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AZIN1 RNA editing confers cancer stemness and enhances oncogenic potential in colorectal cancer

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Adenosine-to-inosine (A-to-I) RNA editing, a process mediated by adenosine deaminases that act on the RNA (ADAR) gene family, is a recently discovered epigenetic modification dysregulated in human cancers. However, the clinical significance and the functional role of RNA editing in colorectal cancer (CRC) remain unclear. We have systematically and comprehensively investigated the significance of the expression status of ADAR1 and of the RNA editing levels of antizyme inhibitor 1 *(AZIN1)*, one of the most frequently edited genes in cancers, in 392 colorectal tissues from multiple independent CRC patient cohorts. Both ADAR1 expression and *AZIN1* RNA editing levels were significantly elevated in CRC tissues when compared with corresponding normal mucosa. High levels of *AZIN1* RNA editing emerged as a prognostic factor for overall survival and disease-free survival and were an independent risk factor for lymph node and distant metastasis. Furthermore, elevated *AZIN1* editing identified high-risk stage II CRC patients. Mechanistically, edited *AZIN1* enhances stemness and appears to drive the metastatic processes. We have demonstrated that edited *AZIN1* functions as an oncogene and a potential therapeutic target in CRC. Moreover, *AZIN1* RNA editing status could be used as a clinically relevant prognostic indicator in CRC patients.

Introduction

Colorectal cancer (CRC) is one of the most frequently occurring malignancies worldwide, and it remains the second leading cause of cancer-related deaths in Western countries (1). CRC pathogenesis is strongly associated with lifestyle choices, such as diet, obesity, and smoking (2). Emerging evidence indicates that these factors have a profound effect on epigenetic alterations, including histone modification (3), DNA methylation (4), and regulation of noncoding RNAs (5). These epigenetic modifications can directly influence key cellular mechanisms, such as gene expression and enhancer activity, to maintain cellular homeostasis and prevent various diseases, including cancer (6).

RNA editing is a recently identified epigenetic alteration that is involved in the posttranscriptional regulation of key genes associated with human cancers (7). Specifically, the conversion of adenosine to inosine (A-to-I), where splicing and translational machineries recognize the inosine residues as guanosines, is emerging as the most frequent type of RNA editing process in humans. Such A-to-I RNA editing is primarily catalyzed by enzymes encoded by the family of adenosine deaminases that act on the RNA (ADAR) genes (ADAR1, ADAR2, and ADAR3) (8). Intriguingly, several recent studies have shown that A-to-I RNA editing by ADAR1 was prominent in several cancers, including hepatocellular carcinoma (HCC), esophageal cancer, and gastric cancer (9–11). Antizyme inhibitor 1 *(AZIN1)* was identified as one of the most frequently

occurring A-to-I RNA alterations in HCC (9). Mechanistically, edited *AZIN1* conferred the gain-of-function phenotype associated with aggressive tumors and promoted ornithine decarboxylase (ODC) and polyamines accumulation (9, 10). In spite of the growing evidence for A-to-I RNA editing in tumorigenesis, the clinical significance and the functional role of *AZIN1* RNA editing in CRC remains unexplored.

Herein, we demonstrate for the first time to our knowledge that *AZIN1* RNA editing levels and ADAR1 expression are significantly increased in CRC and edited *AZIN1* is an independent prognostic factor for overall survival (OS) and disease-free survival (DFS) in patients with this malignancy. Furthermore, edited *AZIN1* appears to function as an oncogene; enhances cellular proliferation, invasion, and migration capabilities; and promotes cancer stem-like cell features. Collectively, we illustrate that RNA editing is dysregulated in CRC and edited *AZIN1* acts as an oncogene in CRC.

Results

The RNA editing gene, ADAR1, and AZIN1 RNA editing levels are frequently dysregulated in CRC. With the hypothesis to determine whether RNA editing levels are dysregulated in CRC, we first assessed the expression levels of ADAR1 and ADAR2 in training ($n = 24$) and validation cohorts ($n = 50$) of patients by analyzing their CRC and matched adjacent normal mucosa tissues (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.99976DS1). Although 3 ADARs (ADAR1, ADAR2, and ADAR3) have been identified as members of the ADAR family in vertebrates, since ADAR3 is primarily expressed in brain cells (12), its expression was excluded in our CRC cohort. We found that ADAR1 expression was significantly upregulated in CRC tissues in both cohorts ($P = 0.0002$ in the training cohort, $P < 0.0001$ in the validation cohort; Figure 1A), while the expression of ADAR2 was significantly downregulated in CRC tissues when compared with normal mucosa ($P < 0.0001$ in both cohorts; Supplemental Figure 2). To further validate the overexpression of ADAR1 in CRC, we used immunohistochemical analysis and observed a strong staining for ADAR1 in CRC cells, which was primarily confined to the cytoplasm, while very limited expression was detected in adjacent normal mucosa, which is consistent with previous reports in other human cancers (10, 13) (Supplemental Figure 3, A and B).

Considering that we have previously identified *AZIN1* as one of the most frequently edited genes in esophageal cancer and HCC (9, 10), we asked whether the dysregulation of ADAR1 corresponds to the alteration of edited *AZIN1* levels in the CRC clinical cohorts. To quantify *AZIN1* RNA editing levels, we employed RNA editing site-specific quantitative PCR (RESSq-PCR), as described previously (14). Consistent with the outcomes of ADAR1 dysregulation, *AZIN1* RNA editing levels were significantly increased in neoplastic tissues when compared with normal mucosa ($P < 0.0001$ in both cohorts; Figure 1B).

Since *AZIN1* RNA is edited by ADAR1 and not by ADAR2 (9), we assessed whether *AZIN1* RNA editing levels were associated with ADAR1 expression in CRC cohorts. As expected, edited *AZIN1* positively correlated with ADAR1 expression in both clinical cohorts ($P < 0.0001$, $\rho = 0.5$ in both cohorts; Figure 1C), suggesting that the dysregulation of ADAR1 might contribute to increased levels of *AZIN1* RNA editing in CRC, highlighting that the dysregulation of RNA editing is a prominent feature in CRC patients.

Edited AZIN1 and aberrant expression of ADAR1 associate with disease progression, recurrence, and prognosis in CRC patients. Next, we evaluated the clinical significance of edited *AZIN1* and the expression level of ADAR1 in a large clinical evaluation cohort (Supplemental Table 1). Surprisingly, although the expression status of ADAR1 was not significantly different across tumor stages, *AZIN1* RNA editing levels were higher in stage IV CRCs compared with stage I CRCs ($P < 0.05$; Figure 2A). Additionally, a time-to-event analysis revealed that highly edited *AZIN1* and a high expression of ADAR1 resulted in poor OS (ADAR1, $P = 0.004$; *AZIN1* RNA editing, $P = 0.0003$; Figure 2B). Furthermore, elevated editing levels of *AZIN1* also correlated with poor DFS (ADAR1, $P = 0.076$; *AZIN1* RNA editing, $P = 0.027$; Figure 2C). The prognostic significant of the results was analyzed by normalizing *AZIN1* expression results with both β-actin as well as GAPDH, and the results were quite similar.

In order to determine the clinical significance of edited *AZIN1* and ADAR1 expression levels, we assessed whether the expression of these genes associated with various clinicopathological factors (Table 1 and Supplemental Table 3). Quantitative profiling analyses revealed that increased *AZIN1* RNA editing correlated significantly with the presence of lymph node metastasis ($P = 0.0054$), hepatic metastasis ($P = 0.0006$), distant metastasis ($P = 0.0028$), and progression of tumor node metastasis (TNM) staging ($P = 0.0003$) in CRC patients (Table 1). Likewise, the overexpression of ADAR1 was associated with male sex ($P = 0.016$), hepatic metastasis ($P = 0.0095$), distant metastasis ($P = 0.016$), and progression of TNM staging ($P = 0.025$; Supplemental Table 3).
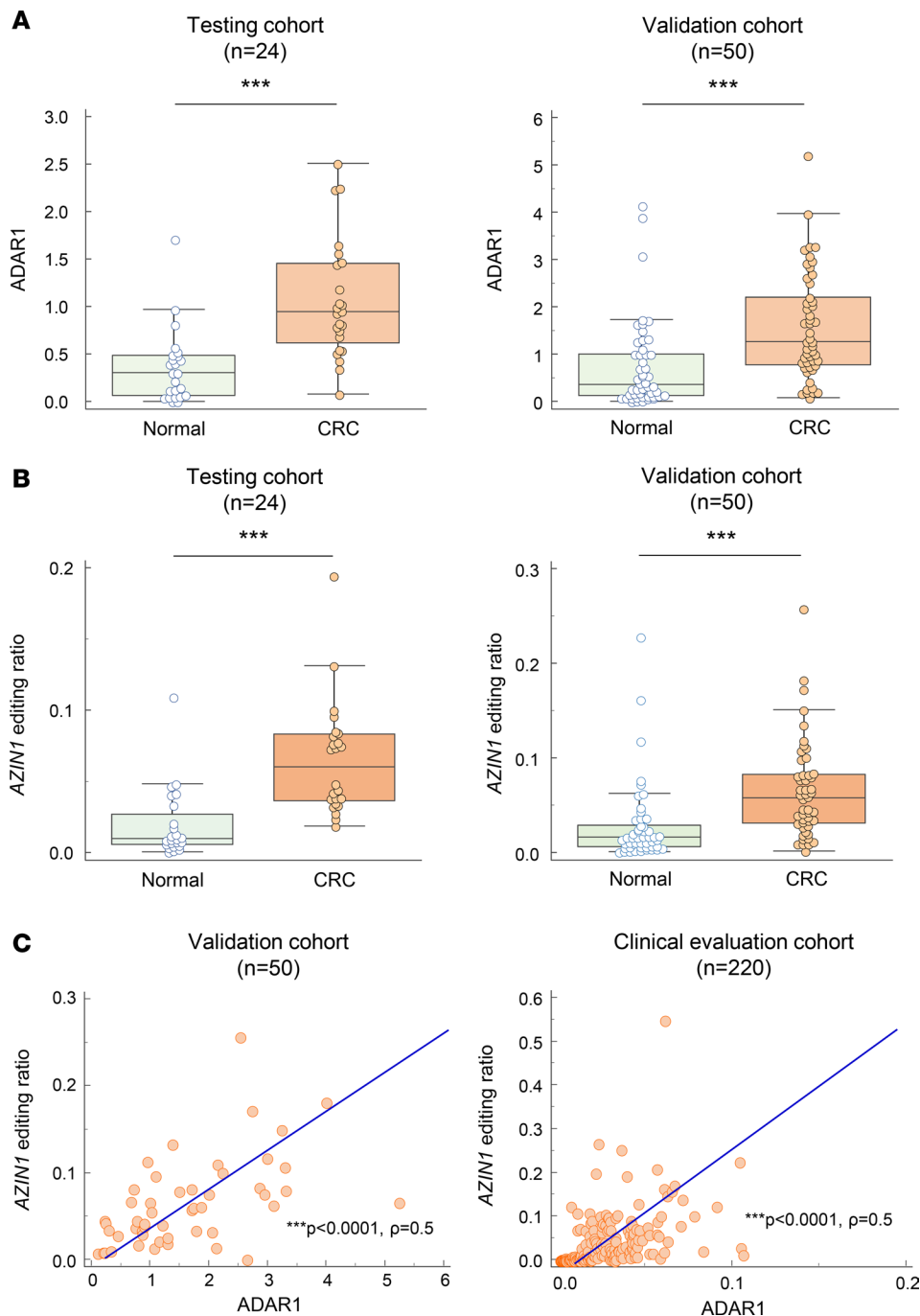
Figure 1. RNA editing gene ADAR1 is deregulated along with *AZIN1* RNA editing levels in CRC. (A) ADAR1 expression levels in CRC tissues compared with that in normal mucosa in the training cohort and validation cohort (Wilcoxon's signed-rank test). **(B)** *AZIN1* RNA editing levels in CRC tissues compared to that of normal mucosa in the training cohort and validation cohort (Wilcoxon's signed-rank test). **(C)** Correlation between *AZIN1* RNA editing and ADAR1 expression levels in the validation cohort and the clinical evaluation cohort (Spearman's rank correlation analysis). ***$P < 0.001$.

To further evaluate the prognostic biomarker potential of *AZIN1* RNA editing, we performed a multivariate Cox regression analysis. In addition to the presence of distant metastasis, high levels of edited *AZIN1* emerged as an independent prognostic factor for OS (hazard ratio [HR], 1.85; 95% CI, 1.03–3.32, $P = 0.041$; Table 2) and DFS in CRC patients (HR, 3.02; 95% CI, 1.23–7.39, $P = 0.016$; Table 2). Moreover, a multivariate analysis revealed that high levels of *AZIN1* editing were an independent predictive factor for lymph node metastasis (odds ratio [OR] 2.15, 95% CI 1.19–3.9, $P = 0.012$), hepatic metastasis (OR 2.96, 95% CI 1.3–6.75, $P = 0.0097$), and distant metastasis (OR 2.33, 95% CI 1.14–4.78, $P = 0.02$; Table 3), suggesting that the dysregulation of edited *AZIN1* may also be involved in metastatic disease progression.

Next, we examined whether *AZIN1* RNA editing levels and the expression status of ADAR1 could be used as predictive biomarkers of recurrence and prognosis in stage II CRC patients. High levels of ADAR expression and edited *AZIN1* were both associated with poor DFS in CRC patients in the clinical
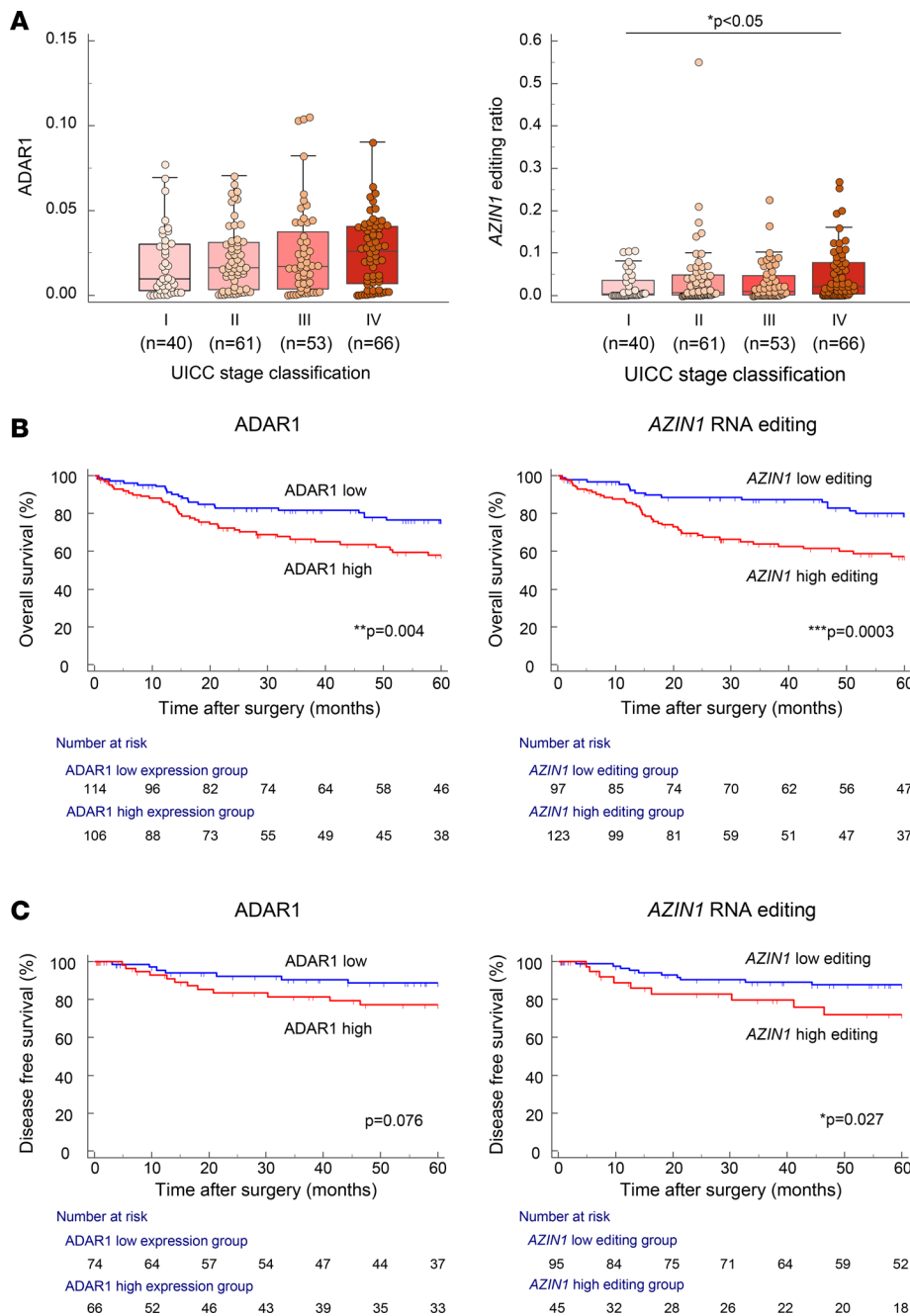
Figure 2. High *AZIN1* RNA editing levels and high expression of ADAR1 correlate with poor prognosis of OS and DFS in the clinical evaluation cohort. (**A**) TNM stage-dependent expression status of ADAR1 and *AZIN1* RNA editing levels in CRC tissues (Kruskal-Wallis test). (**B**) Kaplan-Meier survival curves for OS in CRC patients in the clinical evaluation cohort (*n* = 220) sorted into low and high expression levels of ADAR1 and low and high *AZIN1* RNA editing status (log-rank test). (**C**) Kaplan-Meier survival curves for DFS in CRC patients in the clinical evaluation cohort (*n* = 220) were sorted into low and high expression levels of ADAR1 and low and high *AZIN1* RNA editing status (log-rank test).

evaluation cohort ($P = 0.007$ and $P = 0.03$; Supplemental Figure 4). Furthermore, elevated edited *AZIN1* in CRC tumors was significantly associated with poor OS in stage II disease ($P = 0.016$), suggesting that edited *AZIN1* RNA status could be used to identify high-risk stage II CRC patients.

AZIN1 RNA editing levels are increased in colorectal adenomas. Bearing in mind that edited *AZIN1* was elevated in CRCs, we then assessed the levels of edited *AZIN1* in colorectal adenomas compared to those of matched normal mucosa to determine whether this epigenetic modification has a role in the multistep cascade of progression from adenoma to carcinoma. To our surprise, edited *AZIN1* levels were also elevated in the colorectal adenomas compared with adjacent normal mucosa ($P = 0.016$), suggesting that *AZIN1* RNA editing is an early event and that it may play a key role in cancer initiation (Supplemental Figure 5).

AZIN1 RNA editing promotes cellular proliferation, invasion, and migration in CRC. To gain insights into the biological relevance of edited *AZIN1* in CRC, we overexpressed either wild-type or edited *AZIN1* in HT29

Table 1. Clinicopathological variables and *AZIN1* RNA editing status in the clinical evaluation cohort

Variable		n	High (n = 123)	Low (n = 97)	P value
Sex	Male	129	75	54	0.43
	Female	91	48	43	
Age (yr)	<69[B]	109	64	45	0.41
	≥69	111	59	52	
Location	Colon	155	89	66	0.49
	Rectum	65	34	31	
Histological type	Differentiated	198	108	90	0.22
	Undifferentiated	22	15	7	
Pathological T category	pT1/2	50	22	28	0.05
	pT3/4	170	101	69	
Lymph vessel invasion	Absent	21	8	13	0.08
	Present	199	115	84	
Vascular invasion	Absent	51	28	23	0.87
	Present	169	95	74	
Lymph node metastasis	Absent	122	58	64	0.0054[C]
	Present	98	65	33	
Hepatic metastasis	Absent	174	87	87	0.0006[C]
	Present	46	36	10	
Distant metastasis	Absent	154	76	78	0.0028[C]
	Present	66	47	19	
UICC TNM classification	Stage I	40	15	25	0.0003[C]
	Stage II	61	30	31	
	Stage III	53	31	22	
	Stage IV	66	47	19	

The header "*AZIN1* RNA editing[A]" spans the High, Low, and P value columns.

[A]Adenosine-to-inosine modification of *AZIN1* RNA. [B]The median age at surgery is 69 years in this cohort. [C]$P < 0.05$. UICC, Union for International Cancer Control.

(microsatellite stable) and HCT116 (microsatellite unstable) cell lines ($P < 0.001$ in both cell lines; Figure 3A). Consistent with a previous study (9), immunofluorescence staining revealed localization of wild-type *AZIN1* within the cytoplasm, while edited *AZIN1* was present in both the nucleus and the cytoplasm of the transfected CRC cells (Supplemental Figure 6). We have previously demonstrated that the oncogene ODC is a downstream target of edited *AZIN1* (9). The overexpression of edited *AZIN1* resulted in upregulation of ODC protein in both cell lines, confirming that edited *AZIN1* stabilizes ODC more effectively than wild-type *AZIN1* (Figure 3B). Next, we assessed whether edited *AZIN1* enhances cellular proliferation in CRC using an MTT assay. Overexpression of edited *AZIN1* significantly increased cellular proliferation in both CRC cell lines when compared with wild-type *AZIN1* ($P < 0.01$ in HT29, $P < 0.001$ in HCT116; Figure 3C).

We next performed invasion and migration assays to determine whether edited *AZIN1* promotes the invasive and migratory potential of CRC cells. Overexpression of edited *AZIN1* enhanced invasiveness relative to both empty vector ($P < 0.05$ in both cell lines) and wild-type *AZIN1* ($P < 0.05$ in both cell lines; Figure 3D). Similarly, the overexpression of edited *AZIN1* increased migration levels compared with both the controls ($P < 0.05$ in both cell lines) and wild-type *AZIN1*-overexpressed cells ($P < 0.05$ in both cell lines; Figure 3E). Collectively, these results suggest that *AZIN1* RNA editing plays a critical role in CRC pathogenesis.

AZIN1 RNA editing promotes stemness in CRC cells. Cancer stem cells (CSCs) have been hypothesized as one of the underlying causes of metastasis and disease recurrence (15). Therefore, we assessed whether *AZIN1* editing influences CRC stemness. First, we used spheroids to enrich CSCs in HT29 and HCT116 cell lines (Figure 4A). To validate the enrichment of CSCs in spheroids, we first confirmed overexpression of OCT4 and SOX2, stemness-associated genes, in spheroids in comparison to parental cells ($P < 0.05$; Figure 4B). We thereafter analyzed the expression of ADAR1 between spheroids and parental cells to determine whether ADAR1 activity is higher in CSCs. Interestingly, we found that ADAR1 expression was overexpressed in spheroids when compared with parental cells in both cell lines ($P < 0.05$ in both cell lines; Figure 4C). Consistently, edited *AZIN1* was also overexpressed in the spheroids ($P < 0.05$ in both

Table 2. Univariate and multivariate analysis for predictors of overall survival and disease-free survival in the clinical evaluation cohort

Variables	Univariate			Multivariate		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Overall survival						
Sex (male)	1.46	0.86–2.48	0.16	1.06	0.61–1.84	0.84
Age (≥69 year old)[A]	0.71	0.43–1.18	0.19	0.86	0.51–1.45	0.57
Location (colon)	1.41	0.83–2.38	0.2	1.47	0.85–2.55	0.17
Histological type (differentiated)	0.59	0.29–1.2	0.15	0.71	0.35–1.46	0.35
T classification (pT3/4)	24	3.33–173.4	0.0016[B]	9.26	1.15–74.9	0.037[B]
Vessel involvement (present)	2.66	1.21–5.84	0.015[B]	0.74	0.32–1.69	0.47
Lymphatic vessel involvement (present)	7.91	1.1–57.1	0.04[B]	1.85	0.23–14.9	0.56
Lymph node metastasis (present)	2.89	1.72–4.83	0.0001[B]	1.52	0.89–2.6	0.13
Distant metastasis (present)	10.5	6.02–18.2	< 0.0001[B]	6.88	3.78–12.5	< 0.0001[B]
AZIN1 editing	2.74	1.55–4.84	0.0005[B]	1.85	1.03–3.32	0.041[B]
Disease-free survival						
Sex (male)	1.92	0.75–4.89	0.17	2.33	0.83–6.53	0.11
Age (≥71 year old)[C]	1.03	0.44–2.37	0.95	0.86	0.34–2.14	0.75
Location (colon)	1.31	0.53–3.22	0.55	1.14	0.43–3.03	0.79
Histological type (differentiated)	–	–	0.96	–	–	0.98
T classification (pT3/4)	2.99	1.01–8.87	0.047[B]	1.82	0.52–6.38	0.35
Vessel involvement (present)	–	–	0.95	–	–	0.96
Lymphatic vessel involvement (present)	–	–	0.96	–	–	0.99
Lymph node metastasis (present)	2.94	1.27–6.81	0.012[B]	1.99	0.81–4.93	0.14
AZIN1 editing	2.59	1.07–6.22	0.034[B]	3.02	1.23–7.39	0.016[B]

[A]The median age at surgery is 69 years old. [B]*P* < 0.05. [C]The median age at surgery is 71 years old. HR, hazard ratio.

cell lines; Figure 4C). These results suggest that the overexpression of ADAR1 in CSCs may have resulted in the overexpression of edited *AZIN1*. In order to determine whether edited *AZIN1* drives stemness or whether it is a passenger, we assessed the spheroid-forming capacity of cells overexpressing edited *AZIN1*. CRC cells overexpressing edited *AZIN1* possessed significantly greater spheroid-forming capacity when compared with parental or wild-type *AZIN1*-overexpressing cells (HT29, *P* < 0.01 and HCT116, *P* < 0.001 vs. empty vector and HT29, *P* < 0.001, and HCT116, *P* < 0.01 vs. wild-type *AZIN1*, Figure 4D). Moreover, OCT4 and SOX2 levels were significantly higher in spheroids overexpressing edited *AZIN1* when compared with the overexpressed wild-type *AZIN1* in HT29 (*P* < 0.05) and HCT116 cells (*P* < 0.05; Figure 4E). Recently, a variant isoform of CD44, CD44v6, has been identified as a key CRC CSC marker that is typically overexpressed in cancer tissues (16, 17). Elevated levels of CD44v6 expression are associated with poor patient prognosis in various cancers, including CRC (15, 18–22). Substantial overexpression of CD44v6 was observed in CRC cells overexpressing edited *AZIN1* in comparison with wild-type *AZIN1*-overexpressing cells (Figure 4F), providing more indication that *AZIN1* RNA editing is involved in driving cancer stemness in CRC.

AZIN1 RNA editing promotes tumor growth in a xenograft animal model. To confirm our in vitro findings, we established xenograft tumors using HCT116 cells that were transfected with vectors containing either wild-type or edited *AZIN1*. Mice injected with edited *AZIN1*-transfected cells showed accelerated tumor growth when compared with the wild-type *AZIN1*-transfected group. After 2 weeks of tumor growth, the mice implanted with edited *AZIN1*-transfected cells had both larger tumor volume and greater tumor

Table 3. Univariate and multivariate analysis for lymph node, hepatic, and distant metastases in the clinical evaluation cohort

| Variables	Univariate			Multivariate		
	OR	95%CI	P value	OR	95%CI	P value
Lymph node metastasis						
Sex (male)	0.83	0.48–1.42	0.5	0.79	0.44–1.42	0.42
Age (≥69 years old, median)[A]	0.97	0.57–1.65	0.9	1.01	0.56–1.82	0.96
Tumor location (rectum)	1.1	0.61–1.96	0.76	1.08	0.57–2.05	0.81
Histological type (undifferentiated type)	0.52	0.21–1.27	0.15	0.64	0.24–1.67	0.36
T classification (pT3/4)	5.91	2.62–13.3	< 0.0001[B]	3.5	1.38–8.89	0.008[B]
Lymphatic vessel invasion(present)	8.85	2.01–39.0	0.004[B]	1.58	0.26–9.69	0.62
Vascular invasion (present)	3.86	1.86–8.02	0.0003[B]	2.42	1.02–5.74	0.045[B]
AZIN1 editing	2.2	1.27–3.82	0.005[B]	2.15	1.19–3.9	0.012[B]
Hepatic metastasis						
Sex (male)	1.81	0.9–3.64	0.09	2.01	0.92–4.4	0.08
Age (≥69 years old, median)[A]	0.5	0.26–0.97	0.042[B]	0.45	0.21–0.96	0.038[B]
Tumor location (rectum)	0.92	0.45–1.9	0.83	0.77	0.34–1.74	0.53
Histological type (undifferentiated type)	0.53	0.2–1.38	0.19	0.54	0.19–1.6	0.27
T classification (pT3/4)	5.3	1.57–17.9	0.007[B]	8.72	1.07–71.3	0.043[B]
Lymph vessel invasion(present)	1.65	0.47–5.88	0.44	0.09	0.006–1.4	0.09
Vascular invasion (present)	2.33	0.92–5.85	0.07	1.47	0.43–5.08	0.54
Node involvement (present)	4.87	2.35–10.1	< 0.0001[B]	4.15	1.83–9.43	0.0007[B]
AZIN1 editing	3.68	1.72–7.89	0.0008[B]	2.96	1.3–6.75	0.0097[B]
Distant metastasis						
Sex (male)	1.45	0.81–2.69	0.2	1.63	0.81–3.28	0.17
Age (≥69 years old, median)[A]	0.33	0.18–0.61	0.0004[B]	0.27	0.13–0.55	0.0003[B]
Tumor location (rectum)	0.95	0.5–1.79	0.87	0.8	0.38–1.69	0.56
Histological type (undifferentiated type)	0.58	0.24–1.44	0.24	0.57	0.2–1.6	0.29
T classification (pT3/4)	6.6	2.27–19.2	0.0005[B]	7.31	1.52–35.1	0.013[B]
Lymph vessel invasion(present)	2.78	0.79–9.78	0.11	0.15	0.01–1.58	0.11
Vascular invasion (present)	4.13	1.66–10.2	0.002[B]	3.02	0.91–10.1	0.07
Node involvement (present)	4.08	2.21–7.56	< 0.0001[B]	3.29	1.62–6.68	0.001[B]
AZIN1 editing	2.67	1.42–4.99	0.002[B]	2.33	1.14–4.78	0.02[B]

[A]The median age at surgery is 69 years old. [B]$P < 0.05$. OR, odds ratio.

weight than those implanted with wild-type *AZIN1*-transfected cells (tumor volume, $P < 0.01$; tumor weight, $P = 0.019$), further supporting the oncogenic role of edited *AZIN1* in CRC (Figure 5A). Collectively, our findings suggest that *AZIN1* RNA editing may perform a crucial function in various stages of CRC tumorigenesis (Figure 5B).

Discussion

CRC arises through stepwise, sequential accumulation of genetic and epigenetic alterations in colorectal tissues. RNA editing is a recently discovered epigenetic modification that appears to be frequently dysregulated in various cancers. Although RNA editing was initially considered to be a rare event, limited specifically to coding exons within genes, high-throughput sequencing data have now revealed that it occurs more prevalently (23). In particular, A-to-I RNA editing mediated by ADARs is the most prominent form of RNA editing in humans, and various studies have recently demonstrated cancer-specific dysregulation of ADAR as well as subsequent site-specific RNA editing in several human cancers (9–11, 24). More specifically, *AZIN1* has been identified as one of the most frequently edited genes in HCC and esophageal cancers (9, 10). However, whether RNA editing holds a significant biological purpose in CRC is unknown. In this study, we demonstrated the dysregulation of ADAR1 in CRC, with corresponding *AZIN1* editing alteration using multiple independent CRC cohorts. *AZIN1* RNA editing levels showed strong association with various metastasis-associated parameters. Mechanistically, we used a series of functional validation studies in CRC cell lines and xenograft animal models to demonstrate that edited *AZIN1* acquires oncogenic properties, including enhancement of stem-like characteristics.
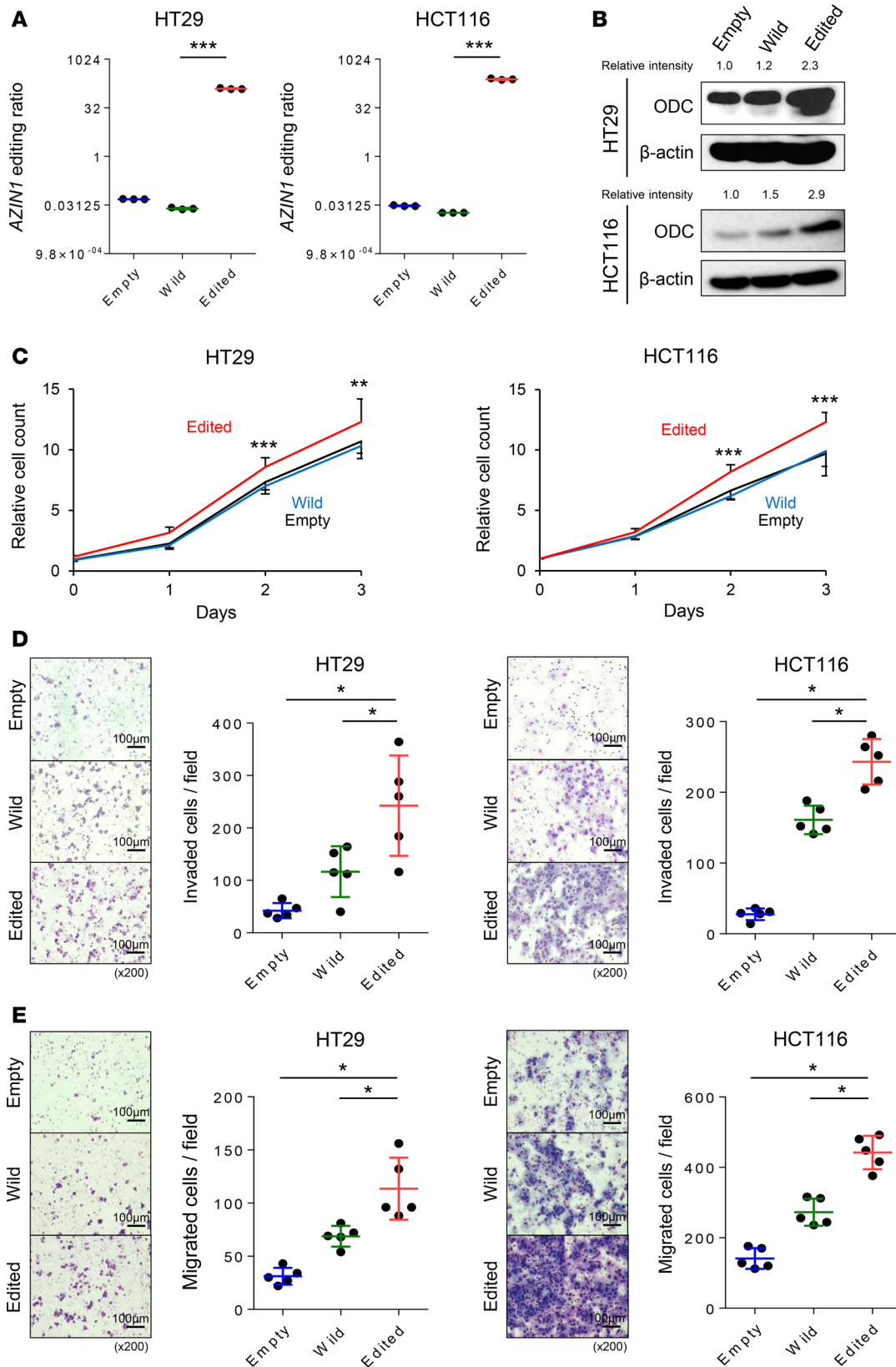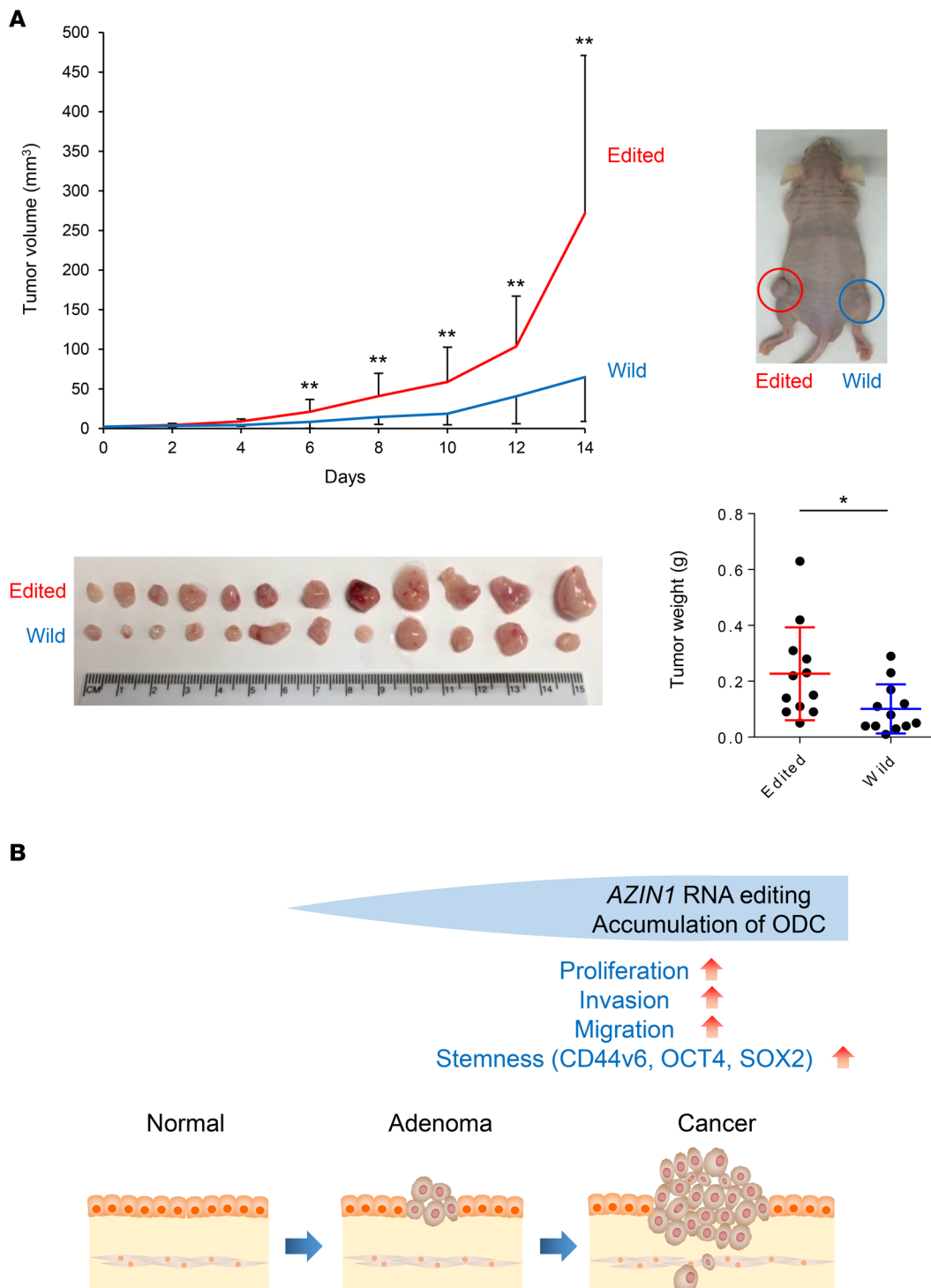
Figure 3. *AZIN1* RNA editing promotes cellular proliferation, invasion, and migration. (**A**) Edited to wild-type *AZIN1* RNA ratios in HT29 and HCT116 cell lines transfected with empty, wild-type, or edited *AZIN1*-containing plasmids, as determined by RESSq-PCR (*n* = 3) (Steel test). (**B**) ODC protein expression levels in the transfected HT29 and HCT116 cell lines analyzed by Western blot. (**C**) Effect on cellular proliferation of HT29 and HCT116 cells after transfection resulting in overexpression of wild-type or edited *AZIN1* (*n* = 16) (Wilcoxon's rank-sum test). (**D**) Effect of overexpression of wild-type or edited *AZIN1* on invasiveness of HT29 and HCT116 cells (*n* = 5) (Steel test). (**E**) Effect of overexpression of wild-type or edited *AZIN1* on migration of HT29 and HCT116 cells (*n* = 5) (Steel test). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bar: 100 μm; original magnification, ×200.
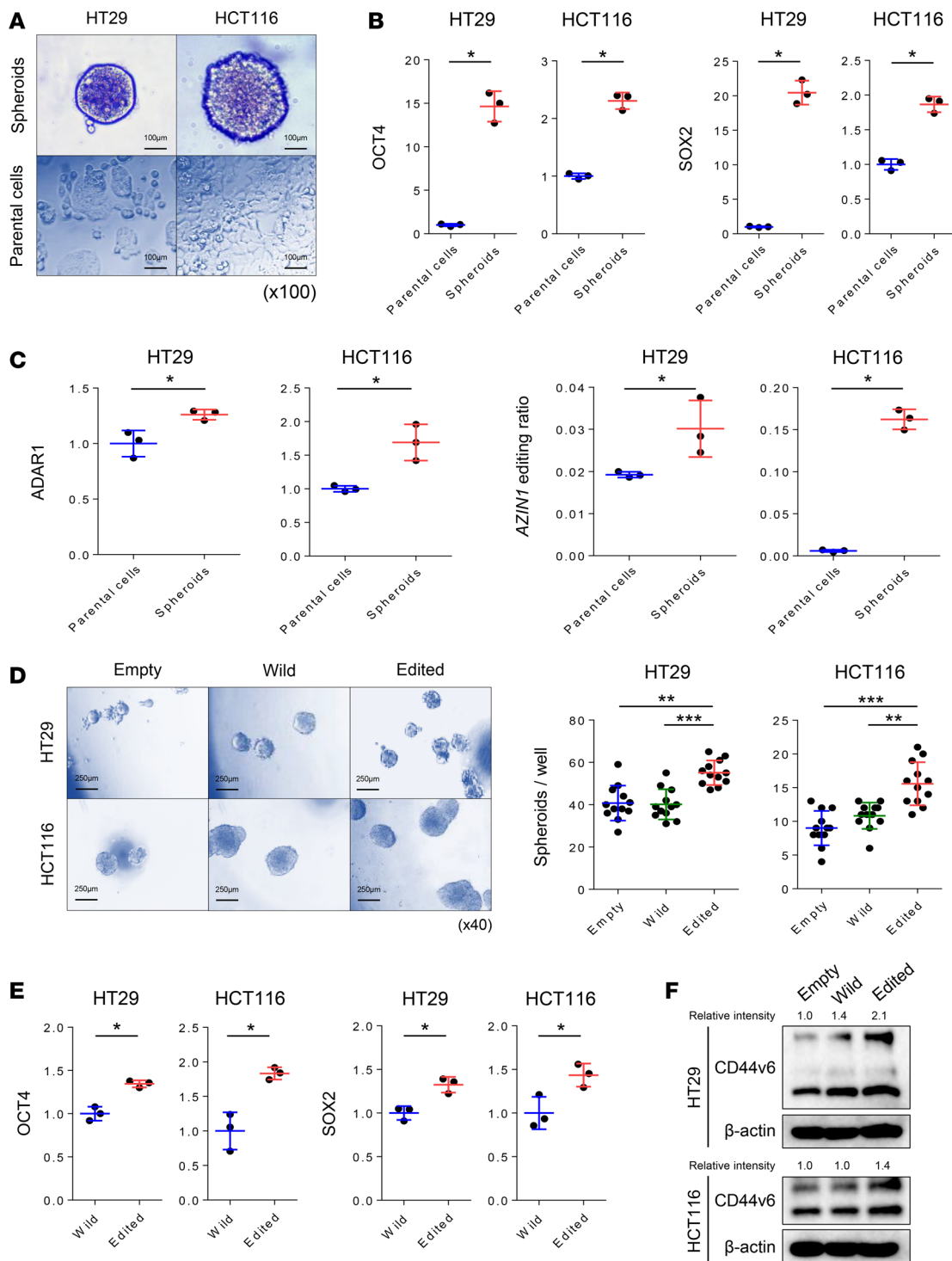
Figure 4. *AZIN1* RNA editing promotes stemness in CRC cells. (**A**) Spheroids established from HT29 and HCT116 cells (scale bar: 100 μm; original magnification, ×100). (**B**) OCT4 and SOX2 RNA expression levels in parental cells and spheroids derived from HT29 or HCT116 cell lines (*n* = 3) (Wilcoxon's rank-sum test). (**C**) *AZIN1* RNA editing levels and ADAR1 expression levels in parental cells and spheroids derived from HT29 and HCT116 cell lines (*n* = 3) (Wilcoxon's rank-sum test). (**D**) Comparison of spheroid formation by HT29 and HCT116 cells overexpressing wild-type or edited *AZIN1* with cells transfected with empty vector (*n* = 12) (Steel test) (scale bar: 250 μm; original magnification, ×40). (**E**) OCT4 and SOX2 RNA expression levels in spheroids derived from either wild-type or edited *AZIN1* overexpressing HT29 and HCT116 cells (*n* = 3) (Wilcoxon's rank-sum test). (**F**) CD44v6 protein expression in HT29 and HCT116 cells transfected with empty, wild-type, or edited *AZIN1*-containing plasmids. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

A

B

Figure 5. *AZIN1* RNA editing promotes tumor growth in a xenograft animal model. (**A**) Development of xenograft tumors was established by injecting mice with HCT116 cells that were transfected with either wild-type or edited *AZIN1*-containing plasmids (Wilcoxon's rank-sum test). (**B**) *AZIN1* RNA editing can promote proliferation, invasion, migration, and stemness in CRC. In combination with our findings from the clinical study, *AZIN1* RNA editing may have a crucial role in the shift from the preneoplastic step to the advanced metastatic step in CRC tumorigenesis. *$P <$ 0.05, **$P <$ 0.01.

In the present study, we demonstrated for the first time to our knowledge that RNA editing is dysregulated in CRC, a finding which is in consonance with previous reports in other cancers (9–11, 25, 26). We showed the dysregulation of ADAR1, with a corresponding increase in *AZIN1* RNA editing levels in cancer tissues when compared with normal mucosa in multiple CRC cohorts. Intriguingly, the enhancement of edited *AZIN1* was also observed in premalignant adenomas, suggesting that aberrant expression of ADAR1 with a concomitant increase in edited *AZIN1* RNA levels may be an important event in the early initiation steps of colorectal carcinogenesis. Furthermore, we showed that high *AZIN1* RNA editing levels, as well as the expression of ADAR1 in CRC tissues, were potential predictors for recurrence and poor prognosis in CRC patients. *AZIN1* RNA edited status showed a significant correlation with metastasis-associated clinical factors, including lymph node, hepatic, and distant metastases in CRC patients. Additionally, high *AZIN1* RNA editing status associated with disease recurrence and poor survival in stage II CRC patients, an important clinical finding considering that a significant proportion of stage II CRC patients (25%–30%) develop recurrence. Accordingly, *AZIN1* RNA editing could be used as a prognostic biomarker for the identification of high-risk stage II CRC patients who could truly benefit from adjuvant chemotherapy (27–29). Collectively, our data indicate that *AZIN1* RNA editing could be used as a prognostic biomarker for disease progression, especially for the metastatic process in CRC.

Using a series of experiments, we interrogated the functional role of edited *AZIN1* in CRC. We demonstrated that *AZIN1* RNA editing promoted the accumulation of ODC and subsequently enhanced the malignant potential of cells through increased proliferation, invasion, and migration. ODC is a key protein that catalyzes the rate-limiting step in polyamine synthesis and controls the rate of the cell cycle (30). *AZIN1* is an ODC homolog, which controls ODC accumulation through the inhibition of antizyme, a negative regulator of ODC (31). Hence, *AZIN1* prevents proteolytic degradation of ODC by sequestering antizyme from ODC. This stabilization of ODC leads to the accumulation of polyamine and increases cellular proliferation. Interestingly, recent studies show that edited *AZIN1* may confer even greater antizyme-binding affinity than standard *AZIN1*, thereby augmenting the effect on cellular proliferation (32, 33). Our results corroborate some of the previous studies reporting the oncogenic potential of edited *AZIN1* in HCC and esophageal cancer (9, 10). Additionally, ODC is known to promote self-renewal of embryonic stem cells via accumulation of polyamine (34). We showed that *AZIN1* RNA editing levels are increased in spheroid-derived cancer stem-like cells and edited *AZIN1* significantly enhanced spheroid formation, with a corresponding increase in stemness markers. Considering that CSCs are thought be involved in metastatic processes, the enhancement of stemness could be a pivotal link to our clinical findings.

One of the limitations of our study is that we did not determine whether edited *AZIN1* may enhance metastatic potential of CRC cells in vivo. We are currently in the process of establishing such an animal model for analyzing the metastatic potential of edited *AZIN1*-transfected cancer cells. Furthermore, considering that we have identified prognostic potential of edited *AZIN1* and demonstrated its involvement in cancer stemness, it would be interesting to determine whether edited *AZIN1* expression is associated with drug resistance.

In summary, our study provides evidence we believe to be novel for the oncogenic role of edited *AZIN1* in CRC. Our study highlights the clinical and biological significance of *AZIN1* RNA editing in CRC, including the enhancement of cancer stem-like features. Therefore, we propose that *AZIN1* RNA editing status could be used as a prognostic indicator in CRC patients and as a potential therapeutic target in CRC.

Methods

Patients and sample collection. In this study, we examined a total of 392 tissue specimens, which included 294 fresh-frozen primary CRCs, 12 adenomas, and 86 normal mucosae collected from 4 independent patient cohorts. These cohorts were enrolled at the National Cancer Center Hospital (training cohort), Okayama University Hospital (validation cohort), Mie University Hospital (clinical evaluation cohort), and Tokushima University Hospital (adenoma cohort), as described in Supplemental Table 1.

The diagnosis of CRC was confirmed for all enrolled patients based on clinicopathological findings. The TNM staging system from the American Joint Committee on Cancer was used for the pathological tumor staging of CRCs. All CRC patients who underwent surgery were followed up for tumor recurrence at regular intervals for up to 5 years. During each annual hospital visit, all patients underwent a chest x-ray, colonoscopy, and abdominal computed tomography. Patients treated with radiotherapy or chemotherapy before surgery were excluded from this study. All patients with stage III/IV disease received 5-fluorouracil–based chemotherapy, whereas no adjuvant chemotherapy was given to stage I and II patients.

RNA extraction and cDNA synthesis. Fresh-frozen surgical specimens were homogenized with a Mixer Mill MM 300 homogenizer (QIAGEN). The total RNA from tissues and cell lines was isolated using the RNeasy Mini kits (QIAGEN) according to the manufacturer's instructions. The cDNA was synthesized from 1.0 μg total RNA using the Advantage RT PCR kit (Clontech Laboratories Inc.).

RESSq-PCR. RNA editing of *AZIN1* was analyzed using the RESSq-PCR method published previously (14). In brief, specific primers for the wild-type and edited *AZIN1* sequences were designed (Supplemental Figure 1A). Based on the difference in the Ct values, the ratios between the edited and wild-type *AZIN1* were calculated using the formula $2^{-(Ct\ edited\ -\ Ct\ wild-type)}$. Primer sequences for the PCRs are shown in Supplemental Table 2. To confirm the reliability of this method for the assessment of *AZIN1* RNA editing levels, we first examined the levels of predetermined mixtures of oligonucleotides derived from wild-type or edited *AZIN1* sequences, ranging from 0% to 100%, and whether PCR can accurately quantify these oligonucleotides. We found that RESSq-PCR could reliably identify the two variants in the mixture, and the observed differences in the Ct values were adequate to generate ratios for edited and wild-type *AZIN1* (Supplemental Figure 1, B and C).

Real-time quantitative PCR analyses for ADAR1, ADAR2, OCT4, and SOX2. Real-time quantitative PCR was performed for gene expression analysis using the StepOne Real Time PCR System and Power SYBR Green Master Mix (Life Technologies), as previously described (35). GAPDH was used as a normalization control. The relative expression of each mRNA was determined using the ΔΔCt method. Primer sequences are shown in Supplemental Table 2.

Cell lines. The HT29 (microsatellite stable) and HCT116 (microsatellite unstable) CRC cell lines were purchased from ATCC. All cell lines were cultured according to the manufacturer's specifications. All cell lines were tested and authenticated every few months using a panel of established genetic markers. All experiments were performed using cells that did not exceed 15–20 passages.

Immunohistochemical analysis. Paraffin-embedded sections were deparaffinized using xylene and ethanol, and endogenous peroxidase activity was eliminated with H_2O_2. Following antigen retrieval by autoclaving the tissues at 121°C for 15 minutes, slides were incubated with an anti-ADAR1 antibody at a 1:100 dilution (Abcam) overnight. The color development was achieved using the EnVision + Dual Link Kit (DAKO), and slides were counterstained with hematoxylin. Negative controls were run in parallel. The level of ADAR1 staining was evaluated using the Allred proportion score (0, none; 1, 1%; 2, 1%–10%; 3, 10%–33%; 4, 33%–67%; and 5, >67% positive cells), measured 3 times by 2 independent investigators who were blinded to the nature of the specimens and antibodies used.

Immunofluorescence analysis. Following fixation by methanol, cultured cells were stained with an anti-AZIN1 antibody at a 1:200 dilution (Abcam) overnight, followed by Alexa Fluor 488–conjugated goat anti-mouse IgG (H+L) secondary antibody at a 1:200 dilution (A-11001, Thermo Fisher Scientific). Immunofluorescence was examined using an upright fluorescence microscope from the Olympus Laboratories.

Wild-type and edited AZIN1 overexpression assays. Plasmids bearing wild-type or edited *AZIN1* cDNA sequences were used to overexpress *AZIN1* (9). These plasmids were ligated into the pLenti6/V5-TOPO vector (9, 10), and each *AZIN1* expression construct was transfected into HT29 or HCT116 cells (2,000 ng per 1 million cells) using Lipofectamine 2,000 (Invitrogen). For all transfections, empty pLenti6/V5-TOPO vector was used as the control vector. Forty-eight hours after transfection, the transfected cells were collected for further experiments.

Western immunoblotting. Western immunoblotting experiments were performed as described previously (36). Anti-ADAR1 (1:2,000 dilution; ab88574, Abcam), anti-ODC (1:2,000 dilution; ab66067, Abcam), and anti-CD44v6 (1:1,000 dilution; ab78960, Abcam) antibodies were used to detect target proteins, and an anti–β-actin antibody (1:5,000 dilution; A5441, MilliporeSigma) was used as the loading control.

Cell proliferation assays. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MilliporeSigma) was performed to measure the effect on cell proliferation following the overexpression of wild-type or edited *AZIN1* using the method described previously (35).

Invasion/migration assays. The invasiveness of cancer cells was evaluated using BioCoat Matrigel Invasion Chambers (Corning Life Sciences) as described previously (35).

Establishment of spheroid-derived CSCs. Spheroid-derived CSCs were generated from HT29 and HCT116 cells in serum-free DMEM/F12 medium containing B27, N2 supplements (Gibco), 10 ng/ml human recombinant basic fibroblast growth factor (Gibco), and 20 ng/ml epidermal growth factor (MilliporeSigma) and cultured in Costar ultra-low attachment flasks (Corning) as described previously (37).

Spheroid formation assays. HT29 and HCT116 cells were cultured in serum-free DMEM/F12 medium, including B27 and N2 supplements, 10 ng/ml human recombinant basic fibroblast growth factor (Gibco), and 20 ng/ml epidermal growth factor (MilliporeSigma), using ultra-low-attachment 96-well plates (Corning). The number of spheroids was counted using a microscope.

Xenograft studies. Male athymic nude mice were obtained from Harlan Laboratories at 5 weeks of age and kept under controlled conditions (12-hour light/dark cycles), with food and water ad libitum. To establish a xenograft tumor model, HCT116 cell lines transfected with wild-type or edited *AZIN1* cDNA sequence plasmids were subcutaneously injected in the left and right flanks of 12 mice (3×10^6 cells/injection site) with 50 μl Matrigel (Corning). Mice were monitored for 14 days following the injection, and subcutaneous tumors were measured every second day (1/2 length × width × height). At 2 weeks after injection, all animals were sacrificed.

Statistics. Results are expressed as mean ± SD. JMP software (version 10.0, SAS Institute Inc.) and MedCalc (version 16.8.4, MedCalc Software) were used to perform statistical analyses. Differences between groups were estimated by Wilcoxon's signed-rank test, Wilcoxon's rank-sum test, the χ^2 test, Steel test, Kruskal-Wallis test, and 1-way ANOVA analysis, as appropriate. The correlation of two groups was estimated by Spearman's rank correlation analysis. For time-to-event analyses, survival estimates were calculated using the Kaplan-Meier analysis, and groups were compared with the log-rank test. Receiver operating characteristic curves were established to determine the cutoff values for analyzing risk factors for prognosis and each metastasis type by Youden's index. OS was measured from the date patients underwent surgery to the date of death, resulting from any cause, or the last known follow-up for patients that were still alive. DFS analysis was measured from the date the patient underwent curative surgery to the date of disease recurrence, death from any cause, or until last contact with the patient.

The Cox's proportional hazards models were used to estimate HRs for recurrence or death. Assumptions of proportionality were confirmed for the Cox proportional hazards analyses by generating Kaplan-Meier survival curves (e.g., high vs. low expression groups) and by ensuring that the two curves did not intersect each other. Multivariate logistic regression models were used to predict factors influencing lymph node metastasis, hepatic metastasis, and distant metastasis. Forced-entry regression was used to include these variables in all multivariable equations in order to analyze whether each of the predictors affected the outcome after adjusting for known confounders. All *P* values were 2 sided, and those less than 0.05 were considered statistically significant.

Study approval. Written informed consent was obtained from each patient, and the institutional review boards of Baylor University Medical Center; Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences; Mie University Graduate School of Medicine; University of Tokushima; National Cancer Center Hospital; and National University of Singapore approved this study. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Baylor Scott & White Research Institute.

Author contributions

KS, YO, ST, JM, and AG conceived and designed experiments. KS, YO, and ST performed experiments. KS, YO, ST, JM, and AG analyzed data. KS, YT, TN, MK, NT, TT, YY, TF, and LC contributed reagents, materials, and other analytical tools. KS, YO, ST, and AG wrote the manuscript.

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