RNA editing in *RHOQ* promotes invasion potential in colorectal cancer

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RNA editing can increase RNA sequence variation without altering the DNA sequence. By comparing whole-genome and transcriptome sequence data of a rectal cancer, we found novel tumor-associated increase of RNA editing in *ras homologue family member Q* (*RHOQ*) transcripts. The adenosine-to-inosine (A-to-I) editing results in substitution of asparagine with serine at residue 136. We observed a higher level of the *RHOQ* RNA editing in tumor compared with normal tissue in colorectal cancer (CRC). The degree of RNA editing was associated with RhoQ protein activity in CRC cancer cell lines. RhoQ N136S amino acid substitution increased RhoQ activity, actin cytoskeletal reorganization, and invasion potential. *KRAS* mutation further increased the invasion potential of RhoQ N136S in vitro. Among CRC patients, recurrence was more frequently observed in patients with tumors having edited *RHOQ* transcripts and mutations in the *KRAS* gene. In summary, we show that RNA editing is another mechanism of sequence alteration that contributes to CRC progression.

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Abbreviations used: CRC, colorectal cancer; HCC, hepatocellular carcinoma; ICE, inosine chemical erasing; *RHOQ*, ras homolog family member Q.

Colorectal cancer (CRC) is a major global health problem as indicated by its incidence and mortality rate around the world. Genetic and epigenetic alterations, including chromosomal instability, microsatellite instability, and CpG island methylation, contribute to development and progression of CRC. Continuous efforts have been made to better understand the genomic signatures of CRC (Wood et al., 2007; Leary et al., 2008). Recently, comprehensive characterization of genomic alterations in CRC was made possible using next-generation sequencing technology (Bass et al., 2011; Seshagiri et al., 2012; Cancer Genome Atlas Network, 2012). The spectrum of genomic information encompasses mutations, copy number alterations, and gene fusions.

RNA editing is a posttranscriptional modification of the RNA sequence that can increase the diversity of the transcriptome repertoire. Adenosine-to-inosine (A-to-I) editing mediated by adenosine deaminase acting on RNA is the predominant form of RNA editing in humans (Nishikura, 2010). Because the edited inosine is recognized as guanosine during the translational process, RNA editing in coding regions can result in amino acid substitution. In normal tissues, RNA editing has been most

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widely studied in nervous system tissue. RNA editing is important in maintaining normal brain function, and defects in RNA editing have been reported in neurological disorders (Mehler and Mattick, 2007).

Dysregulation of RNA editing has also been demonstrated in cancer. Reduced A-to-I editing was observed in several human tumor types, including brain tumors (Maas et al., 2001; Paz et al., 2007). Moreover, restoration of the defective editing activity was able to inhibit proliferation of brain tumor cells (Paz et al., 2007; Cenci et al., 2008). In contrast, increased A-to-I editing in hepatocellular carcinoma (HCC) has been identified in recent studies (Chan et al., 2013; Chen et al., 2013). Increased editing of *AZIN1* transcript resulted in amino acid substitution, which conferred enhanced tumorigenicity in HCC (Chen et al., 2013). Genome-wide detection of potential A-to-I editing site candidates in HCC was made possible by next-generation sequencing of the transcriptome (Chan et al., 2013; Chen et al., 2013). Recent advances in transcriptome sequencing and bioinformatic analysis have facilitated identification of novel RNA editing sites (Ju et al., 2011; Li et al., 2011; Bahn et al., 2012; Ramaswami et al., 2013). These advances will promote future studies that will enhance our understanding of the role of RNA editing in cancer.

In the present study, we identified a novel A-to-I editing of *ras homologue family member Q* (*RHOQ*) transcripts by comparing whole-genome and transcriptome sequencing data. This editing is frequently found in CRC and results in amino acid substitution associated with invasiveness. Our results indicate that RNA editing is another mechanism of sequence alteration contributing to CRC progression.

RESULTS AND DISCUSSION

Identification of RNA editing in *RHOQ* transcripts

We collected fresh-frozen tumor and adjacent normal mucosal tissue samples from a 59-yr-old male patient who underwent low anterior resection for rectal cancer. Pathological examination revealed moderately differentiated adenocarcinoma invading the muscularis propria, without any lymph node metastasis (pT2N0M0).

We performed whole-genome and transcriptome sequencing of both the tumor and normal tissue. Mean coverage of whole-genome sequencing analysis was 22X for the tumor and 24X for the normal mucosa. A total of 30 somatic mutations (27 point mutations and 3 deletions) were identified in the coding sequence (unpublished data). Mutations were detected in *KRAS*, *APC*, and *FBXW7*, which are commonly mutated genes in CRC.

Comparing transcriptome and whole-genome sequencing data, 24 tumor-associated RNA editing events were identified (unpublished data). Among the 13 nonsynonymous RNA editing events, which were validated by Sanger sequencing of genomic DNA (gDNA) and complementary DNA (cDNA) of the tumor and normal tissue, three RNA editing events in three genes were confirmed (Fig. 1 A). The A-to-I RNA editing of *RHOQ* transcripts resulted in amino acid substitution from asparagine (AAT) to serine (AGT). Two other editings were G-to-T (*TEX10*) and G-to-A (*PSMG2*) editing, which resulted in tryptophan (TGG) to cysteine (TGT) substitution and arginine (CGC) to histidine (CAC) substitution, respectively.

Tumor-associated RNA editing in *RHOQ*

We quantitatively analyzed the A-to-I RNA editing of *RHOQ* in 60 pairs of CRC and adjacent normal mucosa samples by measuring the edited sequence in the cDNA using pyrosequencing. The degree of RNA editing was significantly higher in the tumor compared with matched normal tissue ($P < 0.0001$; Fig. 1 B). Compared with normal tissue, the degree of RNA editing increased in 70% (42/60) of the tumors (median absolute difference: +20.7%, range: +0.5–70.0%), and decreased in 30% (18/60) of the tumors (median absolute difference: −5.0%, range: −0.4–17.3%). Using a cutoff value of at least 20% edited sequence, RNA editing of *RHOQ* transcripts was observed in 40% (24/60) of the tumors compared with 10% (6/60) in the normal tissue samples.

We next analyzed the A-to-I editing in 92 cell lines that are commonly used in cancer research. RNA editing of *RHOQ* transcripts was also observed in various types of cancer cell lines, but the prevalence and degree of editing was varied according to tumor type (Fig. 1 C). Higher levels of RNA editing of *RHOQ* transcripts were observed in cell lines from gastric cancer (62%, 8/13), nonsmall cell lung cancer (60%, 9/15), and CRC (38%, 11/29). The lowest level of RNA editing of *RHOQ* transcripts was found in HCC cell lines (8%, 1/13).

To confirm that the guanosine in the cDNA sequence was the result of guanosine replacement of edited inosine in messenger RNA (mRNA) during reverse transcription, we performed an inosine chemical erasing (ICE) assay (Sakurai et al., 2010). We cyanoethylated total RNA from SNU C4 and SNU 81 cells under two different time conditions (Fig. 1 D). After the cyanoethylation and RT-PCR, the guanosine peaks resulting from inosine significantly decreased in a time-dependent manner relative to the adenosine peaks at the RNA-editing site of *RHOQ*. These data demonstrate that the guanosine sequence in the *RHOQ* cDNA is a result of A-to-I editing, and therefore excludes the possibility of guanosine contamination.

Effect of RNA editing on RhoQ protein activity

We examined the correlation between the degree of RNA editing and the active form of the RhoQ protein in a panel of 19 CRC cell lines. Because GTP-RhoQ specifically binds to PAK1, whereas GDP-RhoQ does not, we measured PAK1-bound GTP-RhoQ as the active form using a pull-down assay (Chiang et al., 2002). We found that active PAK1-bound GTP-RhoQ was significantly higher in cell lines with edited *RHOQ* transcripts compared with WT *RHOQ* transcripts (Pearson's $r = 0.625$; $P = 0.004$; Fig. 2 A). We then evaluated whether transfection of plasmid encoding edited *RHOQ* resulted in higher activity of RhoQ protein than transfection of plasmid encoding WT *RHOQ* in cell lines with endogenous

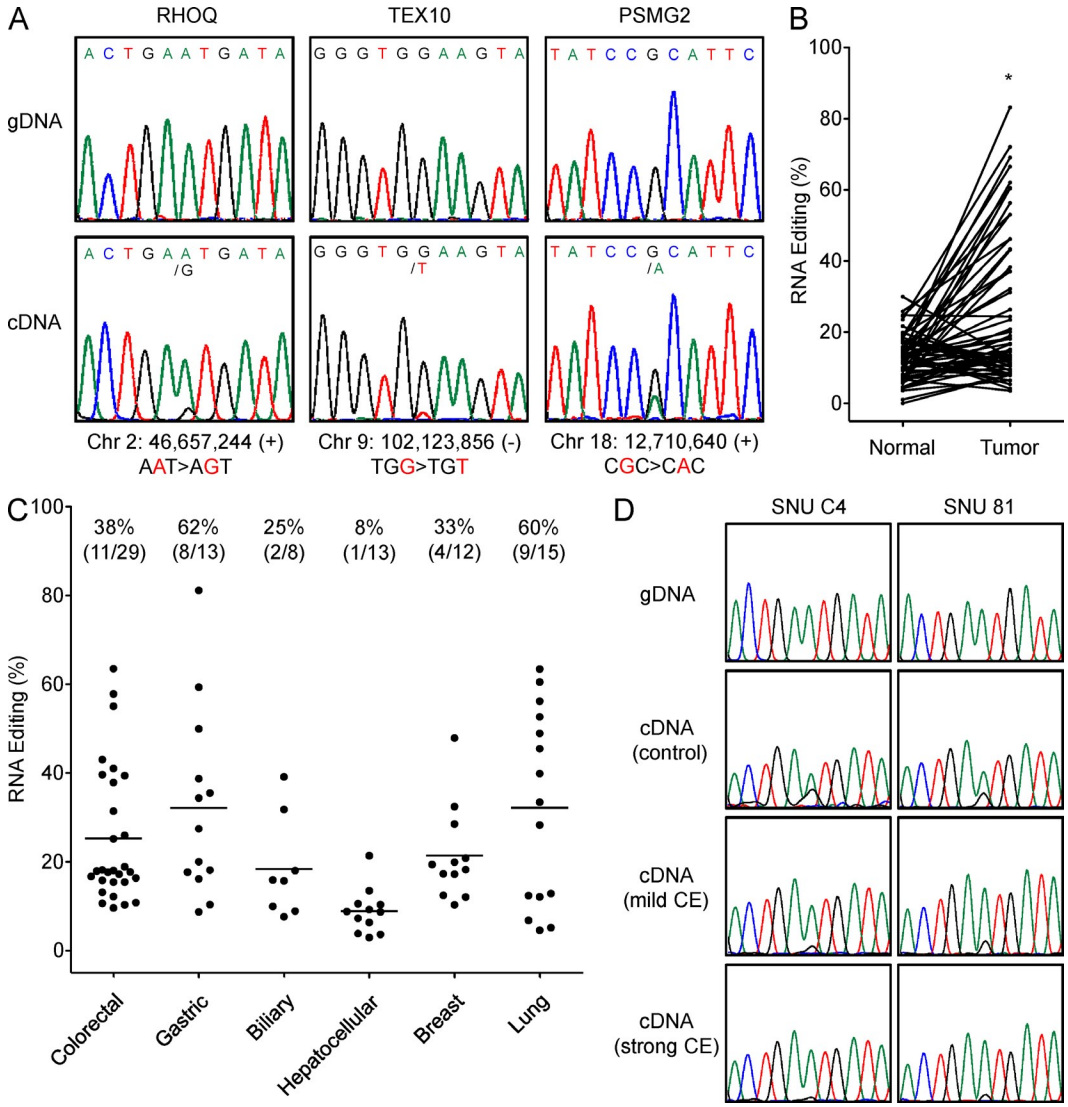

Figure 1. *RHOQ* RNA editing in CRC patients and cancer cell lines. (A) RNA editings of *RHOQ* (chr 2: 46,657,244; A-to-G), *TEX10* (chr 9: 102,123,856; G-to-T), and *PSMG2* (chr 18: 12,710,640; G-to-A) are validated in genomic DNA (top) and cDNA (bottom) by Sanger sequencing. (B) *RHOQ* RNA editing in 60 paired specimens of primary colorectal tumor and adjacent normal mucosa. The percentage of RNA editing was determined using pyrosequencing. The p-value was calculated by paired Student's *t* test. (C) Percentage of *RHOQ* editing in the following cell lines: CRC ($n = 29$), gastric cancer ($n = 13$), biliary cancer ($n = 8$), HCC ($n = 13$), breast cancer ($n = 1$ 2), and nonsmall cell lung cancer ($n = 15$). The percentages and the numbers in parentheses indicate the percentages and numbers of cell lines/total cell lines having *RHOQ* RNA editing using a cut-off of 20% edited sequence determined by pyrosequencing, respectively. (D) Top panels show chromatograms of regions amplified from gDNA in SNU C4 and SNU 81 cells. The bottom three sets of panels show the chromatograms of cDNAs amplified from total RNA under the following conditions: without cyanoethylation (control, 0 M acrylonitrile for 15 min), treated with mild conditions of cyanoethylation (mild CE, 1 mol/liter acrylonitrile for 15 min), or strong conditions of cyanoethylation (strong CE, 1 mol/liter acrylonitrile for 30 min). The G peaks resulting from A-to-I editing decreased in response to the strength of cyanoethylation. Data are representative of three independent experiments. *, $P < 0.001$.

WT *RHOQ* transcripts and low RhoQ protein activity. We transfected COS7, HCT116, and LOVO cell lines with plasmids encoding mock, WT, or edited *RHOQ* (Fig. 2 B). Expression of edited RhoQ protein resulted in a 1.6–2.4-fold increase in PAK1-binding of GTP-RhoQ compared with that in cells expressing WT RhoQ protein.

We also analyzed changes in the subcellular distribution of RhoQ protein using immunofluorescent staining (Fig. 2 C). Expression of edited RhoQ protein led to actin cytoskeletal reorganization in the HCT116 cell line, which was not observed in cells overexpressing WT RhoQ protein. Furthermore, whereas WT RhoQ protein was observed only in the plasma membrane, edited RhoQ protein colocalized with the reorganized actin fibers, in addition to the plasma membrane staining. These data suggest that the edited RhoQ protein is involved in dynamic reorganization of the actin cytoskeleton.

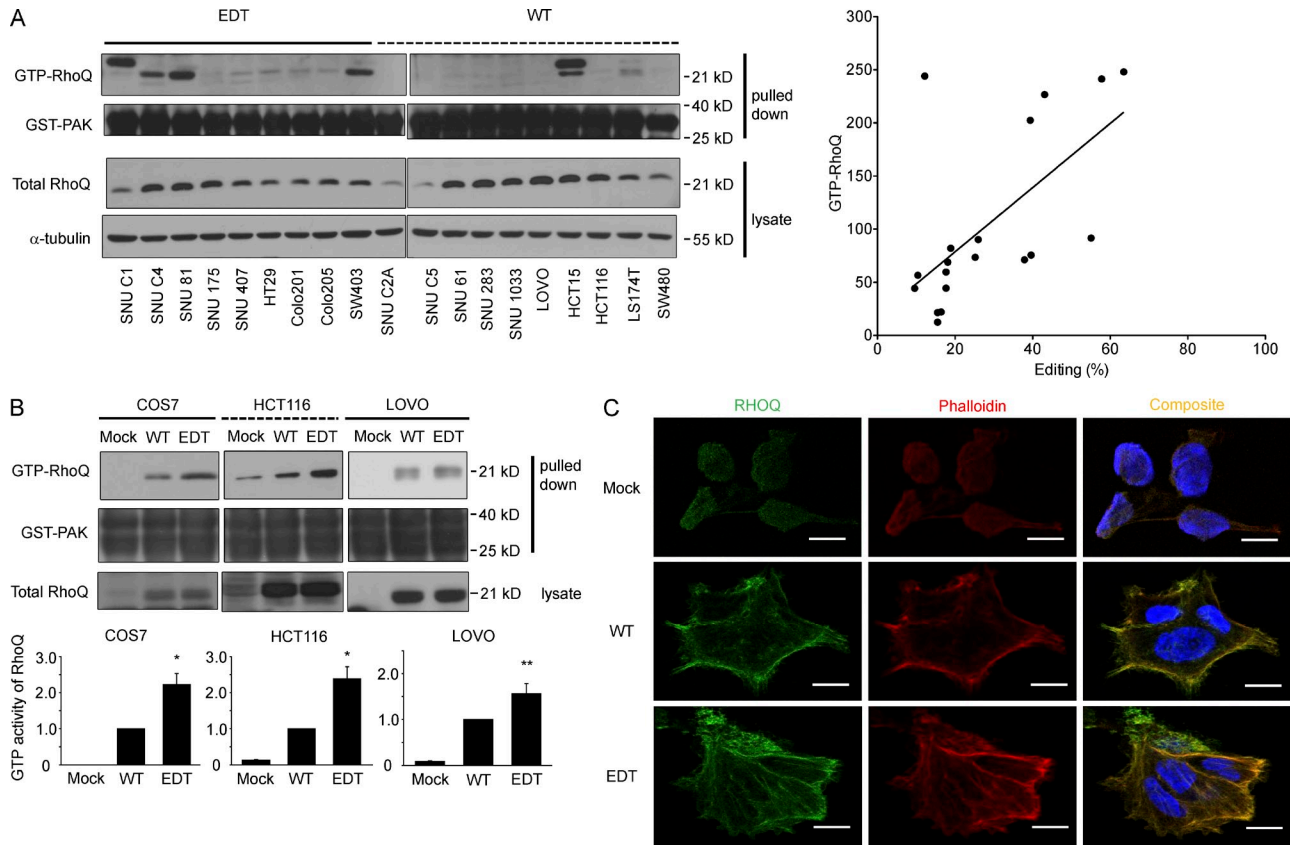

Figure 2. ***RHOQ*** **RNA editing increases the GTP-bound form of RhoQ and regulates F-actin reorganization.** (A) 19 human CRC cell lines were used for the RhoQ activity assay by in vitro GST-PAK1 (76–150) pull-down. Western blot analysis for GTP-RhoQ, GST-PAK, total RhoQ, and α-tubulin are shown for the indicated cells (left). Scatter plot with linear regression line showing amount of GTP-bound RhoQ measured by densitometer versus degree of editing (right; two independent reconstitution experiments). (B) COS7, HCT116, and LOVO cells were transiently transfected with WT *RHOQ*, N136S edited *RHOQ* (EDT), or empty vector control (Mock) and the activity of RhoQ was measured by the RhoQ activity assay after 48 h. Western blotting was performed for GTP-RhoQ, GST-PAK, and total RhoQ. Data represent the mean ± SD of three independent experiments. P-values were determined by Student's *t* test comparing WT and EDT. (C) Stable RhoQ WT or RhoQ N136S (EDT)-expressing HCT116 cells were stained with rhodamine-phalloidin (red) to visualize F-actin and with antibodies to RhoQ (green) and Alexa Fluor 488–conjugated anti-mouse IgG to visualize RhoQ. Areas with overlapping actin (red) and RhoQ (green) staining are seen as yellow in the composite image. Bars, 20 µm. Data are representative of two independent experiments. *, $P < 0.05$; **, $P = 0.069$.

RNA editing of *RHOQ* transcripts and invasion potential

We next evaluated the effect of *RHOQ*-transcript editing on the proliferation and invasion potential of CRC cell lines. We transfected the HCT116 cell line with plasmid encoding mock, WT, or edited *RHOQ* and compared the viability of cells for 5 d using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (Fig. 3 A). No difference in cell proliferation was observed according to *RHOQ* status. Nonetheless, transfection with plasmid encoding edited *RHOQ* significantly increased the invasion potential of HCT116 and LOVO cells (Fig. 3 B). Expression of edited RhoQ protein resulted in a 2.3–5.1-fold increase in invasive cells compared with expression of WT RhoQ protein. In contrast, small interfering RNA-mediated knockdown of RhoQ in the SW403 cell line having edited *RHOQ* significantly decreased invasion potential. There was a 79% reduction in the number of invasive cells (Fig. 3 C). These data provide evidence that the edited form of the RhoQ (N136S) protein plays an important role in promoting the invasive potential of CRC. Moreover, co-transfection of plasmids encoding edited *RHOQ* and mutant *KRAS* (G12D) further increased the invasive potential in SNU C5 cells, which have endogenous WT *RHOQ* and *KRAS*, compared with cells transfected with either edited *RHOQ* alone or mutant *KRAS* alone (Fig. 3 D).

We also analyzed the clinical significance of the *RHOQ* RNA editing (N136S) in a cohort ($n = 129$) of stage II and III CRC patients treated with surgery with curative intent, followed by adjuvant oxaliplatin, 5-fluorouracil, and leucovorin chemotherapy. Edited *RHOQ* RNA was found in 87% of the tumors analyzed. No recurrence was observed in the WT *RHOQ* group ($n = 17$), whereas 10 patients had recurrences in the edited *RHOQ* group ($n = 112$) during a median follow up period of 27 (range, 18–47) months ($P = 0.15$ by log-rank test). Notably, recurrence was more frequently observed in patients with tumors having edited *RHOQ* and mutant *KRAS*

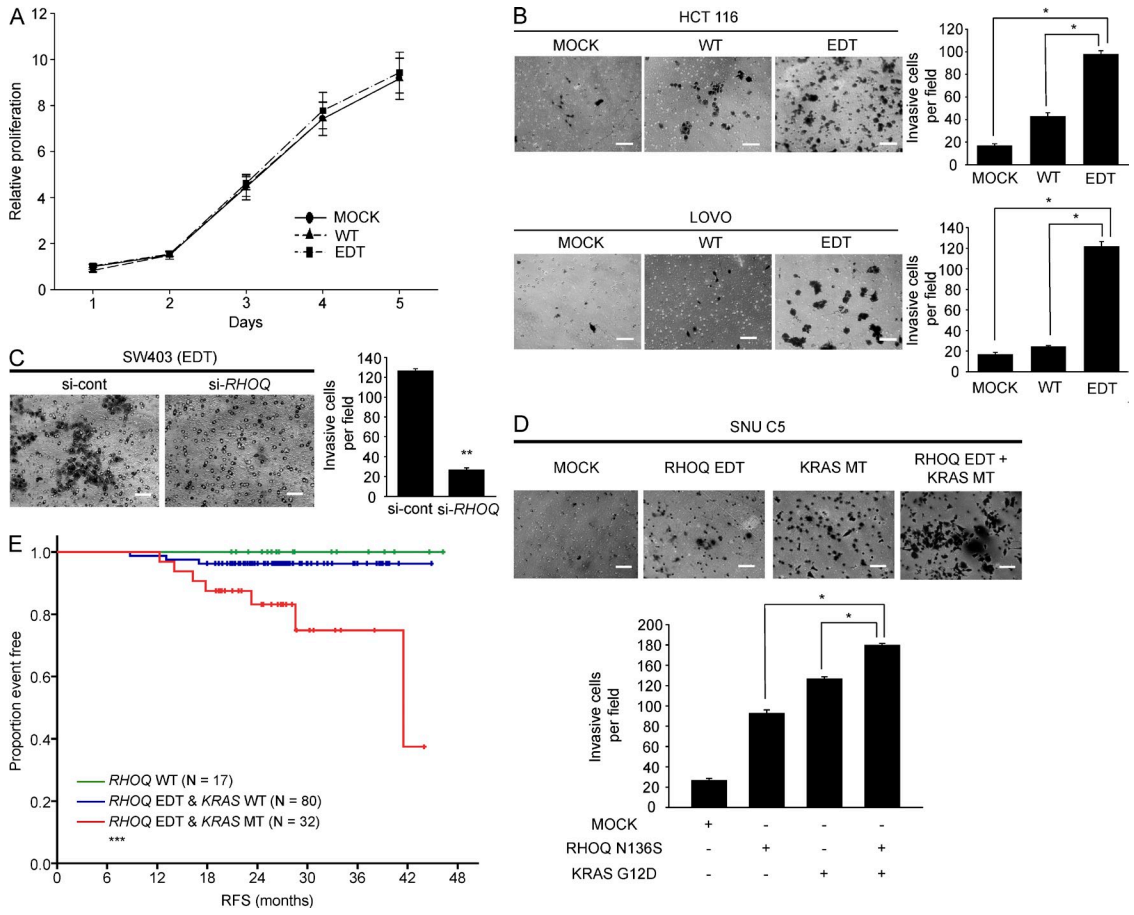

Figure 3. *RHOQ* RNA editing enhances invasion potential. (A) HCT116 cells were transfected with WT *RHOQ*, N136S edited *RHOQ* (EDT), or empty vector control (Mock). The cell viabilities were measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays for 5 d. Data are representative of two independent experiments. (B) HCT116 and LOVO cells were transfected with the indicated plasmids for 72 h. Cell invasion was analyzed via Boyden chamber assay. Bars, 100 µm. Data represent the mean ± SD of three independent experiments. P-values were calculated by the Student's *t* test. (C) SW403 cells expressing edited *RHOQ* were transfected with either *RHOQ* small interfering RNA (si-*RHOQ*) or scramble siRNA (si-cont) and were analyzed with the Boyden chamber assay. Data represent the mean ± SD of three independent experiments. P-value was determined by the Student's *t* test. (D) SNU C5 cells were transfected with WT *RHOQ*, N136S edited *RHOQ* (EDT), *KRAS* mutant (G12D), or empty vector control (Mock) for 72 h. Cell invasion was analyzed with the Boyden chamber assay. Bars, 100 µm. Data represent the mean ± SD of three independent experiments. P-values were determined by the Student's *t* test. (E) Kaplan-Meier curves of relapse-free survival (RFS) according to *RHOQ* and *KRAS* status in stage III and high-risk stage II CRC patients treated with adjuvant chemotherapy. P-value was calculated by the overall comparison log-rank test. *, $P < 0.01$; **, $P < 0.001$; ***, $P = 0.005$.

($P = 0.005$ by overall comparison log-rank test; Fig. 3 E). The clinical significance of *RHOQ* editing and *KRAS* mutation merits further investigation in a larger patient population with longer duration of follow up and higher number of recurrence events.

Collectively, these results show that RhoQ is activated in CRC by RNA editing, which results in substitution of asparagine with serine. The amino acid substitution caused actin cytoskeletal reorganization and increased invasion potential without affecting proliferation. The invasion potential of cells expressing activated RhoQ was enhanced in the presence of *KRAS* mutation. We were also able to confirm the aggressive behavior of CRC having both edited *RHOQ* and mutant *KRAS* in the clinical samples. These findings are in agreement with a previous study demonstrating collaboration between activated RhoQ and the Ras–Raf pathway (Murphy et al., 1999).

Protein structure of edited RhoQ

The thermal stabilities of the recombinant WT and edited RhoQ proteins were measured by thermal denaturation using circular dichroism (Fig. 4 A). The results indicated that the stabilities of the two proteins are rather similar. Also, the x-ray crystal structure of the edited RhoQ protein compared with the previously reported WT RhoQ protein structure (Hemsath et al., 2005) indicated that there are no changes in the global or local structural folds (Fig. 4 B). Hence, we hypothesize that the edited RhoQ (N136S) protein likely disrupts normal protein–protein interactions of RhoQ in the cell. In the complex crystal structure of Ras-related Rap GTPase with its interacting RapGAP (GTPase-activating protein), the region of Rap that corresponds to the helix of RhoQ containing the N136 participates in an interaction with RapGAP in the crystal asymmetric unit

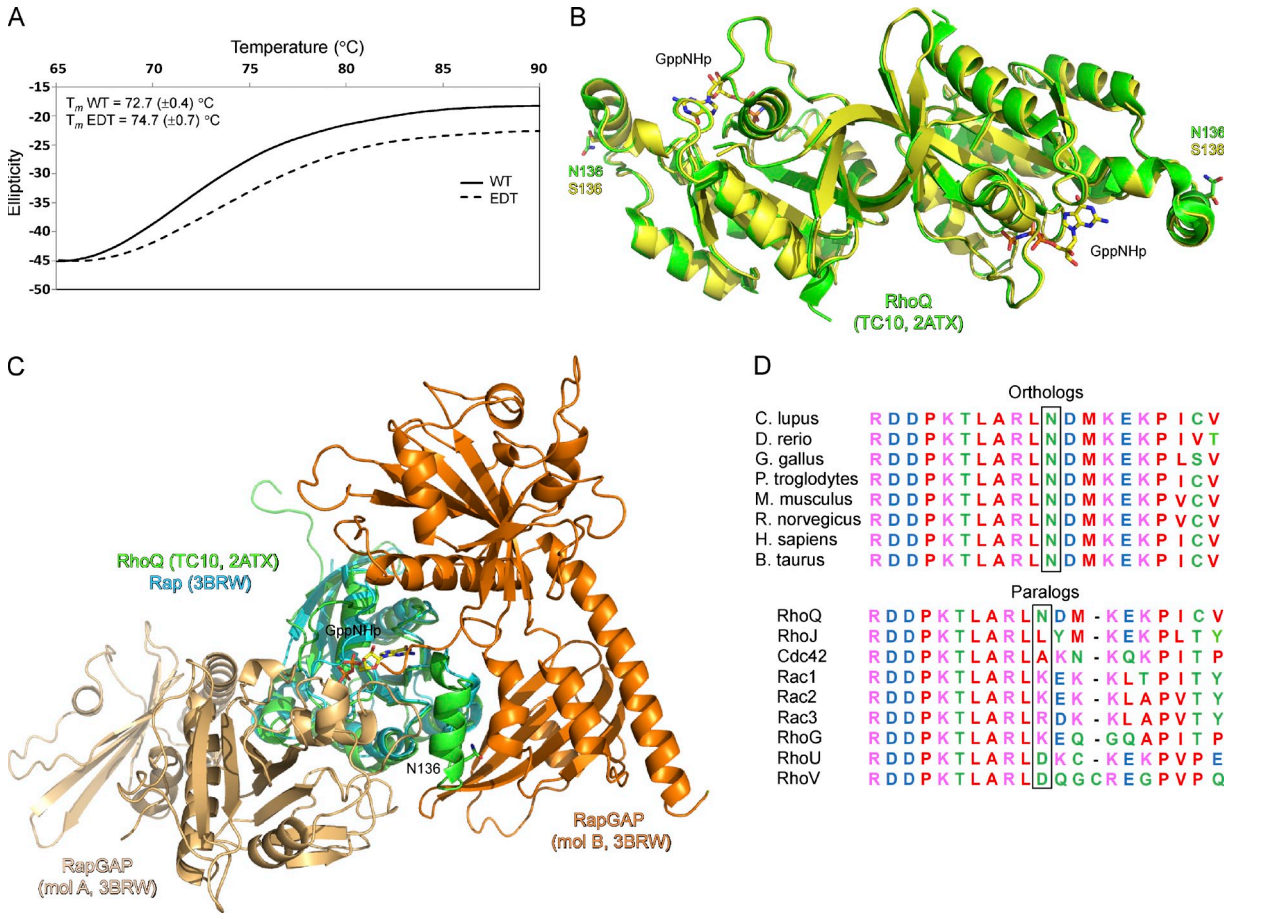

Figure 4. Structure analysis of RhoQ protein. (A) The melting temperature of the WT and edited RhoQ (EDT) are similar. The thermal stabilities of the recombinant RhoQ proteins were measured by thermal denaturation using circular dichroism. The results indicate that the stabilities of the two proteins are rather similar. (B) The crystal structure of the edited (N136S) RhoQ is similar to the WT. The x-ray crystal structure of the edited RhoQ was determined and compared with that of the previously reported WT RhoQ. The analysis indicates that there are no changes in the global or the local structural folds. The protein is shown using a ribbon diagram. The nonhydrolysable GTP analogue (GppNHp) and the side chain of residue 136 are shown in stick models. The WT RhoQ structure is available from the Protein Data Bank (PDB) under accession code 2ATX. (C) The regions around N136 may be important in mediating protein–protein interaction. The structure of RhoQ was superimposed on the Ras-related Rap GTPase of the Rap–RapGAP complex. The analysis suggests that the helix containing N136 may be important in forming the protein interaction surface of RhoQ. RhoQ, Rap, and the two RapGAPs of the crystal asymmetric unit are shown in ribbon diagrams with the GTP analogue. RhoQ and Rap–RapGAP structures are from PDB under accession codes 2ATX and 3BRW, respectively. (D) Amino acid sequence comparison among the orthologues at the *RHOQ* RNA editing site (above). The RhoQ amino acid sequence of *C. lupus*, *D. rerio*, *G. gallus*, *P. troglodytes*, *M. musculus*, *R. norvegicus*, *B. taurus*, and *H. sapiens* are compared. The box indicates the RNA editing site of *RHOQ* (N136). Amino acid sequence comparison among the paralogs at the *RHOQ* RNA editing site (below). The amino acid sequences of human RhoQ, RhoJ, Cda42, Rac1, Rac2, Rac3, RhoG, RhoU, and RhoV are compared. Sequence alignment was performed using ClustalW program. Residues are colored according to the physicochemical properties: small and hydrophobic residues in red (AVFPMILW); acidic residues in blue (DE); basic residues in magenta (RK); and residues containing a hydroxyl, sulfhydryl, or sidechain amide group in green (STYHCNQ).

(Fig. 4 C; Scrima et al., 2008). This data suggests that amino acid substitution of N136S in the edited RhoQ may disrupt WT RhoQ protein–protein interactions. By comparing amino acid sequences among orthologues of RhoQ, we found that amino acid N136 was evolutionally conserved throughout various species. In contrast, none of the paralogs harbored asparagine at the corresponding positions (Fig. 3 D). Collectively, these findings indicate that N136 is important for RhoQ-specific protein–protein interactions. RhoQ interacting proteins that are affected by the N136S substitution remains to be identified and will require further investigation.

Rho GTPases are involved in important cellular functions, including cell polarity, migration, and vesicular trafficking by regulating cytoskeletal dynamics (Heasman and Ridley, 2008). Rho GTPases are present as either the active GTP-bound form or the inactive GDP-bound form. The transition between the two states is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine dissociation inhibitors (GDIs; Cherfils and Zeghouf, 2013).

Activation of Rho GTPases is implicated in development and progression of many types of human malignancies,

including CRC (Leve and Morgado-Díaz, 2012). Among the 22 mammalian Rho GTPases, up-regulation of RhoA, RhoC, Rac1, Rac1b, and Cdc42 has been reported in CRC (Leve and Morgado-Díaz, 2012). Among the Rho GTPases, RhoQ (TC10) is most similar to Cdc42 and RhoJ (TCL) in terms of its sequence (Rojas et al., 2012). RhoQ has been most extensively studied for its central role in insulin-stimulated GLUT4 transport in adipocytes (Kanzaki, 2006). However, little is known about its involvement in cancer. In addition, genetic alterations in Rho GTPase genes, including *RHOQ*, have rarely been found in CRC in the previous cancer genomic studies (Gao et al., 2013). Here, we show that RNA editing is an alternative mechanism of oncogene activation in CRC. The contribution of tumor-associated increase in RNA editing of coding sequence in the progression of cancer has also been demonstrated in HCC (Chen et al., 2013). Considering the progress of genome and transcriptome sequencing studies in various types of cancer, many other oncogenic RNA editings will be discovered in near future. Moreover, therapeutic strategies using the tumor-associated increase of RNA editings as targets merit further research.

In summary, we found frequent RNA editing in the coding sequence of *RHOQ* leading to amino acid substitution that promoted invasion in CRC cells. It will be important to include RNA editing as a component of the genomic alteration repertoire in future studies of cancer.

MATERIALS AND METHODS

Patient tissue. The study protocol was reviewed and approved by the institutional review board of Seoul National University Hospital (SNUH). Written informed consent was received from the patient whose tumor underwent whole-genome and transcriptome sequencing. For comparison of *RHOQ* RNA editing in tumor and normal tissue, 60 pairs of freshly frozen primary tumor and adjacent normal tissue specimens were acquired from the SNUH Tumor Bank. The association between *RHOQ* RNA editing in tumor tissue and clinical outcome was analyzed in patients with stage III and high-risk stage II CRC who had received surgery with curative intent, followed by adjuvant-modified FOLFOX-6 (oxaliplatin, 5-fluorouracil, and leucovorin) chemotherapy at SNUH. This study was performed in accordance with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Cell lines. The cancer cell lines (29 CRC, 13 gastric cancer, 8 biliary cancer, 13 HCC, 12 breast cancer, 15 nonsmall cell lung cancer, and A431 epidermoid carcinoma) and COS7 monkey kidney cells were supplied by the Korean Cell Line Bank (Seoul, Korea) and the American Type Culture Collection. COS7 cell lines were maintained in DMEM culture media supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. The other cell lines were maintained in RPMI-1640 culture media with the same conditions.

DNA and RNA isolation and cDNA synthesis. The gDNA from the patient tissues or the cell lines was isolated using the DNEasy Blood and Tissue kit (QIAGEN) according to the manufacturer's instructions. Total RNA was extracted from the same samples using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized from total RNA using ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions.

Whole-genome and RNA sequencing. The RNA used for sequencing was extracted from tissue using RNAiso Plus (Takara Bio Inc.) and then purified using RNeasy MinElute (QIAGEN). RNA was assessed for quality and quantified using an RNA 6000 Nano LabChip on a 2100 Bioanalyzer (Agilent Technologies). The RNA-seq libraries were generated according to the standard protocol of Illumina Inc. for high-throughput sequencing.

The gDNA used for whole-genome sequencing was extracted from colon cancer and normal tissue samples. The gDNA (2–3 µg) from each sample was sheared and used for the construction of a paired-end sequencing library as described in the protocol provided by Illumina. RNA and whole-genome sequencing were performed with an Illumina SBS kit v2 on a Genome Analyzer IIx (Illumina) to obtain 75- and 101-bp paired-end reads. The image analysis and base calling were performed using the Illumina pipeline (v1.8) with default settings.

Sequence analyses. The RNA and whole-genome sequencing reads were aligned to the NCBI human reference genome assembly (build 36.1) using GSNAP (Wu and Nacu, 2010) with allowance for 5% mismatches. In the same manner, the RNA sequencing reads were also aligned to a cDNA set consisting of 161,250 mRNA sequences obtained from public databases (36,742 RefSeq, 73,671 UCSC, and 161,214 Ensembl) to decrease the false positives and false negatives in variant detection from RNA sequencing data (Ju et al., 2011).

Sanger sequencing analysis for validation of single nucleotide variants (SNVs) and RNA editing sites. Each SNV or RNA editing site was amplified by PCR with target-specific primers in gDNA or cDNA templates. The purified PCR products were sequenced with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and analyzed with a 3730 ABI capillary electrophoresis system (Applied Biosystems).

Pyrosequencing analyses of RHOQ editing. We designed target-specific primers for pyrosequencing analysis. The reverse primer was biotin labeled. Single-stranded biotinylated PCR products were processed for pyrosequencing analysis according to the manufacturer's standard protocol (PyroMark Q96 ID; QIAGEN).

ICE assay. The ICE assay is composed of the following four steps: (1) RNA cyanoethylation, (2) cDNA synthesis by reverse transcription, (3) PCR amplification, and (4) direct sequencing. We cyanoethylated total RNA from SNU C4 and SNU 81 cells containing edited *RHOQ* transcripts using two different conditions (mild condition: 1 mol/liter acrylonitrile for 15 min at 70°C; strong condition: 1 mol/liter acrylonitrile for 30 min at 70°C) compared with control (without cyanoethylation). The ICE assay was performed essentially as previously described (Sakurai et al., 2010).

Edited *RHOQ* plasmid constructs and Western blotting. The cDNA for human *RHOQ* was purchased from Addgene. Point mutations for N136S were introduced into the cDNA using a QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutant plasmid construct was confirmed by sequencing, and then transfected into each cell line using Lipofectamine 2000. Stable cell lines were generated by selection with G418 following the method described by the manufacturer (Invitrogen). The cells were then lysed in radioimmunoprecipitation assay buffer. Antibodies against RhoQ (T307) were purchased from Abcam. Anti-GST and α-tubulin antibodies were obtained from Santa Cruz Biotechnology, Inc.

RhoQ in vitro pull-down assay. RhoQ activity in the cells was examined under various conditions by a pull-down assay using an activation-specific probe, GST-PAK1 (76–150), as described previously (Chiang et al., 2002).

Cell proliferation assay. Cells were transfected with plasmids encoding for WT *RHOQ*, edited *RHOQ*, or an empty vector control. The viability of cells was assessed for 5 d after the transfection using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays (Sigma-Aldrich).

In vitro invasion assay. For the in vitro invasion assay, cells were transfected with plasmids encoding WT *RHOQ* and edited *RHOQ*. Forty-eight hours post-transfection, cells were seeded on a Matrigel-coated membrane matrix (BD) in the insert of a 24-well culture plate. Fetal bovine serum was added to the lower chamber as a chemoattractant. After 24 h, the noninvading cells were gently removed with a cotton swab. Migrating cells located on the lower surface of the chamber were stained with the Diff-Quick Staining Set (Dade) and counted under a Bio-Rad Gel DOC.

Immunofluorescence assay. Cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. Then, cells were incubated for 1 h at room temperature with phalloidin-rhodamine (1:200, Sigma-Aldrich). The cells were then washed with PBS and nano-pure H_2O before mounting. Cell images were obtained using a Leica TCS SP8 STED.

Protein preparations. Bacterial expression constructs of full-length WT *RHOQ* and edited *RHOQ* (N136S) were cloned into pET28 and transformed into the *Escherichia coli* strain BL21 (DE3). Recombinant protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside when OD_{600} = 1.2 (25°C, 27 h, with 25 g/ml kanamycin). The harvested cells were resuspended in 20 mM Tris (pH 7.5, 500 mM NaCl, 5 mM imidazole) and homogenized by sonication. The supernatants were loaded onto Nickel beads. After washing with 20 mM Tris (pH 7.5, 500 mM NaCl, 20 mM imidazole), the bound proteins were eluted with 20 mM Tris (pH 7.5, 500 mM NaCl, 200 mM imidazole), and the N-terminal His_6-tags were removed by adding bovine thrombin (Invitrogen) to the eluents (16 h, 4°C). The proteins were further purified using Superdex200 HR26/60 on ÄKTA FPLC (GE Healthcare) with preequilibration (20 mM Tris, pH 7.5, 2 mM $MgCl_2$, and 2 mM DTT). The final concentrations of WT (∼40 mg/ml) and edited (∼10 mg/ml) RhoQ proteins were estimated by calculated extinction coefficient (180,000 M^{-1} cm^{-1} at λ = 280 nm).

Temperature-induced melting study using circular dichroism. To determine the thermal stabilities of proteins, ellipticity (λ = 222 nm) over temperature was scanned using JASCO spectropolarimeter J-810 using protein concentration of ∼0.3 mg/ml.

Crystallization, x-ray data collection, and structure determination. Crystals of edited RhoQ proteins were grown under previously reported conditions (20% PEG3350, 200 mM NaCl, and 0.1 M MES, pH 6.0) using hanging drop vapor diffusion. Crystals from the drop were transferred to reservoir solutions supplemented with 20% glycerol and mounted under a cryostream at Pohang Accelerator Laboratory (7A). Data were processed according to a previously published method (Otwinowski and Minor, 1997). Then, using a previously reported structure (2ATX), REFMAC5 (Vagin et al., 2004) generated the electron-density map. Finally, the edited structure was analyzed using a crystallographic object-oriented toolkit (COOT; Emsley and Cowtan, 2004).

Data access. The sequencing data are uploaded to the EBI European Nucleotide Archive (http://www.ebi.ac.uk/ena/home) under accession no. ERP004029.

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