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Combination treatment with highly bioavailable curcumin and NQO1 inhibitor exhibits potent antitumor effects on esophageal squamous cell carcinoma

Short title: Antitumor effect of Theracurmin®

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Abstract

Background : Esophageal squamous cell carcinoma (ESCC) is one of the most intractable cancers, so the development of novel therapeutics has been required to improve patient outcomes. Curcumin, a polyphenol from *Curcuma longa*, exhibits various health benefits including antitumor effects, but its clinical utility is limited because of low bioavailability. Theracurmin® (THC) is a highly bioavailable curcumin dispersed with colloidal submicron particles.

Methods : We examined antitumor effects of THC on ESCC cells by cell viability assay, colony and spheroid formation assay, and xenograft models. To reveal its mechanisms, we investigated the levels of reactive oxygen species (ROS) and performed microarray gene expression analysis. According to those analysis, we focused on NQO1, which involved in the removal of ROS, and examined the effects of NQO1-knockdown or overexpression on THC treatment. Moreover, the therapeutic effect of THC and NQO1 inhibitor on ESCC patient-derived xenografts (PDX) was investigated.

Results : THC caused cytotoxicity in ESCC cells, and suppressed the growth of xenografted tumors more efficiently than curcumin. THC increased ROS levels and activated the NRF2–NMRAL2P–NQO1 expressions. Inhibition of NQO1 in ESCC cells by shRNA or NQO1 inhibitor resulted in an increased sensitivity of cells to THC, whereas overexpression of NQO1 antagonized it. Notably, NQO1 inhibitor significantly enhanced the antitumor effects of THC in ESCC PDX tumors.

Conclusions : These findings suggest the potential usefulness of THC and its combination with NQO1 inhibitor as a therapeutic option for ESCC.

Keywords:

esophageal squamous cell carcinoma; curcumin; Theracurmin®; NQO1; NQO1 inhibitor

Abbreviations

AUC, area under the blood concentration–time curve; DCF, 2',7'-dichlorodihydrofluorescein; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; ESCC, esophageal squamous cell carcinoma; FU, fluorouracil; IC₅₀, 50% inhibitory concentration; KEAP1, Kelch-like ECH-associated protein 1; MS, mass spectrometry; NMRAL2P, NmrA-like redox sensor 2 pseudogene; NQO1, NAD(P)H quinone dehydrogenase; NRF2, nuclear factor erythroid 2-like 2; PARP, Poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PDX, patient-derived xenograft; ROS, reactive oxygen species; RT–PCR, real-time reverse transcription–polymerase chain reaction; SABG, senescence-associated β -galactosidase; SE, standard error; ssDNA, single stranded DNA; TCA, tricarboxylic acid; THC, Theracurmin®; 8-OHdG, 8-hydroxy-2'-deoxyguanosine

Introduction

Esophageal squamous cell carcinoma (ESCC) is the major histological type of esophageal cancer [1, 2], which is the sixth leading cause of cancer-related mortality and the eighth most common cancer worldwide [3, 4]. Despite recent progress in systematic therapeutics, ESCC remains one of the most intractable cancers, having an extremely low five-year survival rate [5, 6]. Therefore, the development of novel treatment options has been needed to improve the outcomes for ESCC patients.

Curcumin is a naturally occurring polyphenol derived from the root of *Curcuma longa* that is recognized as a generally safe compound by the Food and Drug Administration [7, 8]. Curcumin demonstrates various biological benefits including antimicrobial and anti-inflammatory actions, and is involved in the regulation of programmed cell death and survival pathways by modulating transcription factors such as nuclear factor- κ B, growth factors, inflammatory cytokines, and receptors [9]. Curcumin has been shown to have antitumor effects on several types of cancer cells including lung cancer [10], glioblastoma [11], colon cancer [12], pancreatic cancer [13], prostate cancer [14], and ESCC [15-17].

Despite the demonstration of the promising antitumor effects of curcumin in preclinical studies, its clinical use is currently limited because of its poor bioavailability in humans [18]. Curcumin is not easily soluble in water [19], and oral administration of curcumin does not achieve sufficient blood concentrations to exert therapeutic efficacy [20-22]. To overcome this limitation, various strategies of drug development have been attempted to improve the bioavailability of curcumin [23-27].

Theracurmin[®] (THC, curcumin content 30% w/w) is an effective preparation of

curcumin dispersed with colloidal submicron particles, making it easily disperse in water [22]. Consequently, the bioavailability of curcumin in THC is much improved, and the area under the blood concentration–time curve (AUC) after the oral administration of THC is more than 40-fold higher than that of curcumin in rats and 27-fold higher than that of curcumin in humans [22]. In fact, THC has been reported to be clinically useful for treating osteoarthritis [28], muscle damage [29], and atherosclerotic hyperlipidemia [30]. With regard to experimental cancer research, the cytotoxicity or antitumor effects of THC have been reported using several cancer cell lines [31, 32], but the effectiveness of THC against ESCC has not been fully clarified.

The purposes of our study were to investigate the antitumor effects of THC on ESCC cells and to compare the effects of curcumin and THC *in vivo*. Here, we found that induction of NAD(P)H quinone dehydrogenase 1 (NQO1), which is the enzyme that scavenges reactive oxygen species (ROS) [33], plays an antagonistic role in THC-induced antitumor effects, and we, therefore, examined the effects on ESCC of a combination treatment with THC and NQO1 inhibitor.

Materials and methods

In vitro assay and analysis

Methods for cell culture, WST-1 cell viability assay, Caspase-Glo® 3/7 assay, spheroid assay, soft agar colony formation assay, microarray hybridization, real-time reverse transcription–polymerase chain reaction (RT–PCR), western blotting, chromatin protein isolation, measurement of intracellular and/or mitochondrial ROS

levels, immunofluorescent staining for 8-hydroxy-2'-deoxyguanosine (8-OHdG), cell cycle assay, senescence-associated β -galactosidase (SABG) assay, viral infections, and metabolite analysis are described in Supplementary materials and methods.

Assessment of bioavailability and antitumor effects of curcumin and THC in vivo

All animal experiments conformed to the relevant regulatory standards and were approved by the Institutional Animal Care and Use Committee of Kyoto University (Med Kyo 18284).

C57BL/6 male mice (CLEA Japan, Inc., Tokyo, Japan) were given either a control diet (without curcumin or THC), a curcumin diet (containing 0.6 g/kg curcumin), or a THC diet (containing 2 g/kg THC that included 0.6 g/kg curcumin). After one week, blood was taken from the heart of mice and placed into heparinized tubes. Plasma was immediately prepared by centrifugation at 1000 *g*, 4 °C for 10 min and stored at –80 °C until use. The plasma concentration of curcumin was measured using high performance liquid chromatography–tandem mass spectrometry (MS)/MS as described previously [22].

To compare the tumor growth-inhibitory effects of curcumin and THC, xenografted tumors derived from TE-11R cells were used. TE-11R cells (1.5×10^6 cells) were suspended in 50% Matrigel (BD Biosciences, San Jose, CA), followed by subcutaneous implantation into the left flank of 6-week-old hairless SCID male mice (Charles River Laboratories Japan Inc. Yokohama, Japan) ($n = 15$, day 0). The mice were randomly assigned to three groups ($n = 5$ each) and received either control, curcumin, or THC diet from day 0 to day 70.

The tumors were measured with a caliper, and tumor volume (mm^3) was

calculated using the following formula: $(\text{length}) \times (\text{width})^2 \times 0.5$.

Assessment of antitumor effects of THC and NQO1 inhibitor in vivo

Patient-derived xenograft (PDX) ESCC tumors were utilized to assess the therapeutic effects of THC and NQO1 inhibitor *in vivo*. All experiments conformed to the relevant regulatory standards and were approved by the Institutional Animal Care and Use Committee of Kyoto University (Med Kyo 18284) and the Ethics Committee of Kyoto University (G0770).

To establish PDX tumors, biopsy specimens taken from human ESCC tissue of primary site were placed in a subcutaneous pocket created by a 5-mm incision in the left flank of 6-week-old hairless SCID male mice ($n = 20$), which was then closed by suturing. Mice were randomly assigned to one of four groups at day 21 ($n = 5$ each), and given either normal water or THC-containing water (5000 ppm) for drinking from day 21 to day 70. Either DMSO (mock) or 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936) (sc-362737, Santa Cruz Biotechnology, Inc., CA, USA) (5 mg/kg) was administered intraperitoneally every other day from day 21 to day 70.

The tumors were monitored with a caliper, and tumor volume (mm^3) was calculated using the following formula: $(\text{length}) \times (\text{width})^2 \times 0.5$.

Immunohistochemical staining

Immunohistochemical staining was performed as described previously [34]. Additional information is given in Supplementary materials and methods.

Statistical analyses

Data are presented as the mean $\pm$ standard error (SE) of triplicate experiments unless otherwise stated. Differences between two groups were analyzed using the 2-tailed Student's *t*-test, and **P* < 0.05 and ***P* < 0.01 were considered significant. All statistical analyses were performed using SPSS 21 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Cytotoxic and antiproliferative effects of THC on ESCC cells

We treated ESCC cells (TE-1, TE-5, TE-6, TE-8, TE-10, TE-11, TE-11R, T.Tn, and HCE-4 cells) with various concentrations of THC for 96 h, then determined cell viability. As shown in **Fig. 1a** and **Supplementary Fig. 1**, THC dose-dependently decreased cell viability in all ESCC cells. The 50% inhibitory concentration (IC₅₀) values of THC for each ESCC cell are shown in **Supplementary Table 1**. We selected 3 cell lines, TE-5, TE-8, and TE-11R cells, for subsequent experiments because TE-5 cells are derived from poorly-differentiated ESCC [35], and TE-8 and TE-11R cells were suitable for spheroid and colony formation assays.

Next, we measured caspase 3 and caspase 7 activity in those cells treated with THC. Treatment with THC significantly increased the caspase activity as well as the cleavage of Poly (ADP-ribose) polymerase (PARP) protein, showing the capability of THC to induce apoptosis (**Fig. 1b, c**).

In addition, we investigated whether THC influences cell cycle and/or senescence in ESCC cells. As shown in **Supplementary Fig. 2a**, THC induced G2/M

cell cycle arrest in ESCC cells. Moreover, THC significantly increased SABG-positive senescent cells in ESCC cells (**Supplementary Fig. 2b**).

Collectively, these results demonstrated the cytotoxic and antiproliferative effects of THC on ESCC cells.

Inhibitory effects of THC for spheroid and colony formation in ESCC cells

We performed spheroid and soft agar colony formation assays to investigate the effect of THC on the stem cell-like properties and anchorage-independent cell growth activities of ESCC cells. THC showed a strong dose-dependent inhibition of spheroid formation (**Fig. 1d**) and colony formation (**Fig. 1e**) in both TE-8 and TE-11R cells.

Bioavailability and antitumor effects of THC against ESCC xenografts in vivo

To examine the difference in bioavailability between curcumin and THC *in vivo*, we fed mice either a curcumin diet (curcumin group) or a THC diet (THC group) containing equal amounts of curcumin for a week. There was no significant difference in the dietary intake of the groups. In addition, there were no obvious abnormalities in their general condition (e.g., adverse hematological effects and body weight loss) (data not shown). We examined plasma curcumin concentrations in these mice, and the THC group showed markedly higher plasma levels of curcumin than the curcumin group (1110.4 ± 199.8 ng/mL *vs* 191.1 ± 64.4 ng/mL) (**Fig. 2a**). To investigate whether this better bioavailability of THC led to a stronger antitumor effect *in vivo*, we compared the tumor growth inhibition in ESCC (TE-11R) xenograft mice treated with THC or curcumin. As shown in **Fig. 2b**, the THC group demonstrated significantly greater tumor growth inhibition than the curcumin

group (43% vs 11% at day 70).

Upregulation of NMRAL2P after THC treatment

Next, we performed microarray gene expression analysis to identify the changes in gene expression patterns in ESCC cells (TE-5 and TE-8 cells) after THC treatment. We identified several genes that showed differential RNA expression after THC treatment. Among these, expression levels of NmrA-like redox sensor 2 pseudogene (NMRAL2P, also known as Loc344887) was elevated in THC-treated TE-5 and TE-8 cells (log2 fold change: TE-5, 4 h; 2.95, 8 h; 4.35, 24 h; 4.77; TE-8, 8 h; 2.17, 24 h; 2.21, respectively) (**Supplementary Fig. 3**). We confirmed by RT-PCR that THC upregulated NMRAL2P expression time-dependently in TE-5, TE-8, and TE-11R cells (**Fig. 3a**).

Activation of NRF2–NMRAL2P–NQO1 pathway by THC

Because NMRAL2P has been shown to be involved in the nuclear factor erythroid 2 like 2 (NRF2) pathway, in which NRF2 regulates NMRAL2P expression and NMRAL2P subsequently regulates expression of NQO1 [36-38], we next evaluated whether THC affected NRF2 activation and NQO1 expression in ESCC cells. As shown in **Fig. 3b**, THC increased nuclear, but not cytosolic, NRF2 protein levels, suggesting that THC treatment activated the NRF2 transcription factor. THC also increased mRNA and protein levels of NQO1 (**Fig. 3c, d**).

Kelch-like ECH-associated protein 1 (KEAP1) is a repressive partner of NRF2 that suppresses activation of the NRF2 pathway [39]. We next performed siRNA-mediated inhibition of KEAP1, NRF2, and NMRAL2P in TE-5 and TE-8 cells, and confirmed the reduced expression levels of the respective genes (**Supplementary Fig.**

4a–d). Knockdown of *KEAP1* resulted in an increased expression of NMRAL2P while knockdown of *NRF2* caused a reduction of NMRAL2P levels in both TE-5 and TE-8 cells with and without THC treatment (**Supplementary Fig. 4e**). As shown in **Supplementary Fig. 4f, g**, knockdown of *NMRAL2P* or *NRF2* attenuated NQO1 mRNA and protein induction by THC. These results suggested that THC promoted the NRF2–NMRAL2P–NQO1 pathway via activation of NRF2 transcription factor.

In addition, we examined the basal KEAP1 mRNA expression level or nuclear NRF2 protein level in ESCC cells, and we assessed the correlation between their expression levels and their differentiation status, morphological phenotype, and sensitivity to THC (**Supplementary Table 1, 2** and **Supplementary Fig. 5a, b**). Although nuclear NRF2 expression level is considered to be associated with the sensitivity to chemoradiotherapy in ESCC [40] and the expression of NRF2/KEAP1 might be associated with oncogenic characteristics in ESCC cells, there were no significant correlations between them in this study (data not shown).

Increased intracellular and mitochondrial ROS levels induced by THC

Because NRF2 plays an important role in the response to oxidative stress [39], we measured the ROS levels in TE-5, TE-8, and TE-11R cells after THC treatment. As shown in **Fig. 4a**, THC significantly increased intracellular ROS levels in a dose-dependent manner. In addition, 8-OHdG, a marker of oxidative DNA damage, was increased by THC treatment (**Fig. 4b**), and mitochondrial ROS levels also showed a dose-dependent elevation after THC treatment (**Fig. 4c**).

Inhibitory effect of THC on the TCA cycle

Because THC increased mitochondrial ROS levels in ESCC cells, we

investigated whether THC affected mitochondrial functions such as the tricarboxylic acid (TCA) cycle. Metabolome analyses revealed that THC sharply decreased the levels of cis-aconitic acid, isocitric acid, and 2-oxoglutaric acid, components of the TCA cycle, indicating the inhibitory effects of THC on the TCA cycle (**Fig. 4d**).

The role of NQO1 in ESCC cells with THC treatment

To investigate how NQO1 influences the cytotoxic effect of THC on ESCC cells, we created *NQO1* knockdown TE-11R cells using three types of shRNAs. As shown in **Fig. 5a, b**, NQO1 expression levels were remarkably reduced by all shRNAs at both mRNA and protein levels. *NQO1* knockdown resulted in an increase of 8-OHdG-
indicated oxidative damage in THC-treated cells (**Fig. 5c**). Although *NQO1* knockdown alone did not affect cell growth (data not shown), susceptibility to THC in *NQO1* knockdown cells was significantly higher than that in control cells (**Fig. 5d**). Conversely, when we overexpressed NQO1 in TE-11R cells via lentivirus infection (**Fig. 5e, f**), NQO1 overexpression resulted in a decrease of 8-OHdG-
indicated oxidative damage after THC treatment (**Fig. 5g**) and was associated with resistance to THC treatment (**Fig. 5h**).

Combination effects of THC and NQO1 inhibitor in vitro

Because of the additive effects of THC and NQO1 inhibition, we next examined whether NQO1 inhibitor enhanced the cytotoxic effects of THC. ESCC cells were treated with THC and ES936, which is a mechanism-based inhibitor of NQO1 [41]. ES936 has been shown not to decrease NQO1 protein expression [42]. The combination of THC and NQO1 inhibitor resulted in an increase in ROS production, 8-OHdG-indicated oxidative damage, and the cleavage of PARP protein (**Fig. 6**).

Combination effect of THC and NQO1 inhibitor against ESCC PDX tumors in vivo

To assess the antitumor effects of THC and NQO1 inhibitor *in vivo*, we evaluated the tumor growth inhibition in ESCC PDX tumors. First, to determine the optimal dose of THC, mice were given either normal water or THC-containing water (2500, 5000, or 10000 ppm), and plasma curcumin concentrations were examined. As shown in **Fig. 7a**, plasma curcumin levels increased THC-dose dependently. When 5000 ppm of THC was administered, the plasma concentration of curcumin reached 3973.8 ng/mL (about 11 μ M), which was similar to the concentration we used in *in vitro* experiments. Therefore, we used water containing 5000 ppm THC for subsequent treatments. As shown in **Fig. 7b**, treatment with the combination of THC and NQO1 inhibitor resulted in a significant inhibition of PDX tumor growth compared with that after control or monotherapy (inhibition at day 70: NQO1 inhibitor 1.9%, THC 40.7%, THC plus NQO1 inhibitor 72.6%). There was no significant difference in the water intake between groups, and no significant adverse hematological effects or weight loss were detected (data not shown). Combination treatment with THC and NQO1 inhibitor significantly decreased Ki67 expression, a marker of cellular proliferation, compared with vehicle control and/or monotherapy with THC (**Fig. 7c, d**). In addition, combination treatment with THC and NQO1 inhibitor and/or THC monotherapy significantly increased single stranded DNA (ssDNA), a marker of apoptosis, compared with vehicle control (**Fig. 7c, e**). Moreover, 8-OHdG, nuclear NRF2, and NQO1 levels were increased by treatment with THC alone as well as combination treatment with THC and NQO1 inhibitor (**Fig. 7c**). As a note, THC treatment did not cause any histological damages in normal esophageal tissues, and it did not increase ROS levels (8-OHdG levels) in normal esophageal

tissues (data not shown).

Discussion

Highly bioavailable curcumin (Theracurmin[®], THC) showed antitumor effects on various types of ESCC cells and xenografted tumors. THC increased ROS levels in accompany with the activation of NRF2–NMRAL2P–NQO1 pathway. Since NQO1 revealed to play an antagonistic and antioxidative role in THC-induced cytotoxicity, we proposed a combination treatment with THC and NQO1 inhibitor. As a result, such a treatment strategy exhibited potent antitumor effects on ESCC PDX tumors.

In this study, biological effects (e.g., cytotoxic effects, ROS production, and sensitization of NQO1 inhibitor) of THC were similar to those of curcumin *in vitro* (**Fig. 1a**, **Fig. 4a**, **Fig. 6c** and **Supplementary Fig. 6**). We suggest this is because THC is identical with curcumin as a component [22]. However, the antitumor effect of THC *in vivo* was much higher than that of an equal dose of curcumin. We suggest that the difference in the antitumor effect between THC and curcumin *in vivo* is caused by their different bioavailability (**Fig. 2a**).

The peak plasma curcumin levels following oral THC administration (5000 ppm) in mice reached 3973.8 ng/mL (**Fig. 7a**), which is roughly equivalent to 11 μ M. In our *in vitro* experiments, we used 5–50 μ M THC and the IC₅₀ values of THC in various ESCC cells were between 7.03 and 34.98 μ M. Because the plasma curcumin concentration after THC administration (400 mg curcumin/day) in humans was reported to be at about 3.7 μ M [43], we presume that the dose of THC used in our experiments does not greatly exceed the achievable physiological range.

In the present study, NMRAL2P was upregulated in ESCC cells treated with THC. NMRAL2P is a long noncoding RNA that acts as a coactivator of NQO1 [38]. Although its precise function remains to be clarified, potential roles for NMRAL2P as a prognostic factor and/or a therapeutic target for cancer have been reported [44, 45]. Our results showed that *KEAP1* knockdown increased expression of NMRAL2P while *NRF2* knockdown reduced it. Moreover, *NMRAL2P* knockdown inhibited NQO1 induction by THC. These results are consistent with previous reports [36-38] and indicate that NMRAL2P is acting downstream of NRF2 and upstream of NQO1 in the NRF2-related signal cascade.

We showed that THC treatment increased intracellular and mitochondrial ROS levels in ESCC cells. These results were consistent with previous reports showing curcumin-mediated ROS generation [46-48] that led to mitochondrial damage [49]. In this study, we also demonstrated that THC treatment affected the TCA cycle, which may retain a role in cancer cell metabolism. In particular, 2-oxoglutaric acid plays critical roles as a precursor of glutamine formation, as a nitrogen transporter for the urea cycle and/or ammonia detoxification, and as a cosubstrate for dioxygenases [50]. In addition, 2-oxoglutaric acid-dependent dioxygenases mediates the demethylation of DNA and histones, which is involved in regulation of the expression of many genes [51, 52], and depletion of 2-oxoglutaric acid was able to cause epigenetic changes [53]. Thus, the decrease in 2-oxoglutaric acid caused by THC may induce a range of aberrations in cellular metabolism.

We showed that THC increased nuclear NRF2 as well as NQO1 expressions. As NRF2 is upregulated by ROS production [39] and NQO1 is a downstream factor of NRF2 [39], we suggest that nuclear NRF2 is increased via THC-mediated ROS

production and NQO1 is upregulated via NRF2 activation. To examine the role of NQO1 in THC-mediated cytotoxicity in ESCC cells, we performed the experiments using cells with *NQO1* gene modification and/or NQO1 inhibitors. Knockdown of *NQO1* or administration of NQO1 inhibitor resulted in an increase of ROS and/or 8-OHdG levels in THC-treated ESCC cells while overexpression of NQO1 resulted in a decrease of 8-OHdG in THC-treated ESCC cells. These results suggest that NQO1 plays an antioxidative role in THC-mediated cytotoxicity. As NQO1 acts as a reductase [33, 54-56], cells undergoing oxidative stress are considered to induce NQO1 to protect cells from those stresses. Accordingly, we thought that inhibition of NQO1 could enhance the antitumor effects of THC and we revealed that the strategy is effective.

We showed that basal levels of NQO1 protein in ESCC cells tended to be high, compared with those in normal esophageal epithelial cells (**Supplementary Fig. 5c**). Therefore, cytotoxic effect of THC may occur efficiently on ESCC cells in the presence of NQO1 inhibitor. As NQO1 inhibitor has not been used clinically, future studies to develop NQO1 inhibitors are warranted.

A limitation of this study is that we could not determine whether NMRAL2P regulates NQO1 directly or indirectly. Moreover, it remains unclear whether the antitumor effect of THC and NQO1 inhibitor is due to the direct effect on tumor cells or indirect effect on microenvironment. Further study will be required to address these questions.

In conclusion, THC exhibits *in vitro* as well as *in vivo* antitumor effects, and showed remarkably higher bioavailability and stronger antitumor effects than curcumin *in vivo*. THC induced ROS in accompany with the activation of NRF2–

NMRAL2P–NQO1 pathway. NQO1 played an antioxidative role in THC-mediated cytotoxicity. Importantly, NQO1 inhibitor enhanced the THC-induced antitumor effects (**Supplementary Fig. 7**). These results suggest the potential usefulness of combination therapy with THC and NQO1 inhibitor for the treatment of ESCC.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure. 1

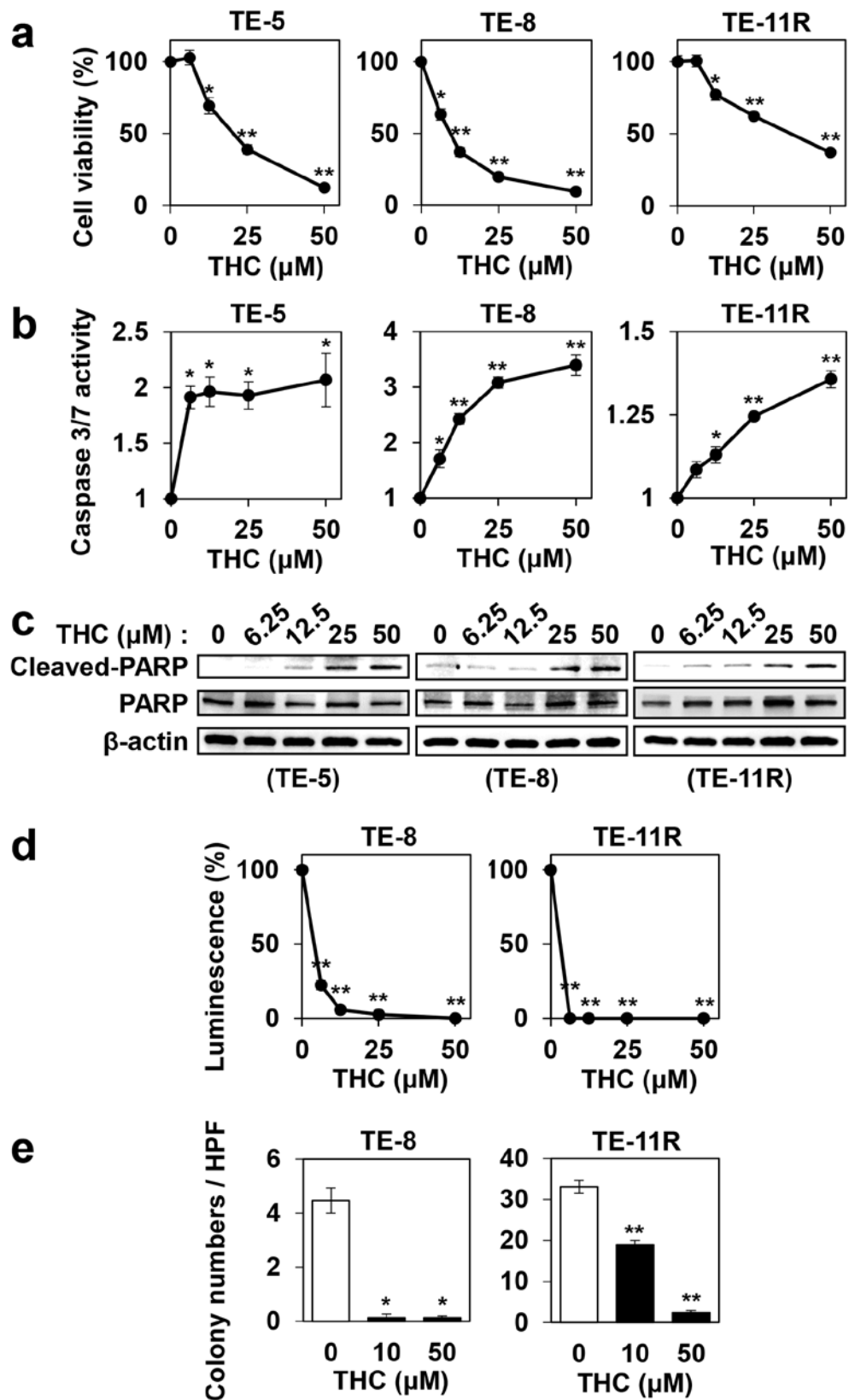


Figure. 2

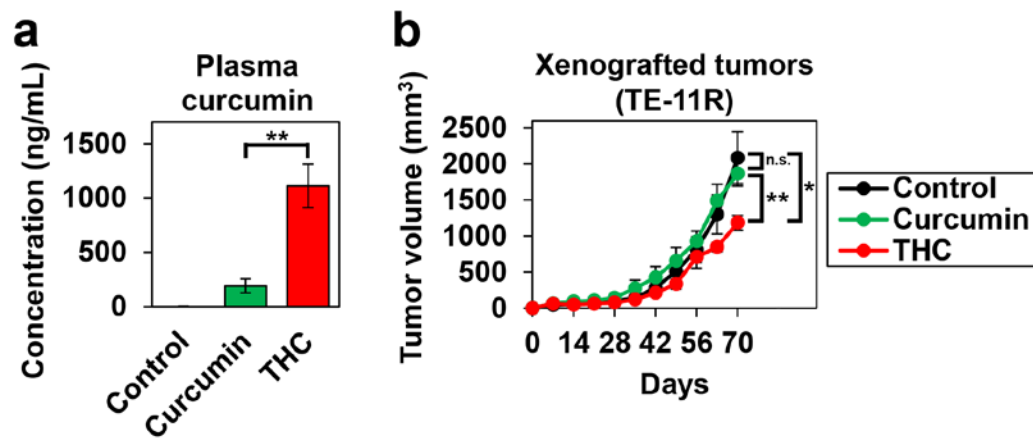


Figure. 3

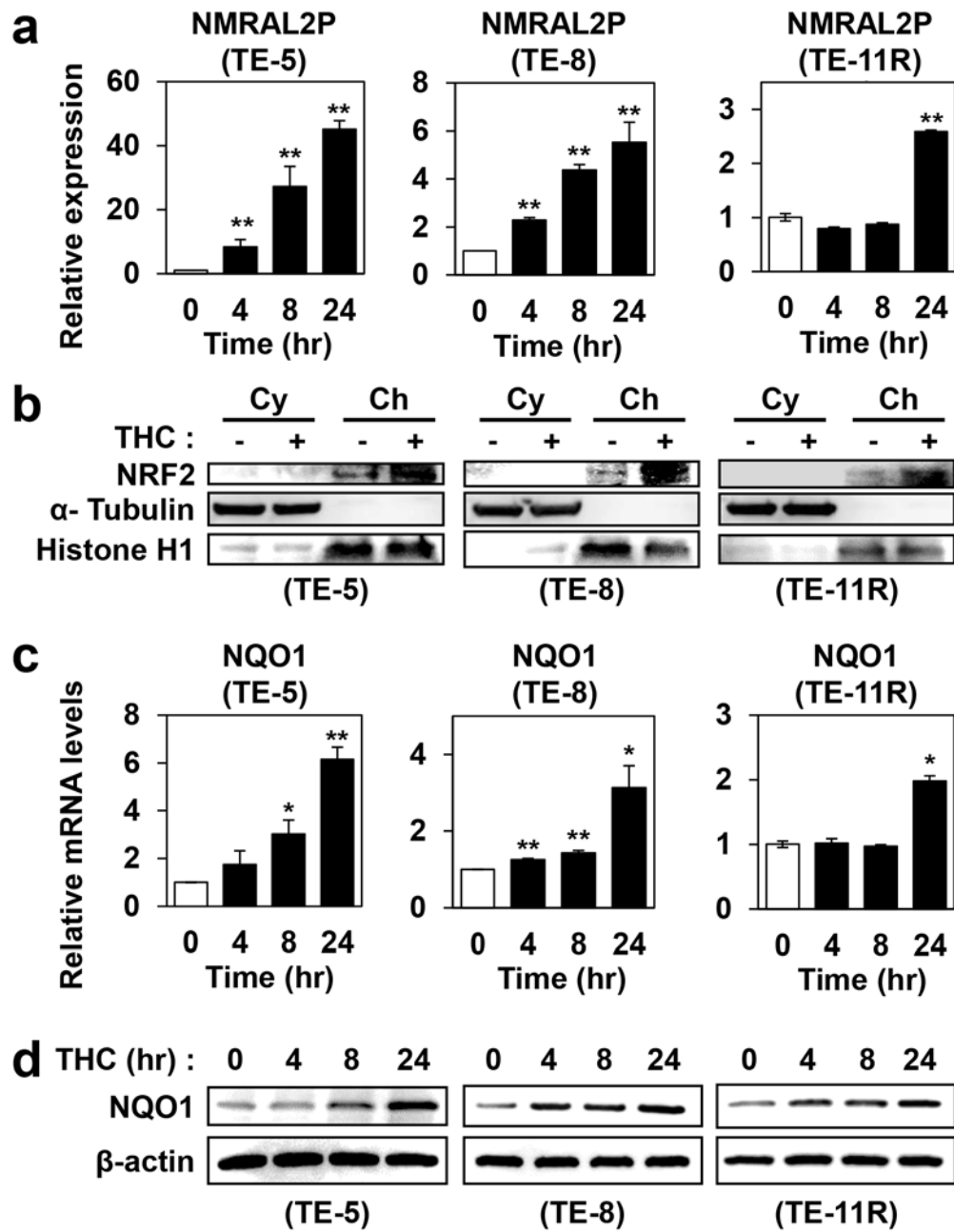


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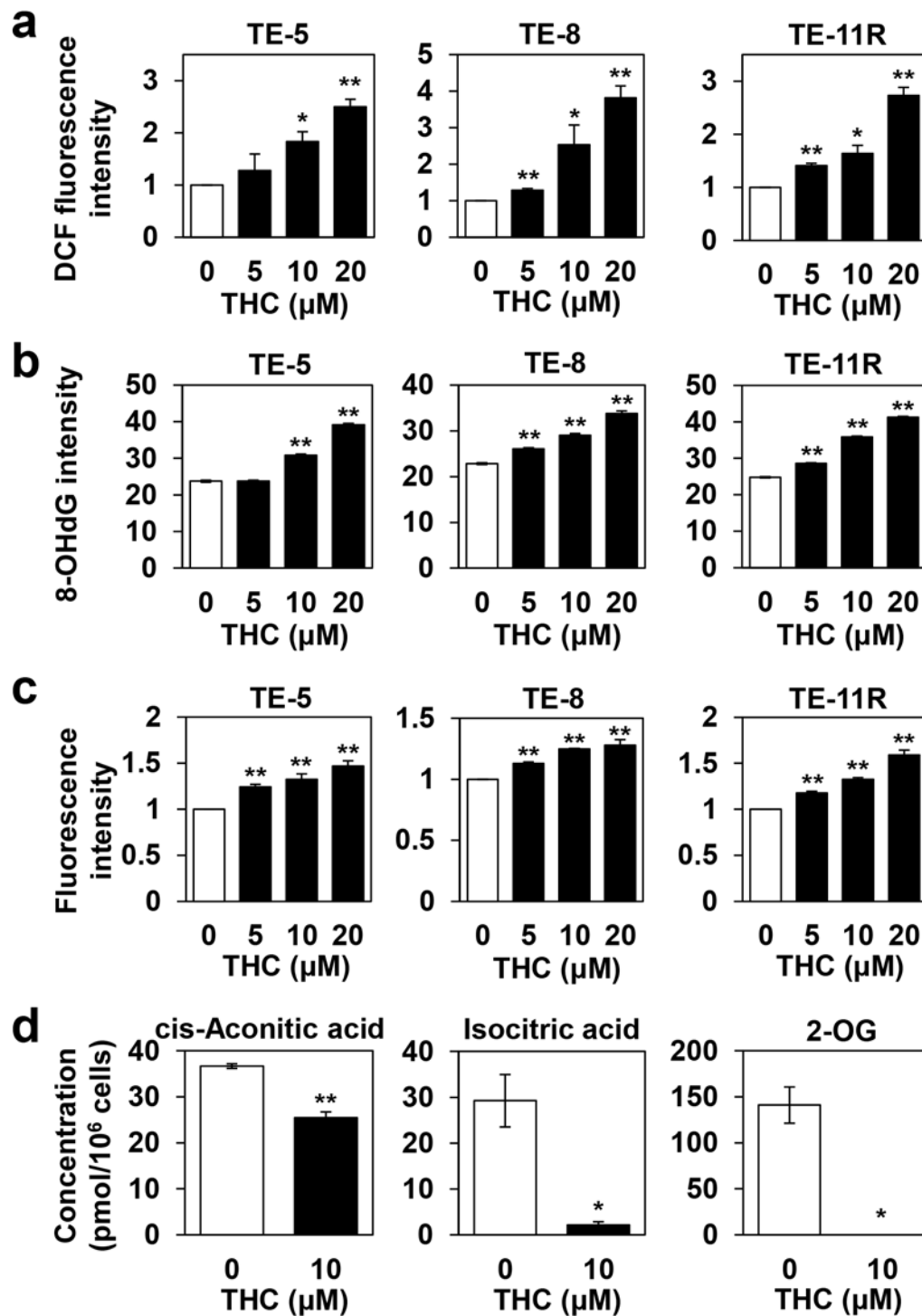


Figure. 5

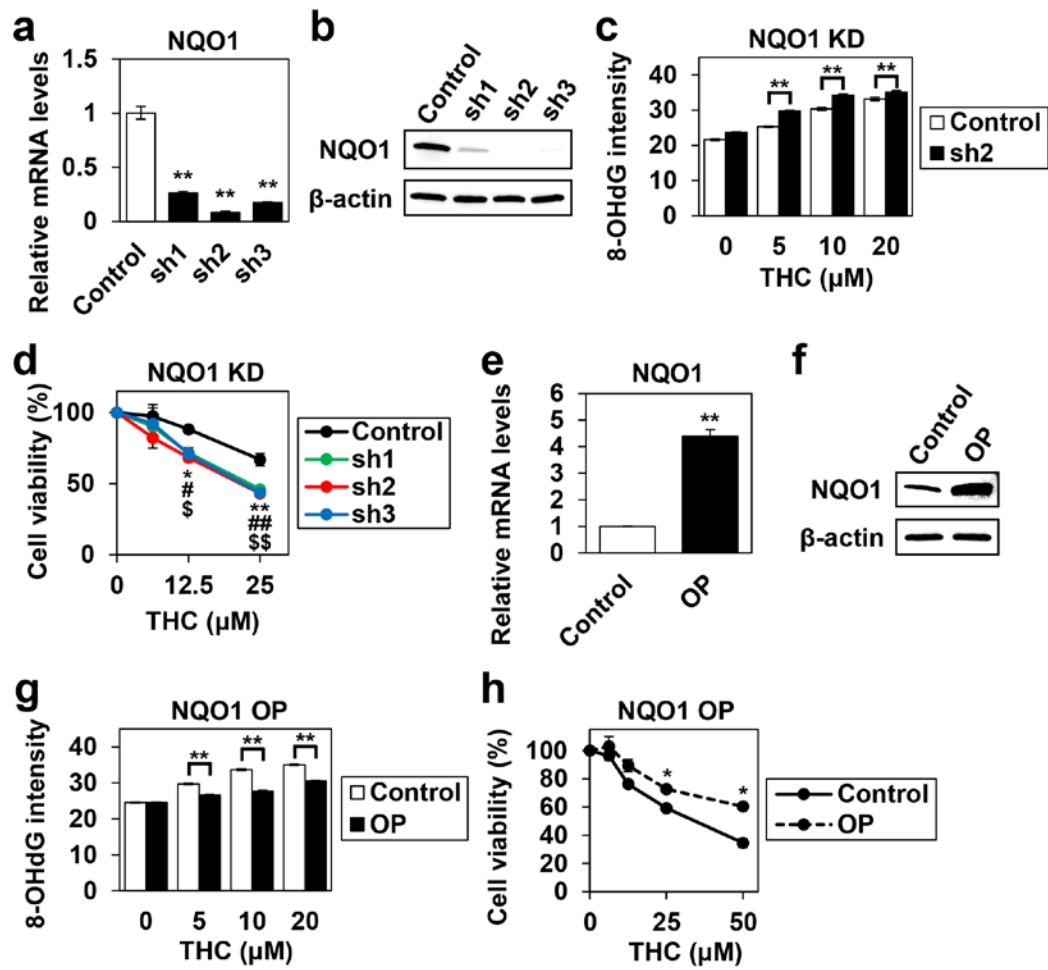


Figure. 6

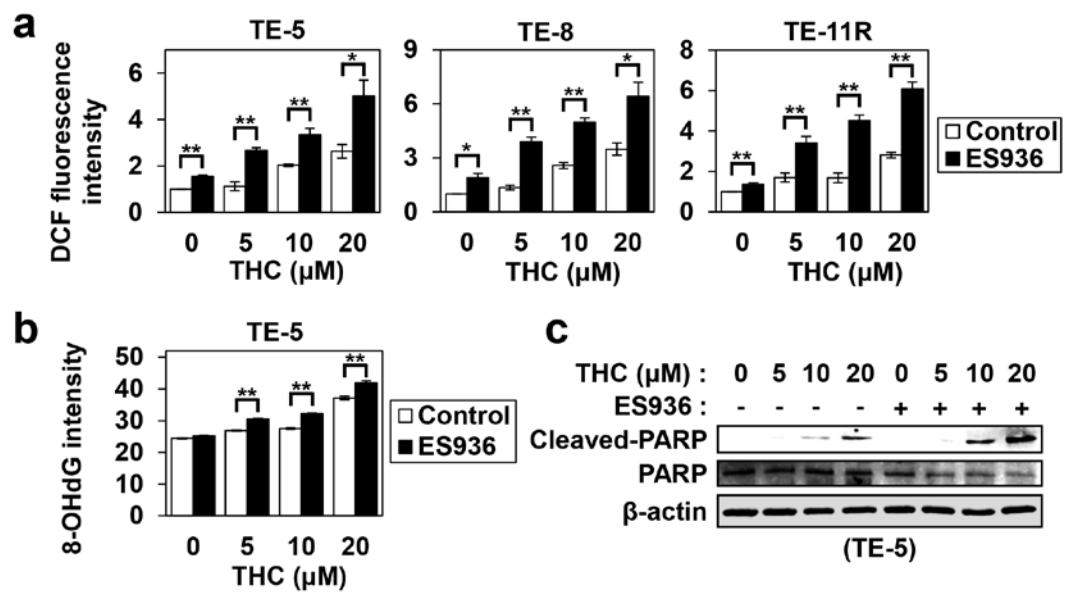


Figure. 7

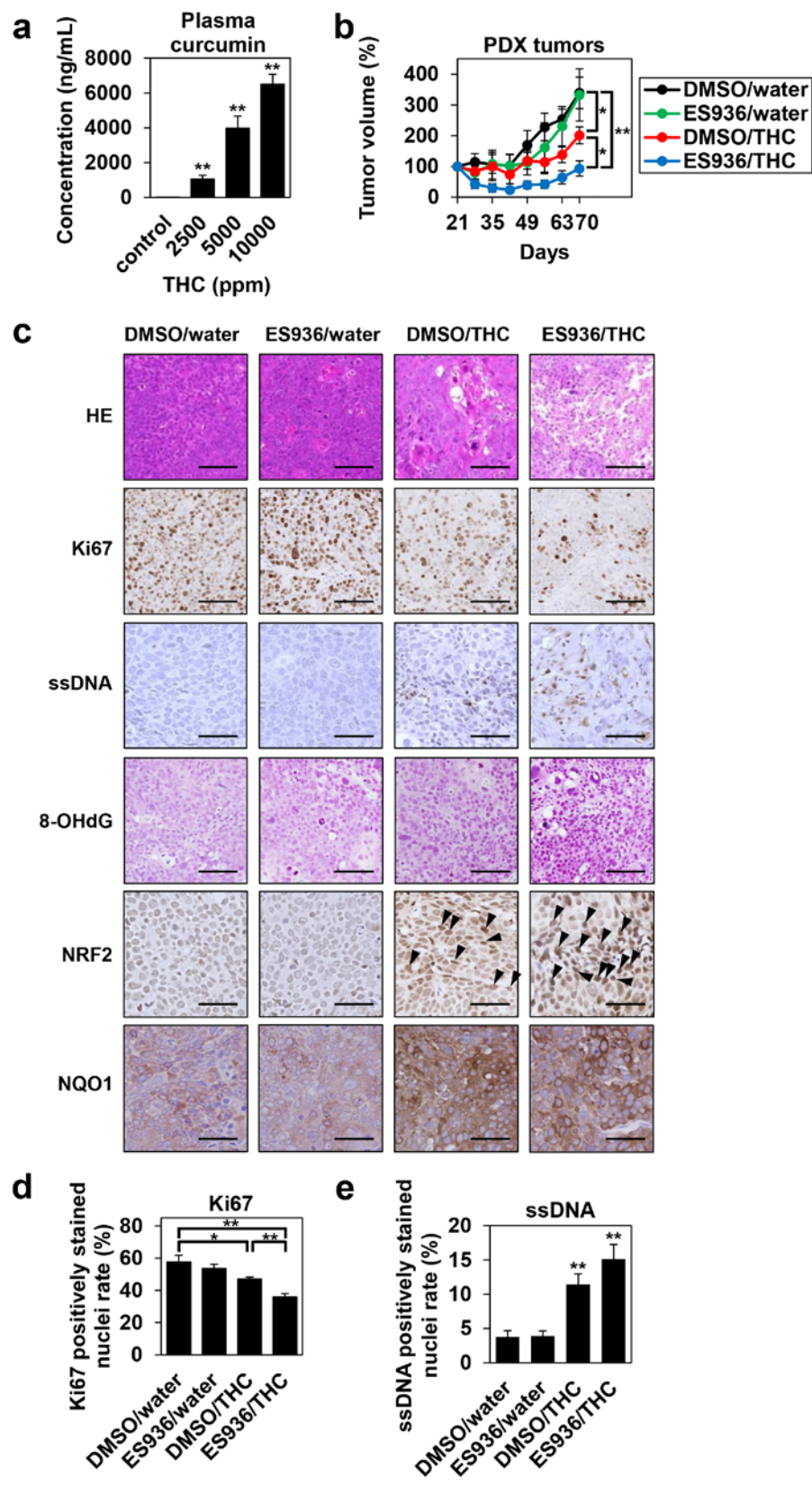


Figure legends

Figure. 1

Cytotoxic effects of Theracurmin® on ESCC cells. (a) Cell viability: ESCC cells were cultured with the indicated concentrations (0–50 μ M) of Theracurmin® (THC) for 96 h. Cell viability was measured using the WST-1 assay. Viability of each cell type treated with THC relative to that of untreated cells is indicated. (* P < 0.05, ** P < 0.01, *vs* untreated cells; n = 3). (b) Caspase 3/7 activity: ESCC cells were cultured with the indicated concentrations (0–50 μ M) of THC for 12 h (TE-5) or 24 h (TE-8 and TE-11R). Caspase 3/7 activity was measured using the Caspase-Glo 3/7 Assay. Relative Caspase 3/7 activity of each cell type treated with the indicated concentrations of THC relative to that of untreated cells is indicated. (* P < 0.05, ** P < 0.01, *vs* untreated cells; n = 3) (c) Assessment of apoptosis: ESCC cells were cultured with the indicated concentrations (0–50 μ M) of THC for 24 h. The cleavage of PARP was investigated by western blotting. β -actin served as a loading control. (d) Spheroid assay: Spheres were generated by TE-8 and TE-11R cells treated with the indicated concentrations (0–50 μ M) of THC for 2 weeks. Spheroid formation level was measured using the CellTiter-Glo 3D Cell Viability Assay. Spheroid formation by each cell type treated with THC relative to that of untreated cells is indicated. (** P < 0.01, *vs* untreated cells; n = 3) (e) Colony formation assay: Colonies were generated by TE-8 and TE-11R cells with the indicated concentrations (0–50 μ M) of THC for 2 weeks (TE-11R) or 3 weeks (TE-8). Colony numbers per high-power field (HPF) were counted in 15 random fields to determine the mean colony-forming units for each sample. (* P < 0.05, ** P < 0.01, *vs* untreated cells)

Figure. 2

Comparison of blood curcumin concentration and antitumor effect on ESCC after ingestion of curcumin and Theracurmin® diet. (a) Plasma concentration of curcumin: C57BL/6 mice received control, curcumin (0.6 g/kg) or Theracurmin® (THC) (2 g/kg: containing 0.6 g/kg curcumin) diet for one week. Plasma concentration of curcumin was measured. (** $P < 0.01$, *vs* curcumin diet; $n = 5$) (b) Xenografted-tumor growth: TE-11R cells (1.5×10^6 cells) were injected into hairless SCID mice that received control, curcumin, or THC diet for 70 days. The tumor volume was measured with a caliper. (* $P < 0.05$, ** $P < 0.01$, n.s. (non significant, $P > 0.05$), *vs* control or curcumin diet; $n = 5$)

Figure. 3

Activation of NRF2, and induction of NMRAL2P and NQO1 in ESCC cells treated with Theracurmin®. (a) NMRAL2P expression levels: ESCC cells were cultured with Theracurmin® (THC, 10 μ M) for the indicated times. NMRAL2P expression level was measured by RT-PCR. Relative NMRAL2P RNA levels of each cell type treated with THC for the indicated time relative to that of untreated cells are indicated. β -actin served as an internal control. (** $P < 0.01$, *vs* untreated cells; $n = 3$) (b) NRF2 activation: ESCC cells were cultured with THC (TE-5: 10 μ M, TE-8 and TE-11R: 50 μ M) for 24 h. Cellular protein was divided into cytosolic fraction (Cy) and chromatin fraction (Ch). Western blotting was performed to reveal NRF2 activity. α -Tubulin or histone H1 served as a loading control for cytosolic or chromatin extracts, respectively. (c) NQO1 mRNA levels: ESCC cells were cultured with THC (10 μ M) for

the indicated times. NQO1 mRNA level was measured by RT-PCR. Relative NQO1 mRNA levels of each cell type treated with THC for the indicated time relative to that of untreated cells are indicated. β -actin served as an internal control. (* P < 0.05, ** P < 0.01, *vs* untreated cells; $n = 3$) (d) NQO1 protein levels: ESCC cells were cultured with THC (TE-5: 10 μ M, TE-8 and TE-11R: 50 μ M) for the indicated times. NQO1 protein level was measured by western blotting. β -actin served as a loading control.

Figure. 4

Theracurmin®-mediated oxidative damage in ESCC cells. (a) ROS production: ESCC cells were cultured with the indicated concentrations (0–20 μ M) of Theracurmin® (THC) for 1 h. Intracellular ROS levels were measured using the DCF assay. Relative DCF fluorescence intensity of each cell treated with the indicated concentrations of THC relative to that of untreated cells is indicated. (* P < 0.05, ** P < 0.01, *vs* untreated cells; $n = 3$) (b) Oxidative DNA damage: ESCC cells were cultured with the indicated concentrations (0–20 μ M) of THC for 24 h. Oxidative DNA damage was measured by the intensity of immunofluorescent staining of 8-OHdG. (** P < 0.01, *vs* untreated cells; $n = 3$) (c) Mitochondrial ROS levels: ESCC cells were cultured with the indicated concentrations (0–20 μ M) of THC for 1 h. Mitochondrial ROS level was measured. Relative fluorescence intensity of each cell type treated with the indicated concentrations of THC relative to that of untreated cells is indicated. (** P < 0.01, *vs* untreated cells; $n = 3$) (d) Metabolite analysis: TE-5 cells were cultured with THC (10 μ M) for 8 h. The amounts of cis-aconitic acid, isocitric acid, and 2-OG were

measured. (* $P < 0.05$, ** $P < 0.01$, *vs* untreated cells; $n = 3$).

Figure. 5

The role of NQO1 in Theracurmin®-treated ESCC cells. (a) NQO1 mRNA levels in *NQO1* knockdown cells: RT-PCR showed efficient *NQO1* knockdown using shRNA in TE-11R cells. Relative NQO1 mRNA level of each cell type relative to that of control cells is indicated. β -actin served as an internal control. (** $P < 0.01$, *vs* the control cells; $n = 3$) (b) NQO1 protein levels in *NQO1* knockdown cells: Western blotting showed efficient *NQO1* knockdown using shRNA in TE-11R cells. β -actin served as a loading control. (c) Oxidative DNA damage in *NQO1* knockdown cells: *NQO1* knockdown cells were cultured with the indicated concentrations (0–20 μ M) of THC for 24 h. Oxidative DNA damage was measured by the intensity of immunofluorescent staining for 8-OHdG. (** $P < 0.01$, *vs* untreated cells) (d) Cell viability in *NQO1* knockdown cells: *NQO1* knockdown cells were cultured with the indicated concentrations of THC (0–25 μ M) for 96 h. Cell viability was measured using the WST-1 assay. Viability of each cell type treated with the indicated concentrations of THC relative to that of untreated cells is indicated. (sh1, * $P < 0.05$, ** $P < 0.01$; sh2, # $P < 0.05$, ## $P < 0.01$; sh3, \$ $P < 0.05$, \$\$ $P < 0.01$; *vs* untreated control cells; $n = 3$) (e) NQO1 mRNA levels in NQO1-overexpressing cells: RT-PCR showed efficient NQO1 overexpression in TE-11R cells. NQO1 mRNA level of each cell type relative to that of control cells is indicated. β -actin served as an internal control. (** $P < 0.01$, *vs* control cells; $n = 3$) (f) NQO1 protein levels in NQO1-overexpressing cells: Western blotting showed efficient NQO1 overexpression in TE-11R cells. β -

actin served as a loading control. (g) Oxidative DNA damage in NQO1-overexpressing cells: NQO1-overexpressing cells were cultured with the indicated concentrations (0–20 μ M) of THC for 24 h. Oxidative DNA damage was measured by the intensity of immunofluorescent staining for 8-OHdG. (** $P < 0.01$, *vs* untreated cells) (h) Cell viability in NQO1-overexpressing cells: NQO1-overexpressing cells were cultured with the indicated concentrations of THC (0–50 μ M) for 96 h. Cell viability was measured using the WST-1 assay. Viability of each cell type treated with the indicated concentrations of THC relative to that of untreated cells is indicated. (* $P < 0.05$, *vs* untreated control cells; $n = 3$) sh1-3: *NQO1* knockdown cells using shRNA 1-3, OP: NQO1-overexpressing cells.

Figure. 6

Combination effects of Theracurmin[®] and NQO1 inhibitor *in vitro*. (a) ROS production in ESCC cells treated with both Theracurmin[®] (THC) and NQO1 inhibitor: ESCC cells were cultured with the indicated concentrations (0–20 μ M) of THC and ES936 (50 nM) for 1 h. ROS level was measured using the DCF assay. DCF fluorescence intensity of each cell type treated with THC and ES936 relative to that of untreated cells is indicated. (* $P < 0.05$, ** $P < 0.01$, *vs* the cells untreated with ES936; $n = 3$) (b) Oxidative DNA damage in TE-5 cells treated with both THC and NQO1 inhibitor: ESCC cells were cultured with the indicated concentrations (0–20 μ M) of THC and ES936 (50 nM) for 24 h. Oxidative DNA damage was measured by the intensity of immunofluorescent staining for 8-OHdG. (** $P < 0.01$, *vs* the cells untreated with ES936; $n = \text{approx. } 350$) (c) Assessment of apoptosis: TE-5 cells were

cultured with the indicated concentrations (0–20 μM) of THC and ES936 (50 nM) for 24 h. The cleavage of PARP was investigated by western blotting. β -actin served as a loading control.

Figure. 7

Antitumor effect of Theracurmin® and NQO1 inhibitor against ESCC PDX tumors.

(a) Plasma concentration of curcumin after ingesting Theracurmin® (THC)-containing water: C57BL/6 mice received water containing 2500, 5000, or 10000 ppm THC for two days. Plasma concentration of curcumin was measured. (** $P < 0.01$, *vs* control; $n = 5$) (b) PDX tumor growth: Growth kinetics of subcutaneous ESCC PDX tumors treated with or without THC and/or NQO1 inhibitor. (* $P < 0.05$, ** $P < 0.01$, *vs* DMSO/water or DMSO/THC; $n = 5$) (c) Hematoxylin and eosin (HE) and immunohistochemical staining: HE and immunohistochemical staining for Ki67, ssDNA, 8-OHdG, NRF2, and NQO1 in ESCC PDX tumors treated with or without THC and/or NQO1 inhibitor. Scale bar = 100 μm (HE, Ki67, and 8-OHdG) and 50 μm (ssDNA, NRF2, and NQO1). Arrowheads indicate nuclear NRF2 positive cells. (d) Ki67 positively stained nuclei rate: Ki67 positively stained nuclei rate were counted in 6 random fields. (* $P < 0.05$, ** $P < 0.01$, *vs* DMSO/water or DMSO/THC). (e) ssDNA positively stained nuclei rate: ssDNA positively stained nuclei rate were counted in 6 random fields. (* $P < 0.05$, ** $P < 0.01$, *vs* DMSO/water).

Supplementary materials and methods

Cell lines and cell culture

We used human ESCC cell lines TE-1, TE-5, TE-6, TE-8, TE-10, and TE-11, which were obtained from Riken BioResource Center (Ibaraki, Japan), T.Tn, which was purchased from JCRB (Osaka, Japan), and HCE-4, which was provided by the Cell Culture Core of the University of Pennsylvania. TE-11R is 5-fluorouracil (FU)-resistant ESCC cells that have been described previously [1, 2]. The characteristics of these cells are summarized in **Supplementary Table 2** [3, 4]. HEK293T, a cell line derived from human embryonic kidney cells, was used to produce lentivirus, and was purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Two independent immortalized normal human esophageal keratinocyte cell lines, EPC1-hTERT and EPC2-hTERT, were obtained from the Cell Culture Core of the Center for Molecular Studies in Digestive and Liver Diseases at the University of Pennsylvania [5, 6]. ESCC cells except HCE-4 were cultured in RPMI1640 medium (Life Technologies Corp., Grand Island, NY, USA). HCE-4 and HEK293T cells were cultured in DMEM medium (Life Technologies). These media were supplemented with 10% fetal bovine serum (Life Technologies), 100 µg/mL of streptomycin and 100 units/mL of penicillin (Life Technologies). EPC1-hTERT and EPC2-hTERT cells were cultured in Keratinocyte Serum-Free medium (Life Technologies), supplemented with 40 µg/mL of Bovine Pituitary Extract, 1 ng/mL Epidermal Growth Factor, 100 µg/mL of streptomycin, and 100 units/mL of penicillin (Life Technologies). All cells were cultured at 37 °C in a 5% CO₂ incubator.

WST-1 cell viability assay

Cell viability was determined using the WST-1 assay (Roche Applied Science, Upper Bavaria, Germany) according to the manufacturer's instructions. Cells ($1-5 \times 10^3$ cells/well) were seeded in 96-well plates and exposed to the indicated concentrations (0–50 μM) of THC (Theravalues Corporation, Tokyo, Japan) or curcumin (Nacalai tesque, Inc., Kyoto, Japan) for 96 h. All data were obtained with a multiwell plate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland) at wavelengths of 450 nm and 630 nm.

Caspase-Glo® 3/7 assay

The caspase-3 and caspase-7 activities of ESCC cells were evaluated using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells (2×10^3 cells/well) were plated in 96-well plates. After 12-h (TE-5) or 24-h (TE-8 and TE-11R) treatment with THC (0–50 μM), the Caspase-Glo® 3/7 reagent was added to each well, the plates were incubated for 45 min at 37 °C, then the luminescence was measured using a plate-reading luminometer (GloMax®-Multi Detection System, Promega).

Spheroid assay

Cells (100 cells/well) were seeded in 96-well ultra-low attachment plates (Corning, New York, NY, USA) in MEGM™ Mammary Epithelial Cell Growth Medium (Lonza, Basal, Switzerland) and cultured with the indicated concentrations (0–50 μM) of THC for 2 weeks. The CellTiter-Glo® 3D Cell Viability reagent (Promega) was added to each well, and the plates were incubated for 30 min at room temperature, then the luminescence was measured using a plate-reading

luminometer (GloMax®-Multi Detection System).

Soft agar colony formation assay

Anchorage-independent growth activities were determined using a soft agar colony formation assay. This assay was performed in 6-well plates with a base of 1.5 mL of medium containing 1% NuSieve™ GTG™ Agarose (Lonza). Cells (2.5×10^4 cells/well) were seeded in 1.5 mL of medium containing 0.67% agar and layered onto the base, then 2 mL of medium was added on top of the agar gel. Colonies were stained with 0.02% Giemsa Stain Solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) at day 14 (TE-11R) or day 21 (TE-8) and 15 photographs of colonies were taken for each density using a Keyence BIOREVO BZ-9000 microscope (Keyence Corp., Osaka, Japan). Colonies larger than 50 μm were counted in the phase-contrast images.

Microarray hybridization

Cells (5×10^5 cells) were treated with 10 μM THC for 4 h, 8 h, or 24 h. Isolated RNA from each group was reverse transcribed, amplified, and labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Quantification of total RNA was performed using a NanoDrop® 2000 (ThermoFisher Scientific, Grand Island, NY, USA) and the quality of the total RNA was assessed using TapeStation 2200 (Agilent Technologies). Quantification and specific activity of labeled cRNA were determined using the NanoDrop® 2000 (ThermoFisher Scientific). A total of 600 ng of cRNA was then applied to the SurePrint G3 Human Gene Expression v2 8×60 K Microarray Kit (#G4858A-039494, Agilent Technologies) (reference sequence genes targeted: 24588) as per the manufacturer's

instructions and hybridized in a rotating oven for 17 h at 65 °C. Arrays were washed and then scanned using a Microarray Scanner G2565CA (Agilent Technologies). Feature extraction software was utilized to extract gene expression data (Agilent Technologies). We used GPL16699 platform (Agilent-039494 SurePrintG3 Human GE v2 8 × 60K Microarray 039381, Feature Number version). The signal values were corrected using background signal and processed quantile normalization and log 2 transformation. After the filtering with the quality of signal, differential expression genes (DEGs) were detected using limma [7]. The comparisons were made between ESCC cells (TE5/TE8 without THC treatment, and TE5/TE8 after 4 h, 8 h and 24 h THC treatment) to investigate the differences of gene expression pattern after THC treatment. The detected DEGs for each comparison were filtered to those having a false discovery rate (FDR) < 0.05 and log2 fold changes > 2.

Real-time reverse transcription–PCR

RNA extraction, cDNA synthesis, and real-time reverse transcription–polymerase chain reaction (RT–PCR) was performed as described previously [4]. The relative expression of each mRNA was normalized to β -actin as an internal control. The primers used in this study were as follows: *KEAPI*: forward 5'-GGCCAAGCAAGAGGAGTTCT-3'; reverse 5'-GGGGTTGTAACAGTCCAGGG-3'; *NRF2*: forward 5'-GCTTGAGGCTCATCTCACA-3'; reverse 5'-TGCCATCTCTTGTTTGCTGC-3'; *NMRAL2P*: forward 5'-CTGGAGAACGTCAAGCGACT-3'; reverse 5'-GGGATGCCAATGGACCAGAA-3'; *NQO1*: forward 5'-AAAGGACCCTTCCGGAGTAA-3'; reverse 5'-AGGCTGCTTGAGCAAAATA-3'; *ACTB*: forward 5'-

TTGTTACAGGAAGTCCCTTGCC-3'; reverse 5'-ATGCTATCACCTCCCCTGTGTG-3'.

Western blotting

Whole cell lysates were collected and western blotting was performed as described previously [4]. Primary antibodies and titers were as follows: rabbit polyclonal anti-PARP antibody (#9542, Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1000), rabbit monoclonal anti-NRF2 antibody (#ab62352, Abcam Inc., Cambridge, MA, USA; 1:1000), mouse monoclonal anti-NQO1 antibody (A180, #3187, Cell Signaling; 1:1000), rabbit polyclonal anti- α -tubulin antibody (#ab4074, Abcam; 1:400), rabbit polyclonal anti-histone H1 antibody (FL-219, #sc-10806, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:200), and rabbit monoclonal anti- β -actin antibody (13E5, #4970, Cell Signaling; 1:2000). The secondary antibodies and titers were HRP-linked whole Ab donkey anti-rabbit IgG, (#NA-934, GE Healthcare, Little Chalfont, UK; 1:2000) and HRP-linked whole Ab sheep anti-mouse IgG, (#NA-931, GE Healthcare; 1:2000).

Chromatin protein isolation

Chromatin protein isolation was performed as previously described [8]. Cells were treated with 10 μ M THC for 24 h or siRNA for 48 h and resuspended (2×10^6 cells/mL) in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 1 mM PMSF). Triton X-100 (0.1%) was added and samples centrifuged at 3500 *g*, 4 °C for 5 min. This supernatant was defined as the cytosolic fraction. The pellet was washed once in buffer A, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM PMSF). Insoluble chromatin was

collected by centrifugation at 3500 *g*, 4 °C for 5 min, washed once in buffer B, and centrifuged again under the same conditions. The final chromatin pellet was resuspended in 2 × SDS–PAGE sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 10% 2-mercaptoethanol) and was defined as the chromatin fraction. To perform western blotting, the cytosolic fraction was mixed with an equal volume of 2 × SDS–PAGE sample buffer.

siRNA transfection

Gene-specific siRNAs or a MISSION® siRNA Universal Negative Control (Sigma–Aldrich, St. Louis, MO, USA) were transfected into cells using Lipofectamine® RNAiMax transfection reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The sequences of the siRNAs used in this study were as follows: *KEAP1*: GUGUUACGACCCAGAUACA; *NRF2*: CAAACAGAAUGGUCCUAAA; *NMRAL2P*: GCCAAUAUGUGUCGUUUCU.

Measurement of intracellular ROS levels

The generation of intracellular reactive oxygen species was measured using an OxiSelect™ Intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was added to cells 1 h after treatment with THC (0–20 μM), curcumin(0–20 μM) and/or ES936 (50 nM), and intracellular ROS levels were quantified by detecting oxidized fluorescent 2',7'-dichlorodihydrofluorescein (DCF) using a multiwell fluorometric plate reader (Infinite 200 Pro) at 485/535 nm.

Measurement of mitochondrial ROS levels

The generation of mitochondrial ROS was measured using an OxiORANGE™ Mitochondrial ROS assay kit (GORYO Chemical, Inc., Sapporo, Japan). An oxidation-sensitive probe was added, and then cells were treated with THC (0–20 μ M) for 1 h. Mitochondrial ROS levels were quantified by detecting oxidized fluorescent probe using a multiwell fluorometric plate reader (Infinite 200 Pro) at 535/590 nm.

Immunofluorescent staining for 8-OHdG

DNA oxidation was assessed by 8-OHdG [9]. The cells were fixed in 4% paraformaldehyde and treated with RNase (100 mg/mL) in Tris buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 0.4 M NaCl) at 37 °C for 1 h. After rinsing with phosphate-buffered saline (PBS), cells were treated with proteinase K (10 mg/mL) at room temperature for 7 min. After another rinse with PBS, DNA was denatured by treatment with 4 N HCl for 7 min at room temperature. The pH was adjusted with 50 mM Tris-HCl for 5 min at room temperature. Cells were rinsed with 0.2% Triton X and 0.5% goat serum in PBS, and stained with a monoclonal anti-8-OHdG antibody (15A3, #sc-66036, Santa Cruz; 1:50). Alexa Fluor 594-conjugated goat anti-mouse IgG (#8890, Cell Signaling; 1:500) was used as the secondary antibody. The coverslips were mounted with Vectashield mounting solution including DAPI (Vector Labs, CA, USA). Fluorescence images were captured and the fluorescent intensity of 8-OHdG from approximately 350 cells was measured using a Keyence BIOREVO BZ-9000 microscope (Keyence Corp., Osaka, Japan).

Cell cycle assay

Cells (2.5×10^4 cells/well) were seeded in 24-well plates and exposed to the indicated concentrations (0–50 μM) of THC for 24 h. The Cell-Clock Cell Cycle Assay kit (Biocolor Life Science Assays, Antrim, UK) was used to stain cells, which were scored by counting at least 100 cells high-power field ($n = 6$) under light microscopy.

Senescence-associated β -galactosidase (SABG) assay

Cells (1×10^5 cells/well) were seeded in 6-well plates and exposed to the indicated concentrations (0–50 μM) of THC for 24 h. The Senescence β -Galactosidase Staining kit (Cell Signaling) was used to stain senescent cells, which were scored by counting at least 100 cells high-power field ($n = 6$) under light microscopy.

Metabolite analysis

Metabolite analysis was performed using C-SCOPE (Human Metabolome Technologies, Yamagata, Japan), according to the recommended protocol [10]. Cells (5×10^5 cells) were treated with 10 μM THC for 8 h, washed twice with 5% mannitol solution, and treated with methanol and 4 μM internal standard. After centrifugation at 2300 g , 4 $^{\circ}\text{C}$ for 5 min, the supernatants were collected for centrifugal filtration through a 5-kDa-cutoff filter at 9100 g , 4 $^{\circ}\text{C}$ for 3 h. Concentrations of all charged compounds were measured by capillary electrophoresis time-of-flight mass spectrometry and capillary electrophoresis tandem mass spectrometry as described previously [10–12].

Vectors and viral infections

The following lentiviral vectors were used in this study: GIPZ nonsilencing

Lentiviral shRNA control (control for *NQO1* knockdown), V3LHS_331229 (shNQO1-1), V3LHS_331230 (shNQO1-2), V3LHS_331231 (shNQO1-3), LentiORF control (control for *NQO1* overexpression), and PLOHS_100005522 (*NQO1* overexpression) (GE Healthcare Dharmacon, Lafayette, CO). They were transfected into HEK293T to produce viral particles using the Trans-Lentiviral shRNA Packaging Kit (GE Healthcare Dharmacon). TE-11R cells were incubated with RPMI1640 medium containing these viral particles and 10 µg/mL hexamethrine bromide, then, centrifuged at 1800 rpm, 32 °C for 1 h. After centrifugation, the medium was replaced with fresh RPMI1640 medium containing viral particles and 10 µg/mL hexamethrine bromide, and centrifuged again under the same conditions. After centrifugation, the medium was changed to normal RPMI1640. 1.5 µg/mL puromycin (knockdown) or 20 µg/mL Blasticidin S (overexpression) were used for cell selection.

Immunohistochemical staining

Mice were painlessly sacrificed under appropriate anaesthesia with the inhalation of isoflurane (Escain, Mylan Pharmaceuticals, Tokyo, Japan) and cervical dislocation, and tissue samples were collected in 10% formaldehyde phosphate buffer solution (Wako Pure Chemical Industries, Ltd.). Incubation and washing procedures were carried out at room temperature. After deparaffinization and antigen retrieval, endogenous peroxidase activity was blocked by 0.3% H₂O₂ in methyl alcohol for 30 min (Ki67, ssDNA, NRF2 and NQO1). The glass slides were washed in PBS (6 times, 5 each min) and mounted with 1% horse normal serum in PBS for 30 min. Subsequently primary antibody was applied overnight at 4 °C. They were incubated with biotinylated horse anti-mouse serum (second antibody) diluted to 1:300 in PBS

for 40 min, followed by washes in PBS (6 times, 5 min). Avidin-biotin-peroxidase complex (Ki67, ssDNA, NRF2 and NQO1) or avidin-biotin-alkaliphosphatase complex (8-OHdG) (Vector Laboratories, Burlingame, CA) at a dilution of 1:100 in BSA was applied for 50 min. After washing in PBS (6 times, 5 min), coloring reaction was carried out with DAB (Ki67, ssDNA, NRF2 and NQO1) or Fast red (8-OHdG), and nuclei were counterstained with hematoxylin.

Primary antibodies and titers were as follows: mouse anti-Ki67 antibody (#NCL-L-Ki67-MM1, Leica Biosystems, Nussloch, Germany; 1:100), rabbit anti-single stranded DNA antibody (#18731, Immuno-Biological Laboratories Co., Ltd., IBL, Tochigi, Japan; 1:600), rabbit polyclonal anti-NRF2 antibody (#GTX103322, GeneTex, LA, USA; 1:200), goat polyclonal anti-NQO1 antibody (#ab2346, Abcam; 1:200), and mouse anti-8-OHdG antibody (N45.1, #MOG-020P, Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd, JalCA, Shizuoka, Japan; 10 µg/ml).

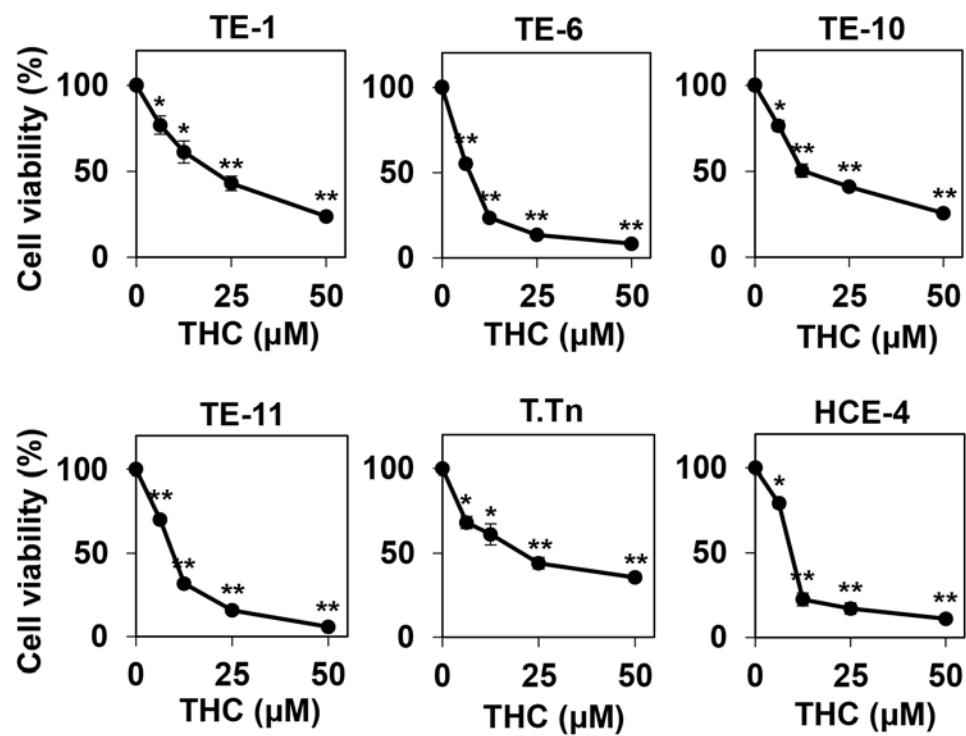
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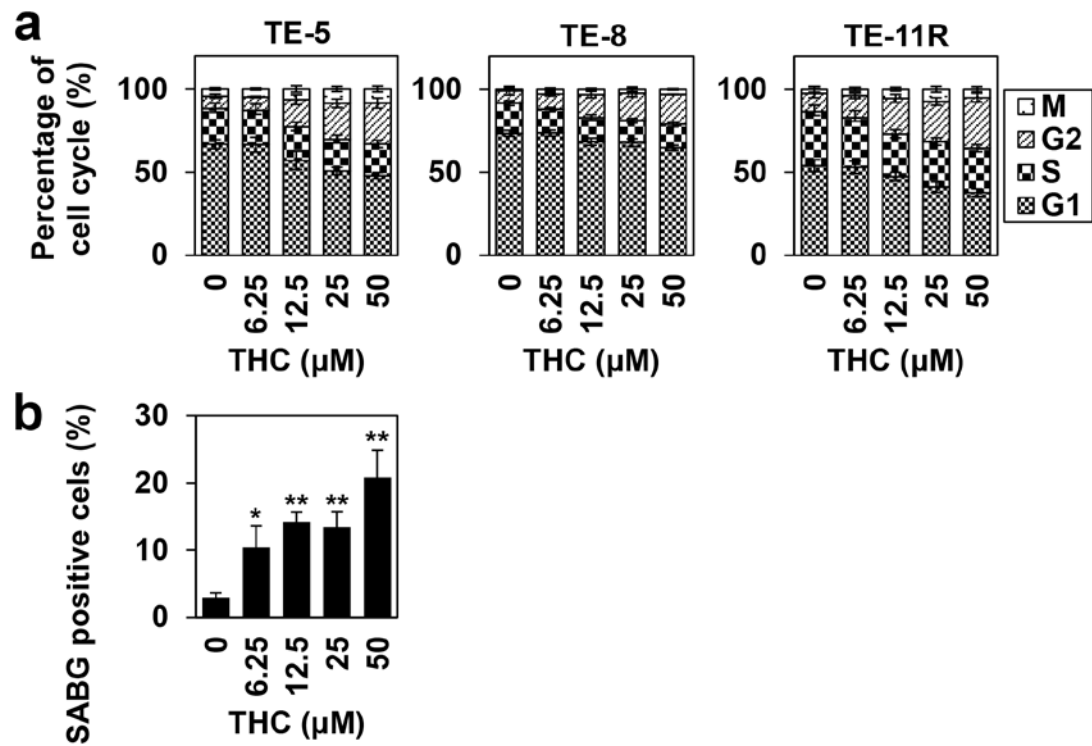
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Supplementary Figure. 1



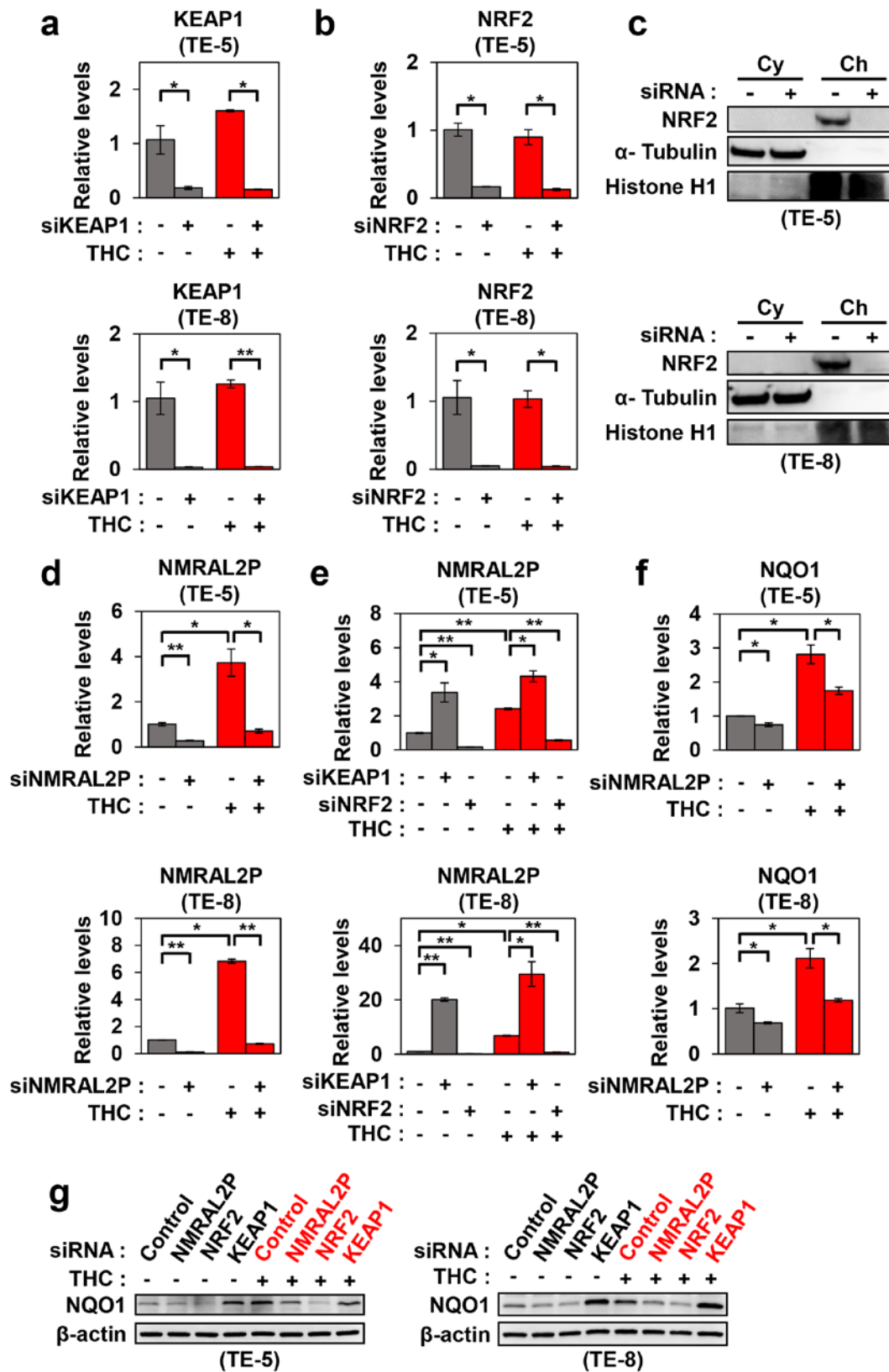
Supplementary Figure. 2



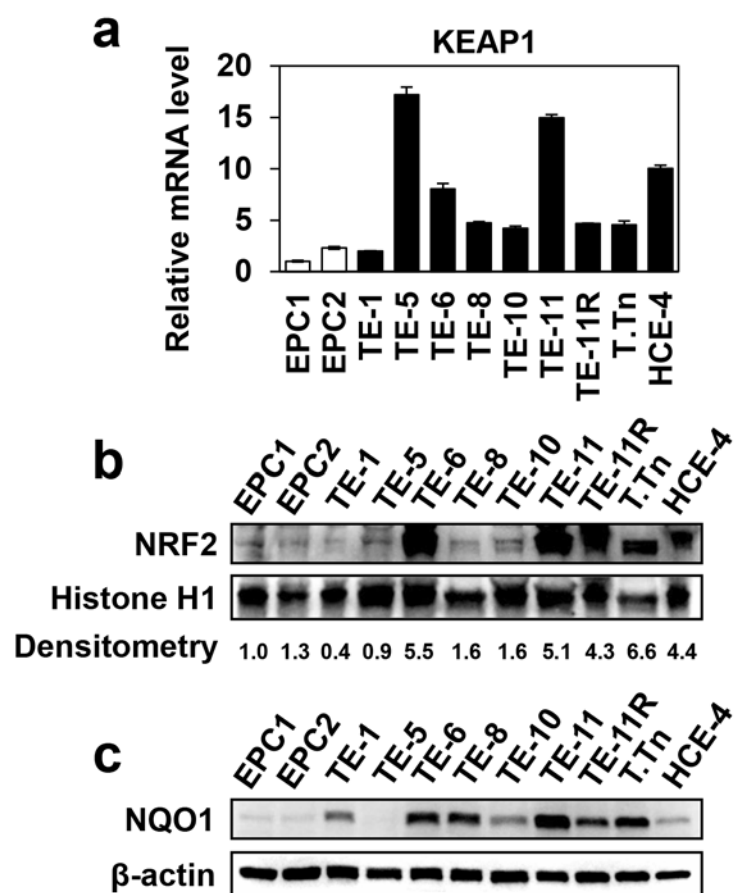
Supplementary Figure. 3

TE-5 \ TE-8	4 h	8 h	24 h
4 h	-	NMRAL2P	NMRAL2P
8 h	-	NMRAL2P	NMRAL2P AKR1C1 AKR1C8P
24 h	-	NMRAL2P	NMRAL2P AKR1C1 AKR1C8P

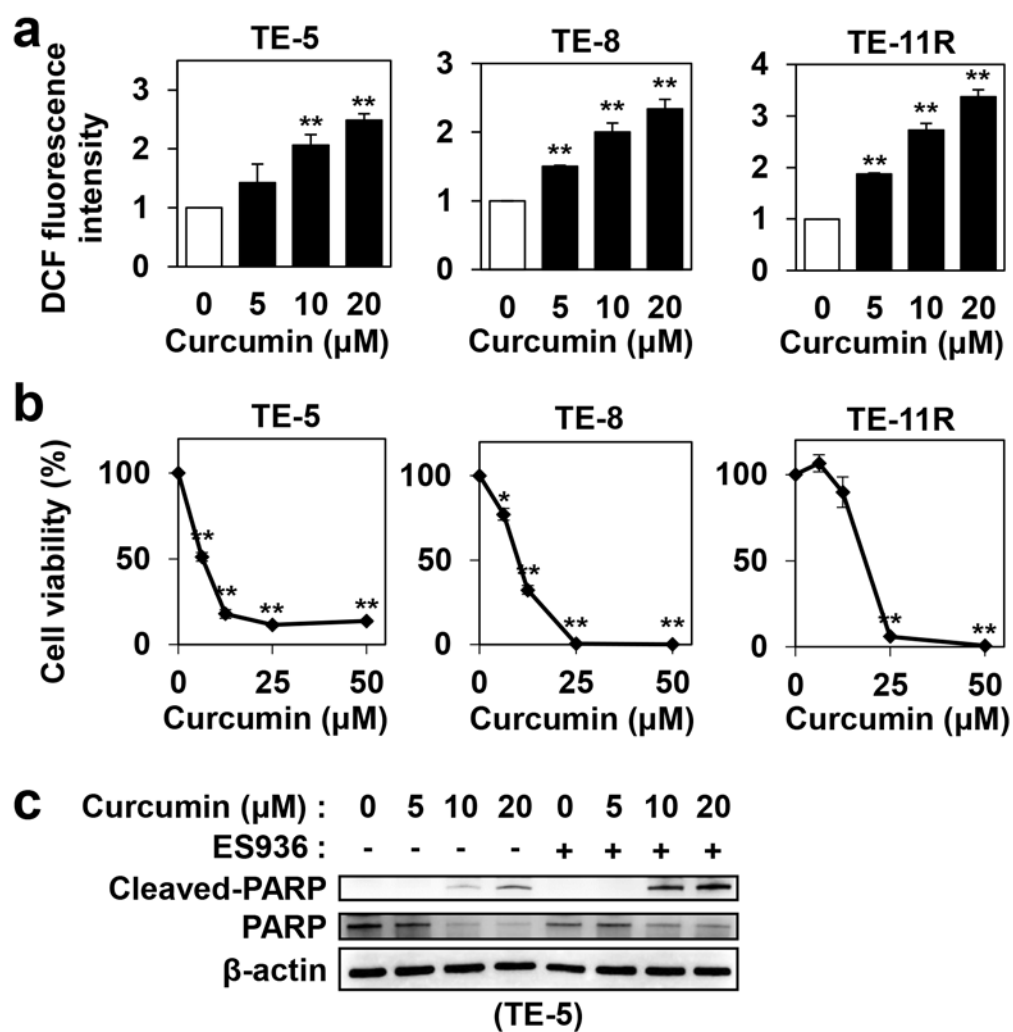
Supplementary Figure. 4



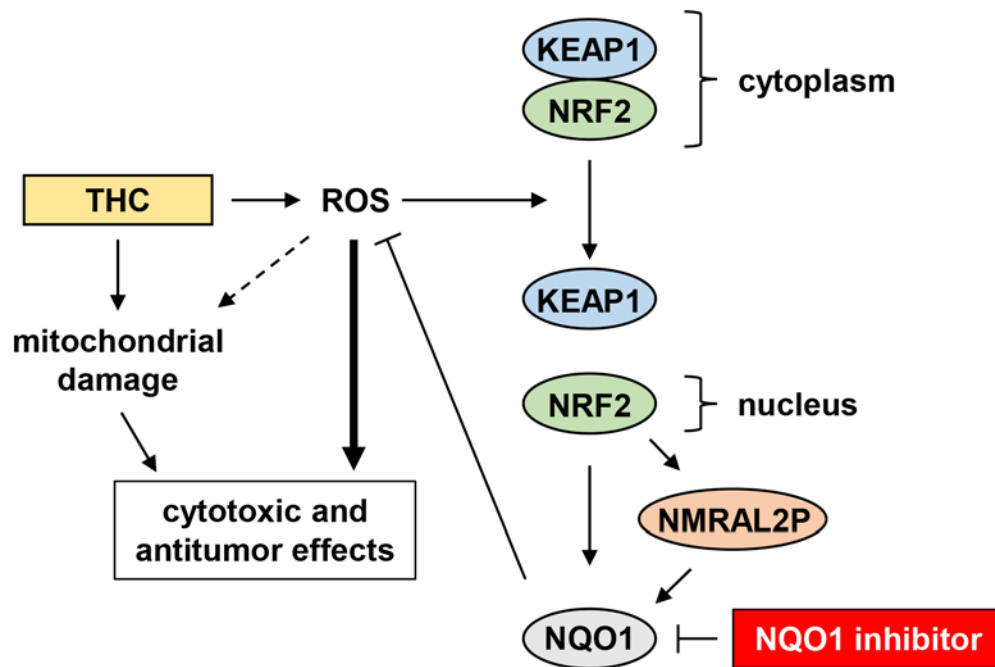
Supplementary Figure. 5



Supplementary Figure. 6



Supplementary Figure. 7



Supplementary Figure legends

Supplementary Figure. 1

Cytotoxic effects of Theracurmin® against ESCC cells. Cell viability: ESCC cells were cultured with the indicated concentrations (0–50 μ M) of Theracurmin® (THC) for 96 h. Cell viability was measured using the WST-1 assay. Viability of each cell type treated with THC relative to that of untreated cells is indicated. (* $P < 0.05$, ** $P < 0.01$, vs untreated cells; $n = 3$)

Supplementary Figure. 2

Effect of Theracurmin® on cell cycle and senescence. (a) Cell cycle: ESCC cells (TE-5, TE-8, and TE-11R cells) were cultured with the indicated concentrations (0–50 μ M) of Theracurmin® (THC) for 24 h. Cell cycle was measured using the cell-clock cell cycle assay. Cells per high-power field (HPF) were counted in 6 random fields. (b) Cell senescence: ESCC cells (TE-8 cells) were cultured with the indicated concentrations (0–50 μ M) of THC for 24 h. Cell senescence was assessed with senescence-associated β -galactosidase (SABG) assay. Cells per HPF were counted in 6 random fields. (* $P < 0.05$, ** $P < 0.01$, vs untreated cells)

Supplementary Figure. 3

Upregulation of NMRAL2P after Theracurmin® treatment. Genes upregulated in Theracurmin® (THC)-treated ESCC cells: Microarray gene expression analysis identified several genes that were upregulated in ESCC cells (TE-5 and TE-8 cells)

treated with THC (10 μ M) for 4 h, 8 h, or 24 h.

Supplementary Figure. 4

Activation of NRF2–NMRAL2P–NQO1 pathway by THC. (a) KEAP1 mRNA levels in *KEAP1* knockdown cells treated with or without Theracurmin® (THC): RT–PCR showed efficient *KEAP1* knockdown using siRNA in TE-5 and TE-8 cells. KEAP1 mRNA level of each cell type relative to that of control cells untreated with THC is indicated. β -actin served as an internal control. (* P < 0.05, ** P < 0.01, *vs* the control cells; n = 3) (b) NRF2 mRNA levels in *NRF2* knockdown cells treated with or without THC: RT–PCR showed efficient *NRF2* knockdown using siRNA in TE-5 and TE-8 cells. NRF2 mRNA level of each cell type relative to that of control cells untreated with THC is indicated. β -actin served as an internal control. (* P < 0.05, *vs* the control cells; n = 3). (c) NRF2 protein levels in *NRF2* knockdown cells: Cellular protein was divided into cytosolic fraction (Cy) and chromatin fraction (Ch). Western blotting showed efficient nuclear *NRF2* knockdown using siRNA in TE-5 and TE-8 cells. α -Tubulin or histone H1 served as a loading control for cytosolic or chromatin extracts, respectively. (d) NMRAL2P expression levels in *NMRAL2P* knockdown cells treated with or without THC: RT–PCR showed efficient *NMRAL2P* knockdown using siRNA in TE-5 and TE-8 cells. NMRAL2P level of each cell type relative to that of control cells untreated with THC is indicated. β -actin served as an internal control. (* P < 0.05, ** P < 0.01, *vs* the control cells; n = 3). (e) NMRAL2P expression levels in *KEAP1* or *NRF2* knockdown cells treated with or without THC: RT–PCR showed that siRNA-mediated knockdown of *KEAP1* and *NRF2* changed the expression level of

NMRAL2P. NMRAL2P level of each cell type relative to that of control cells untreated with THC is indicated. β -actin served as an internal control. (* $P < 0.05$, ** $P < 0.01$, *vs* the control cells; $n = 3$). (f) NQO1 mRNA levels in *NMRAL2P* knockdown cells treated with or without THC: RT-PCR showed that siRNA-mediated knockdown of *NMRAL2P* changed the mRNA levels of NQO1 in TE-5 and TE-8 cells. NQO1 mRNA level of each cell type relative to that of control cells untreated with THC is indicated. β -actin served as an internal control. (* $P < 0.05$, *vs* the control cells; $n = 3$). (g) NQO1 protein levels in *NMRAL2P*, *NRF2*, or *KEAP1* knockdown cells treated with or without THC: Western blotting showed that siRNA-mediated knockdown of *NMRAL2P*, *NRF2*, and *KEAP1* changed the protein level of NQO1 in TE-5 and TE-8 cells. β -actin served as a loading control. THC: treatment with THC (10 μ M) for 24 h.

Supplementary Figure. 5

The levels of KEAP1 mRNA, nuclear NRF2 protein, and NQO1 protein in ESCC cells under the basal-line condition. (a) KEAP1 mRNA levels: RT-PCR showed KEAP1 mRNA levels in immortalized normal esophageal epithelial cells (EPC1-hTERT and EPC2-hTERT cells) and ESCC cells (TE1, TE5, TE6, TE8, TE10, TE11, TE11R, T.Tn, and HCE4 cells) under the base-line condition. KEAP1 mRNA level of each cell type relative to that of EPC1-hTERT is indicated. β -actin served as an internal control. ($n = 3$) (b) NRF2 protein levels in chromatin fraction: Western blotting with protein of chromatin fraction showed NQO1 protein levels in immortalized normal esophageal epithelial cells and ESCC cells under the base-line condition. Histone H1

served as a loading control for chromatin extracts. Densitometry is the relative intensities of NRF2 after normalization for the levels of histone H1. (c) NQO1 protein levels: Western blotting showed NQO1 protein levels in immortalized normal esophageal epithelial cells and ESCC cells under the base-line condition. β -actin served as a loading control.

Supplementary Figure. 6

Cytotoxic effects of curcumin on ESCC cells. (a) ROS production: ESCC cells were cultured with the indicated concentrations (0–20 μ M) of curcumin for 1 h. Intracellular ROS levels were measured using the DCF assay. Relative DCF fluorescence intensity of each cell treated with the indicated concentrations of curcumin relative to that of untreated cells is indicated. (** $P < 0.01$, *vs* untreated cells; $n = 3$). (b) Cell viability: ESCC cells were cultured with the indicated concentrations (0–50 μ M) of curcumin for 96 h. Cell viability was measured using the WST-1 assay. Viability of each cell type treated with curcumin relative to that of untreated cells is indicated. (* $P < 0.05$, ** $P < 0.01$, *vs* untreated cells; $n = 3$). (c) Assessment of apoptosis: TE-5 cells were cultured with the indicated concentrations (0–20 μ M) of curcumin and ES936 (50 nM) for 24 h. The cleavage of PARP was investigated by western blotting. β -actin served as a loading control.

Supplementary Figure. 7

Working model of Theracurmin® effects on ESCC cells. Theracurmin® (THC) induces ROS and mitochondrial damage, which leads to cytotoxic and antitumor effects in

ESCC cells. Here, THC upregulates the NRF2–NMRAL2P–NQO1 pathway, which attenuates ROS production. NQO1 inhibitor can reinforce the antitumor effects of THC.

Supplementary Table 1. The IC₅₀ values of Theracurmin® on ESCC cells.

Cell line	IC ₅₀ (μM)
TE-1	19.23
TE-5	19.45
TE-6	7.03
TE-8	8.88
TE-10	12.91
TE-11	8.98
TE-11R	34.98
T. Tn	19.66
HCE-4	8.94

The IC₅₀ values of Theracurmin® on each ESCC cell line are calculated from the cell survival shown in Fig. 1a and Supplementary Fig.1.

Supplementary Table 2. The characteristics of the ESCC cells used in the current study.

Cell	Differentiation	Phenotype	5-FU resistant
TE-1	well	epithelial	no
TE-5	poor	epithelial	no
TE-6	well	epithelial	no
TE-8	moderate	mesenchymal	no
TE-10	well	epithelial	no
TE-11	moderate	epithelial	no
TE-11R	moderate	epithelial	yes
T.Tn	–	epithelial	no
HCE-4	–	mesenchymal	no

Cellular characteristics in terms of differentiation, phenotypes, and 5-FU resistance are summarized. poor: poorly differentiated, moderate: moderately differentiated, well: well differentiated, –: unknown.