

Effects of 5-aza-2'-deoxycytidine on *RECK* gene expression and tumor invasion in salivary adenoid cystic carcinoma

X.Q. Zhou^{1,2,3*}, S.Y. Huang^{3*}, D.S. Zhang^{1,3*}, S.Z. Zhang³, W.G. Li³, Z.W. Chen³ and H.W. Wu³

¹Department of Oral and Maxillofacial Surgery, School of Stomatology, Shandong University, Jinan, China

²Department of Oral and Maxillofacial Surgery, The First People's Hospital of Jining, Shandong, China

³Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital, Affiliated to Shandong University, Jinan, China

Abstract

Reversion-inducing cysteine-rich protein with kazal motifs (*RECK*), a novel tumor suppressor gene that negatively regulates matrix metalloproteinases (MMPs), is expressed in various normal human tissues but downregulated in several types of human tumors. The molecular mechanism for this downregulation and its biological significance in salivary adenoid cystic carcinoma (SACC) are unclear. In the present study, we investigated the effects of a DNA methyltransferase (DNMT) inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), on the methylation status of the *RECK* gene and tumor invasion in SACC cell lines. Methylation-specific PCR (MSP), Western blot analysis, and quantitative real-time PCR were used to investigate the methylation status of the *RECK* gene and expression of *RECK* mRNA and protein in SACC cell lines. The invasive ability of SACC cells was examined by the Transwell migration assay. Promoter methylation was only found in the ACC-M cell line. Treatment of ACC-M cells with 5-aza-dC partially reversed the hypermethylation status of the *RECK* gene and significantly enhanced the expression of mRNA and protein, and 5-aza-dC significantly suppressed ACC-M cell invasive ability. Our findings showed that 5-aza-dC inhibited cancer cell invasion through the reversal of *RECK* gene hypermethylation, which might be a promising chemotherapy approach in SACC treatment.

Key words: *RECK*; Salivary adenoid cystic carcinoma; 5-aza-2'-deoxycytidine; Methylation; Transwell assay

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common subtypes of malignant tumors. It accounts for only 1% of all head and neck malignancies, but it is well known for both distant metastasis and perineural invasion (1,2). Despite aggressive surgery, long-term survival is only 39.6% after 15 years (3). The underlying molecular mechanisms of carcinogenesis are still unclear.

The reversion-inducing cysteine-rich protein with kazal motifs (*RECK*) gene was identified by screening an expression library for cDNAs that could reverse the round morphology associated with v-Ki-ras-transformation of 3T3 cells to the nontransformed flat morphology (4). It is thought to be a novel matrix metalloproteinase (MMP) inhibitor that can inhibit tumor angiogenesis, invasion, and metastasis. *RECK* is expressed in various normal human tissues, but downregulated in several types of human cancers, including pancreatic (5), colorectal (6), breast (7), and hepatocellular carcinoma (8). In a previous study (9), we

found that *RECK* expression was significantly lower in SACC than in normal tissues, but the mechanism was not clear.

DNA methylation changes are frequently found in human cancers (10,11). Hypomethylation of oncogenes can result in aberrant activation, and hypermethylation of suppressor genes can lead to silencing. Several methylation-regulated candidate genes have been identified in SACC, including aurothiazin (SBSN) (12), aquaporin 1 (AQP1) (13), phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (14), cyclin-dependent kinase inhibitors (15), RAS-associated domain family protein 1A (RASSF1) (16), and death-associated protein kinase (DAPK) (17), but the methylation of *RECK* in SACC has not yet been reported.

In the present study, we examined the methylation status of the *RECK* gene and determined the effects of 5-aza-2'-deoxycytidine (5-aza-dC) treatment on its methylation status and expression level in human adenoid cystic carcinoma cell lines. The overall aim was the development

Correspondence: Dong-sheng Zhang: <ds63zhang@sdu.edu.cn>.

*These authors contributed equally to this study.

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of new strategies for cancer prevention and therapeutic interventions.

Material and Methods

Cell lines and cell cultures

Two human salivary adenoid cystic carcinoma cell lines, ACC-2 and ACC-M, were used. Both cell lines were kindly provided by Professor Wantao Chen (Department of Oral and Maxillofacial Surgery, Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University, Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Hyclone, USA) and antibiotics in a humidified 37°C incubator containing 5% CO₂.

Cell viability assay

Briefly, cells were seeded on 96-well plates and treated with 5-aza-dC in triplicate. After 24, 72, or 144 h incubation, the medium was replaced with fresh medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 4 h, the supernatants were removed and the resulting MTT formazan was solubilized in DMSO and measured spectrophotometrically at 570 nm.

5-aza-dC treatment

We treated the SACC cell lines in triplicate with 5-aza-dC (Sigma, USA) to determine dose- and time-dependent changes as described previously (18). Briefly cells were diluted to a low density (5×10^5 /100 mm dish) 24 h before 5-aza-dC treatment. Stock solutions of 5-aza-dC were dissolved in DMSO. Cells were treated with 1, 5, and 10 μ M 5-aza-dC for 144 h, or 10 μ M 5-aza-dC for 24, 72, or 144 h. Baseline expression was established in mock treatment of cells with the same volume of DMSO in triplicate.

Bisulfite modification and methylation-specific PCR (MSP)

Genomic DNA was isolated and modified using the CpGenome™ Direct Prep Bisulfite Modification kit (Millipore, USA) according to the manufacturer's instructions. MSP reactions were performed using 2 × Taq PCR MasterMix (Tiangen, China) in 25 mL volumes under the following conditions: 94°C for 3 min, then 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and 5 min at 72°C. The PCR product lengths for methylated and unmethylated RECK are 195 and 199 bp. Universal Methylated DNA (Millipore) and normal human blood DNA were used as positive controls for the methylated and unmethylated conditions. Water blanks were used as a negative control. Positive and negative controls were used in each round of MSP. All assays were performed in triplicate. For each DNA sample, primer sets for methylated (M) and unmethylated (U) DNA were used for analysis (Table 1). The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Table 1. RECK primer sequences for methylation-specific PCR.

<i>RECK</i> gene	Primer
Methylated	
Forward	5'-ATAAAGAGTTTTGGTACGGGGTAC-3'
Reverse	5'-AAA ACCGCGAAATACTCGAA-3'
Unmethylated	
Forward	5'-TAAAGAGTTTTGGTATGGGGTATGT-3'
Reverse	5'-CTCCAAACCACAAAATACTCAA-3'

RNA extraction and quantitative real-time PCR

Total RNA was isolated from cultured cells using a Trizol reagent kit (TaKaRa, China) according to the manufacturer's protocol. The quantity and quality of the RNA samples were measured by spectrophotometry and electrophoresis. cDNA was synthesized from 1 μ g of total RNA using PrimeScript™ RT reagent kits with gDNA Eraser (TaKaRa). Quantitative real-time PCR reactions were performed using SYBR® Premix Ex Taq™ (TaKaRa) in 20 mL volumes under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. The RECK and GAPDH primers are shown in Table 2. The $2^{-\Delta\Delta C_T}$ method was used for analysis of quantitative real-time PCR data, and $\Delta\Delta C_T$ (threshold cycle) = (C_T RECK, treated group - C_T GAPDH, treated group) - (C_T RECK gene, untreated group - C_T GAPDH, untreated group). The change in RECK mRNA expression was calculated as $2^{-\Delta\Delta C_T}$. All reactions were performed in triplicate with water controls.

Western blot analysis

Total cellular proteins were extracted, and were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (19). Proteins were transferred from gels to polyvinylidene fluoride membranes followed by incubation with the primary anti-RECK and anti-GAPDH antibodies. GAPDH was used as a loading control on each membrane. Enhanced chemiluminescence reagents were used to detect the proteins on the membranes. Anti-RECK antibody was purchased from Abcam (USA, at 1:1000 dilution) and anti-GAPDH antibody

Table 2. Primer sequences for quantitative real-time PCR.

Gene	Primer
<i>RECK</i>	
Forward	5'-TGCAAGCAGGCATCTTCAA-3'
Reverse	5'-ACCGAGCCATTTTCATTCTG-3'
<i>GAPDH</i>	
Forward	5'-GCAGGGGGGAGCCAAAAGGG-3'
Reverse	5'-TGCCAGCCCCAGCGTCAAAG-3'

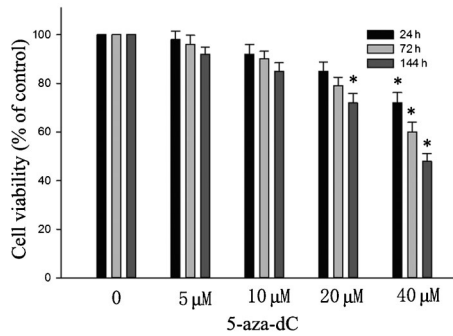


Figure 1. Effect of 5-aza-2'-deoxycytidine (5-aza-dC) on viability of ACC-M cells. Cells were treated with various concentrations of 5-aza-dC for 24, 72, and 144 h. Cell viability is reported as means \pm SD of 3 independent experiments. * $P < 0.05$ compared with control (ANOVA).

was obtained from Santa Cruz Biotechnology (USA, at 1:2500 dilution).

In vitro invasion assay

The invasiveness of cells exposed to 5-aza-dC was assayed using Matrigel (BD, USA) coated 8 mm pore size filter inserts in 24-well plates (Sigma-Aldrich, USA). Cells exposed to 5-aza-dC (1, 5, 10 μ M) or DMSO for 72 h were collected and 1×10^5 cells in 200 μ L of medium were placed in the upper part of the Transwell unit and allowed to invade for 24 h. The lower part of the Transwell unit was filled with 500 μ L medium containing 10% FBS. After incubation, noninvasive cells on the upper part of the membrane were removed with a cotton swab. Invasive cells on the bottom surface of the membrane were fixed in formaldehyde for 20 min, stained with crystal violet for 5 min, and observed by light microscopy at $100\times$ magnification. The number of cells in 5 randomly selected fields were counted; all assays were performed in triplicate.

Statistical analysis

SPSS (Statistic Package for Social Sciences) 13.0 for Windows (SPSS Inc., USA) was used to analyze data. Differences between groups were assessed by ANOVA

with Dunnett's *post hoc* test. Statistical significance was defined as $P < 0.05$.

Results

Cytotoxic effect of 5-aza-dC in ACC-M cells

The cytotoxic effect of 5-aza-dC in human adenoid cystic carcinoma ACC-M cells is shown in Figure 1. It is demonstrated that treatment with 40 μ M for 24 h, or 20 μ M 5-aza-dC for 144 h significantly decreased the viability of ACC-M cells. Treatment with 5-aza-dC at doses of 10 μ M or less for 144 h did not cause cytotoxicity of ACC-M cells.

Methylation status of RECK gene in ACC-2 and ACC-M cell lines

MSP assays showed that the ACC-M cell lines contained both unmethylated and methylated promoters, but that ACC-2 had only a strongly unmethylated promoter (Figure 2A). Normal human blood DNA (NBD) was used as a positive control for unmethylated status and universal methylated DNA (UMD) as a positive control for methylated status, H_2O was a negative control.

Reversal of hypermethylation status in ACC-M cells by 5-aza-dC

The time- and dose-dependent effects of 5-aza-dC in ACC-M cell lines were investigated in cells treated with 1, 5, or 10 μ M of 5-aza-dC for 144 h. Methylation-specific RECK gene bands still existed but had a very weak appearance; however, the unmethylation-specific bands appeared to be enhanced. After treating the cells with 10 μ M of 5-aza-dC for 24, 72, or 144 h, unmethylation-specific bands of the RECK gene became increasingly intensive, and methylation-specific bands of the RECK gene became increasingly weaker (Figure 2B).

5-aza-dC enhanced RECK mRNA and protein expression in ACC-M cell lines

To confirm the effect of 5-aza-dC on expression of RECK mRNA, real-time quantitative PCR was performed in ACC-M cell lines after treatment with different concentrations of

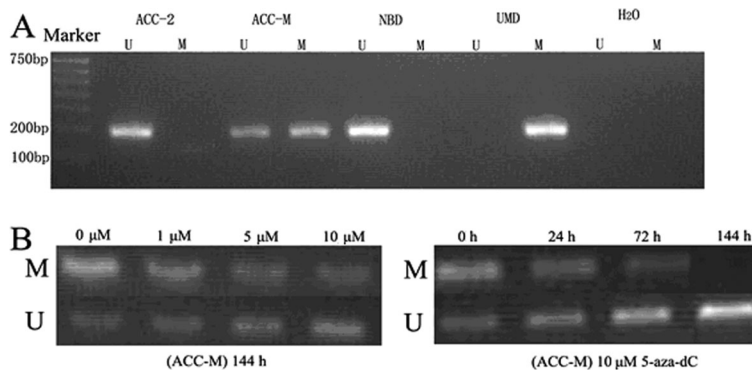


Figure 2. A, Methylation status of the RECK gene in ACC-2 and ACC-M cell lines. M: methylation-specific band; U: unmethylation-specific band; NBD: normal blood DNA as positive control for unmethylated status; UMD: universal methylated DNA as positive control for methylated status; H_2O : negative control. B, Alterations of methylation status of the RECK gene in ACC-M cell lines after treatment with 1, 5, 10 μ M 5-aza-2'-deoxycytidine (5-aza-dC) for 144 h and treated for 24, 72, and 144 h with 10 μ M 5-aza-dC.

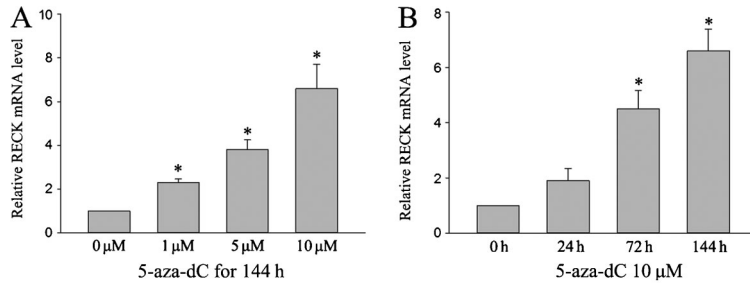


Figure 3. Relative mRNA expression levels of *RECK* gene by quantitative real-time PCR in ACC-M cell lines. 5-aza-2'-deoxycytidine (5-aza-dC) enhanced *RECK* mRNA expression in a dose- and time-dependent manner. All expression levels are shown relative to the untreated sample. Data are reported as means \pm SD (n=3). *P<0.05 compared with control (ANOVA).

5-aza-dC for different times (Figure 3). The results showed that the relative amounts of *RECK* mRNA expressed increased in a dose- and time-dependent manner with significant effects at 1, 5, and 10 μ M (P<0.05) and at 72 and 144 h (P<0.05).

As shown in Figure 4, *RECK* protein expression was low in ACC-M cells. After treatment with different doses of 5-Aza-dC for 144 h, or with 10 μ M of 5-Aza-dC for different times, *RECK* protein expression increased. These findings suggest that inhibition of *RECK* protein expression may be associated with *RECK* promoter methylation.

DNA methyltransferase (DNMT) inhibitor 5-aza-dC reduced the invasive ability of human ACC-M cells

As shown in Figure 5, treatment with 1, 5, or 10 μ M 5-aza-dC significantly suppressed the invasive ability of ACC-M cells. We conclude that restoration of *RECK* expression by 5-aza-dC is important for the inhibition of cell invasion by ACC-M cells.

Discussion

In the present study, we demonstrated that 5-aza-dC enhanced expression of *RECK* mRNA and protein by reversal of the hypermethylation status of the *RECK* promoter in a time- and dose-dependent manner, and could inhibit cancer cell invasion in ACC-M cell lines.

RECK is an important MMP inhibitor and is involved in the regulation of various physiological and pathological processes. Many studies have demonstrated that *RECK*

mRNA and protein are frequently highly expressed in both human tissue and untransformed cells, but are lost or not detectable in most tumor cells. Several hypotheses have been advanced to explain the mechanism of low *RECK* expression in tumor tissue. Epigenetic mechanisms have been shown to play an important role in the regulation of gene expression. Hsu et al. (20) reported that the oncogene *HER-2/neu* represses *RECK* by inducing the binding of Sp1 and Sp3 proteins as well as histone deacetylase (HDAC) to the Sp1-binding site. Sasahara et al. (21) reported that the HDAC inhibitor trichostatin A inhibited the interaction between HDAC and Sp1, which prevented binding to the Sp1 site, thereby restoring *RECK* expression.

DNA methylation of the *RECK* promoter and histone acetylation/deacetylation have been studied to uncover the underlying mechanisms of *RECK* expression (22). DNA methylation, a crucial epigenetic alteration, is associated with the silencing of tumor suppressor genes in several cancers. Cho et al. (23) found that downregulation of *RECK* mRNA and protein expression in colon tumor tissues significantly correlated with methylation of the *RECK* promoter. Chang et al. (24) suggested that downregulation of the metastasis suppressor *RECK* is caused by promoter methylation in non-small-cell lung cancer. In our previous research, we found *RECK* expression in SACC was significantly lower than in normal tissues, but the underlying mechanism by which *RECK* was downregulated in tumors has never been reported. In our present research, the methylation status of the *RECK* promoter assayed by MSP in ACC-M cell lines, characterized by frequent

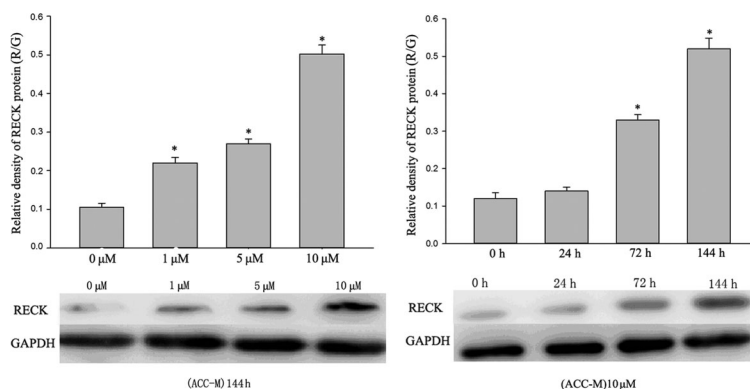


Figure 4. Western blotting results of *RECK* in ACC-M cells with DMSO or 1, 5, 10 μ M 5-aza-2'-deoxycytidine (5-aza-dC) treatment for 144 h and treated for 24, 72, and 144 h with 10 μ M 5-aza-dC. R/G represents the brightness ratio of the *RECK* protein (106 kDa) vs GAPDH (36 kDa). *P<0.05 compared with control (ANOVA).

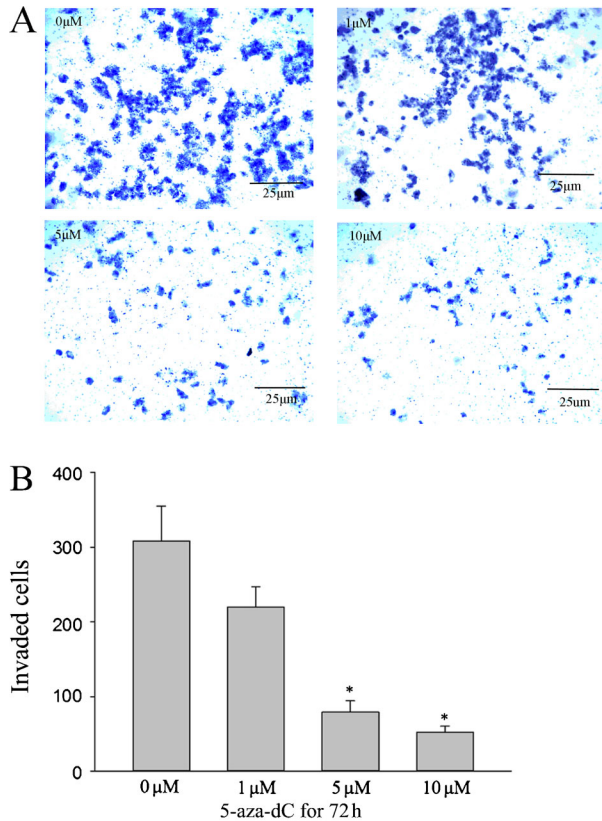


Figure 5. 5-aza-2'deoxyctidine (5-aza-dC) inhibited the invasion ability of ACC-M in a dose-dependent manner. Cells were incubated with DMSO or different doses of 5-aza-dC for 72 h. After incubation for 24 h, invaded cell numbers were counted as described previously (100× magnification). * $P < 0.05$ compared with control (ANOVA).

pulmonary metastasis and screened in ACC-2 cells, was both unmethylated and methylated, but ACC-2 had only unmethylated bands. The present study thus suggests that a decrease in or deficiency of RECK expression in ACC-M cells may be caused by the methylation of CpG islands in the RECK promoter region.

5'-Azacytidine has been approved by the FDA for the treatment of myelodysplastic syndrome (25), which, as a classic DNMT inhibitor, was reported to restore RECK mRNA and protein expression by demethylation of the RECK promoter. Epigallocatechin-3-gallate (EGCG) is the major polyphenol and possibly the key active ingredient in green tea. Previous studies (26-29) have shown that EGCG inhibited DNMT and blocked the hypermethylation of newly synthesized DNA strands, resulting in the reversal of the hypermethylation and re-expression of silenced genes with reduced side effects and toxicity. This study demonstrated that almost no RECK protein was expressed in ACC-M cells (isolated from advanced lung metastases),

which are characterized by high pulmonary metastasis and are isolated from ACC-2 lines. After ACC-M cells were treated with 5-aza-dC, the expression of RECK mRNA and protein was increased in a dose- and time-dependent manner. The invasiveness of ACC-M cells was significantly reduced by 5-aza-dC treatment.

Degradation of extracellular matrix (ECM) is an important step during cell invasion and is usually mediated by extracellular proteases, such as MMPs. The inhibition of MMP-2 is a potential means for the prevention of the metastasis of cancer cells (30,31). RECK acts as an inhibitor of MMPs and can inhibit tumor angiogenesis, invasion and metastasis (32). The expression of RECK in SACC is lower than that in normal tissue counterparts, and in our previous research decreased RECK was correlated with a poor prognosis (9). Enhancement of RECK expression may suppress cancer cell invasion (33). In the present study, 5-aza-dC had a significant effect on the invasive capability of ACC-M that was associated with increasing expression of RECK.

The mitogen-activated protein kinase (MAPK) pathway is known to participate in various signaling cascades that play an important regulatory role in cell growth, apoptosis, differentiation, and metastasis (34). Inhibition of the MAPK pathway may potentially prevent angiogenesis, proliferation, invasion, and metastasis in a wide range of tumors (35,36). Metastasis is also regulated by the phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) signaling pathway, which is involved in many cellular processes, including survival, adhesion, and metastasis (37,38). The inhibition of the MAPK and PI3K/Akt pathways may potentially prevent cancer cell proliferation, invasion, and metastasis. Yan (39) reported that tomatidine inhibits the invasion of A549 cells by reducing the expression of MMPs, and also inhibits extracellular signal-regulated (ERK) and Akt signaling pathways and nuclear factor kappa light-chain enhancer of activated B cells (NF- κ B) activity. Although the effect of 5-aza-dC on suppressing invasiveness in ACC-M cells was demonstrated, the signal pathway remains unclear. In future studies, we will investigate the signal pathway to better understand the metastasis of SACC.

In conclusion, our findings are the first to show that 5-aza-dC can inhibit cancer cell invasion through reversal of RECK gene hypermethylation, which could serve as a promising chemotherapeutic strategy for SACC treatment. Additional studies are needed to find a more tolerable and effective drug, and to uncover the mechanism underlying RECK hypermethylation in SACC.

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