Abstract. Epigallocatechin-3-gallate (EGCG) is an active and major constituent of green tea. As a non-nucleoside inhibitor of DNA methylation, EGCG is able to inhibit the hypermethylation of newly synthesised DNA, resulting in the reversal of hypermethylation and recovery in expression of the silenced genes. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a novel tumour suppressor gene, which negatively regulates matrix metalloproteinases, and inhibits tumour invasion, angiogenesis and metastasis. The present study aimed to examine the effects of EGCG on the methylation status of the RECK gene and tumour invasion in a salivary adenoid cystic carcinoma (SACC) cell line in vitro. Marked levels of methylated and weak levels of unmethylated RECK promoter were detected in the SACC83 cells, which was determined using methylation-specific polymerase chain reaction (PCR). In addition, the treatment of SACC83 cells with EGCG partially reversed the hypermethylation status of the RECK gene. Western blot analysis and reverse transcription-PCR demonstrated that EGCG significantly enhanced the protein and mRNA expression levels of RECK, and significantly reduced the invasive ability of the SACC83 cells, as determined using a Transwell assay. These results suggested that EGCG possesses novel anti-metastatic therapeutic potential for the treatment of SACC.

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common types of malignancy, which is known to be associated with persistent slow growth, perineural invasion, high rates of recurrence and the formation of distant metastases (1). Despite the use of aggressive surgery, the 5-year survival rate of SACC is ~75%, whereas the long-term survival rate is only 39.6% (2). The underlying molecular mechanisms of SACC carcinogenesis remain to be fully elucidated.

Changes in DNA methylation is commonly observed in human cancer (3,4). The hypomethylation of oncogenes can result in their aberrant activation, and hypermethylation of tumour suppressor genes can result in their silencing (5). DNA methylation inhibitors can be divided into two categories: Nucleoside inhibitors and non-nucleoside inhibitors. Nucleoside inhibitors, including 5-aza-2'-deoxycytidine, are associated with substantial toxic effects and have short half-lives in aqueous solutions (6). Epigallocatechin-3-gallate (EGCG) is a non-nucleoside inhibitor (7), which mediates the inhibition of DNA methylation by binding to the catalytic pocket of human DNA methyltransferase (DNMT) and inhibit the hypermethylation of newly synthesised DNA, resulting in the reversal of hypermethylation and the re-expression of silenced genes.

Several oncogene and tumour suppressor gene candidates have been suggested to be involved in SACC, including supra-basin (8), aquaporin 1 (9), phosphatase and tensin homolog deleted on chromosome 10 (10), cyclin-dependent kinase inhibitors (11) and RAS-associated domain family protein 1A (12). The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene is anchored to the cell surface by glycosylphosphatidylinositol, which can suppress tumour invasion and metastasis via degradation of the extracellular matrix (13). RECK is expressed in various types of normal human tissue, and is downregulated in several types of human cancer, including pancreatic cancer (14), colorectal cancer (15), breast cancer (16) and hepatocellular carcinoma (17). Our previous study demonstrated that the expression of RECK was significantly lower in SACC, compared with normal tissue (18); however, the underlying mechanism remains to be fully elucidated.

The present study aimed to determine the methylation status of the RECK promoter in the human SACC83 SACC...
cell line, and aimed to investigate the effects of EGCG on the expression of RECK and the invasiveness of the SACC83 cells.

Materials and methods

Cell lines and cell culture. The SACC83 SACC cell line [kindly provided by Professor Shenglin Li (Department of Oral and Maxillofacial Surgery, School of Stomatology, Beijing University, Beijing, China)] was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), 50,000 units penicillin and 50 mg streptomycin (both from Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified incubator containing 5% CO₂.

EGCG treatment. To determine the dose- and time-dependent changes associated with EGCG treatment, the SACC83 cells were treated in triplicate with EGCG (Wako Pure Chemical Industries, Ltd., Osaka, Japan), as described previously (19). Briefly, the cells were seeded at low density (5x10⁴/100 mm dish) 24 h prior to treatment with EGCG. To determine the dose-dependent changes, the cells were treated with 0, 5, 15 or 45 μM EGCG for 6 days. EGCG was added, in new culture medium, to the cells on days 1, 3 and 5. For the assessment of time-dependent changes, the cells were treated with 45 μM EGCG for 0, 36, 72 or 144 h.

Bisulphite modification and methylation-specific polymerase chain reaction (PCR; MSP). Genomic DNA was isolated and modified using a CpGenome™ DirectPrep Bisulfite Modification kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. The DNA concentration was determined using spectrophotometry. For MSP, the following primer sets (Takara Bio Inc., Dalian, China) were used for methylated DNA: M_RECK, forward 5'-ATCAAAGGTTTTGAGGCTGGTAC-3' and reverse 5'-AAAAACCCGCAAATCTCGAA-3'; and the primer sets for unmethylated DNA were as follows: U_RECK, forward 5'-TAAAGGTTTTGAGGCTGGTAC-3' and reverse 5'-CTCCTAAAAACACAAATTACCTTCAAA-3'. The MSP reactions were performed in a mixture of 12.5 µl 2X Taq PCR Master mix (Tiangen Biotech, Co., Ltd., Beijing, China) reverse primers (1 µl), DNA (~1 µg) and distilled water in 25-µl volumes in a 2X Master Mix (10 µl), forward and reverse primers (1 µl), cDNA (2 µl) and distilled water under the following conditions: 95°C for 10 min; followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; and finally 7 min at 72°C. GAPDH was used as an internal control to estimate the efficiency of cDNA synthesis using the primer: GAPDH, forward 5'-GGCACACACTGCTGTA-3' and reverse 5'-TGTTGAGTTGCTCCTTCCA-3'. The predicted size for the PCR products of RECK and GAPDH were 477 and 512 bp, respectively. The PCR products were separated on 2% agarose gel, stained with EtBr and visualised under ultraviolet light and images were captured using a Bio-Rad VersaDoc 3000 Imaging system (Bio-Rad Laboratories, Inc.). Image J software (version 1.48u; National Institutes of Health, Bethesda, MD, USA) was used for grey value analysis. The relative mRNA expression levels of RECK were normalised against GAPDH.

Western blot analysis. Following treatment with EGCG, the SACC83 cells were washed twice with cold phosphate-buffered saline (PBS) and treated with extraction buffer [50 M Tris-HCl (pH 7.4), 150 M NaCl, 2 M EDTA and 1% NP-40]. The cell extractions were subsequently centrifuged at 12,000 x g for 15 min at 4°C, and the supernatants were collected. The protein concentration was quantified using a Bicinchoninic Acid Protein Measurement kit (Shenneng Bocai Biology, Co., Ltd., Shanghai, China). Equal quantities (40 μg) of cellular proteins were subjected to 8% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were blocked with 5% (w/v) non-fat milk in PBS containing 0.1% Tween-20 (Shenneng Bocai Biology, Co., Ltd., Shanghai, China) and then incubated with primary antibodies (mouse anti-human RECK polyclonal, cat. no. ab88249; anti-GAPDH, cat. no. sc-365062) at 1:1,000 and room temperature for 1 h. Subsequently, the membranes were incubated with an appropriate horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody (cat. no. sc-2005) at room temperature for 1 h. The immuno-detected proteins were visualised using enhanced chemiluminescence. Image J software was used for grey value analysis. The anti-RECK antibody was purchased from Abcam (Cambridge, MA, USA) and the anti-GAPDH and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc.

RNA extraction and reverse transcription (RT)-PCR. Total RNA was extracted using a Total RNA Extraction kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer’s instructions. The quantity and quality of the RNA samples were measured using a spectrophotometer and electrophoresis. RECK cDNA was synthesised from 1 μg of total RNA using a PrimeScript™ RT Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). The following primers (Takara Bio Inc.) were used for PCR: RECK, forward 5'-CCTCAGTGACAGCTTACACAG-3' and reverse 5'-GCAAGCACACACTGCTGTA-3'. Reactions were performed in 20 µl volumes containing 2X Master Mix (10 µl), forward and reverse primers (1 µl), cDNA (2 µl) and distilled water under the following conditions: 95°C for 10 min; followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; and finally 7 min at 72°C. GAPDH was used as an internal control to estimate the efficiency of cDNA synthesis using the primer: GAPDH, forward 5'-GCACACACTGCTGTA-3' and reverse 5'-TGTTGAGTTGCTCCTTCCA-3'. The predicted size for the PCR products of RECK and GAPDH were 477 and 512 bp, respectively. The PCR products were separated on 2% agarose gel, stained with EtBr and visualised under ultraviolet light and images were captured using a Bio-Rad VersaDoc 3000 Imaging system (Bio-Rad Laboratories, Inc.). Image J software (version 1.48u; National Institutes of Health, Bethesda, MD, USA) was used for grey value analysis. The relative mRNA expression levels of RECK were normalised against GAPDH.

In vitro invasion assay. To assess the invasive ability of the cells treated with EGCG, Matrigel invasion assays were performed using 8-mm pore filter inserts in 24-well plates (Sigma-Aldrich) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The cells were incubated with EGCG (0, 5, 15 or 45 μM)
for 72 h, and were subsequently collected, washed three times with PBS and re-suspended in serum-free DMEM. A total of $1 \times 10^5$ cells/200 µl medium were plated in the upper chamber of the Transwell unit and allowed to invade for 24 h at 37°C. The lower chamber of the Transwell unit was filled with 500 µl medium supplemented with 10% FBS. At the end of the incubation period, the non-invaded cells on the upper surface of the membrane were carefully removed using a cotton swab. The invaded cells on the bottom surface of the membrane were fixed in 4% formaldehyde for 20 min and stained with 0.1% crystal violet for 5 min. Subsequently, the invaded cells on the lower surface of the membrane were visualised in five randomly-selected fields under a microscope (Olympus BH2; Olympus, Tokyo, Japan; magnification, x200). All assays were performed in triplicate and the mean number of invaded cells was used for analysis.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Differences between the groups were assessed using one-way analysis of variance with Dunnett’s post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Methylation status of the RECK gene in SACC83 cells. The RECK gene in SACC83 cells exhibited marked expression of methylated and weak expression of unmethylated promoter, determined using MSP (Fig. 1A). Normal human blood DNA was used as a positive control for unmethylation, and Universal Methylated DNA was used as a positive control for methylation. H2O was used as a negative control.

EGCG reverses the hypermethylation status of RECK in the SACC83 cell line. The present study aimed to examine the time-and-dose-dependent effects of EGCG in SACC83 cells.

Figure 1. Detection of the methylation status of the RECK gene in SACC83 cells. (A) Methylation status of the RECK gene in SACC83 cells, NBD positive control for unmethylation, UMN positive control for methylation and H2O negative control groups. (B) Changes in the methylation status of the RECK gene in SACC83 cells following treatment with 0, 5, 15 or 45 µm EGCG for 144 h, or treatment with 45 µm EGCG for 0, 36, 72 or 144 h. RECK, reversion-inducing cysteine-rich protein with Kazal motifs; EGCG, epigallocatechin-3-gallate; SACC, salivary adenoid cystic carcinoma; M, methylation-specific band; U, unmethylation-specific band; NBD, normal blood DNA; UMD, Universal Methylated DNA; Bp, base pairs.

Figure 2. Relative mRNA expression levels of RECK, determined using reverse transcription-quantitative polymerase chain reaction in SACC83 cells. Treatment with EGCG enhanced the mRNA expression of RECK in a (A) dose- and (B) time-dependent manner. The relative mRNA expression levels of RECK were normalised to GAPDH. Band density was determined using densitometry. Data are presented as the mean ± standard deviation (n=3). *$P<0.05$, compared with the control. RECK, reversion-inducing cysteine-rich protein with Kazal motifs; EGCG, epigallocatechin-3-gallate; SACC, salivary adenoid cystic carcinoma.
Following treatment of the cells with 0, 5, 15 or 45 µm EGCG for 144 h, methylation-specific bands of the RECK gene were weak in appearance, whereas unmethylation-specific bands of the RECK gene appeared markedly enhanced. Following treatment of the cells with 45 µm EGCG for 0, 36, 72 or 144 h, the unmethylation-specific bands of RECK were more marked, whereas the methylation-specific bands of RECK were almost undetectable (Fig. 1B).

Treatment with EGCG enhances the mRNA and protein expression levels of RECK in SACC83 cells. To determine the effects of EGCG on the mRNA expression of RECK, RT-qPCR was
performed to detect the mRNA expression levels of RECK in the SACC83 cells following treatment with a range of concentrations of EGCG for different durations (Fig. 2). The relative mRNA expression levels of RECK increased in a dose- and time-dependent manner (Fig. 2A and B).

As shown in Fig. 3, the protein expression levels of RECK were relatively low in the untreated SACC83 cells. Following treatment of the cells with different doses of EGCG for 6 days, and with 45 µm EGCG for different periods of time, the protein expression levels of RECK increased in the SACC83 cells (Fig. 3A and B).

**EGCG reduces the invasiveness of human SACC83 cells.** The present study also investigated the effects of EGCG on cell invasion. As shown in Fig. 4, treatment with 5, 15 or 45 µm EGCG markedly suppressed the invasive ability of the SACC83 cells in a dose-dependent manner. These results suggested that restoration of the expression of RECK by EGCG is important for inhibiting the invasiveness of SACC83 cells.

**Discussion**

RECK is an important matrix metalloproteinase inhibitor, which is involved in the regulation of various physiological and pathological processes. Oh et al (20) previously reported that mice lacking the expression of RECK succumb to mortality *in utero* due to developmental defects in blood vessels, the neural tube and mesenchymal tissues. In addition, RECK has been identified as a target of myogenic regulatory factors and is involved in the control of myogenesis (21). Several studies have demonstrated that RECK mRNA and protein are highly expressed in human tissue and untransformed cells (22); however, the expression of RECK is lost or undetected in the majority of tumour cells. Several hypotheses have been suggested regarding the mechanism underlying the low expression levels of RECK in tumour tissues. DNA methylation, which is a critical epigenetic alteration, is associated with the silencing of tumour suppressor genes in several types of cancer. Cho et al (22) demonstrated that downregulation of the mRNA and protein expression levels of RECK in colon tumour tissue are significantly correlated with methylation of the RECK promoter. Furthermore, Chang et al (23) reported that downregulation of the metastasis suppressor RECK is due to promoter methylation in non-small cell lung cancer. In the present study, the methylation status of the RECK promoter was determined using MSP, and the SACC83 cells were found to exhibit weak expression levels of unmethylated promoter and marked expression levels of methylated promoter. These results suggested that a decrease or deficiency in the expression of RECK in SACC83 cells may be caused by the methylation of CpG islands in the RECK promoter region.

EGCG is a major polyphenol in green tea, and a key active ingredient (19). Previous studies (24,25) have demonstrated that EGCG inhibits DNMT and inhibits the hypermethylation of newly synthesized DNA, resulting in the reversal of hypermethylation and the re-expression of silenced genes, with fewer side effects and toxicity. The present study demonstrated that almost no RECK protein was detected in untreated SACC83 cells. However, following treatment of the cells with EGCG, the mRNA and protein expression levels of RECK increased in a dose- and time-dependent manner in the SACC83 cells. Furthermore, following treatment of SACC83 cells with EGCG, their invasive capability was significantly reduced.

In conclusion, the results of the present study demonstrated that EGCG inhibited cancer cell invasion through reversal of the hypermethylation status of RECK. This may offer a potential therapeutic strategy for the chemotherapeutic treatment of SACC. Further investigations are required to fully elucidate the underlying molecular mechanisms by which EGCG inhibits tumour invasion.

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**References**


