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## Activation of *AZIN1* RNA editing is a novel mechanism that promotes invasive potential of cancer-associated fibroblasts in colorectal cancer

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### Abstract

Adenosine-to-inosine (A-to-I) RNA editing is a recently described epigenetic modification, which is believed to constitute a key oncogenic mechanism in human cancers. However, its functional role in cancer-associated fibroblasts (CAFs) within the tumor microenvironment (TME) and its clinical significance remains unclear. Herein, we systematically analyzed a large cohort of 627 colorectal cancer (CRC) specimens, and investigated the expression pattern of ADAR1 and its biological significance on the *antizyme inhibitor 1* (*AZIN1*) RNA editing levels. Both ADAR1 expression and *AZIN1* RNA editing levels were significantly elevated in CRC tissues vs. normal mucosa, and these findings correlated with the increased expression of mesenchymal markers, Vimentin ( $\rho=0.44$ ) and Fibroblast activation protein ( $\rho=0.38$ ). Intriguingly, ADAR1 expression was specifically upregulated in both cancer cells and fibroblasts from cancerous lesions. Conditioned medium from cancer cells led to induction of ADAR1 expression and activation of *AZIN1* RNA editing in fibroblasts ( $p<0.05$ ). Additionally, edited *AZIN1* enhanced the invasive potential of fibroblasts. In conclusion, we provide novel evidence that hyper-editing of *AZIN1*

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enhances the invasive potential of CAFs within the TME in colon and is an important predictor of tumor invasiveness in CRC.

### Keywords

*AZINI*; RNA editing; cancer associated fibroblasts; colorectal cancer; biomarker; ADAR

## INTRODUCTION

Colorectal cancer (CRC) is a common malignancy worldwide and remains the second leading cause of cancer-related deaths in Western countries[1]. CRC pathogenesis is strongly associated with lifestyle choices, such as smoking, diet, alcohol, and obesity[2, 3]. Emerging evidence demonstrates that such lifestyle choices profoundly impact various epigenetic modifications, including DNA methylation[4], histone alterations[5], and regulation of the expression of noncoding RNAs[6]. Dysregulation of these epigenetic processes can directly influence oncogenic signaling pathway, and lead to cancer[7].

RNA editing is a recently identified epigenetic mechanism that regulates posttranscriptional activity of key oncogenes by altering their amino acid sequence and leading to changes in oncogenic function[8]. For example, recent studies have determined that dysregulation of RNA editing is involved in the development of hepatocellular carcinoma, gastric cancer, and colorectal cancer (CRC)[9–12]. Such a RNA editing process, wherein the conversion of an adenosine (A) to inosine (I) in primary RNA transcripts (A-to-I editing) is mediated by Adenosine Deaminase family Acting on RNA (ADAR), leading to diversification of the transcriptome in human cells[13]. One of the targets of RNA editing, *Antizyme inhibitor 1* (*AZINI*), confers a gain-of-function phenotype frequently through A-to-I conversions, which can promote ornithine decarboxylase (ODC) and polyamines accumulation – conditions that are associated with aggressive tumors[9, 10]. Intriguingly, *AZINI* is also an important target of ADAR1 in various cancers[9–12].

From a mechanistic perspective, conditions that promote cancer progression and metastasis are frequently influenced by cancer-associated fibroblasts (CAFs) within the tumor microenvironment (TME). Invasion by CAFs within the TME frequently guides invasion by the cancer cells[14]. Although CAFs can be deemed to be important prognostic markers and potential therapeutic targets[15], the clinical significance of RNA editing in CAFs remains unexplored.

This study is the first to investigate the clinical significance of ADAR1 expression and the degree of *AZINI* RNA editing in colorectal CAFs, along with the determination the functional role of *AZINI* RNA editing in this malignancy. We discovered that ADAR1 is frequently overexpressed in fibroblasts from CRC specimens, and discovered that conditioned medium derived from cancer cells induces ADAR1 expression and promotes *AZINI* RNA editing in fibroblasts. Furthermore, we identified a novel mechanism where *AZINI*, that has undergone A-to-I editing, promotes the invasive potential in fibroblasts. Collectively, we illustrate that dysregulation and activation of RNA editing is a salient

feature of the oncogenic cascade that promotes the invasive potential of fibroblasts within the TME in colon cancer.

## METHODS AND MATERIALS

### Patients and Sample Collection

This study examined a total of 627 clinical specimens, which included a testing cohort of 115 matched CRCs and normal mucosa procured from Okayama university and a validation cohort of 512 CRC specimens from TCGA dataset as described in Table 1[16–18]. The labels for the TCGA primary CRC consensus molecular subtypes (CMS) were obtained from Sage Bionetworks Synapse (syn4978511)[19–21]. The diagnosis of each CRC was confirmed for all enrolled patients based on clinicopathological findings. The Tumor Node Metastasis (TNM) staging system from the American Joint Committee on Cancer was used for pathology staging. Written, informed consent was obtained from each patient, and the institutional review board approved this study.

### RNA extraction and cDNA synthesis

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

### RNA editing site-specific quantitative PCR (RESSq-PCR)

The degree of editing of *AZINI* RNA was analyzed using the RNA editing site-specific quantitative PCR (RESSq-PCR) method published previously[22]. In brief, specific primers for the wild-type and edited *AZINI* sequences were designed. Based on the difference in the Ct values, the ratios between the edited and wild-type *AZINI* were calculated using the formula  $2^{-(Ct_{\text{Edited}} - Ct_{\text{Wild-type}})}$ . Primer sequences for the PCRs are shown in Supplementary Table 1.

### Real-time quantitative PCR analyses for ADAR1

The 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, Carlsbad, CA, USA), as previously described[23]. GAPDH was used as a normalization control. The relative expression of each mRNA was determined using the Ct method. Primer sequences are shown in Supplementary Table 1.

### Cell lines

The HCT116, HT29, and RKO CRC cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). 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[12]. 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<sub>2</sub>O<sub>2</sub>. Following antigen retrieval by autoclaving the tissues at 121°C for 15 minutes, slides were incubated overnight with an anti-ADAR1 antibody, at a 1:100 dilution (Abcam, Cambridge, MA, USA). The color development was achieved using EnVision + Dual Link Kit (DAKO, Carpinteria, CA, USA) 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 three times by two independent investigators who were blinded to the nature of the specimens and antibodies used.

### Immunofluorescence analysis

Cultured cells were fixed in methanol prior to staining with anti-*AZIN1* antibody at a 1:200 dilution (ab57169, Abcam) overnight, followed by Alexa Fluor 488-conjugate Goat anti-Mouse IgG (H+L) secondary antibody at a 1:200 dilution (A-11001, Thermo Fisher Scientific, Waltham, MA, USA). Immunofluorescence labeling was examined using an upright fluorescence microscope from the Olympus laboratories.

### Wild-type and RNA edited *AZIN1* overexpression assays

Plasmids bearing wild type or RNA 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 a fibroblast cell line, WI38 (2000 ng per one million cells) using Lipofectamine 2000 (Invitrogen). For all transfections, empty pLenti6/V5-TOPO®vector was used as the control vector. Forty-eight hours after transfection, the fibroblasts were collected for further experiments.

### Western immunoblotting

Western immunoblotting experiments were performed as described previously[24]. Anti-ODC (1:2000 dilution; ab66067, Abcam) antibodies were used to detect target proteins, and an anti-β-actin antibody (1:5000 dilution; A5441, Sigma) was used as the loading control.

### Invasion/migration assays

The invasiveness of cancer cells was evaluated using BioCoat Matrigel Invasion Chambers (Corning Life Sciences, Tewksbury, MA, USA) as described previously[23].

### Statistical analysis

Results are shown as means ± standard deviation (SD). JMP software (ver. 10.0, SAS Institute Inc., Cary, NC, USA) was used to perform statistical analyses. Differences between groups were estimated by Wilcoxon's rank sum test, the  $\chi^2$  test, and Steel test, as appropriate. Correlations of two groups were 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. Overall survival (OS) was measured from the date patients underwent surgery until their date of death resulting from

any cause, or last known follow-up for patients that were still alive. All p values were 2-sided, and those less than 0.05 were considered statistically significant.

## RESULTS

The ADAR1 expression is upregulated and the degree of *AZINI* RNA editing is increased in CRC ADAR1 is reported to be dysregulated in several cancer[9–12]. We first measured the expression of ADAR1 in both our testing and validation cohorts. ADAR1 was significantly overexpressed in cancerous tissues when compared to adjacent normal mucosa ( $p < 0.0001$  in testing cohort,  $p = 0.0439$  in validation cohort; Figure 1A). ADAR1 can catalyze specific A-to-I editing in RNA. We confirmed that the degree *AZINI* RNA editing was significantly elevated in CRC tissues when compared to corresponding normal mucosa ( $p < 0.0001$ ; Figure 1B). Our results demonstrate that activation of RNA editing is a key feature of CRCs.

### Mesenchymal markers are frequently overexpressed in Consensus Molecular Subtype 4 colorectal cancers

Consensus Molecular Subtype (CMS) classification represents CRC heterogeneity at the gene-expression level[19]. We assessed whether RNA editing associated with any specific CMS subgroups. First, we confirmed the clinical significance of various CMS in CRC. As reported previously, the CMS4 group exhibited substantially worse survival compared with other groups ( $p = 0.10$ ; Figure 1C). The CMS4 subtype is characterized as mesenchymal, therefore Vimentin (VIM) and Fibroblast activation protein (FAP) were significantly upregulated in CMS4 cancers vis-à-vis other groups ( $p < 0.0001$ ; Figure 1D). Other mesenchymal markers including N-cadherin, Snail, and Twist were upregulated in CMS4 CRCs. On the other hand, E-cadherin was downregulated in CMS4 CRCs (Supplementary Figure 1). Our results suggest that CMS4 CRCs might be influenced by the epithelial-mesenchymal transition (EMT) or accumulation of CAFs within the TME.

### ADAR1 is overexpressed in CMS4 CRCs and correlates with mesenchymal markers

To investigate the relationship between RNA editing enzyme, ADAR1, we analyzed ADAR1 expression in each of the four CMS subgroup. Intriguingly, ADAR1 expression was significantly upregulated in CMS4 vs. CMS2 ( $p < 0.0001$ ) and CMS3 ( $p < 0.0001$ ; Figure 1E) cancers. Interestingly, ADAR1 was overexpressed in CMS1 CRCs, which is not surprising because these lesions are characterized by very diffused immune infiltrates; while increased number of immune cells are responsible for enhanced interferon secretion and induced ADAR1 expression[25].

ADAR1 expression in CRC tissues positively correlated with Vimentin ( $p < 0.0001$ ,  $\rho = 0.44$ ) and FAP ( $p < 0.0001$ ,  $\rho = 0.38$ ), suggesting that RNA editing is affected by EMT or fibroblast accumulation (Figure 1F). Although our data show that ADAR1 expression and mesenchymal characteristics correlate in CRC microenvironment, the key cause of upregulation of RNA editing enzyme remains unknown.

### ADAR1 is overexpressed in mesenchymal fibroblasts in CRC

We next determined whether RNA editing is induced in EMT-cancer cells or mesenchymal fibroblasts. First, we analyzed ADAR1 expression in EMT-induced cancer cell lines. However, neither ADAR1 expression nor RNA editing were upregulated in EMT-induced cancer cells (data not shown). These findings indicated that upregulation of ADAR1 and RNA editing does not originate from EMT-induced cancer cells. Next, we used clinical specimens from CRC patients to determine whether ADAR1 was overexpressed in mesenchymal cells.

Immunohistochemical analysis revealed a strong staining for ADAR1, not only in CRC cells but also in mesenchymal fibroblasts when compared with adjacent normal mucosa (Figure 2A). Intriguingly, ADAR1-positive fibroblasts were significantly more concentrated within the cancerous lesions, and ADAR1 expression was much higher in CRC ( $p<0.0001$ ) and fibroblasts ( $p<0.0001$ ) vs. normal epithelium (Figure 2B).

### ADAR1 targets *AZIN1* editing in fibroblasts

The ability of ADAR1 to target *AZIN1* was investigated *in vitro* by modulating the RNA expression. The efficient suppression of ADAR1 via RNA interference resulted in reduced *AZIN1* RNA editing ( $p=0.0495$ ; Figure 3A). The inverse experiment was performed by overexpression of ADAR1 using ADAR1-carrying plasmids in WI38 fibroblasts. The efficient overexpression of ADAR1 manifested with an increase *AZIN1* RNA editing ( $p=0.0495$ ; Figure 3B). Collectively, these results suggest that *AZIN1* RNA editing is controlled by ADAR1 in fibroblasts.

### Conditioned medium from cancer cells induced upregulation of ADAR1 and enhanced *AZIN1* RNA editing in fibroblasts

Analysis of clinical specimens revealed that ADAR1 is expressed at higher levels in fibroblasts surrounding cancer cells. However, there is lack of any direct evidence indicating any interactions between cancer cells and fibroblasts. We next assessed whether cancer cells can promote ADAR1 expression and enhance *AZIN1* RNA editing in fibroblasts. Fibroblasts were cultured in the conditioned medium derived from CRC cells. Fibroblasts cultured with such conditioned medium expressed ADAR1 at significantly higher levels when compared to controls ( $p=0.0495$  in HCT116, HT29, and RKO medium; Figure 3C). Likewise, the overexpression of ADAR1 was associated with corresponding increase in *AZIN1* RNA editing ( $p=0.0495$  in HCT116, HT29, and RKO medium; Figure 3D). Collectively, these results suggest that cancer cells promote *AZIN1* RNA editing in fibroblasts via induction of ADAR1 RNA editing enzyme. Our results are consistent with the clinical evidence that CMS4 mesenchymal subtype shows higher ADAR1 expression (Figure 1E).

### Edited *AZIN1* promotes invasion and migration in fibroblasts

To better understand the biological significance of edited *AZIN1* RNA in fibroblasts, we overexpressed either wild-type or edited *AZIN1* RNA in WI38 fibroblast cells. The overexpression of *AZIN1* was confirmed by immunohistochemistry and RT-qPCR (Figure 4A). We next performed invasion and migration assays to determine whether edited *AZIN1* RNA promotes the invasive and migratory potential in fibroblasts. Overexpression of edited

*AZINI* RNA enhanced invasiveness of fibroblasts relative to wild-type *AZINI* ( $p=0.0079$ ; Figure 4B). Similarly, the overexpression of edited *AZINI* RNA increased the migratory potential of fibroblasts when compared to wild-type *AZINI* overexpressed cells ( $p=0.0079$ ; Figure 4C). Collectively, these results suggest that edited *AZINI* RNA in fibroblasts may facilitate cancer progression and worse prognosis in CMS4 CRCs.

Previously the oncogene, ODC, was identified as a downstream target of edited *AZINI*[9]. The overexpression of edited *AZINI* RNA resulted in upregulation of ODC protein in fibroblasts, confirming that edited *AZINI* RNA stabilizes ODC more effectively than its wild-type counterpart in fibroblasts (Figure 4D). In this previous study, ODC was reported to be the promoter of invasive potential in fibroblasts[26]. Our results suggest that edited *AZINI* RNA may promote invasion of fibroblasts via accumulation of ODC (Figure 4E).

Taken together, findings from our study suggest that overexpression of ADAR1 in cancer lesion promotes malignant potential in CRC. We present novel evidence that ADAR1 is overexpressed in fibroblasts and may facilitate invasive potential in cancerous lesions in CRC microenvironment (Figure 5).

## DISCUSSION

CRC originates from the accumulation of a series of genetic and epigenetic alterations in colorectal tissues. RNA editing has emerged as a critical epigenetic modification responsible for the evolution and disease progression in various cancers. Adenosine-to-inosine (A-to-I) RNA editing in the context of oncogenes or tumor suppressor genes can alter tumor characteristics to promote a more aggressive phenotype. One of the major targets of the RNA editing enzyme ADAR1, which itself is dysregulated in various cancers, is *AZINI*. Emerging evidence indicates that *AZINI* is highly edited in various cancers including HCC, esophageal cancer, and CRC[9, 10, 12]; and edited version of *AZINI* is highly oncogenic.

Fibroblasts influence the TME by remodeling the extracellular matrix and creating tracks for collective invasion of cancer cells[27, 28]. In particular, invasion of TME by CAFs frequently precedes invasion by cancer cells, often termed as ‘CAF-led cancer cell invasion’ [14]. Therefore, anti-tumor therapies are touted to eventually include targeting of CAFs[28–30]. However, whether RNA editing has a significant biological impact on fibroblasts in the TME remains unknown. In this study, we demonstrated that ADAR1 levels are frequently dysregulated in CRC, which leads to enhanced editing of its target gene, *AZINI* RNA. Furthermore, we observed that ADAR1 overexpression is not exclusive to cancer cells but also occurs in CAFs as well. Intriguingly, ADAR1 expression is significantly higher in mesenchymal colorectal tumors – those designated as mesenchymal subgroup of CRCs (CMS4) in this study. We performed a series of functional validation studies in fibroblast cells to determine how edited *AZINI* contributes to cancer invasiveness.

Ours is the first report to demonstrate that the RNA editing enzyme ADAR1 is frequently overexpressed in mesenchymal subgroup CMS4 CRCs. We also reveal that ADAR1 overexpression correlated with enhanced *AZINI* RNA editing in CRC tissues. Intriguingly, ADAR1 expression correlated with the mesenchymal markers Vimentin and FAP, which

further supports our argument that *AZINI* RNA editing facilitates the invasive potential in CRC-associated fibroblasts. By discovering a correlation between ADAR1 and mesenchymal markers, we were encouraged to investigate the significance of RNA editing in mesenchymal cells, specifically CAFs, which are important components of the TME landscape.

To the best of our knowledge, no prior report has demonstrated how *AZINI* RNA editing facilitates the invasive potential of CRC-associated fibroblasts. In addition, none of the previous studies have tested the hypothesis behind the cause of upregulated ODC in fibroblasts. Our findings fill in a very important gap in knowledge. To answer this question, we examined the functional role of edited *AZINI* RNA in fibroblasts by using clinical data obtained from CRC cohorts. We demonstrated that conditioned medium derived from cancer cells stimulated ADAR1 expression and *AZINI* RNA editing in fibroblasts. Additionally, *AZINI* RNA editing promoted the accumulation of ODC followed by enhanced invasiveness in fibroblasts. ODC is a key protein that catalyzes the first and rate-limiting step in polyamine synthesis[31, 32]. *AZINI* is an ODC homologue, which controls ODC accumulation through the inhibition of antizyme, a negative regulator of ODC[33]. Edited *AZINI* RNA has a greater binding affinity for the antizyme vs. the wild-type *AZINI*; which can help stabilize ODC more efficiently.

We are the first to propose that the *AZINI* RNA editing-ODC accumulation axis is exploited by fibroblasts to promote invasiveness. Enhanced *AZINI* RNA editing leads to ODC accumulation. ODC then promotes invasiveness of fibroblasts - a characteristic feature of cancer cells[26]. We are also the first to demonstrate the invasive potential of edited *AZINI* RNA with ODC accumulation in fibroblasts. We conclude that a pathway that includes ADAR1, *AZINI* editing, and ODC is triggered by cancer cells and may activate and promote fibroblasts to invade the tumor microenvironment.

One of the limitations of our study is our inability to identify the soluble factor from cancer cells that stimulates RNA editing. However, considering that we have uncovered the invasive potential of edited *AZINI* RNA in CAFs, our findings support further investigations into whether ADAR1 inhibition is a viable therapy for CRC.

In summary, our study provides novel evidence for the tumor-supportive role of edited *AZINI* RNA in fibroblasts in CRC. Our study highlights the biological and clinical significance of *AZINI* RNA editing in fibroblasts and its impact on enhancing invasion within the TME in colon cancer. Our findings suggest that *AZINI* RNA editing levels are not only important prognostic biomarkers, these may also serve as a potential therapeutic target in colorectal cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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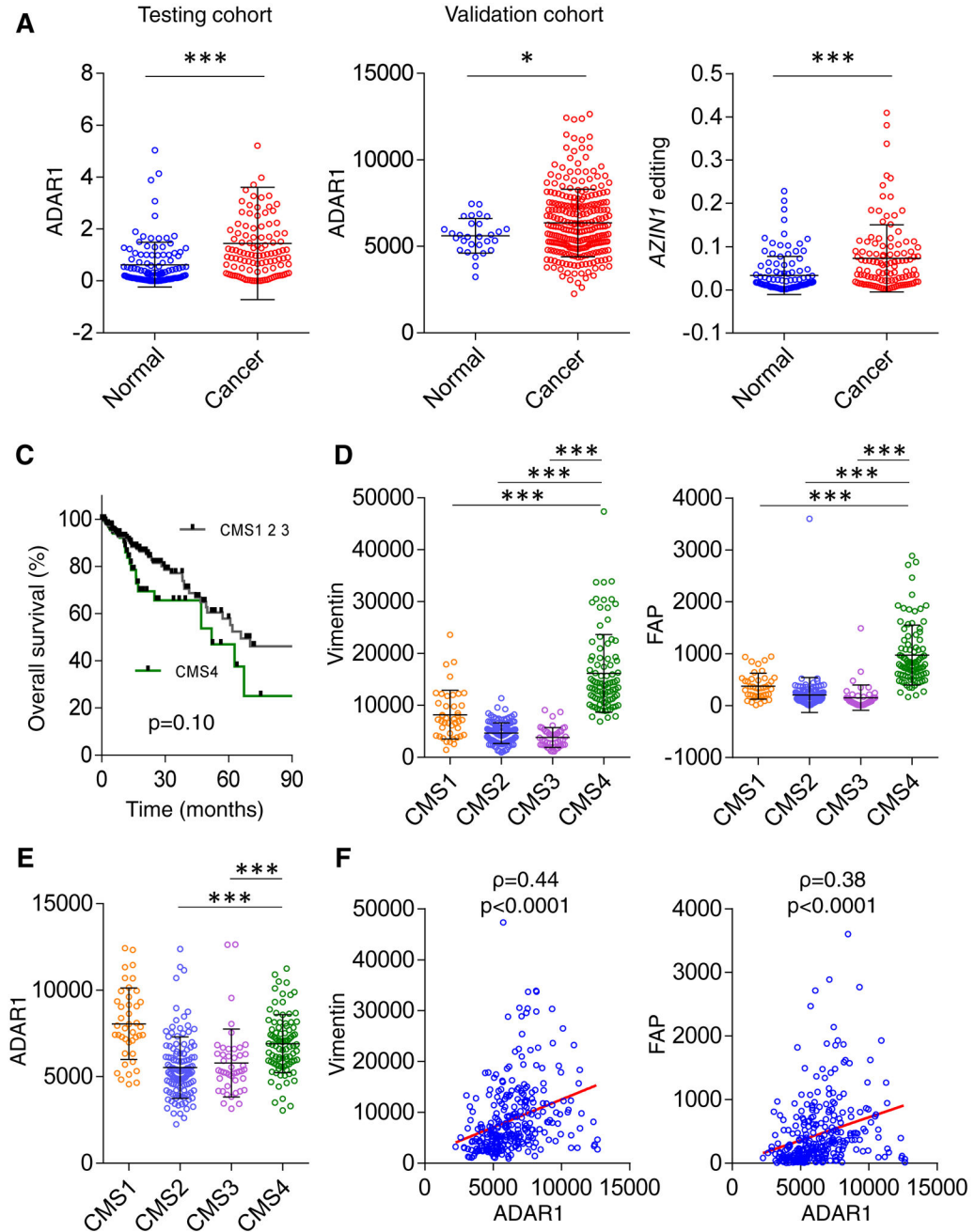
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### HIGHLIGHTS

- Hyper-editing of AZIN1 RNA is a frequent event in colorectal cancer fibroblasts.
- Increased AZIN1 editing promotes invasion in colorectal cancer tumor microenvironment.
- AZIN1 may be a potential therapeutic target in colorectal cancer.



**Figure 1: ADAR1 and AZIN1 RNA editing are upregulated in CMS4 CRC**

(A) ADAR1 expression levels in CRC tissues compared with normal mucosa in testing and validation cohorts. Wilcoxon's signed rank test. (B) AZIN1 RNA editing levels in CRC tissues compared with normal mucosa in the testing cohort. Wilcoxon's signed rank test. (C) Kaplan-Meier survival curves for OS in CRC patients in validation cohort sorted into CMS4 and other subgroups. Log-rank test. (D) CMS-dependent expression status of Vimentin and FAP in CRC tissues. Steel test. (E) CMS-dependent expression status of ADAR1 in CRC

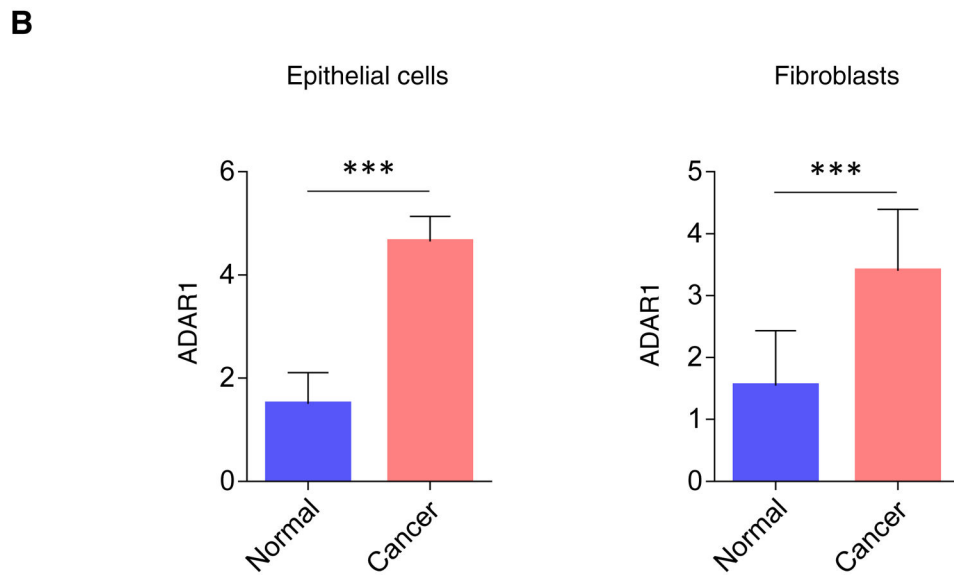
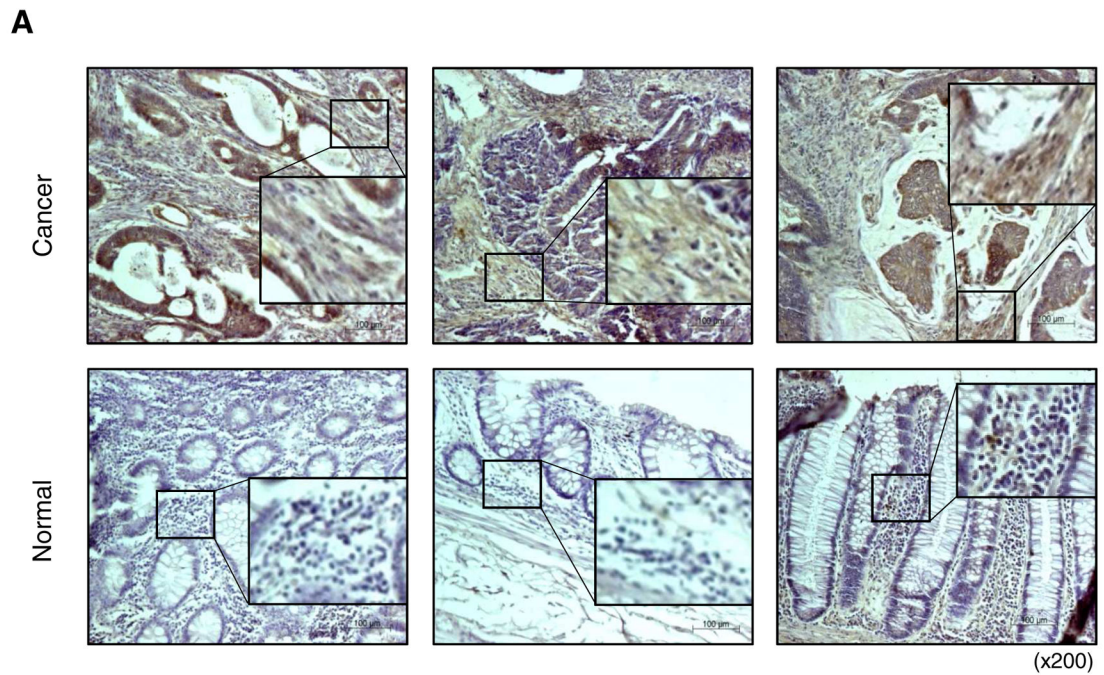
tissues. Steel test. **(F)** Correlation between Vimentin, FAP, and ADAR1 expression levels.  
\*p<0.05, \*\*\*p<0.001.

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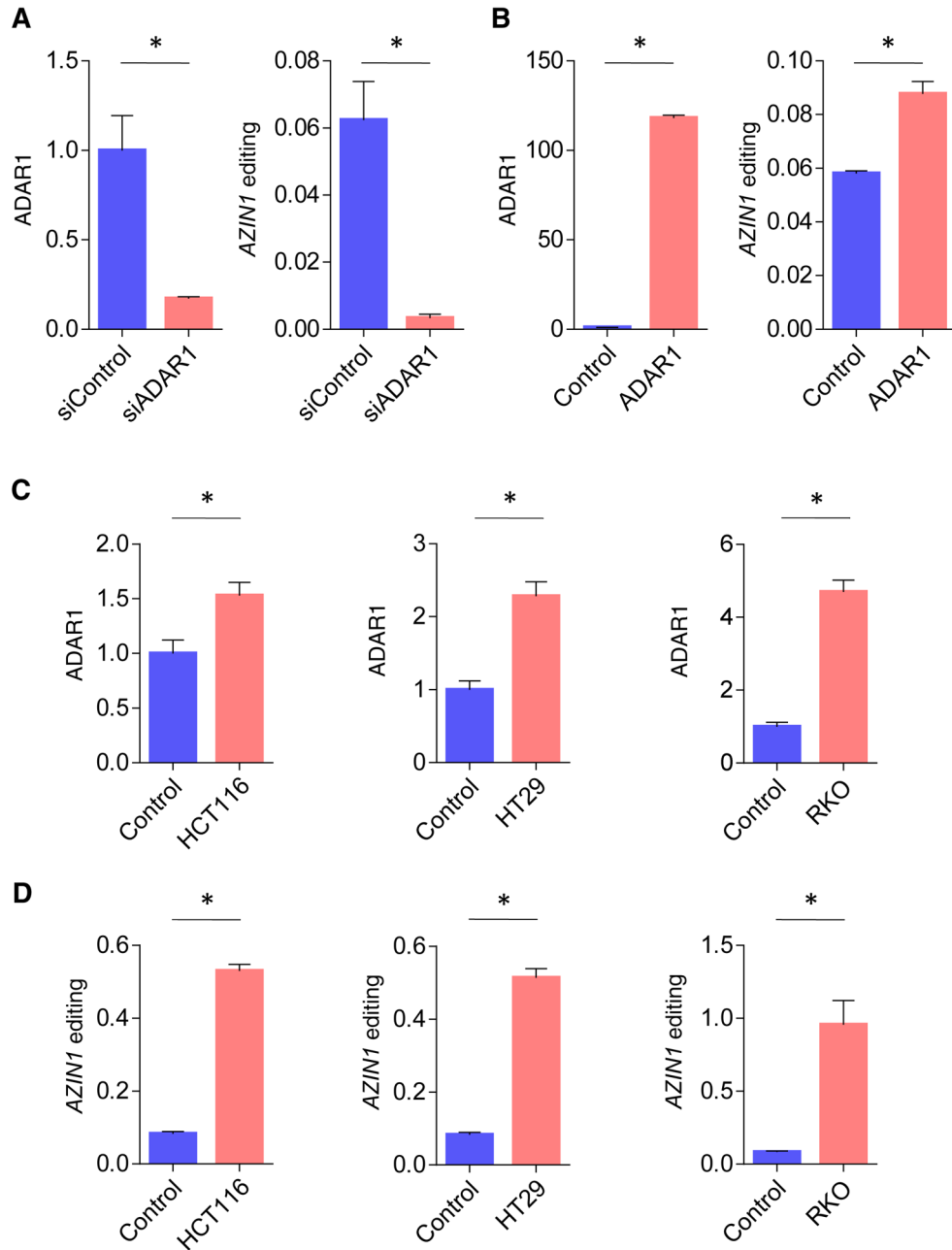
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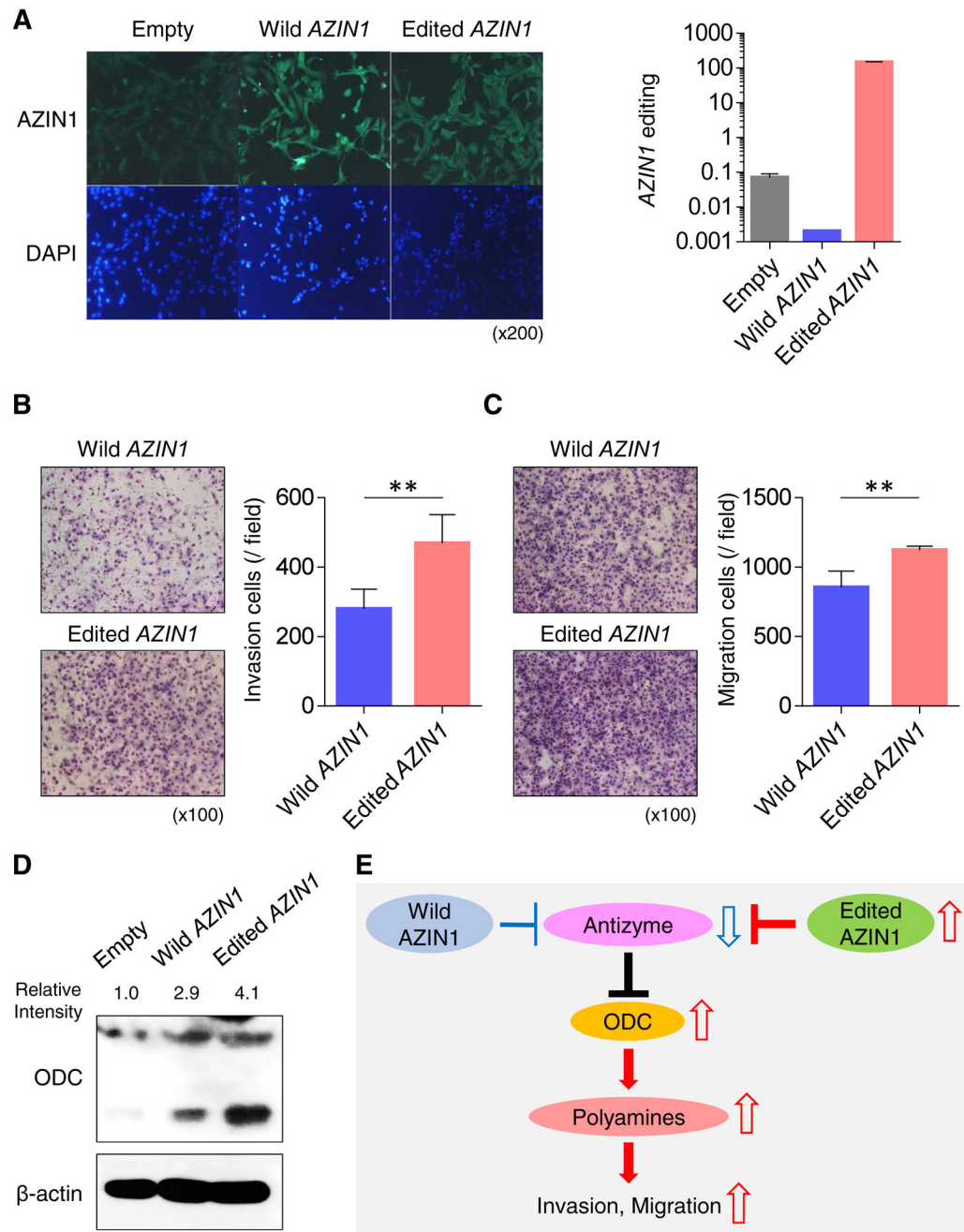
**Figure 2: ADAR1 is overexpressed in mesenchymal fibroblasts in CRC lesions**  
**(A)** Immunohistochemical staining of ADAR1 in CRC clinical specimens. **(B)** ADAR1 expression in epithelial cells or fibroblasts in CRC. Wilcoxon's signed rank test. \*\*\* $p < 0.001$ .



**Figure 3: Culture medium of cancer cells induced upregulation of ADAR1 and AZIN1 RNA editing in fibroblasts**

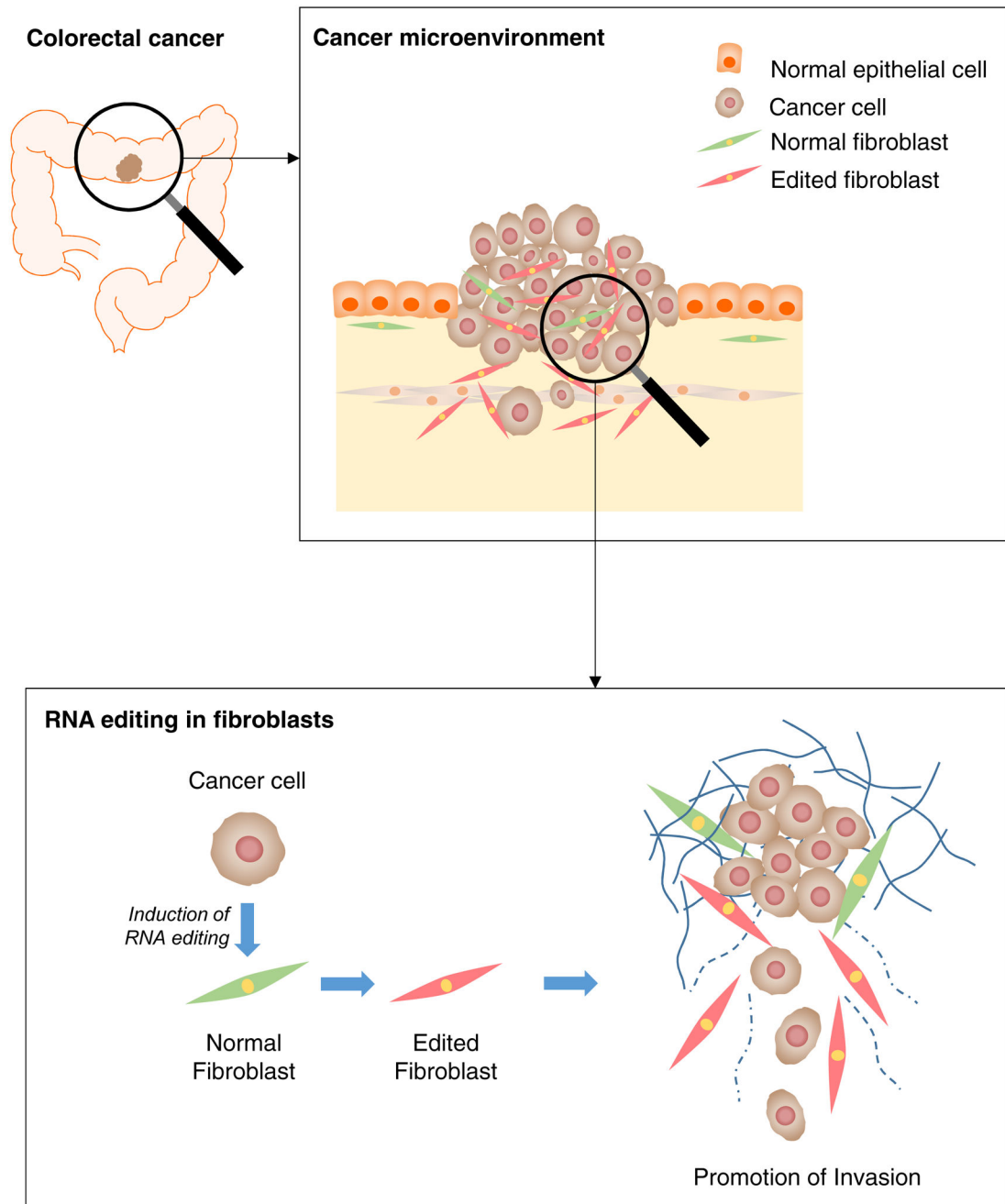
(A) ADAR1 expression levels in WI38 fibroblast cell line transfected with siADAR1 or siControl. Wilcoxon's signed rank test. (B) RNA edited to wild-type *AZIN1* RNA ratios in HT29 and HCT116 cell lines transfected with wild type or edited *AZIN1* RNA containing plasmids as determined by RESSq-PCR. Wilcoxon's signed rank test. (C) ADAR1 expression levels in WI38 fibroblast cell line cultured in cancer medium. Wilcoxon's signed rank test. (D) RNA Edited to wild type *AZIN1* RNA ratios in WI38 fibroblast cell line cultured in cancer medium by RESSq-PCR. Wilcoxon's signed rank test. \* $p < 0.05$ .





**Figure 4: RNA edited *AZIN1* promotes invasion and migration in fibroblasts**

(A) ADAR1 expression levels and RNA edited to wild type *AZIN1* RNA ratios in WI38 fibroblast cell line transfected with wildtype or edited *AZIN1* RNA containing plasmids. (B) Effect of overexpression of wild type or edited *AZIN1* RNA on invasiveness of WI38 cell line. Wilcoxon's signed rank test. (C) Effect of overexpression of wild type or edited *AZIN1* RNA on migration of WI38 cell line. Wilcoxon's signed rank test. (D) ODC protein expression levels in the transfected WI38 cell line analyzed by western blot. (E) Promotion of invasion and migration by RNA edited *AZIN1* in fibroblasts. \*\* $p < 0.01$ .



**Figure 5:** Upregulation of ADAR1 in cancer-associated fibroblasts may promote cancer progression. ADAR1 is also overexpressed in fibroblasts and may facilitate the invasive potential of cancerous lesions in CRC.

Table 1:

Clinicopathological variables and *AZIN1* RNA editing status in validation cohort

Variable	n	CMS1 (n=76)	CMS2 (n=221)	CMS3 (n=72)	CMS4 (n=143)	P value
Gender						0.7547
Male	239	37	123	38	75	
Female	273	39	98	34	68	
Age (y)						0.0074
<68 <sup>#</sup>	249	24	109	36	80	
≥68	263	52	112	36	63	
Pathological T category						<0.0001
pT1/2	108	14	60	22	12	
pT3/4	404	62	161	50	131	
Lymph node metastasis						<0.0001
Absent	296	56	125	52	63	
Present	216	20	96	20	80	
Distant metastasis						0.0089
Absent	442	72	185	67	118	
Present	69	4	36	4	25	
UICC TNM classification						<0.0001
Stage I/II	287	55	118	51	63	
Stage III/IV	225	21	103	21	80	
ADAR1						<0.0001
Low	148	8	83	28	29	
High	148	34	37	17	60	
Vimentin						<0.0001
Low	148	14	93	41	0	
High	148	28	27	4	89	
Fibroblast activation protein						<0.0001
Low	148	17	90	39	2	
High	148	25	30	6	87	

<sup>#</sup>The median age at surgery is 68 years in this cohort.