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A potential proliferative gene, NUDT6, is down-regulated by green tea catechins at the post-transcriptional level

Mugdha Sukhthankar^a, Chang K. Choi^a, Anthony English^b, Jong-Sik Kim^c, and Seung Joon Baek^{a,*}

^aLaboratory of Environmental Carcinogenesis, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA

^bDepartment of Mechanical, Aerospace & Biomedical Engineering, College of Engineering, University of Tennessee, Knoxville, TN 37996, USA

^cDepartment of Biology, Andong National University, Andong, Korea

Abstract

The main aims of this study were to elucidate the effect of green tea catechins on NUDT6 suppression and to characterize NUDT6 biological activity. Our microarray data showed that the green tea component epicatechin gallate (ECG) suppressed NUDT6 expression, and this was confirmed by RT-PCR. Subsequently, the use of different catechins showed that the effect of epigallocatechin gallate (EGCG) was stronger than that of other catechins. At the post-transcriptional level, EGCG decreased the RNA stability of *NUDT6*, indicating it as a potential mechanism of NUDT6 suppression. Further cloning of 3'UTR of human *NUDT6* mRNA resulted in a reduced luciferase activity by EGCG treatment. This effect was at least in part mediated by ERK and p38MAPK pathways. Finally, increased cell proliferation and cell growth in soft agar were observed in NUDT6 overexpressing cells. These findings provide a novel mechanism for suppression of the proliferative gene *NUDT6* by green tea catechins in human colorectal cancer.

Keywords

NUDT6; GFG; EGCG; catechin; mRNA stability

1. Introduction

Multiple components in fruits, vegetables, herbs and spices have been found to inhibit tumor formation in experimental animals, and many researchers have focused on polyphenolic compounds in this regard because of their potent biological properties [1]. Green tea (*Camellia sinensis*) is the second most commonly consumed beverage in the world (an estimated 18–20 billion cups daily) and represents a particularly important source of anti-tumorigenic polyphenols [2]. The most active constituents of green tea are catechins, including

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*To whom correspondence should be addressed: Seung Joon Baek, Ph.D., Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, Phone: (865) 974-8216, Fax: (865) 974-5616, sbaek2@utk.edu.

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epigallocatechin 3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC). The anti-tumor effects of green tea catechins have been studied at the cell biological level, and the major cellular phenomena were found to be apoptosis and cell cycle arrest [3,4]. Potential mechanisms induced by catechins have been suggested to include induction of tumor suppressor proteins [5,6], inhibition of angiogenesis [7], activation of p53 tumor suppressor protein [8], and inhibition of telomerase activity [9]. While the preponderance of data strongly indicates significant anti-tumorigenic benefits of green tea, the molecular mechanisms by which catechins affect anti-tumorigenesis need to be elucidated in detail.

One of these mechanisms apparently involves NUDT6 (also known as GFG), a member of nudix hydrolases superfamily. The nudix superfamily is widespread among many species, and consists mainly of pyrophosphohydrolases that act upon substrates of general structure nucleoside diphosphates linked to another moiety, X, to yield NMP plus P×X [10]. Interestingly, *NUDT6* is an antisense product of the bidirectional transcribed gene locus, 4q26, whose sense product is basic fibroblast growth factor (bFGF/FGF-2) [11]. As a consequence, Murphy et al have shown that *NUDT6* regulates the expression of *FGF-2* in mammalian cells because it is the antisense of *FGF-2* [12]. However, it has also been shown that *NUDT6* may have a role in modulating proliferation in response to growth signals via the protein itself [13].

In the present study, we found that *NUDT6* was one of the down-regulated genes affected by catechin treatment. EGCG modulates RNA stability of the *NUDT6* transcript through p38MAPK and ERK pathways. In addition, our data also suggest that NUDT6 is a cell proliferative protein as assessed by cell growth, soft agar assay and cell impedance experiments.

2. Materials and methods

2.1. Cell culture, reagents, and plasmid construction

Human colorectal cancer cells HCT-116, SW480, HT-29 and LoVo were purchased from the American Type Culture Collection (Manassas, VA). HCT-116 and HT-29 cells were grown in McCoy 5A, whereas SW-480 and LoVo cells were maintained in RPMI1640 and Ham's F-12 media, respectively. Staurosporine was purchased from Biomol International (Plymouth Meeting, PA). All other chemicals were purchased from Fisher Scientific, unless otherwise specified. V5 and Actin antibodies were obtained from Invitrogen (Carlsbad, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The luciferase-*NUDT6* 3'-untranslated region (3'UTR) hybrid construct was generated by PCR using two primers designed to contain the XbaI restriction enzyme site (underlined) as follows: Forward: 5'-gctctagattcacatttatatgttttag-3' and Reverse: 5'-gctctagacgaaaagaggcttttaaaat-3'. A PCR product using cDNA from HCT-116 cells was digested with XbaI enzyme, followed by ligation into pGL3-promoter vector digested with XbaI. The full-length human *NUDT6* cDNA (915 bp) was isolated by RT-PCR from HCT-116 cells using forward (5'-ggacgaattaagcgcgtggaga-3') and reverse (5'-atcaattccttcatagttttat-3') primers, obtained from the reported human *NUDT6* cDNA sequence (GenBank #NM_007083). Amplified PCR products were then cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) and named as pcDNA 3.1/*NUDT6* for correct orientation and pcDNA 3.1/CONTROL for reverse orientation.

2.2. RNA isolation and RT-PCR

RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA), and cDNA was prepared with an i-script synthesis kit (Bio-Rad Laboratories, Hercules CA). RT-PCR was carried out as previously described [6]. The PCR primers used were as follows : human *NUDT6*, forward (5'- catcctccaagccgattta-3') and reverse (5'-aactctcgaaccgctgtgt-3');

human *NAG-1*, forward (5'-ctccagattccgagagttgc-3') and reverse (5'-agagatacgaggtgcaggt-3'); human *ATF3*, forward (5'-gtttgaggatttgcacacgtgac-3') and reverse (5'-agctgcaatcttatttcttcgt-3'); human *NR4A1*, forward (5'-cacagcttgcttgatgt-3') and reverse (5'-tcttgcaatgatgggtgga-3'); human *ID1*, forward (5'-cggatctgaggagaacaag-3') and reverse (5'-ctgagaagcaccaacgtga-3') and human *ID2*, forward (5'-cgtgaggctcgtaggaaaa-3') and reverse (5'-atagtgggatgcgagtcag-3'). The *GAPDH* levels were used for the normalization of RNA using these primers: forward (5'-gggctgctttaactctgt-3') and reverse (5'-tggcaggttttctagacgc-3'). The thermal cycle settings used on a Master Cycler Gradient (Eppendorf) were as follows: 94°C for 2 min as initial denaturation, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Amplification products (25–30 cycles) were analyzed on 1.2% agarose gel, and the bands were visualized using ethidium bromide as an intercalating agent.

2.3. RNA stability and cycloheximide experiments

For the mRNA stability experiment, HCT-116 cells were grown in 6-cm plates and then treated with DMSO and EGCG for 1 h. Actinomycin D (Fisher Bioreagents, Fairlawn, NJ) was added at the dose of 5 µM to terminate transcription. For the *de novo* protein synthesis experiment, HCT-116 cells were pretreated with 10 µg/ml cycloheximide or DMSO for 1 h in serum-free media followed by treatment with EGCG (50 µM) for 24 h.

2.4. Transient transfection and luciferase reporter assays

HCT-116 cells were plated in 12-well plates at 2×10^5 cells per well and grown for 16 h. Plasmid mixtures containing 0.5 µg of reporter vector and 0.05 µg of pRL-null (Promega) were transfected by LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After transfection, the media were replaced with serum-free media, and the indicated reagents were added. The cells were harvested in 1x luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using a Dual-Glo luciferase assay system (Promega).

2.5. Stable cell lines

HCT-116 cells were plated in 6-cm plates and transfected with either NUDT6 expression vector (pcDNA3.1/V5-His-TOPO/NUDT6) or CONTROL vector (pcDNA3.1/V5-His-TOPO/CONTROL) using LipofectAMINE (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. After 24 h, the cells were then transferred to a 10 cm plate with G418 (500 µg/ml) (Stratagene, Santa Clara, CA). Selection with G418 was carried out for 3 weeks, and then Western blot was carried out to check the stable cell lines.

2.6. Western blot analysis

The stable cells were grown to 60–80% confluence in 6-cm plates followed by 24 h treatment in the presence of indicated compounds. Total cell lysates were isolated using RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and Western blot was carried out as previously mentioned [6].

2.7. Cell proliferation analysis, soft agar cloning assay, and impedance assay

Cell proliferation was carried out using pcDNA3.1/NUDT6 and pcDNA 3.1/CONTROL cell lines with or without staurosporine (5 µM) using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells were seeded at a concentration of 1,000 cells/well in 96-well tissue culture plates in six replicates. The cells were then treated with 5 µM of staurosporine for 1, 2, 3 and 4 days. At 0, 1, 2, 3 and 4 days, 20 µl of CellTiter96 Aqueous One solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Winooski, VT). Soft agar assays were performed to compare the

clonogenic potential of the two stable cells in semi-solid medium as previously described [14]. Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotriazolium violet (Sigma, St. Louis, MO) under the luminescent image analyzer (Fujifilm Co.). Colonies were counted using the Multi Gauge Program (Fujifilm Co.) according to the instructions. For the impedance experiment, preliminary naked scans were performed to optimize sensitivity and to check for any electrode debris or defects. The electrodes were then inoculated with 400 μ L McCoy5A, seeding NUDT6 or CONTROL stable cancer cells at a concentration of 2.4×10^4 cells/well. During the cellular micro-impedance scans, data were acquired at a rate of 32 Hz every 16 s using a 30 ms filter time constant and 12 dB/decade roll off for approximately 100 h. Averages and standard deviation estimates were obtained from the 512 sampled data points over the 16 s time intervals. During the experiments, cell-inoculated electrodes were kept in a cell culture incubator at a temperature of 37°C and 5% CO₂.

2.8. Statistical analysis

SAS for Windows (9.1.3) (SAS Institute., Cary, NC) statistical analysis software was used. For quantitative analyses, analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare mean values. The Student *t* test was also used to analyze the differences between samples. $p < 0.05$ was considered statistically significant and represented with one asterisk. Similarly, two and three asterisks are used to show $p < 0.01$ and $p < 0.001$, respectively.

3. Results

3.1. Green tea catechins alter gene expression in human colorectal cancer cells

To elucidate the gene profiles affected by green tea catechins in human colorectal cancer cells, we performed microarray analysis using HCT-116 cells treated with epicatechin gallate (ECG). Among those genes altered by ECG, *NUDT6* was one of several down regulated genes (data not shown). We selected 3 up regulated and 3 down regulated genes, *ATF3*, *NAG-1*, *NR4A1* and *NUDT6*, *ID2*, *ID1*, respectively. HCT-116 cells were treated with ECG (50 μ M) for 24 h and RNA was isolated; cDNA was made and subjected to RT-PCR. We found that ECG (50 μ M) increased the expression of *ATF3*, *NAG-1* and *NR4A1* mRNA and decreased the expression of *NUDT6*, *ID2* and *ID1* mRNA (Fig. 1A, left panel). The gel densitometry data from three independent experiments are shown in Fig. 1A, right panel. Since we have recently reported that a major green tea catechin EGCG suppresses bFGF at the post translational level [7] and that the *NUDT6* gene is an antisense of *bFGF mRNA* [11], we decided to further investigate *NUDT6* in this study. *NUDT6* belongs to the family of Nudix hydrolases but its function is not well known. We carried out RT-PCR for *NUDT6* in other colorectal cancer cells including HT-29, SW480 and LoVo. As shown in Fig. 1B, ECG down regulated *NUDT6* in these other colorectal cancer cells. We further investigated the dose- and time-dependence. HCT-116 cells were treated with ECG (50 μ M) for 0, 6, 12 and 24 h (Fig. 1C, top panel) and ECG (10, 50 and 100 μ M) for 24 h (Fig. 1C, bottom panel). As shown in Fig. 1C, *NUDT6* mRNA expression was suppressed at 6 h and at a dose of 10 μ M, with almost complete suppression at 100 μ M. Finally, we examined whether other catechins suppressed *NUDT6* by carrying out RT-PCR at a dose of 50 μ M each. We found that of the major catechins of green tea, EGCG had a stronger effect on suppressing *NUDT6* mRNA expression, compared to other catechins (Fig. 1D). Therefore, we decided to use EGCG for further experiments.

3.2. Posttranscriptional regulation of NUDT6 by EGCG

To gain insight into the mechanisms responsible for inhibition of *NUDT6* mRNA by the green tea catechin EGCG, we examined post-transcriptional regulation of *NUDT6*. Posttranscriptional control in eukaryotic gene expression can also provide a regulatory process by determining the abundance of a particular protein [15]. To determine the effects of EGCG

on *NUDT6* mRNA stability, the half-life of the mRNAs were first determined by adding EGCG (50 μ M) to HCT-116 cells for 1 h and then treating them with actinomycin D at different time points. After treatment, mRNA was isolated at 0, 1, 2, 4 and 8 h and subjected to RT-PCR (Fig. 2A, top panel). As a result, the half-life of *NUDT6* mRNA from vehicle and EGCG-treated cells was estimated to be around 7 and 2 h, respectively (Fig. 2A, bottom panel). These results suggest that EGCG may affect *NUDT6* mRNA at the post-transcriptional level. Next, HCT-116 cells were pretreated with or without 10 μ g/ml cycloheximide and vehicle (DMSO) for 1 h, followed by treatment with EGCG (50 μ M) (Fig. 2B, top panel). The decreased mRNA expression of *NUDT6* was found to be insensitive to cycloheximide, suggesting that the mechanism of stabilization by EGCG is not involved in *de novo* protein synthesis (Fig. 2B, bottom panel).

3.3. Involvement of the 3'UTR sequence of *NUDT6* in mRNA stability and MAPK pathways

The stability of mRNA is determined in many cases by interactions between specific RNA-binding proteins and *cis*-acting sequences located in the 3'UTR of the mRNA [15]. The AU-rich element (ARE), which targets mRNA for rapid degradation, is well known and characterized as a *cis*-acting sequence [16–18]. We found one ARE consensus sequence and seven ARE-like sequences [19] in the 3'UTR of *NUDT6*. The 3'UTR of the *NUDT6* gene was cloned into a pGL3-promoter vector, downstream from the luciferase reporter gene (pSV40-LUC-*NUDT6* 3'UTR; Fig. 3A). After transfection, the cells were treated with different doses of EGCG (10, 50 and 100 μ M), and luciferase activity was measured. As shown in Fig. 3B, the luciferase activity was decreased in a dose-dependent manner. This result clearly shows that the 3'UTR of *NUDT6* plays an important role in maintaining the stability of *NUDT6* and that EGCG affects the 3'UTR region in a dose-dependent manner.

We also checked the effect of different catechin treatments on the 3'UTR of *NUDT6* (Fig. 3C), and the result was similar to RT-PCR data shown in Fig. 1D. Although EC and EGC decreased the luciferase activity compared to vehicle, the effect was not statistically significant. Next, we examined whether kinase pathways might be involved in EGCG-induced *NUDT6* suppression. ERK, p38MAPK and JNK have been shown to be involved in ARE-driven mRNA turnovers [20]. Thus, we examined the effects of MAPK pathway inhibitors on EGCG-affected *NUDT6* mRNA stability. HCT-116 cells were transfected with pSV40-LUC-*NUDT6* 3'UTR construct for 24 h, pretreated with U0126 (ERK inhibitor; 5 μ M), SB203580 (p38 inhibitor; 15 μ M) and SP600125 (JNK inhibitor; 30 μ M) for 30 min and treated with EGCG (50 μ M) for 24 h in serum-free media. The treatment of U0126 and SB203580 with EGCG did not decrease luciferase activity compared to vehicle treated samples (Fig. 3D), suggesting the potential role of ERK and, to some extent, p38MAPK in the post transcriptional regulation of *NUDT6* by EGCG.

3.4. Biological activity of *NUDT6*

Biological activity of *NUDT6* has not been elucidated in detail, although there are some reports indicating that *NUDT6* functions as a tumor suppressor and a tumor promoter [12,13]. Commercially available *NUDT6* antibody did not work properly in Western analysis (data not shown). Therefore, we amplified the full-length *NUDT6* gene and were successful in getting the forward and reverse clones of *NUDT6* in expression vector pCDN3.1/V5-His-TOPO (Fig. 4A). We then made stable cell lines for *NUDT6* in both forward and reverse orientations. The stable cell lines, pcDNA 3.1/*NUDT6* and pcDNA 3.1/CONTROL were plated in 6-cm plates and were harvested and subjected to Western analysis. First, *NUDT6* expression was confirmed using antibody against V5 tag (Fig. 4A). Cell proliferation was carried out to determine whether *NUDT6* expression affects cell growth. Staurosporine was also added to induce apoptosis, and absorbance was measured on days 0, 1, 2, 3 and 4. *NUDT6*-overexpressing cells increased cell proliferation in the presence of staurosporine significantly at 2, 3 and 4 days; however, a

significant increase was also seen at 0 and 1 day without staurosporine (Fig. 4B). The pro-tumorigenic activity of NUDT6 was evaluated by determining whether NUDT6 expression would affect cell growth in agarose because the ability to form colonies in soft agar is reflective of tumorigenesis [14]. The NUDT6 stable cell line resulted in a dramatic increase of the clonogenic capacity of the cells (Fig. 4C). To further confirm the biological activity of NUDT6, an electrical impedance assay was carried out, and the resistance and reactance were recorded as described in the Materials and Methods section. The pcDNA 3.1/NUDT6 cells showed increased resistance and reactance compared to pcDNA 3.1/CONTROL cells (Fig. 4D, top and bottom panel, respectively). These data also suggest that NUDT6 may have oncogenic activity. Overall, our data suggest that NUDT6 may act like a cell-proliferating factor in human colorectal cancer cells, and green tea catechins suppress its expression at the post-transcriptional level.

4. Discussion

Colorectal cancer is the third leading cause of cancer-related deaths in the United States [21]. Prevention of colorectal cancer is gaining more attention than its treatment because therapeutic drugs generate many side-effects and discomforts. Dietary compounds have been known to have fewer side effects and play an important role in pathogenesis and prevention of cancer [22]. Green tea catechins are among such dietary compounds, and they are well established as anti-cancer agents [23,24]. The green tea catechins are known to inhibit cell growth, angiogenesis, and metastasis and to promote apoptosis accompanied by alterations in gene expression of tumor suppressor proteins NAG-1 and EGR-1, and oncogenic proteins such as bFGF [5,7,25]. In this paper, we have provided a new mechanism by which EGCG/ECG acts as an anticancer agent. EGCG affects the post-transcriptional regulation of the cell proliferative gene *NUDT6* through ERK and p38MAPK pathways.

Regulation of gene expression by post-transcriptional modification of mRNA stability is an important mechanism used in the control of cell growth. Namely, the rate of mRNA degradation determines the extent and duration of gene expression. The 3'UTR of some mRNAs contain AREs, which regulate degradation of mRNAs and provide an effective way to control protein expression by regulation of mRNA half-life and translation [26]. In particular, cellular events such as differentiation, proliferation, apoptosis, and inflammation are known to be related to modifications of the rate of RNA degradation mediated by the ARE motif [16,26]. Based on their sequences and their effects on mRNA stability, three classes of AREs have been defined [27]. Class I AREs contain scattered copies of the AUUUA sequence within a U-rich region. Class II AREs contain overlapping AUUUA motifs within a U-rich region. Class III AREs contain U-rich sequences rather than the AUUUA motif. Emerging evidence suggests that there is another novel ARE that does not fit in any of the three classes. Examination of sequences revealed that the 3'UTR of *NUDT6* mRNA contains a highly conserved ARE (Fig. 3A) and 7 ARE-like sequences, and these AREs should indicate the rapid degradation of mRNA. Indeed, insertion of the 3'UTR of *NUDT6* into the reporter vector caused a dramatic reduction in luciferase activity in the presence of EGCG (Fig. 3B). In addition, EGCG affected the mRNA stability of *NUDT6* and degraded the mRNA as quickly as 2 h compared to 7 h in vehicle (DMSO) (Fig. 2A, top and bottom panel). There was however no involvement of any *de novo* protein synthesis as depicted in the cycloheximide study (Fig. 2B, top and bottom panel), indicating an involvement of signaling pathways that do not require new protein synthesis.

The MAPK pathway could play a role in the post-transcriptional regulation of genes because a number of ARE-binding proteins require phosphorylation by MAPK for activation [28]. We have shown that treatment with JNK inhibitor and EGCG did not affect luciferase activity of the pSV40-LUC-*NUDT6* 3'UTR construct (Fig. 3D); however, luciferase activity was even increased and restored when U0126 and SB203580 were added, respectively. Interestingly,

U0126 treatment with EGCG increased luciferase activity (Fig. 3D), indicating that the ERK pathway may affect not only the 3'UTR of *NUDT6* but also SV40 promoter activity located in the promoter of this reporter vector. Luciferase activity was decreased in the presence of U0126, compared to the vehicle-treated samples, clearly suggesting that the ERK pathway is involved in stabilizing mRNA of *NUDT6*. Overall, our data suggest that EGCG modulates the ERK and p38MAPK pathways and leads to degradation of *NUDT6* mRNA.

NUDT6 belongs to the Nudix hydrolase family and is hypothesized to have both pro- and anti-proliferative effects [10]. There was a 4.44 fold decrease in the *NUDT6* gene expression in our microarray data treated with ECG. To confirm the microarray data, we selected three up regulated genes, *ATF3*, *NAG-1* and *NR4A1*, and three down regulated genes, *NUDT6*, *ID2* and *ID1*. We previously reported that *NAG-1* and *ATF3* are induced by green tea catechins, and *NR4A1*, *ID1*, *ID2* are known to play a role in tumorigenesis [29–31]. In contrast, the biological function of *NUDT6* and its role in tumorigenesis have not been well elucidated [11]. To elucidate the biological function of *NUDT6* in human colorectal cancer cells, we generated stable cell lines with V5/His tags. The cell proliferation assay showed that the *NUDT6* cells had an increased rate of cell growth compared to control at the early time points (Fig. 4B, 0 and 1 day); however, cell proliferation was seen at the later time points (Fig. 4B, 2, 3, and 4 days) when an apoptosis inducer was added to the cells. This suggests that *NUDT6* may contribute to the initiation of cell proliferation and that its expression is less sensitive to the apoptosis inducer. Cell proliferative properties of *NUDT6* are more evident by soft agar cloning and impedance assays. The *NUDT6* stable cell line had more clonogenic properties compared to control cells, as shown in Fig.4C. The impedance technique has been applied to a number of biological studies that deal with cellular barrier function, attachment, spreading, and adhesion [32]. In addition, frequency-dependent electrical impedance measurements have been used to evaluate the model parameters associated with cell-cell and cell-matrix junction formation [33]. In the present study, micro-impedance measurements were used to quantitatively examine proliferation and morphological changes such as cell-cell adhesion and cell-substrate adhesion under *NUDT6* expression. The electrical impedance assay confirmed that *NUDT6* expression resulted in cell proliferation with enhanced cell motility (Fig. 4D top and bottom panels). All these results show that EGCG decreased the expression of pro-tumorigenic *NUDT6* by modulating the ERK and p38MAPK pathways at the post transcriptional level. Further studies are required to determine the ERK and p38MAPK pathway-related ARE-binding proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

UTR	untranslated region
ARE	AU-rich elements
DMSO	dimethyl sulfoxide
EGCG	epigallocatechin 3-gallate
EGC	epigallocatechin
ECG	epicatechin-3-gallate
EC	epicatechin
ERK	extracellular signal-regulated kinase

FGF-2 fibroblast growth factor 2
 NUDT6 (nudix [nucleoside diphosphate linked moiety X]-type motif 6)

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Fig. 1. *NUDT6* is suppressed in the presence of catechins. (A, left panel) HCT-116 cells were treated with 50 μ M of epicatechin gallate (ECG) for 24 h, and RT-PCR was performed for *NUDT6*; *ATF3*, activating transcription factor 3; *NAG-1*, NSAID-activated gene-1; *NR4A1*, nuclear receptor subfamily 4, group A, member 1; *ID2*, inhibitor of DNA binding 2; *ID1*, inhibitor of DNA binding 1. (A, right panel) Gel densitometry results from three independent experiments for the respective genes from left panel. Significance was set at p = *0.05, **0.01, and ***0.001. (B) Different human colorectal cancer cells were treated with ECG (50 μ M) for 24 h, and *NUDT6* RNA transcripts were measured by RT-PCR. (C) HCT-116 cells were treated with ECG at different time points and at different doses. (D) HCT-116 cells were treated with different catechins (50 μ M each), and *NUDT6* gene expression was determined by RT-PCR.

Fig. 2.

Post-transcriptional regulation of *NUDT6*. (A) EGCG facilitates *NUDT6* mRNA degradation. HCT-116 cells were incubated for 24 h with vehicle or EGCG at a concentration of 50 μ M and subsequently treated with actinomycin D (5 μ M). At the indicated times, total RNA was isolated, and *NUDT6* transcripts were measured by RT-PCR. The relative level of *NUDT6* mRNA was calculated, and the results were plotted as the percentage of the mRNA level present at time 0 of actinomycin D treatment (bottom panel). (B) HCT-116 cells were pretreated with 10 μ g/ml cycloheximide and DMSO for 1 h in serum-free media followed by treatment with EGCG (50 μ M) for 24 h. The data are representative of three independent experiments.

*** p < 0.001.

Fig. 3.

Involvement of 3'UTR sequences in EGCG-induced *NUDT6* mRNA suppression. (A) Schematic diagram of the pSV40-LUC-*NUDT6* 3'UTR construct. (B) The construct was transfected (0.5 μ g) along with pRL-null vector (0.05 μ g) into HCT-116 cells, and the cells were treated with different doses of EGCG (10, 50 and 100 μ M). After 24 h treatment, luciferase activity was measured. All other values were normalized to the control (Veh) value set as 1.0. Tukey's multiple comparison test was used to express results as mean $\pm$ SD of three independent transfections, and different letters indicate significant difference ($p < 0.05$). (C) After transfection, HCT-116 cells were treated with 4 different catechins (EGCG, ECG, EC and EGC at a dose of 50 μ M) and luciferase activity was measured. The values obtained from vehicle-treated samples (Veh) were defined as 1.0, and data was analysed using Tukey's multiple comparison test. (D) HCT-116 cells were plated in a 12-well plate, transfected with the construct mentioned in Fig. 3A, pretreated with inhibitors for the ERK (U0126, 5 μ M), p38MAPK (SB203580, 15 μ M) and JNK pathways (SP600125, 30 μ M) for 30 min and then treated with EGCG (50 μ M) for 24 h. The y-axis represents luciferase activity measured by relative luciferase unit (RLU). ** $p < 0.01$ and *** $p < 0.001$, based on Student *t* tests. All experiments were performed in triplicate with columns representing means and bars representing SD.

Fig. 4.

Characterization of Biological activity of NUDT6 in human colorectal cancer cells. (A) The stable cell lines pcDNA3.1/NUDT6 and pcDNA3.1/CONTROL were made as described in materials and methods section. These clones contained tag proteins, V5 and histidine. Western blot was carried out using anti V5 antibody to confirm the integration of plasmid. Actin served as a loading control. (B) Cell growth curve for CONTROL and NUDT6 cells. CONTROL and NUDT6 cells were plated into a 96-plate (1 day before treatment) and either vehicle or 5 μ M staurosporine added at 0 day. Cell growth was measured using the CellTiter96 Aqueous One Solution Cell Proliferation Assay. Values are expressed as mean $\pm$ SD of six replicates. * p <0.05, ** p <0.01, *** p <0.001 versus control cells. (C) Soft agar assay. The stable cell lines were grown for 2–3 weeks in 0.4% soft agar and stained with *p*-iodonitrotetrazolium violet solution. The results are representative of three different experiments. The data represent mean $\pm$ SD. (D) Micro-impedance measurement of HCT-116 cells with stable cell lines. The corresponding normalized resistance (R) and reactance (X) were measured in HCT-116 cells over a 3.5-day period. The subscripts c and n indicate cell-covered and naked scans, respectively. For the sake of clarity, symbols represent 20 data intervals. The starting cell concentration used here was 2.4×10^4 cells/ml.