Received: 17 December 2018 | Accepted: 3 January 2020

DOI: 10.1002/jcp.29451

Journal of Cellular Physiology WILEY

ORIGINAL RESEARCH ARTICLE

EGCG regulates CTR1 expression through its pro-oxidative property in non-small-cell lung cancer cells

Aochang Chen | Pan Jiang | Falak Zeb | Xiaoyue Wu | Chuyue Xu | Lijun Chen | Qing Feng

Key Laboratory of Toxicology, Department of Nutrition and Food Hygiene, School of Public Health, Nanjing Medical University, Nanjing, Jiangsu, China

Correspondence
Qing Feng, PhD, School of Public Health, Nanjing Medical University, 818 Tianyuan East Rd, Nanjing, 211166 Jiangsu, China.
Email: qingfeng@njmu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81472977

Abstract

Copper transporter 1 (CTR1) plays an important role in increasing cisplatin intake. Our previous studies showed that CTR1 expression was upregulated by (−)-epigallocatechin-3-gallate (EGCG), a green tea polyphenol, therefore enhanced cisplatin sensitivity in ovary cancer and non-small-cell lung cancer (NSCLC) cells. In the current study in the non-small-cell lung cancer cells, we uncovered a potential mechanism of EGCG-induced CTR1 through its pro-oxidative property. We found that EGCG increased reactive oxygen species (ROS) generation, while in the presence of ROS scavenger *N*-acetyl-cysteine (NAC), ROS production was eliminated. Changes of CTR1 expression were consistent with the ROS level. Simultaneously, EGCG down-regulated ERK1/2 while upregulated lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) through ROS to induce CTR1 expression. Besides, in a nude mouse xenografts model, EGCG treatment raised ROS level, expression of CTR1 and NEAT1 in tumor tissue. Also, ERK1/2 and p-ERK1/2 were suppressed as well. Taken together, these results suggested a novel mechanism that EGCG mediated ROS to regulate CTR1 expression through the ERK1/2/NEAT1 signaling pathway, which provided more possibilities for EGCG as a natural agent in adjuvant therapy of lung cancer.

KEYWORDS
CTR1, EGCG, ERK1/2, NEAT1, ROS

1 | INTRODUCTION

Globally, lung cancer is the most prevalent cancer in both genders especially non-small-cell lung cancer (NSCLC; Torre, Siegel, Ward, & Jemal, 2016). Many platinum-based agents such as cisplatin (CDDP) have been used to treat NSCLC as the first-line chemotherapy, whereas drug resistance caused by long-term cisplatin administration continuously leads to cancer treatment failure (Chang, 2011). Copper transporter protein 1 (CTR1; SLC31A1), a high-affinity membrane transport protein, is a critical determinant to the intracellular accumulation of platinum drugs (Konishi et al., 2018). Several in vitro and in vivo studies showed that high CTR1 level correlated with high Pt content while deletion of CTR1 led to a decrease in Pt and cisplatin accumulation as well as cisplatin sensitivity in many types of cancer (Kilari, Guancial, & Kim, 2016). CTR1 expression had been shown to be modulated by specificity protein 1 (Sp1), whose zinc finger domain and transactivation domains were copper sensors (Liang, Tsai, Lee, Savaraj, & Kuo, 2012). Elevated glutathione (GSH) levels upregulated CTR1 expression and conferred cellular sensitization to the cisplatin toxicity through reducing bioavailable pool of Cu (Chen et al., 2008). Our previous work showed that CTR1 could be upregulated by (−)-epigallocatechin-3-gallate (EGCG), a noticeable tea polyphenol, in ovarian cancer cells and NSCLC cells (Jiang, Wu, Wang, Huang, & Feng, 2016; Wang et al., 2015).

Reactive oxygen species (ROS) are initially considered as oxidative stress implicated in cell growth, tumor occurrence and organism immunity. Normal levels of ROS accumulation are balanced by oxidants and antioxidants whereas excessive ROS production is

recognized as an inducer of cellular damage (Franchina, Dostert, & Brenner, 2018). Large quantities of research revealed how ROS works in significant biological signaling events such as NF-κB and extracellular-regulated kinase (ERK) 1/2 pathways (Deng et al., 2017; Schroyer, Stimes, Abi Saab, & Chadee, 2018). Many ROS-generating agents with other molecules targeting important cancer cell phenotypes led them to be clinical useful drugs (Teixeira et al., 2018). It was reported that EGCG induced apoptosis, cell death and cellular DNA breakage in various types of cancer cells could be related to ROS produced by EGCG (Farhan et al., 2016; Min et al., 2012). In addition, our previous study found that EGCG regulated cellular ROS formation in human bronchial epithelial cells (Jiang et al., 2015). However, the interaction between ROS and CTR1 in NSCLC remains barely known.

Because we have proved that EGCG induced CTR1 expression in NSCLC cells through upregulating lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) in previous work (Jiang et al., 2016), herein, we discussed how EGCG modulated NEAT1. Many lncRNAs involved in oxidative stress responses suggested that NEAT1 might be regulated by EGCG produced ROS, thus we started to put our eyes on the relationship between ROS and NEAT1 to further explore why CTR1 was regulated.

In the current study, we found that ROS modulated ERK1/2 and NEAT1 involved in EGCG upregulated CTR1 expression in NSCLC cells. EGCG inhibited ERK1/2 while enhanced NEAT1 expression in vitro and in vivo through ROS production. Besides, correlation between ERK1/2 and NEAT1 was also investigated. These findings indicated that EGCG had great significance as an adjuvant therapeutic agent in cancer treatment.

2 | MATERIALS AND METHODS

2.1 | Reagents

EGCG powder was purchased from Sigma-Aldrich Ltd. (St. Louis, MO), PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) and *N*-acetyl-cysteine (NAC) were purchased from Beyotime (Haimen, China), Hydrogen peroxide (30%, H_2O_2) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). CTR1 and NEAT1 primers were purchased from RiboBio (Guangzhou, China).

2.2 | Cell lines

A549 and NCI-H460 cells were purchased from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank (Shanghai, China), and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

2.3 | ROS generation assay

A549 and NCI-H460 cells were collected from the mid dishes and incubated with DCFH-DA (diluted with serum-free medium to a concentration of 1:1000) for 30 min at 37°C after the indicated treatments. The cells were then washed three times with serum-free medium, once with phosphate-buffered saline (PBS), then resuspended in 350 μl PBS, and analyzed using a flow cytometer. Protein quantitative results were processed by Image J software. Representative fluorescence images of intracellular ROS in two cancer cells were photographed by fluorescence microscope.

2.4 | Western blot analysis

Cell lysates were obtained by RIPA buffer containing phenylmethylsulfonyl fluoride (protease and phosphatase inhibitors) and the protein concentrations were measured with acid protein assay kit (Beyotime, Haimen, China). After electrophoresis in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the protein samples were transferred to nitrocellulose filter membranes. Membranes were subsequently blocked in 5% defatted milk for 1 hr at room temperature, then incubated with primary antibodies overnight at 4°C. The membranes were washed several times with TBST and then were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr at room temperature. The protein bands were detected by Eagle Eye II software. Primary antibodies included anti-CTR1 (1:1000; Abcam, Cambridge, UK), anti-β-actin (1:1000; Boster, Wuhan, China), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2; 1:1000; Cell Signaling Technology, Danvers, MA), anti-GAPDH (Boster, Wuhan, China). Secondary antibodies included: HRP-Conjugated AffiniPure Goat Anti-Rabbit IgG and HRP-Conjugated AffiniPure Goat Anti-Mouse IgG (1:2000, ZSGB-BIO, Beijing, China).

2.5 | RNA extraction, reverse transcription, and real-time RT-PCR

Total RNA from the treated cells was extracted using RNAiso Plus (TaKaRaBio Technology, Dalian, China) follow the instructions of the protocol. RNA was reverse-transcribed by the Prime Script TM RT Master Mix (Takara Biotechnology). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Premix Ex Taq II (Takara Biotechnology) and Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster, CA). The relative expression levels of NEAT1 were compared with control using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the reference gene.

2.6 | siRNA and plasmid transfection

Human ERK1/2, NEAT1, or their corresponding control (RiboBio, Guangzhou, China) siRNAs were transfected into A549 and H460

cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The human NEAT1 or control siRNAs, NEAT1 plasmid or the control plasmid (Invitrogen) were transfected with Lipofectamine 2000.

2.7 | In vivo studies

Ten mice (BALB/c, nude, female, aged 4–5 weeks, weighed 16–18 g) were purchased from Shanghai Animal Laboratory Center and maintained in the Experimental Animal Center at Nanjing Medical University. Mice were injected subcutaneously in the front dorsum with exponentially-growing A549 cells (5×10^6 each). A549 xenografts were randomized into two groups, control group and EGCG group's treatments were as follows: control (normal saline, 0.1 ml/10 g), EGCG (20 mg/kg). Drugs were given every 3 days through intraperitoneal injection. After 2 weeks of treatments, all mice were killed by cervical dislocation, and tumor tissues were isolated.

2.8 | Immunohistochemistry staining

Immunohistochemistry staining of Ki-67 was performed by the Department of Pathology, Nanjing Medical College Affiliated Nanjing Hospital. The results of immunohistochemistry staining were analyzed by Image-Pro Plus software (Version 6.0; Media Cybernetics, Bethesda, MD).

2.9 | ROS detection

ROS level of tumor tissues was detected by ELISA kit of Jiangsu Baolai Biotechnology Co. Ltd (Yancheng, China).

2.10 | Data analysis and graphic processing

The SPSS 19.0 software (SPSS Inc. IBM, Chicago, IL) and GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA) were used for statistical analysis. All data were presented as the mean ± standard deviation (*SD*) of at least three independent experiments. Comparisons between quantitative variables were assessed using Student's *t* test and one-way analysis of variace. *p* Value less than .05 was considered statistically significant. ROS graph was plotted by Flowjo V10 softtware.

3 | RESULTS

3.1 | EGCG increased ROS generation in NSCLC cells

The ability of EGCG to promote ROS accumulation was evidenced by many investigators (Hou et al., 2005). In the present study, intracellular ROS levels of A549 and H460 cells after EGCG treatment were detected. DCFH-DA assay revealed that ROS levels were increased significantly with the various concentrations of EGCG (Figure 1a). Figure 1b showed fluorescence intensity of ROS production. Their representative fluorescence images performed same results (Figure 1c). We found that EGCG increased the intracellular ROS levels in A549 and H460 cells. In the presence of specific ROS scavenger *N*-acetyl-cysteine (NAC), the ROS produced by EGCG was decreased in the cells (Figure 1d–f). These results indicated that EGCG increased ROS generation and NAC reversed ROS level in A549 and H460 cells after its 24 hr treatment.

3.2 | EGCG regulated CTR1 through modulation of redox status in lung cancer cells

As our previous studies, the mRNA and protein expression of CTR1 was elevated by EGCG treatment in a dose-dependent manner in A549 and H460 cells (Figure 2a,b). To test if CTR1 expression could be changed in various redox status, EGCG treatment was used alone or combined with NAC in lung cancer cells. Figures 2c and 2d showed that EGCG at a concentration of 40 μM in A549 cells and 30 μM in H460 cells upregulated CTR1 expression, respectively, while 2 mM of NAC changed CTR1 levels induced by EGCG. Next, treatment with pro-oxidant hydrogen peroxide (H_2O_2) increased CTR1 expression (Figure 2e,f) whereas antioxidant NAC showed the opposite effects (Figure 2g,h). Figure 2e–h results indicated that the regulation to the CTR1 was associated with different redox status. Figure 1 and 2 results together showed us that EGCG exerted its pro-oxidative property in regulating CTR1 expression.

3.3 | EGCG suppressed ERK1/2 expression to regulate CTR1 through ROS production in lung cancer cells

Mitogen-activated protein kinase (MAPK) pathways modulated by oxidative stress had been known to all (Huang, Yu, & Chai, 2015; Koinzer, Reinecke, Herdegen, Roider, & Klettner, 2015). We discussed in our study, whether ROS modulated MAPK involved in the regulation to the CTR1. Three inhibitors including ERK1/2 inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125 were used to demonstrate whether CTR1 expression was regulated by MAPK signaling pathway. Figure 3a showed that inhibition of phosphorylated ERK1/2 significantly induced expression of CTR1 rather than p38 and JNK inhibitors, which indicated that ERK1/2 was involved in CTR1 expression. Similarly, three ERK1/2 siRNAs and siRNA controls were transfected into the lung cancer cells and the ERK1/2 siRNA-02 had the best knockdown effect (Figure 3b). ERK1/2 knockdown increased CTR1 level demonstrated its inhibitory effect on the CTR1 expression (Figure 3b). EGCG decreased p-ERK1/2 expression in a dose-dependent manner (Figure 3c) while NAC increased its expression (Figure 3d). Besides, ERK1/2 inhibitor

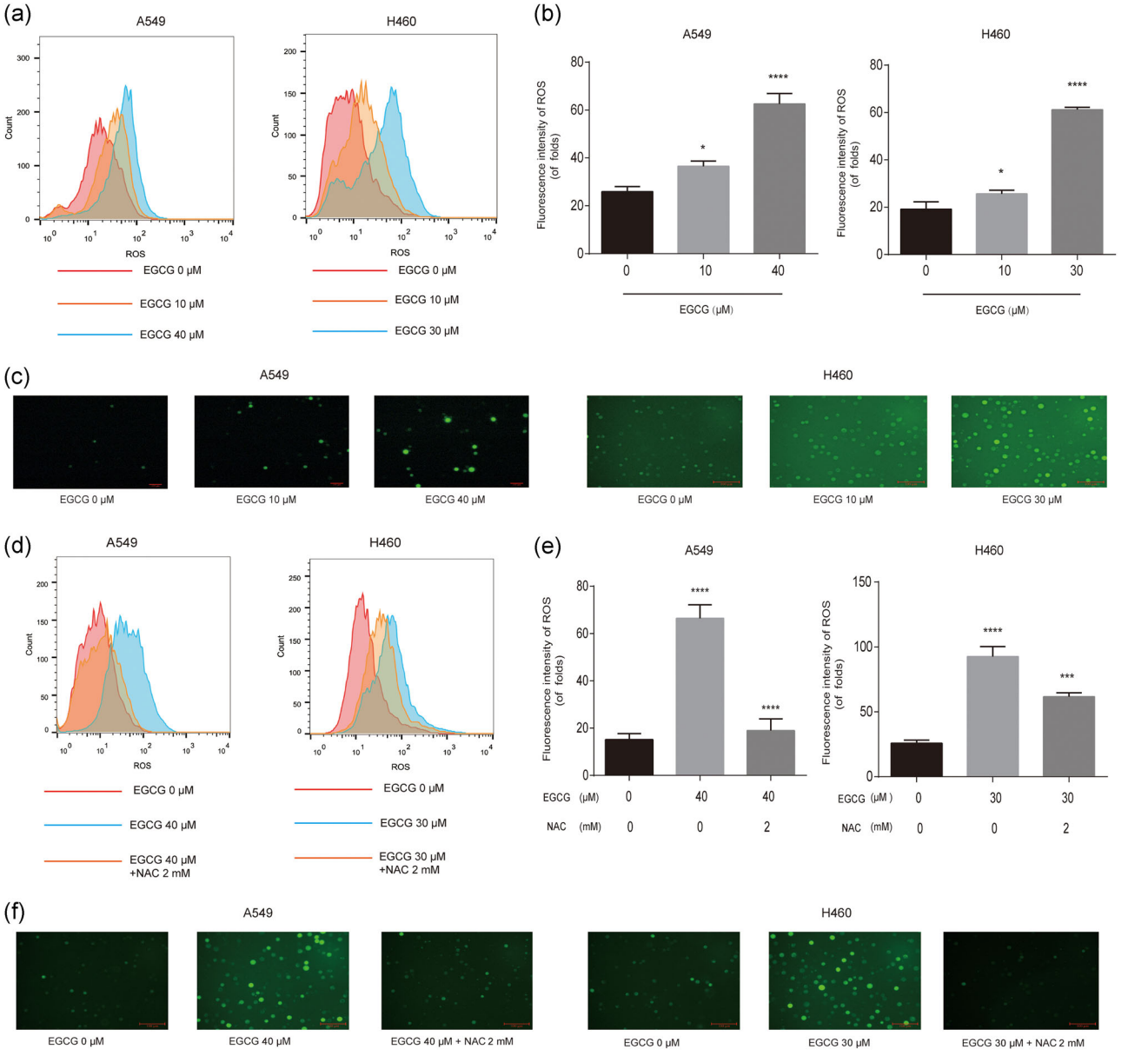

FIGURE 1 EGCG increased ROS generation in A549 and H460 cells. (a,b) ROS levels in A549 and H460 cells. Cells were treated with various doses of EGCG for 24 hr. Flow cytometry was used to detect ROS levels. (c) Representative fluorescence images of intracellular ROS in the cells. (d-e) ROS production in lung cancer cells. Cells were indicated with EGCG alone or EGCG combined with NAC for 24 hr. (f) Fluorescence images of ROS production in A549 and H460 cells incubated with EGCG or EGCG and NAC in combination. Data are representative results from three independent experiments and error bars represent the means ± *SD*. EGCG, (−)-epigallocatechin-3-gallate; NAC, *N*-acetyl-cysteine; ROS, reactive oxygen species. **p* < .05, ****p* < .001, *****p* < .0001 versus control group

combined with EGCG enhanced CTR1 expression while inhibitor combined with NAC suppressed CTR1 expression (Figure 3e,f). Above all indicated that EGCG caused redox status modulated ERK1/2 signaling pathway afterward CTR1 expression in A549 and H460 cells.

3.4 | EGCG enhanced NEAT1 to regulate CTR1 expression through ROS generation in NSCLC cells

First, we verified our former results that NEAT1 regulated CTR1 expression positively in A549 and H460 cells. CTR1 expression was upregulated (Figure 4b) when NEAT1 plasmids (Figure 4a) transfected into the cells. Similarly, CTR1 expression was downregulated (Figure 4d) when NEAT1 siRNAs (Figure 4c) transfected into the cells. Meanwhile, EGCG enhanced NEAT1 expression in a dose-dependent manner (Figure 4e). In the presence of NAC, NEAT1 expression was repressed (Figure 4f). Treatment with H_2O_2 increased NEAT1 expression (Figure 4g) whereas NAC showed the opposite effects (Figure 4h). In addition, CTR1 expression was repressed by NEAT1 siRNA nevertheless enhanced after H_2O_2 treatment in A549 and H460 cells (Figure 4i–l). These results indicated that NEAT1 was an intermediate in the EGCG-induced CTR1 expression through ROS.

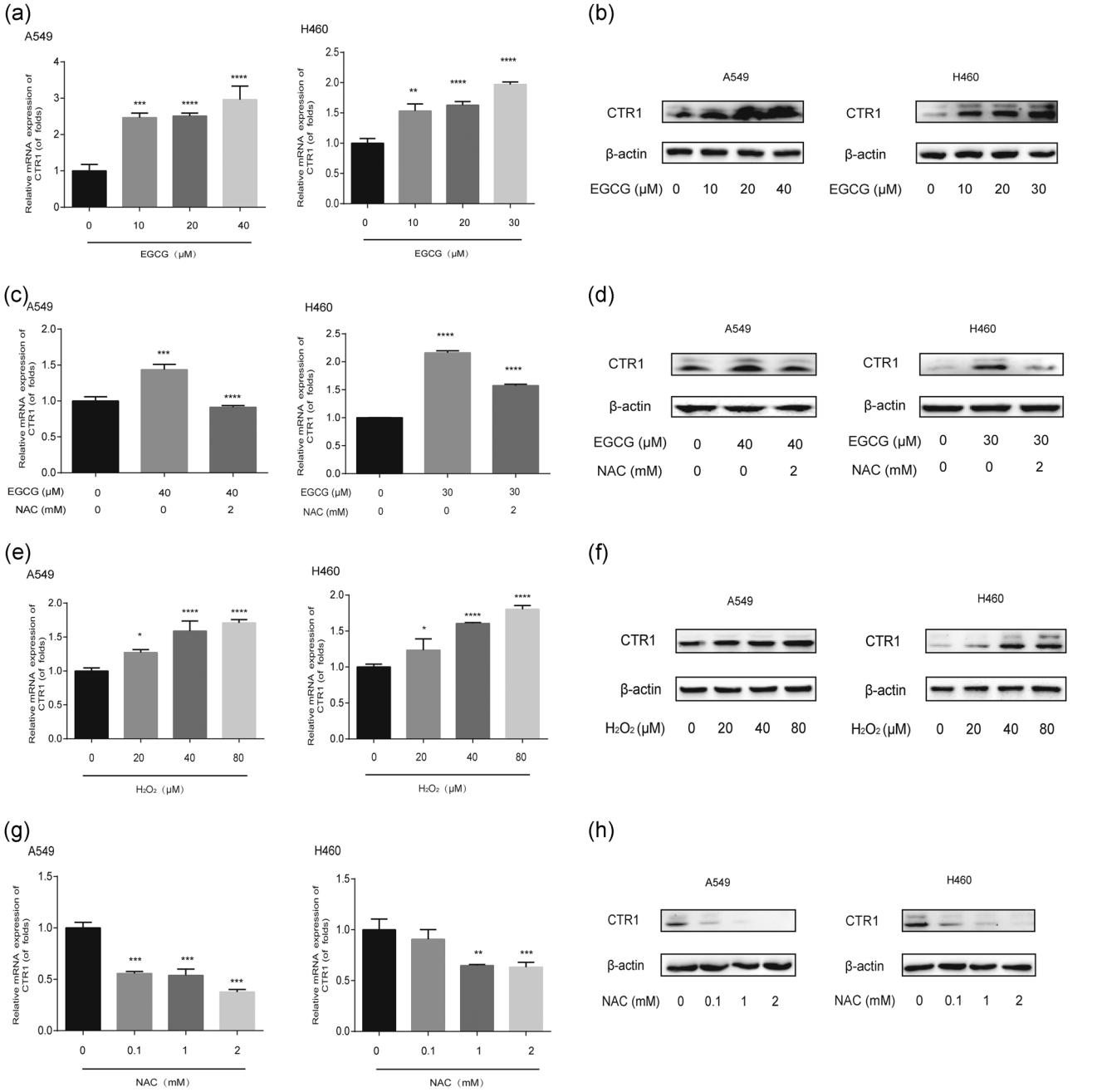

FIGURE 2 EGCG increased CTR1 expression through triggering redox status. Effects of different dosages of EGCG to the CTR1 expression in mRNA level (a) and protein level (b) for 24 hr. β-Actin served as a loading control. Cells were incubated in NAC combined with EGCG. Real-time PCR analyzed the CTR1 expression with GAPDH as an internal control (c), Western blot analysis results showed the protein level of CTR1 (d). Effects of H_2O_2 on CTR1 mRNA level (e) and protein level (f). Effects of NAC were also detected (g–h). CTR1, copper transporter protein 1; EGCG, (−)-epigallocatechin-3-gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; NAC, *N*-acetyl-cysteine; PCR, polymerase chain reaction. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ versus control group

3.5 | The interplay between ERK1/2 and NEAT1 in non-small-cell lung cancer cells

Several studies found that ERK1/2 and NEAT1 could influence each other in different states (Liu, Tai, & Ma, 2018; Ruan et al., 2018). To further explore the relationship between ERK1/2 and NEAT1 in A549 and H460 cells in our study, ERK1/2 inhibitor and siRNA were used. Western blot analysis and real-time PCR results showed that NEAT1 levels were elevated when p-ERK1/2 was inhibited (Figure 5a,b) or ERK1/2 was knocked down (Figure 5c,d). Similarly, when NEAT1 siRNA (Figure 5e) or NEAT1 plasmid (Figure 5g) were transfected into the cells, the p-ERK1/2 was enhanced (Figure 5f) or suppressed (Figure 5h) respectively. These results showed us the mutual inhibition between NEAT1 to ERK1/2 in EGCG regulated CTR1 expression in lung cancer cells.

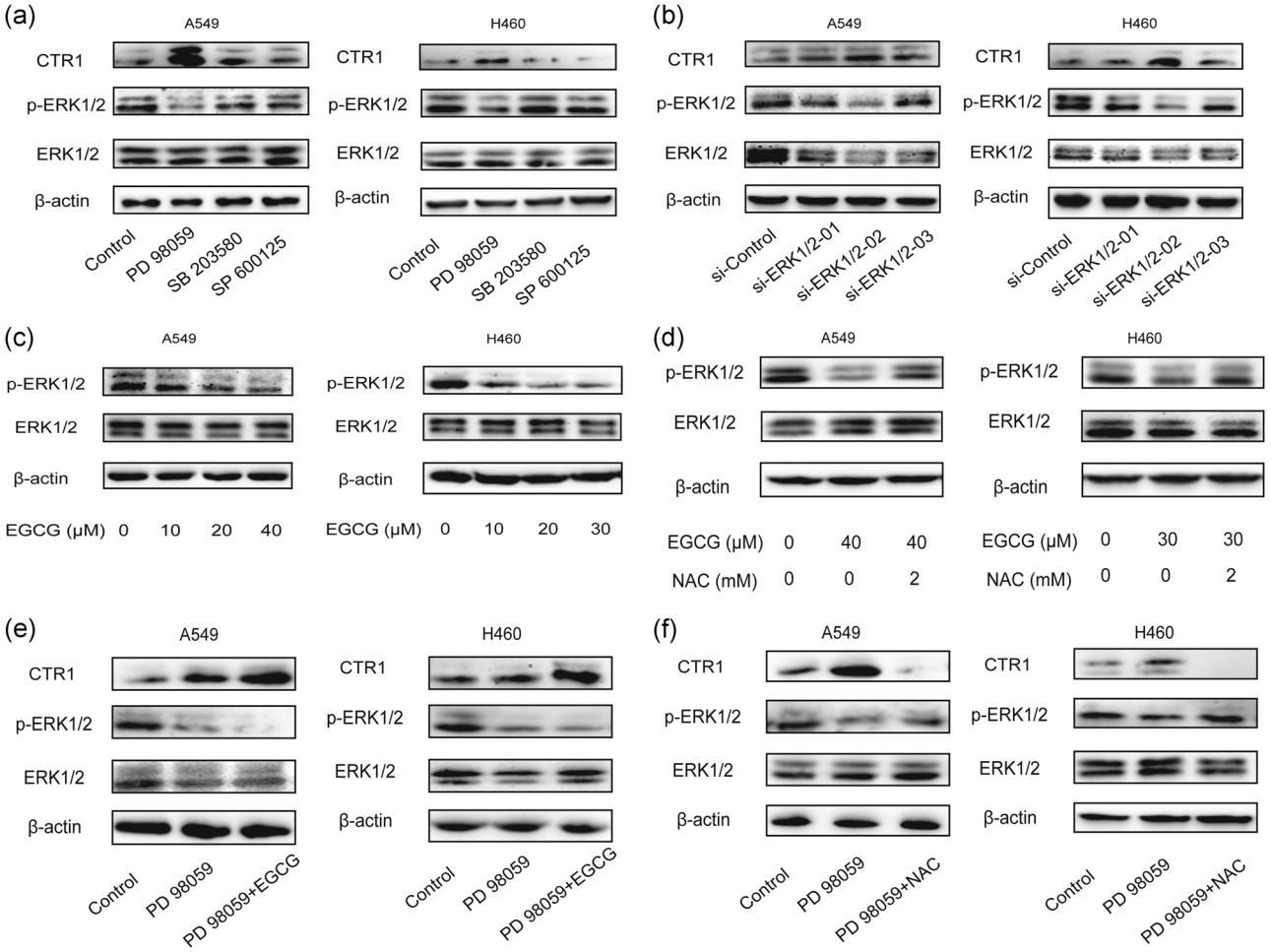

FIGURE 3 Effects of redox status and EGCG on the ERK1/2 to the CTR1. (a) 20 μM ERK1/2 inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125 were used to verify their effects on CTR1 expression for 24 hr. (b) A549 and H460 cells were transfected with three ERK1/2 siRNAs or siRNA control. siRNA-02 showed the best knockdown effect. (c) A549 and H460 cells were treated with indicated concentrations of EGCG for 24 hr. (d) Combination effect of EGCG and NAC was also detected. β-Actin was used as a loading control. ERK1/2 inhibitor combined with EGCG (e) and NAC (f), Western blot analysis were used to detect the protein level of ERK1/2, p-ERK1/2, and CTR1. CTR1, copper transporter protein 1; EGCG, (−)-epigallocatechin-3-gallate; ERK, extracellular-regulated kinase; JNK, Jun N-terminal kinase; NAC, *N*-acetyl-cysteine; siRNA, small interfering RNA

3.6 | EGCG raised ROS level, expression of CTR1 and NEAT1, decreased ERK1/2 expression in A549 cell nude mouse xenografts

An animal model of A549 cell nude mouse xenograft was used to determine whether EGCG had the same effect on the regulation to the ERK1/2 and NEAT1 in vivo. Ten mice were divided into two groups and some indicators were detected. Immunohistochemistry (IHC) showed that the tumor from EGCG group displayed fewer Ki-67-positive cells compared with control group (Figure 6a), which indicated the inhibiting effect of EGCG on the cancer cell proliferation. EGCG increased the ROS generation compared with the control group (Figure 6b). In vivo, NEAT1 and CTR1 were enhanced according to the western blot and real-time PCR results, while ERK1/2 and p-ERK1/2 expression were both well suppressed by EGCG (Figure 6c–e), which is different from the regulation effect of EGCG on p-ERK1/2 in vitro. We will explore the reason for the difference in further experiments.

4 | DISCUSSION

CTR1 functioning as the major platinum-based drug influx, therefore, modulating the cisplatin resistance has been largely investigated (Holzer & Howell, 2006). Previously, we observed that EGCG enhanced the efficacy of cisplatin in NSCLC A549 cells and inhibited cancer stem cell-like properties in A549-cisplatin-resistant cells (Jiang et al., 2018; Zhou, Wang, & Feng, 2014). Besides, CTR1 played a significant role in EGCG-enhanced cisplatin sensitivity in ovary cancer and lung cancer cells (Jiang et al., 2016; Wang et al., 2015). In the current study, EGCG regulated CTR1 through exerting its pro-oxidant characteristics in NSCLC cells. Moreover, ERK1/2 was inhibited while NEAT1 was enhanced by ROS, which both involved in EGCG induced CTR1 expression (Figure 7). To our knowledge, this is the first report providing us a novel mechanism identifying polyphenol caused ROS generation to regulate ERK1/2 and NEAT1 thus promoting CDDP transporter in NSCLC cells.

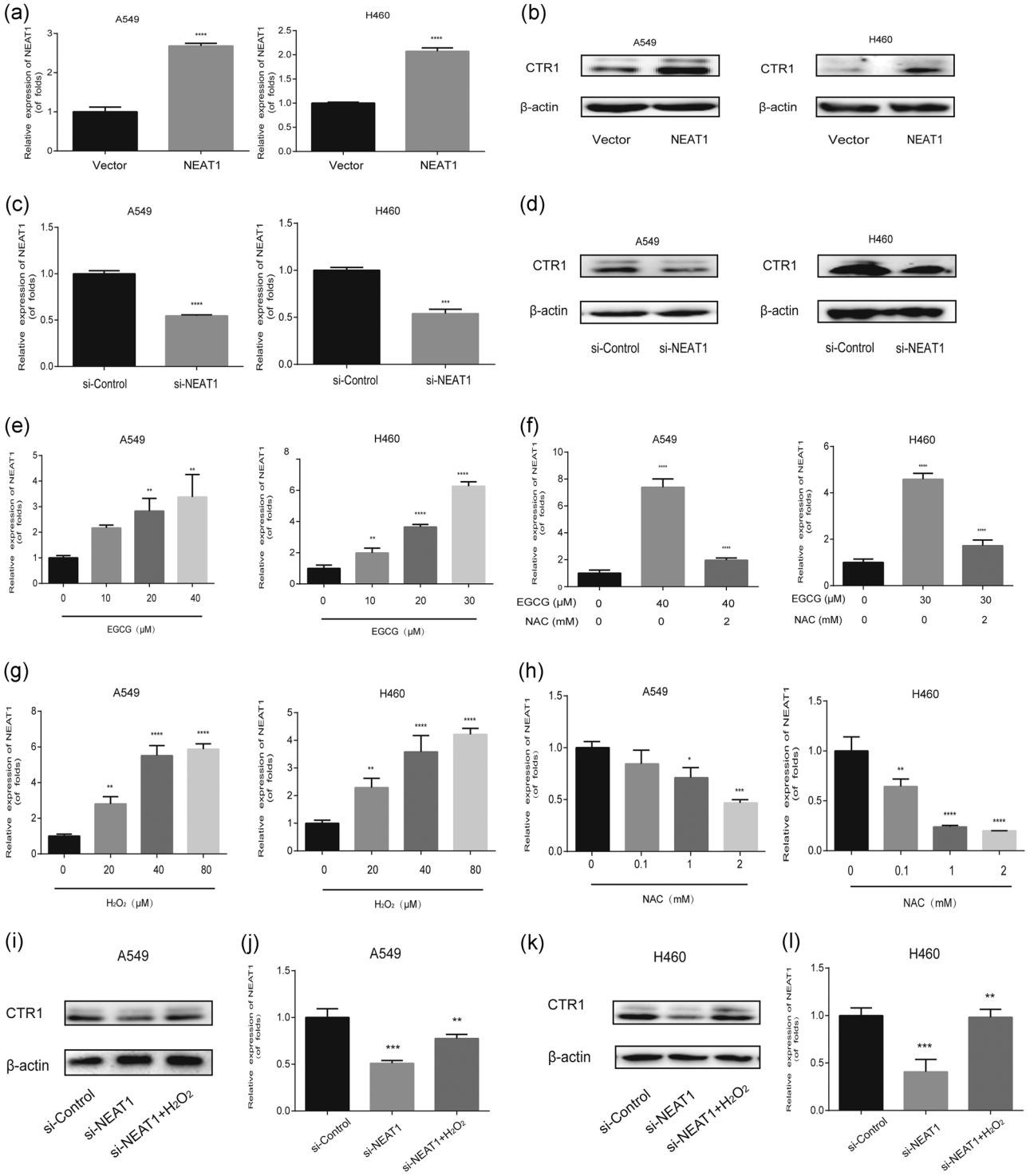

FIGURE 4 Role of NEAT1 in EGCG-regulated CTR1 expression. (a,b) Cells were transfected with vectors or NEAT1 plasmids for 24 hr. The NEAT1 and CTR1 expression were evaluated by real-time PCR and western blot analysis. (c,d) Real-time PCR was used to verify the knockdown effect of NEAT1 siRNA and western blot analysis was performed to detect CTR1 protein level after NEAT1 was knocked down. A549 and H460 cells were incubated with different dosage of EGCG (e). Effects of NAC combined with EGCG on the CTR1 were also performed (f). NEAT1 mRNA levels were also changed by H_2O_2 (g) and NAC (h). (i–l) CTR1 expression was showed after NEAT1 siRNA with H_2O_2 treatment for 24 hr. CTR1, copper transporter protein 1; EGCG, (−)-epigallocatechin-3-gallate; NAC, N-acetyl-cysteine; NEAT1, nuclear paraspeckle assembly transcript 1; PCR, polymerase chain reaction; siRNA, small interfering RNA. Data were expressed as means ± *SD*. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$ versus control group

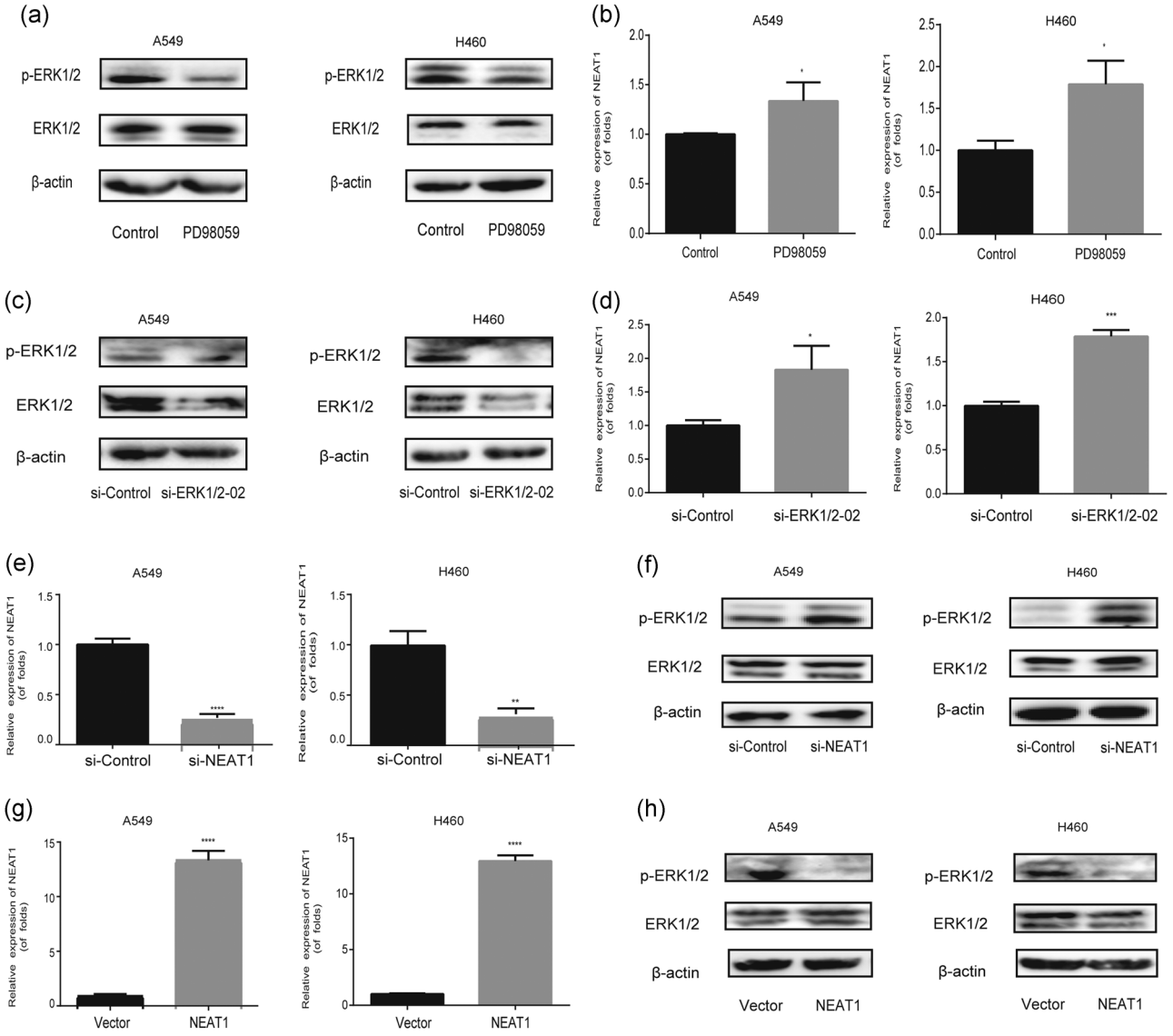

FIGURE 5 An interplay between ERK1/2 and NEAT1 in vitro. (a) ERK1/2 inhibitors were used and (c) siRNAs were transfected into A549 and H460 cells for 24 hr. Western blot analysis was conducted to measure p-ERK1/2 and ERK1/2 protein level, real-time PCR was used to detect mRNA of NEAT1 (b,d) after treatment with β-actin as a loading control. (e–h) Real-time PCR was used to detect NEAT1 with GAPDH as a loading control. Western blot analysis results showed changes of ERK1/2 and p-ERK1/2. Error bars represent the mean ± *SD* of at least three experiments. ERK, extracellular-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; NEAT1, nuclear paraspeckle assembly transcript 1; PCR, polymerase chain reaction; siRNA, small interfering RNA. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ versus control group

Most studies focused on the antioxidative effects of EGCG in various types of disease (Budisan et al., 2017). Many antitumor agents based on the antioxidant activities of EGCG targeting oncogenes were used (Cerezo-Guisado et al., 2015). Notably, EGCG is rather stable in range of 2.0–5.5 of pH (Hong et al., 2002), whereas underwent autoxidation (Akagawa, Shigemitsu, & Suyama, 2003), donated hydrogen atoms and led to the generation of superoxide and oxidized products (Zeng et al., 2018) under neutral and alkaline conditions. For example, EGCG exhibited its auto-oxidation effect in HT-29 cells in typical cell culture conditions at pH 7.2–7.4 and caused maximum H_2O_2 formation at 2 hr in McCoy's 5A medium when 50 μM of EGCG was added (Hong et al., 2002). Increasing concentrations of EGCG as a potent pro-oxidant DNA cleaving agent led to a progressively increased formation of hydroxyl radicals (Kim, Quon, & Kim, 2014). Besides auto-oxidized to cause oxidative stress in the culture medium, EGCG also directly stimulated cells to produce intracellular ROS. EGCG acted as a pro-oxidant by stimulating ROS generation, leading to the activation of AMP-activated protein kinase (AMPK) and exerting its antiobesity effect in vitro had been proved (Collins et al., 2007; Yang, Zhang, Zhang, Huang, & Wang, 2016). Furthermore, the pro-oxidant ability of EGCG may due to cellular increase of ROS through its inhibition to catalase, an antioxidant oligomeric enzyme, in protecting cells from oxidative damage (Pal, Dey, & Saha, 2014). Dual effects in certain conditions made EGCG an effective assistant therapeutic agent to be used.

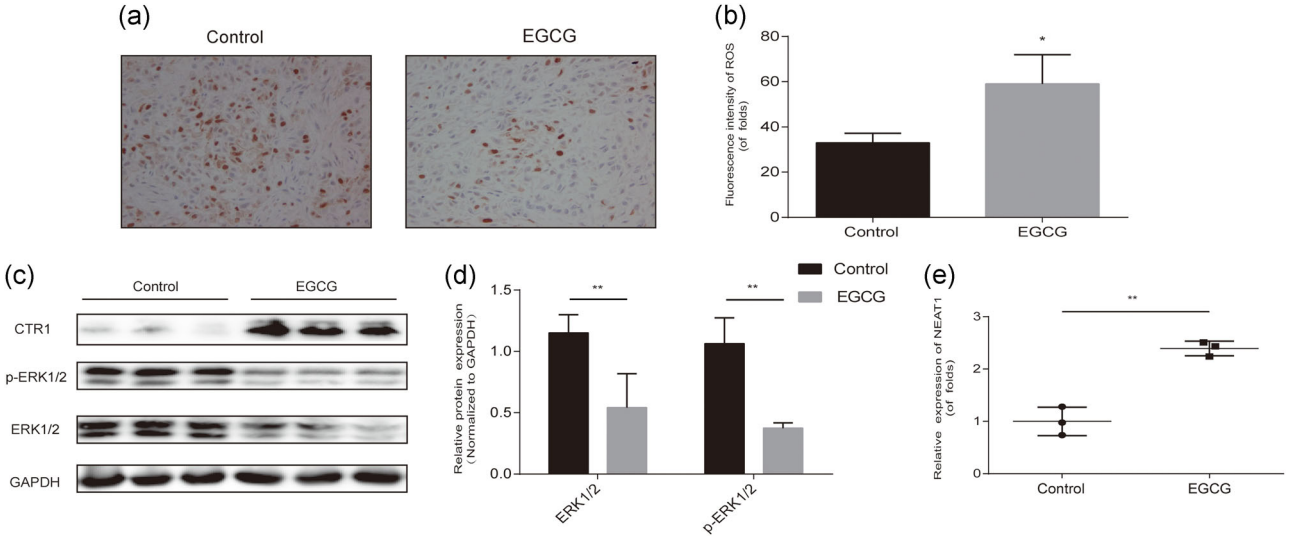

FIGURE 6 EGCG induced CTR1 expression via ERK1/2 and NEAT1 in vivo. Ten 4–5-week-old female BALB/c nude mice were implanted with 5×10^6 A549 cells. They were divided into two groups (control group and EGCG group). (a) IHC staining of Ki-67 in tumor tissues between the two groups was displayed. (b) ROS levels in tumor tissues were detected by the ELISA kit. (c) Western blot analysis was used to assess levels of CTR1, p-ERK1/2, and ERK1/2 in tumor tissues, with GAPDH as an internal control. (d) The intensity of each band was quantified by quantitative analysis. All protein expression levels were normalized to the internal control GAPDH. (e) NEAT1 levels were also tested by real-time PCR. Error bars represent the mean ± *SD* of at least three experiments. CTR1, copper transporter protein 1; EGCG, (−)-epigallocatechin-3-gallate; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry; NEAT1, nuclear paraspeckle assembly transcript 1; PCR, polymerase chain reaction; ROS, reactive oxygen species. *$p < .05$, **$p < .01$ versus control group

ROS, a group of ions and molecules, are produced by both endogenous and exogenous stimuli such as hypoxia, radiation and chemical agents (Bae, Oh, Rhee, & Yoo, 2011). It was thought to be detrimental and associated only with pathological status (Zou, Chang, Li, & Wang, 2017). In fact, cancer cells are more sensitive to ROS than normal cells. H_2O_2 alone as prodrug (Kuang, Balakrishnan, Gandhi, & Peng, 2011) or coupled with ROS-activated agents (Chen et al., 2014) induced DNA cross-linking and apoptosis in various cell types. In psoriasis treatment, the ROS caused by metformin also inactivated ERK1/2 MAP kinase and the oxidative stress sensor Nrf2 to promote HaCaT cell apoptosis (Wang, Li, Zhao, Yu, & Sun, 2018).

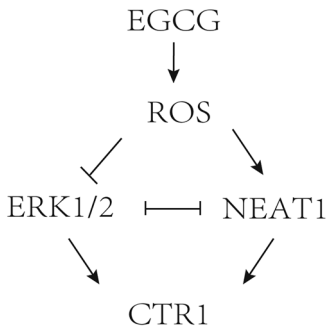

FIGURE 7 EGCG-induced CTR1 expression through ROS production afterward ERK1/2 and NEAT1. A schematic diagram of EGCG-regulated CTR1 through ERK1/2 and NEAT1 in NSCLC cells. CTR1, copper transporter protein 1; EGCG, (−)-epigallocatechin-3-gallate; ERK, extracellular-regulated kinase; NEAT1, nuclear paraspeckle assembly transcript 1; PCR, polymerase chain reaction; NSCLC, non-small-cell lung cancer; ROS, reactive oxygen species

Simultaneously, ROS produced by gambogic acid upregulated initiation-related protein beclin-1 and conversed LC3 I to LC3 II (autophagosome marker) to induce autophagy in the NCI-H441 cells (Ye et al., 2018). ROS caused necroptosis (Villena et al., 2008) and ferroptosis (Cao & Dixon, 2016) were also announced. In our current study, we found that various concentrations of EGCG could produce ROS after 24 hr incubation in A549 and H460 cells, regulated ERK1/2/NEAT1 pathway afterward enhancing CDDP transporter expression.

ERK1/2 belongs to the subfamilies of the MAPKs. MAPK signaling pathway plays a vital contribution in cell proliferation, differentiation, migration, and apoptosis (Sun et al., 2015). It is well known that the MAPK signaling pathway can be stimulated by ROS to modulate its downstream molecules (Oliveira et al., 2018). In this study, EGCG produced ROS inhibited ERK1/2 expression in the cells. When three MAPK kinases inhibitors were given, only ERK1/2 was found to contribute to CTR1 enhancement in NSCLC cells. Then siRNAs of ERK1/2 validated its regulating function to the CTR1 expression. The data showed us that ERK1/2 was the negative regulator of CTR1.

NEAT1 has a significant role in various tumors (Dong et al., 2018). The interaction between NEAT1 and ROS has been reported in several previous studies. For example, the expression of NEAT1 was upregulated by the transcription factor HSF1 in the heat shock response in breast cancer MCF7 cells. Sulforaphane (SFN) mimicked oxidative stress in cells rapidly induced the expression of NEAT1, while pretreatment with NAC, the effect of SFN on NEAT1 expression was counteracted (Lellahi et al., 2018). In H_2O_2-treated

HUVEC, NEAT1 also could be activated by oxidative stress through p53 pathway (Fuschi et al., 2017). In the present study, certain concentrations of EGCG were given to increase the ROS production thereafter induced NEAT1 expression, which means NEAT1 could be regulated by ROS in A549 and H460 cells.

NEAT1 could also interact with ERK1/2 in many disease and treatment. For instance, high expression of NEAT1 specifically activated ERK1/2 in the intervertebral disc degeneration disease (Ruan et al., 2018). Besides, ERK/MAPK pathway could be modulated by NEAT1/let-7a-5p crosstalk in nasopharyngeal carcinoma (Liu et al., 2018). Under the control condition and neuronal activity condition in the brain, the regulation of MEK/ERK signaling to the two spliced variants of NEAT1, NEAT1_1, and NEAT1_2, was diverse (Bluthgen, van Bentum, Merz, Kuhl, & Hermey, 2017). Here in our study, ERK1/2 and NEAT1 could influence each other in NSCLC cells. Inhibition of ERK1/2 via inhibitor and siRNA enhanced NEAT1 expression. When NEAT1 expression was inhibited and enhanced, ERK1/2 expression was upregulated and downregulated, respectively. NEAT1 and ERK1/2 inhibited mutual expression in EGCG induced CTR1 in NSCLC cells. Up to now, we still need more studies to uncover the systematic connection and regulation between NEAT1 and ERK1/2 signaling pathway in a variety of cancers.

Because of excessive copper level could induce CTR1 trafficking from the plasma membrane to the endosomal/lysosomal compartments, where CTR1 may be degraded (Guo, Smith, Lee, Thiele, & Petris, 2004), it was reported that GSH, a crucial cellular antioxidant, and detoxification system in the body, upregulated CTR1 expression to confer cisplatin sensitivity through functioning as an intracellular Cu-chelator (Chen et al., 2008). In contrast to this context in many cases including our study, GSH system might play in enhancing cellular resistance to cisplatin (Kasherman, Sturup, & Gibson, 2009; Lan et al., 2018). GSH could bound to the cisplatin stably in cytoplasm to prevent its transferring to the nucleus and mitochondria, thereafter the complex was readily exported to the out of the cells, which limited the amount of drug reacting with DNA and reduced cisplatin sensitivity. Strong depletion of total GSH content was detected in the elevated accumulation of intracellular ROS condition or oxidative stress-inducing agent (Zulato et al., 2018), while NAC, a cysteine source for GSH synthesis, increased multidrug resistance (Tai et al., 2012). These explained why many chemotherapy drugs under oxidative stress could get better efficiency.

In conclusion, our study indicated possibilities of ROS being the principal modulator in NSCLC. We reported that EGCG could upregulate CTR1 expression through inducing ROS level and involving ERK1/2/NEAT1 pathway. Nevertheless the deep and extensive mechanism of how ROS regulates CTR1 need further investigation and discussion in subsequent experiments

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (81472977) and by funding from the Priority Academic Program Development of Jiangsu Higher Education Institutions. We thank all participants who contributed to this study.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Q. F. and A. C. conceived and designed the experiments, analyzed the data and prepared the manuscript. P. J. and F. Z., participated in revising the manuscript. X. W., C. X, and L. C. contributed to the experiments.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during the present study are included in this article.

ETHICS STATEMENT

This study was approved by the institutional review board of Nanjing Medical University.

ORCID

Qing Feng http://orcid.org/0000-0001-7686-2713

REFERENCES

Akagawa, M., Shigemitsu, T., & Suyama, K. (2003). Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Bioscience, Biotechnology, and Biochemistry*, *67*(12), 2632–2640.

Bae, Y. S., Oh, H., Rhee, S. G., & Yoo, Y. D. (2011). Regulation of reactive oxygen species generation in cell signaling. *Molecules and Cells*, *32*(6), 491–509.

Bluthgen, N., van Bentum, M., Merz, B., Kuhl, D., & Hermey, G. (2017). Profiling the MAPK/ERK dependent and independent activity regulated transcriptional programs in the murine hippocampus in vivo. *Scientific Reports*, *7*, 45101.

Budisan, L., Gulei, D., Zanoaga, O. M., Irimie, A. I., Sergiu, C., Braicu, C., … Berindan-Neagoe, I. (2017). Dietary intervention by phytochemicals and their role in modulating coding and non-coding genes in cancer. *International Journal of Molecular Sciences*, *18*, 6.

Cao, J. Y., & Dixon, S. J. (2016). Mechanisms of ferroptosis. *Cellular and Molecular Life Sciences: CMLS*, *73*(11-12), 2195–2209.

Cerezo-Guisado, M. I., Zur, R., Lorenzo, M. J., Risco, A., Martin-Serrano, M. A., Alvarez-Barrientos, A., … Centeno, F. (2015). Implication of Akt, ERK1/2 and alternative p38MAPK signalling pathways in human colon cancer cell apoptosis induced by green tea EGCG. *Food and Chemical Toxicology*, *84*, 125–132.

Chang, A. (2011). Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. *Lung Cancer*, *71*(1), 3–10.

Chen, H. H., Song, I. S., Hossain, A., Choi, M. K., Yamane, Y., Liang, Z. D., … Kuo, M. T. (2008). Elevated glutathione levels confer cellular

sensitization to cisplatin toxicity by up-regulation of copper transporter hCtr1. *Molecular Pharmacology*, *74*(3), 697–704.

Chen, W., Balakrishnan, K., Kuang, Y., Han, Y., Fu, M., Gandhi, V., & Peng, X. (2014). Reactive oxygen species (ROS) inducible DNA cross-linking agents and their effect on cancer cells and normal lymphocytes. *Journal of Medicinal Chemistry*, *57*(11), 4498–4510.

Collins, Q. F., Liu, H. Y., Pi, J., Liu, Z., Quon, M. J., & Cao, W. (2007). Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5′-AMP-activated protein kinase. *The Journal of Biological Chemistry*, *282*(41), 30143–30149.

Deng, H., Kuang, P., Cui, H., Luo, Q., Liu, H., Lu, Y., … Zhao, L. (2017). Sodium fluoride induces apoptosis in mouse splenocytes by activating ROS-dependent NF-kappaB signaling. *Oncotarget*, *8*(70), 114428–114441.

Dong, P., Xiong, Y., Yue, J., Hanley, S. J. B., Kobayashi, N., Todo, Y., & Watari, H. (2018). Long non-coding RNA NEAT1: A novel target for diagnosis and therapy in human tumors. *Frontiers in Genetics*, *9*, 471.

Farhan, M., Khan, H. Y., Oves, M., Al-Harrasi, A., Rehmani, N., Arif, H., … Ahmad, A. (2016). Cancer therapy by catechins involves redox cycling of copper ions and generation of reactive oxygen species. *Toxins*, *8*(2), 37.

Franchina, D. G., Dostert, C., & Brenner, D. (2018). Reactive oxygen species: Involvement in T cell signaling and metabolism. *Trends in Immunology*, *39*(6), 489–502.

Fuschi, P., Carrara, M., Voellenkle, C., Garcia-Manteiga, J. M., Righini, P., Maimone, B., … Martelli, F. (2017). Central role of the p53 pathway in the noncoding-RNA response to oxidative stress. *Aging*, *9*(12), 2559–2586.

Guo, Y., Smith, K., Lee, J., Thiele, D. J., & Petris, M. J. (2004). Identification of methionine-rich clusters that regulate copper-stimulated endocytosis of the human Ctr1 copper transporter. *The Journal of Biological Chemistry*, *279*(17), 17428–17433.

Holzer, A. K., & Howell, S. B. (2006). The internalization and degradation of human copper transporter 1 following cisplatin exposure. *Cancer Research*, *66*(22), 10944–10952.

Hong, J., Lu, H., Meng, X., Ryu, J. H., Hara, Y., & Yang, C. S. (2002). Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (-)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. *Cancer Research*, *62*(24), 7241–7246.

Hou, Z., Sang, S., You, H., Lee, M. J., Hong, J., Chin, K. V., & Yang, C. S. (2005). Mechanism of action of (−)-epigallocatechin-3-gallate: Auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. *Cancer Research*, *65*(17), 8049–8056.

Huang, Y. C., Yu, H. S., & Chai, C. Y. (2015). Roles of oxidative stress and the ERK1/2, PTEN and p70S6K signaling pathways in arsenite-induced autophagy. *Toxicology Letters*, *239*(3), 172–181.

Jiang, A., Li, Y., Wang, P., Shan, X., Jiang, P., Wang, X., & Feng, Q. (2015). Mechanism of dose-dependent regulation of UBE1L by polyphenols in human bronchial epithelial cells. *Journal of Cellular Biochemistry*, *116*(8), 1553–1562.

Jiang, P., Wu, X., Wang, X., Huang, W., & Feng, Q. (2016). NEAT1 upregulates EGCG-induced CTR1 to enhance cisplatin sensitivity in lung cancer cells. *Oncotarget*, *7*(28), 43337–43351.

Jiang, P., Xu, C., Chen, L., Chen, A., Wu, X., Zhou, M., … Feng, Q. (2018). EGCG inhibits CSC-like properties through targeting miR-485/CD44 axis in A549-cisplatin resistant cells. *Mol Carcinog*, *57*(12), 1835–1844.

Kasherman, Y., Sturup, S., & Gibson, D. (2009). Is glutathione the major cellular target of cisplatin? A study of the interactions of cisplatin with cancer cell extracts. *Journal of Medicinal Chemistry*, *52*(14), 4319–4328.

Kilari, D., Guancial, E., & Kim, E. S. (2016). Role of copper transporters in platinum resistance. *World Journal of Clinical Oncology*, *7*(1), 106–113.

Kim, H. S., Quon, M. J., & Kim, J. A. (2014). New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. *Redox Biology*, *2*, 187–195.

Koinzer, S., Reinecke, K., Herdegen, T., Roider, J., & Klettner, A. (2015). Oxidative stress induces biphasic ERK1/2 activation in the RPE with distinct effects on cell survival at early and late activation. *Current Eye Research*, *40*(8), 853–857.

Konishi, M., Imai, A., Fujii, M., Sugimoto, K., Katakami, N., Imai, Y., & Kamoshida, S. (2018). Correlation of expression levels of copper transporter 1 and thymidylate synthase with treatment outcomes in patients with advanced non-small cell lung cancer treated with S-1/carboplatin doublet chemotherapy. *Asian Pacific Journal of Cancer Prevention*, *19*(2), 435–441.

Kuang, Y., Balakrishnan, K., Gandhi, V., & Peng, X. (2011). Hydrogen peroxide inducible DNA cross-linking agents: Targeted anticancer prodrugs. *Journal of the American Chemical Society*, *133*(48), 19278–19281.

Lan, D., Wang, L., He, R., Ma, J., Bin, Y., Chi, X., … Cai, Z. (2018). Exogenous glutathione contributes to cisplatin resistance in lung cancer A549 cells. *American Journal of Translational Research*, *10*(5), 1295–1309.

Lellahi, S. M., Rosenlund, I. A., Hedberg, A., Kiaer, L. T., Mikkola, I., Knutsen, E., & Perander, M. (2018). The long non-coding RNA NEAT1 and nuclear paraspeckles are upregulated by the transcription factor HSF1 in the heat shock response. *The Journal of Biological Chemistry*, *293*, 18965–18976.

Liang, Z. D., Tsai, W. B., Lee, M. Y., Savaraj, N., & Kuo, M. T. (2012). Specificity protein 1 (sp1) oscillation is involved in copper homeostasis maintenance by regulating human high-affinity copper transporter 1 expression. *Molecular Pharmacology*, *81*(3), 455–464.

Liu, F., Tai, Y., & Ma, J. (2018). lncRNA NEAT1/let-7a-5p axis regulates the cisplatin resistance in nasopharyngeal carcinoma by targeting Rsf-1 and modulating the Ras-MAPK pathway. *Cancer Biology & Therapy*, *19*(6), 534–542.

Min, N. Y., Kim, J. H., Choi, J. H., Liang, W., Ko, Y. J., Rhee, S., … Lee, K. H. (2012). Selective death of cancer cells by preferential induction of reactive oxygen species in response to (−)-epigallocatechin-3-gallate. *Biochemical and Biophysical Research Communications*, *421*(1), 91–97.

Oliveira, M. S., Barbosa, M. I. F., de Souza, T. B., Moreira, D. R. M., Martins, F. T., Villarreal, W., … Bezerra, D. P. (2018). A novel platinum complex containing a piplartine derivative exhibits enhanced cytotoxicity, causes oxidative stress and triggers apoptotic cell death by ERK/p38 pathway in human acute promyelocytic leukemia HL-60 cells. *Redox Biology*, *20*, 182–194.

Pal, S., Dey, S. K., & Saha, C. (2014). Inhibition of catalase by tea catechins in free and cellular state: A biophysical approach. *PLoS One*, *9*(7), e102460.

Ruan, Z., Ma, H., Li, J., Liu, H., Jia, H., & Li, F. (2018). The long non-coding RNA NEAT1 contributes to extracellular matrix degradation in degenerative human nucleus pulposus cells. *Experimental Biology and Medicine*, *243*(7), 595–600.

Schroyer, A. L., Stimes, N. W., Abi Saab, W. F., & Chadee, D. N. (2018). MLK3 phosphorylation by ERK1/2 is required for oxidative stress-induced invasion of colorectal cancer cells. *Oncogene*, *37*(8), 1031–1040.

Sun, Y., Liu, W. Z., Liu, T., Feng, X., Yang, N., & Zhou, H. F. (2015). Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *Journal of Receptor and Signal Transduction Research*, *35*(6), 600–604.

Tai, D. J., Jin, W. S., Wu, C. S., Si, H. W., Cao, X. D., Guo, A. J., & Chang, J. C. (2012). Changes in intracellular redox status influence multidrug resistance in gastric adenocarcinoma cells. *Experimental and Therapeutic Medicine*, *4*(2), 291–296.

Teixeira, J., Amorim, R., Santos, K., Soares, P., Datta, S., Cortopassi, G. A., … Oliveira, P. J. (2018). Disruption of mitochondrial function as mechanism for anti-cancer activity of a novel mitochondriotropic menadione derivative. *Toxicology*, *393*, 123–139.

Torre, L. A., Siegel, R. L., Ward, E. M., & Jemal, A. (2016). Global cancer incidence and mortality rates and trends—An update. Cancer epidemiology, biomarkers & prevention: A publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive. *Oncology*, *25*(1), 16–27.

Villena, J., Henriquez, M., Torres, V., Moraga, F., Diaz-Elizondo, J., Arredondo, C., … Quest, A. F. (2008). Ceramide-induced formation of ROS and ATP depletion trigger necrosis in lymphoid cells. *Free Radical Biology & Medicine*, *44*(6), 1146–1160.

Wang, X., Jiang, P., Wang, P., Yang, C. S., Wang, X., & Feng, Q. (2015). EGCG enhances cisplatin sensitivity by regulating expression of the copper and cisplatin influx transporter CTR1 in ovary cancer. *PLos One*, *10*(4), e0125402.

Wang, X., Li, R., Zhao, X., Yu, X., & Sun, Q. (2018). Metformin promotes HaCaT cell apoptosis through generation of reactive oxygen species via Raf-1-ERK1/2-Nrf2 inactivation. *Inflammation*, *41*(3), 948–958.

Yang, C. S., Zhang, J., Zhang, L., Huang, J., & Wang, Y. (2016). Mechanisms of body weight reduction and metabolic syndrome alleviation by tea. *Molecular Nutrition & Food Research*, *60*(1), 160–174.

Ye, L., Zhou, J., Zhao, W., Jiao, P., Ren, G., & Wang, S. (2018). Gambogic acid-induced autophagy in nonsmall cell lung cancer NCI-H441 cells through a reactive oxygen species pathway. *Journal of Cancer Research and Therapeutics*, *14*(Suppl.), S942–S947

Zeng, J., Xu, H., Cai, Y., Xuan, Y., Liu, J., Gao, Y., & Luan, Q. (2018). The effect of ultrasound, oxygen and sunlight on the stability of (−)-epigallocatechin gallate. *Molecules*, *23*(9), 2394.

Zhou, D. H., Wang, X., & Feng, Q. (2014). EGCG enhances the efficacy of cisplatin by downregulating hsa-miR-98-5p in NSCLC A549 cells. *Nutrition and Cancer*, *66*(4), 636–644.

Zou, Z., Chang, H., Li, H., & Wang, S. (2017). Induction of reactive oxygen species: An emerging approach for cancer therapy. *Apoptosis*, *22*(11), 1321–1335.

Zulato, E., Ciccarese, F., Agnusdei, V., Pinazza, M., Nardo, G., Iorio, E., … Indraccolo, S. (2018). LKB1 loss is associated with glutathione deficiency under oxidative stress and sensitivity of cancer cells to cytotoxic drugs and gamma-irradiation. *Biochemical Pharmacology*, *156*, 479–490.

How to cite this article: Chen A, Jiang P, Zeb F, et al. EGCG regulates CTR1 expression through its pro-oxidative property in non-small-cell lung cancer cells. *J Cell Physiol*. 2020;1–12. https://doi.org/10.1002/jcp.29451