cells were collected and fixed with 70% ethanol overnight at 4°C. Cells were washed with PBS and stained with propidium iodide (PI) using Cell Cycle Analysis Kit (Beyotime, C1052) for 30 min at 37°C. After staining, samples were subjected immediately to flow cytometry analysis. For cell apoptosis analysis, YF®488-Annexin V and PI Apoptosis Kit (US Everbright, Y6002) was used following the manufacturer's instructions. Cells were collected and stained with YF®488-Annexin V and

PI for 20 min at room temperature away from light. To avoid disturbance from GFP fluorescence in shNC and shADAR1 cells, we used Annexin V-APC and 7AAD doubling staining method (Annexin V-APC and 7AAD Apoptosis Kit, US Everbright, A6030) to determine their apoptosis. Samples were subjected immediately to flow cytometry analysis. Flow cytometry was conducted using FACS Celesta (BD) and FACS Calibur (BD) and data was analyzed using FlowJo V10 software.

ADAR1 deaminase activity determination

The ability of compounds to inhibit purified ADAR1 protein deaminase activity was determined by Adenosine Deaminase (ADA) Activity Assay Kit (Fluorometric) (Abcam, ab204695) following the manufacturer's instructions. Measure output on a fluorescent microplate reader at Ex/Em = 535/587 nm in a kinetic mode, every 2 minutes, for at least 60 minutes at 37°C protected from light. The fluorescent value in two time points in compounds treated group relative to control group was regarded as remaining ADAR1 deaminase activity.

Flow Cytometry

Tumors from xenograft mice were ground mechanically, digested, and filtered with 70-µm screen to provide single cell suspensions. Fresh spleens collected from xenograft mice were subjected to erythrocytes lysis (Biolegend, 420301). Mice cells were blocked with anti-mouse CD16/32 (TruStain FcX™, Biolegend, 101319), followed by cell surface staining with various labeled antibodies for 20 min at 4°C in the dark. MNCs isolated from BM blood of AML patients were blocked with Fc receptor blocking solution (Human TruStain FcX™ Biolegend, 422301). Subsequently, cells were centrifuged and resuspended in Cell Staining Buffer (Biolegend, 420201), and were subjected to flow cytometry directly. Anti-mouse antibodies used were as follows: FITC-CD3, APC-CD8a, FITC-CD11b, APC-Gr-I (Iy6C/Iy6G). Anti-human antibodies used were as follows: FITC-CD11b, APC-CD15. Flow cytometry was conducted using FACS Celesta (BD) and FACS Calibur (BD) and data was analyzed using FlowJo V10 software.

Immunohistochemistry (IHC)

Tumors and key organs from prostate cancer xenograft mice were fixed in 4% PFA followed by embedded in paraffin wax, sectioned in slides. Sections were incubated with 3% H₂O₂ for 10 min to quench endogenous peroxidase activity followed by antigen retrieval using unmasking solution. Nonspecific binding was blocked with 2% goat serum for 30 min. These sections were then subjected to ADAR1, Ki-67, Tunel and hematoxylin and eosin (H&E) staining. Organs from mice in acute toxicity study were carried out for H&E staining. A human prostate cancer tissue microarray was purchased from Servicebio technology Co., Ltd. (Wuhan, China). This slide was stained with anti-ADAR1 (1:200 dilution) antibody. Immunohistochemistry staining intensity was measured by Image-Pro Plus 6.0 software. The staining H-Score was calculated by the following formula: H-SCORE = (percentage of cells of weak intensity) × 1 + (percentage of cells of moderate intensity × 2) + percentage of cells of strong intensity × 3). Slide was visualized using CaseViewer2.2 software. Clinicopathologic information of each patient was listed in Supplementary Table S1.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Tumor was lysed in RIPA lysis buffer. IFN- γ level in lysate or plasma was detected using Mouse IFN- γ ELISA Kit (Beyotime, PI508) or Human IFN- γ ELISA Kit (Beyotime, PI511) according to manufacturer's instructions. Briefly, samples were added into pre-immobilized IFN- γ antibody 96-well plate and incubated for 2 h in r.t. IFN- γ biotinylated antibodies were added into plate and incubated for 1 h in r.t. Subsequently, horseradish peroxidase labeled streptavidin was added into plate with incubation for 20 min followed by adding stop solution, and then immediately measured in 450 nm. The IFN- γ content in tumor or plasma was calculated based on standard curve.

Determination of editing level in cells

Cells were extracted using RNA-easy Isolation Reagent (Vazyme, R701-01) following the manufacturer's instructions. RNA was reversely transcribed to cDNA using the HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, R312-02). To precisely measure the A-to-G levels in RNA target, NestPCR was performed to amplify cDNA product preventing mismatching. cDNA product was subjected to PCR amplification for 25 cycles followed by the second PCR for 35 cycles using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, P505-d1) (Primers used for NestPCR in Supplementary Table S2). cDNA products were purified by agarose gel electrophoresis, and then subjected to Sanger Sequencing. The editing level was quantified based on peak height ratio (%G/(A + G)) using BioEdit software.

Quantitative Real-Time PCR (qRT-PCR) and RNA editing site-specific qRT-PCR (RESSqPCR)

Total RNA was extracted using RNA-easy Isolation Reagent according to the manufacturer's instructions. RNA was reversely transcribed to cDNA using HiScript® III RT SuperMix (Vazyme, R323-01) and then subjected to qRT-PCR using ChamQ SYBR qPCR Master Mix (Low ROX premix) (Vazyme, Q331-02). All qPCR assays were run in an ABI 7500 Fast Real-Time PCR system (Applied Biosystem, USA). In this study, each sample was run in triplicates. Relative gene expressions were calculated by $2^{-\Delta\Delta C_t}$ method and *GAPDH* was used as endogenous control. To determine editing level with qPCR method, RESSqPCR was used to confirm the reduced editing level of known target of ADAR1 as previously reported. Relative RNA editing ratios (Relative edit/WT RNA) were calculated using the following formula: $2^{-(C_t \text{ Edit} - C_t \text{ WT})}$. The primers used for qPCR and RESSqPCR were listed in Supplementary Table S2.

Animal studies

Four- to five-week-old wild-type female BALB/c mice were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. We subcutaneously implanted 1×10^6 tumor cells (mixed 1:1 v/v with matrigel) into the right flank of BALB/c mice age-matched to 6- to 8-week-old. Drug treatment was started when xenograft tumor size reached to around 70–100 mm³. ZYS-1 was administrated into mice via intraperitoneal injection twice every day before euthanatizing mice. Tumor volumes were measured every two days using the formula: $L \times W^2/2$. There were 8 mice in the vehicle group and 12 mice in both low dose group (20 mg/kg/day) and high dose group (40 mg/kg/day). At the end point of experiment, we euthanized the mice, and dissected, photographed, and weighed tumors and key organs. Tumors and organs were either

preserved in 4% PFA or carried out directly to further analysis. All animal studies conformed to the guideline of experimental animal of China Pharmaceutic University.

Acute toxicity assay

Six- to eight-week-old wild-type female ICR mice were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. Mice were intraperitoneally or orally administrated with various doses of ZYS-1 for single time, and body weights and conditions were recorded every two days for two consecutive weeks. Each dosing group contained 10 mice. At the end time, all mice were euthanized and key organs were dissected, weighed and kept in 4% PFA for H&E staining. LD₅₀ was calculated using GraphPad Prism 8.0 software.

RNA immunoprecipitation (RIP) assay

Cells were lysed in lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM Hepes pH 7.0, 1 mM DTT, 50 units/mL RNase out, 1 × protease inhibitor cocktail, 1 × PBS) at 4°C for 2 h. Volume of 10% lysate was subjected to RNA isolation as input. 10 mg Protein A-Agrose beads (Sigma-Aldrich, P1406-250MG) was pre-treated with PBS three times and 2% BSA for 30 min and then incubated with 10 µL IgG control, ADAR1 or RPL22 antibody at 4°C for 2 h. Subsequently, beads-antibody complex was incubated with cell lysate on rotation at 4°C overnight. Beads were washed with PBS and then subjected to total RNA isolation. Purified RNA was reversely transcribed followed by qRT-PCR. For anti-ADAR1 RNA immunoprecipitation, extracted RNA was subjected to library construction and RNA-sequencing (Guangzhou Genedenovo Biotechnology Co., Ltd).

Microscale thermophoresis assay (MST)

Purified ADAR1 was first buffer-exchanged with spin-filter concentrator (amicon 30 kDa cutoff) to the dilution buffer of following composition: 25 mM HEPES, 200 mM NaCl pH7.5. Then the protein was labeled with the Monolith NT Protein Labeling Kit RED (NanoTemper, MO-L001) according to the manufacturer's labeling protocol. Compounds were sequentially diluted covering the range of appropriate concentrations from 0.5 mM to 1 µM. The labeled protein was mixed with the same volume of diluted compound. Samples were loaded into Monolith standard-treated capillaries and the thermophoresis was measured at 22°C using 100% LED and 20% MST power on a Monolith NT.115 instrument (NanoTemper Technologies, München, Germany). Data analyses were performed with Mo.Affinity Analysis v2.2.4 and GraphPad Prism 8.0 software.

Structure-Based Virtual Screening

The three-dimensional structure of ADAR1 protein referred to the previously reported literature (48), and Discovery Studio 2021 (Accelrys, CA, USA) was employed to screen the combined ligand database, which was filtered by Lipinski's rule of five, Veber's rule and the 'pan-assay interference compounds'. The structure-based virtual screening procedure contains two programs, LibDock and CDOCKER, LibDock is a molecular docking program based on a high-throughput algorithm, while the CDOCKER is a more

accurate docking method that employs ligands flexibly to match the binding pocket. Based on the virtual screening procedure, we obtained 20 compounds from the ligand database for experimental study.

Statistical analysis

The unpaired, two-tailed Student's *t* test was used to compare difference between two groups carried on SPSS 22.0 software, if not specifically indicated in figure legends. For bioinformatics analysis, Student's *t* test and Mann-Whitney Wilcoxon test were used to compare continuous variables between two groups. Analysis of variance (ANOVA) was utilized to test the variance among multiple groups. Paired, two-tailed Student's *t* test was used to compare ADAR1, ADAR2, and editing levels between 16 pairs matched tumor and normal tissues in TCGA, carried on GraphPad Prism 8.0 software. Kaplan-Meier plots and log-rank tests were used for relapse-free survival analysis. The values were expressed as mean $\pm$ SD with the numbers of replicates listed in figure legends. $P < 0.05$ was considered statistically significant.

Declarations

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Authors' Disclosures

The authors declare no competing financial interests.

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Figures

Figure 1

Figure 1

ADAR1 is highly expressed in prostate cancer and associated with poor survival. (A) Analysis of A-to-G editing genes in three matched tumor and normal tissues from PCa patients. Those A-to-G editing level higher in tumor group are labeled in red, and higher in normal tissues are labeled in blue. (B-E) Comparison of ADAR1 expression between tumor and normal tissues (B), different Gleason grade patient groups (C), age < 65 or > 65 group (D), high and intermediate risk group (E) in TCGA database. (F) Kaplan-

Meier survival plot of high (red line, n = 50) and low (blue line, n = 50) ADAR1 expression PRAD patients. Log-rank test, P = 0.0059. High and low expression patients represent top 10% and bottom 10% ADAR1 expression patients, respectively. (G and H) Immunohistochemical analysis of ADAR1 expression in prostate cancer patients. Representative pictures show ADAR1 expression in tumor and normal tissues, and in different grades (G). Scale bars, 200 μ m (top) and 50 μ m (bottom). Dot plot shows different ADAR1 expression profile between tumor (n = 38) and normal (n = 27) tissue group (H). Two-tailed unpaired Student's t test. (I) ADAR1 protein levels of tumor and normal tissues in three PCa patients. (J) ADAR1 protein levels of five different PCa cell lines. *, P < 0.05; **, P < 0.01; ***, P < 0.001; Error bar, mean $\pm$ SD.

Figure 2

Figure 2

Depletion of ADAR1 inhibits tumor cells proliferation in vitro and in vivo via induction of cell cycle arrest and apoptosis. (A) Protein level of ADAR1 in DU-145 cells that were infected with lentiviral shNC and two independent shADAR1. (B) A-to-G editing frequency of known ADAR1 targets GLI1 on DU-145 shADAR1 and shNC cells, detected by Sanger sequencing; the triangle indicated the double peak A/G, labeled with the percentage of edit allele burden (%G/(A + G)). (C) Cell viability of DU-145 shADAR1 and shNC cells

during a 5-day course. (D) Protein level of ADAR1 in DU-145 cells that were overexpressed with empty vector (EV), Flag-tagged ADAR1 wild type (WT), and ADAR1 E912A mutant (Mut). (E) A-to-G editing frequency of GLI1 on DU-145 cells that were overexpressed with EV, ADAR1 WT, and ADAR1 Mut. (F) Cell viability of DU-145 ADAR1 overexpressed and control cells during a 5-day course. (G) Cell cycle phase distribution of DU-145 ADAR1 KD cells determined by flow cytometry. (H) Representative flow cytometry plot for quantifying apoptosis in DU-145 ADAR1 KD and control cells, analyzed by 7-AAD and Annexin V staining. Labeled number indicates cell percentages of each area. (I) Migration and invasion ability of DU-145 shADAR1 and shNC cells. Representative images of migrated and invaded cells were shown. Scale bars, 500 μ m. (J-L) BALB/c mice was implanted subcutaneously with DU-145 shADAR1 and shNC cells (n = 6 in each group). Tumor mass were photographed (J) and weighted (K) after executing mice. Body weights of mice were measured every two days (L). *, P < 0.05; **, P < 0.01; ***, P < 0.001; Unpaired two-tailed Student's t test. Error bar, mean $\pm$ SD, if not indicated, n = 3.

Figure 3

Figure 3

RNA Sequencing combined validation assays to identify affected signaling pathways of ADAR1 loss in PCa cells. (A) Volcano plot of significantly affected (absolute fold change > 2, P value < 0.05) genes in DU-145 shADAR1 group relative to shNC group revealed by RNA-seq. Both shADAR1 and shNC groups contain two biological replicates. (B and C) Gene set enrichment analysis (GSEA) was performed to analyze the signaling pathways enrichment in shADAR1 and shNC group. NES, normalized enrichment

score. FDR, false discovery rate. ES, enrichment score. (D) The core-enriched pathways were listed according to their NES value. (E) Quantitative PCR validation of increased or decreased genes in RNA-seq data. (F) Immunoblotting analysis of indicated protein on DU-145 shADAR1 and shNC cells. (G) Quantification of mouse IFN- γ concentration in peripheral blood in mice bearing DU-145 shADAR1- or shNC cell-derived tumor by ELISA at the endpoint. (H) Quantification of proportion of CD8⁺ T cells in spleen of mice bearing DU-145 shADAR1- or shNC cell-derived tumor by flow cytometry at the endpoint. Data are from three mice in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Unpaired two-tailed Student's t test. Error bar, mean $\pm$ SD, $n = 3$.

Figure 4

Figure 4

MTDH is a novel critical target of ADAR1 in PCa. (A) Relative enrichment of MTDH mRNA in ADAR1-RIP over IgG (control) determined by qPCR assays. GAPDH expression was used as a negative control. 10% volume of cell lysate was used as input. (B) Validation of editing sites (suggested by RNA-seq) of MTDH by Sanger sequencing. (C) Protein and mRNA level of MTDH between DU-145 shADAR1 and shNC cells. (D) Western blotting analysis showing MTDH level changes after overexpressing EV, ADAR1-Mut, or

ADAR1-WT in DU-145 shADAR1 over control (shNC + EV) cells. (E) Cell viability of control cells and DU-145 shADAR1 cells overexpressed EV, ADAR1-Mut, or ADAR1-WT during a 5-day course. (F) Percentage of remaining MTDH mRNA in DU-145 shADAR1 and shNC cells after actinomycin D (5 µg/mL) treatment for 4, 8, and 12 hours. (G) Validation of RPL22-RIP assay by western blotting. 1% volume of cell lysate was used as input. (H) Relative enrichment of MTDH mRNA in RPL22-RIP over IgG (control) determined by qPCR assays. HPRT1 expression was used as negative control. 10% volume of cell lysate was used as input. (I) Protein level change of p-PKR, PKR, p-EIF2α and EIF2α after ADAR1 knockdown in DU-145 cells. (J) MTDH was overexpressed in DU-145 shADAR1 cells as examined by immunoblot. (K) Cell viability of control cells and DU-145 shADAR1 cells overexpressed EV or MTDH during a 5-day course. (L) Representative images and quantification of invaded cells of control (shNC + EV) cells and DU-145 shADAR1 cells overexpressed EV or MTDH. Scale bar, 500 µm. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; Unpaired two-tailed Student's t test. Error bar, mean $\pm$ SD, $n = 3$.

Figure 5

Figure 5

Discovery of ZYS-1, a potent small-molecule inhibitor of ADAR1. (A) Brief flowchart of the procedure to identify ADAR1 inhibitors from the libraries. (B) Binding mode of compound ZYS-1 in the catalytic pocket of ADAR1. Hydrogen bonds are shown as green dashed lines, σ -hole interactions are magenta, while ZYS-1 is shown in cyan stick. Distances are labeled (Å). The ADAR1 protein was built by homology modeling referred to a previous study. (C) IC₅₀ values of ZYS-1 against cell viability of five PCa cell lines. (D) Effect

of ZYS-1 on inhibition of catalytic activity of purified ADAR1 protein. (E) Representative immunoblot image (left) from CETSA and quantification of band intensity (right) relative to input indicating thermal shift of ADAR1 protein in DU-145 cells. Data are from three independent experiments. (F) Binding of ZYS-1 to ADAR1 protein was detected by MST assay. $KD \pm SD$ values are calculated from three independent experiments. (G) Effect of ADAR1 knockdown and ZYS-1 treatment on DU-145 cell viability. (H) Effect of ZYS-1 treatment (50 nM) on DU-145 shNC and shADAR1 cells, of which cell viability was normalized to that of DMSO-treated shNC and shADAR1 cells. (I) Sanger sequencing showing editing level changes on GLI1 in DU-145 and VCaP cells after treatment with 1 or 2 μM ZYS-1 for 48 h. (J) RESSqPCR was performed to detect GLI1 editing level changes in DU-145 and VCaP cells after treatment with 1 or 2 μM ZYS-1 for 48 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Unpaired two-tailed Student's t test. Error bar, mean $\pm$ SD, $n = 3$.

Figure 6

Figure 6

ZYS-1 strongly suppresses cell survival and invasion via cell cycle arrest and apoptosis, and modulates similar signaling to ADAR1 knockdown. (A) Representative images and quantification of colony numbers. Before plated on 24-well plate for colony formation, DU-145 cells were treated with DMSO or ZYS-1 for 48 h. (B) Cell cycle phase distribution of DU-145 cells after treatment with DMSO or ZYS-1 for 48 h determined by flow cytometry. (C) Apoptosis of DU-145 cells treated with DMSO or ZYS-1 for 48 h

determined by flow cytometry. (D) Representative images and quantification of migration and invasion ability of DU-145 cells after treatment with DMSO or ZYS-1 for 48 h. Scale bar, 200 μ m. (E) ADAR1 protein (top) and mRNA (bottom) level changes in DU-145 and VCaP cells after treatment with ZYS-1 for 48 h. (F) Immunoblot of indicated proteins in DU-145 and VCaP cells after treatment with DMSO or ZYS-1 for 48 h. (G) Immunoblot of MTDH, p-PKR, and PKR, and qPCR determination of MTDH level in DU-145 cells after treatment with DMSO or ZYS-1 for 48 h. (H) Sanger sequencing showing editing level changes on MTDH in DU-145 cells after treatment with 2 μ M ZYS-1 for 48 h. (I) Cell viability of DU-145 MTDH overexpression cells after treatment with 200 nM ZYS-1 for 48 h, detected by CCK-8 assay (OD450 nm). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; Unpaired two-tailed Student's t test. Error bar, mean $\pm$ SD, $n = 3$.

Figure 7

Figure 7

ZYS-1 shows potent anti-tumor efficacy and tolerable favorable safety profile in vivo. (A-C) BALB/c mice were implanted subcutaneously with DU-145 cells. When tumors grew about 70-100 mm³, the mice were injected intraperitoneally with either vehicle (n = 8 mice) or ZYS-1 (20 and 40 mg/kg, n = 12 in each group) twice daily for about a month. Tumor volumes were measured every two days (B). After euthanizing mice, tumors were excised followed by photographed (A) and weighted (C). (D)

Representative images of H&E, Ki-67, TUNEL and ADAR1 immunohistochemical staining of paraffin section of tumor from mice treated with vehicle, 20 or 40 mg/kg ZYS-1. Scale bar, 100 μ m. (E) Quantification of Ki-67 staining positive cells (brown) as a percentage of the total number of cells from three sections of tumor. (F) Quantification of TUNEL staining positive cells (green) as a percentage of the total number of cells from three sections of tumor. Nucleus are stained as blue with DAPI. (G) Quantification of ADAR1 staining (brown) intensity from three sections of tumor, as calculated by H-score. (H) Quantification of human IFN- γ concentration in tumor lysate from mice treated with vehicle, 20 or 40 mg/kg ZYS-1 by ELISA. (I and J) Representative flow cytometry plot (I) and quantification (J) of proportion of CD8⁺ T cells in tumor and spleen from mice treated with vehicle, 20 or 40 mg/kg ZYS-1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Unpaired two-tailed Student's t test. Error bar, mean $\pm$ SD, $n = 3$.

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