Human cancer stem cells are a target for cancer prevention using (−)-epigallocatechin gallate

Hirota Fujiki1 · Eisaburo Sueoka1 · Anchalee Rawangkan2 · Masami Suganuma2

Abstract

Purpose Our previous experiments show that the main constituent of green-tea catechins, (−)-epigallocatechin gallate (EGCG), completely prevents tumor promotion on mouse skin initiated with 7,12-dimethylbenz(a)anthracene followed by okadaic acid and that EGCG and green tea extract prevent cancer development in a wide range of target organs in rodents. Therefore, we focused our attention on human cancer stem cells (CSCs) as targets of cancer prevention and treatment with EGCG.

Methods The numerous reports concerning anticancer activity of EGCG against human CSCs enriched from cancer cell lines were gathered from a search of PubMed, and we hope our review of the literatures will provide a broad selection for the effects of EGCG on various human CSCs.

Results Based on our theoretical study, we discuss the findings as follows: (1) Compared with the parental cells, human CSCs express increased levels of the stemness markers Nanog, Oct4, Sox2, CD44, CD133, as well as the EMT markers, Twist, Snail, vimentin, and also aldehyde dehydrogenase. They showed decreased levels of E-cadherin and cyclin D1. (2) EGCG inhibits the transcription and translation of genes encoding stemness markers, indicating that EGCG generally inhibits the self-renewal of CSCs. (3) EGCG inhibits the expression of the epithelial-mesenchymal transition phenotypes of human CSCs. (4) The inhibition of EGCG of the stemness of CSCs was weaker compared with parental cells. (5) The weak inhibitory activity of EGCG increased synergistically in combination with anticancer drugs.

Conclusions Green tea prevents human cancer, and the combination of EGCG and anticancer drugs confers cancer treatment with tissue-agnostic efficacy.

Keywords CSCs · EGCG · Parental cells · Self-renewal · Stemness

Abbreviations

ABC ATP-binding cassette
AFM Atomic force microscopy
ALDH Aldehyde dehydrogenase
CSCs Cancer stem cells
CRC Colorectal cancer
DMBA 7,12-Dimethylbenz(a)anthracene
EGCG (−)-Epigallocatechin gallate
EGFR Epidermal growth factor receptor
EMT Epithelial-mesenchymal transition
ER Estrogen receptor

Introduction

Cancers develop from a small subset of cancer stem cells (CSCs). A CSC, as defined by attendees of an American Association for Cancer Research Workshop on Cancer Stem Cells (Clarke et al. 2006), is “a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor”. In a simple model, cells of the epidermis are classified into two types of proliferating cells as follows: stem cells with high
proliferative potential (self-renewal) and transit amplifying cells (progenitor cells) with a low capacity for self-renewal and a high probability of undergoing terminal differentiation (Jones and Watt 1993). The role of human cancer stem cells (CSCs) is now a topic of cancer prevention, and inhibitors of CSCs are attracting much attention as potential therapeutics (Yoshida and Saya 2016).

We found that repeated applications of the green-tea component 5 mg (−)-epigallocatechin gallate (EGCG) before each treatment with 1 μg okadaic acid, a potent tumor promoter and inhibitor of protein phosphatases 1 and 2A (Fujiki and Suganuma 1993), completely prevents tumor promotion on mouse skin in two-stage carcinogenesis experiments initiated using 7,12-dimethylbenz(a)anthracene (DMBA). In contrast, tumors formed in 73.3% of mice treated with DMBA plus okadaic acid at week 20 (Yoshizawa et al. 1992; Fujiki and Okuda 1992).

Breast cancer patients who consume >5 cups of green tea per day (average eight cups) experience lower recurrence rates compared with patients who consume <4 cups daily (average three cups) (Nakachi et al. 1998). Further, drinking 10 Japanese-size cups (120 ml/cup) of green tea per day delays cancer onset by 7.3 years in female patients drinking 10 Japanese-size cups (120 ml/cup) of green tea supplemented with green tea tablets prevents the recurrence of 51.6% of colorectal adenomas (Shimizu et al. 2008).

Human CSCs differentially express stemness markers compared with their parental cells

Human CSCs enriched from primary and secondary spheroids are capable of self-renewal. It is important to study...
the quantitative differences between the levels of stemness markers between CSCs and parental cells, because CSCs redifferentiate into parental cells when cultured in parental growth conditions, and parental cell lines do not form tumorspheres (Dalla Pozza et al. 2015).

This section reviews studies of six human CSCs and their respective parental cells, which were derived from colorectal cancer (CRC), nasopharyngeal cancer, neuroblastoma, and glioblastoma (Table 1). The human CRC, spheroid-derived CSCs, designated HCT116-SDCSCs, express significantly higher levels of stem cell markers (approximately 4.5-fold and 3.2-fold for Oct4 and Nanog, respectively, compared with the parental cells) (Toden et al. 2016). Oct, Nanog, and Sox2 are transcription factors required for the maintenance of pluripotency by coordinated networks of transcription factors (Kashyap et al. 2009; Sarkar and Hochedlinger 2013; Boumahdi et al. 2014). Moreover, the expression levels of the surface marker CD44 and self-renewal markers Notch, Bmi-1, CD133, and ALDH1 are higher in HCT116-SDCSCs compared with parental cells (Table 1). Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme that catalyzes the oxidation of retinal to retinoic acid (Duester 2000) and is used to isolate stem-like breast cancer cells, which are characterized by their enhanced tumorigenicity and ability to self-renew (Ginestier et al. 2007). The results indicate that HCT116-SDCSCs comprise a higher CSC population compared with that of the parental cell population (Toden et al. 2016).

Human nasopharyngeal sphere-derived cells CSCs, designated TW01, express relatively high levels of the stem cell markers Sox2, Oct4, and KLf-4, plus EMT markers including Twist, Snail, and vimentin, along with N-cadherin, compared with parental cells. The relative increases of mRNA levels decreases in the order vimentin, Snail, Oct4, Sox2, Twist, KLf-4, and N-cadherin (Table 1). However, decreased expression of E-cadherin by TW01 sphere-derived cells was detected (Lin et al. 2012). The results indicate that TW01 sphere-derived cells express EMT phenotypes and self-renewal activity associated with invasive and migratory properties more strongly compared with those of parental cells. Similarly, the human nasopharyngeal spheroid cell lines CNE2-SCs and C666-1-SCs express increased levels of the mRNAs of the stemness markers CD44 and Bim-1, the mesenchymal markers N-cadherin and vimentin, and the transcription factor Twist compared with those of their respective parental cells (Table 1). Further, decreased levels of the epithelial marker E-cadherin are expressed by CNE2-SCs and C666-1-SCs. The expression of the CSC marker CD44 by CNE2-SCs and C666-1-SCs is significantly increased compared with that of their respective parental cells (Li et al. 2015). CD44 is an adhesion molecule that binds to osteopontin and hyaluronic acid (Goodison et al. 1999), and CD44+ cells in a tumor express high levels of nuclear Bim-1, which identifies CSCs (Prince et al. 2007). These results show that CNE2-SCs and C666-1-SCs have a stronger ability to self-renew and migrate compared with their respective parental cells, suggesting that the tumor spheroid cells are enriched with malignant phenotypes (Li et al. 2015).

Human neuroblastoma BE(2)-C spheres express increased levels of Nanog and Oct4 (approximately 50-fold and 5.0-fold, respectively) compared with the parental cells (Nishimura et al. 2012), consistent with findings for human HCT116-SDCSCs (Toden et al. 2016). The expression levels of markers of neuronal differentiation, Cu2+-transporting ATPase alpha polypeptide (ATP7A) and dickkopf homolog 2 (DKK2), are slightly decreased in BE(2)-C spheres (Table 1). These results indicate that BE(2)-C spheres are

### Table 1: Human CSCs Differentially Express Stemness Markers

| Cancers and Names of CSCs | Markers of Increased Expression | Markers of Decreased Expression | References |
|---------------------------|--------------------------------|---------------------------------|------------|
| Colorectal Cancer         |                                |                                 |            |
| HCT116-SDCSCs             | mRNAs: Oct4, Nanog,            |                                 | Toden et al. (2016) |
|                           | Proteins: CD44, Notch, Bmi-1, CD133, ALDH1 |                                 |            |
| Nasopharyngeal Cancer     |                                |                                 |            |
| TW01 sphere               | mRNAs: Sox2, Oct4, KLf-4, Twist, Snail, vimentin, N-cadherin | E-cadherin | Lin et al. (2012) |
| CNE2- & C666-1-SCs        | mRNAs: CD44, Bmi-1, N-cadherin, vimentin, Twist | E-cadherin | Li et al. (2015) |
|                           | Proteins: CD44                 |                                 |            |
| Neuroblastoma BE(2)-C sphere | mRNAs: Nanog, Oct4,             | ATP7A, DKK2                     | Nishimura et al. (2012) |
| Glioblastoma U87 GSLCs    | mRNAs: ALDH1, CD133,           | GFAP                            | Zhang et al. (2015) |
|                           | Proteins: CD133, ALDH1,        | Cyclin D1                       |            |

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enriched in tumor-initiating cells as well as CSCs that are responsible for resistance to chemotherapy and tumor recurrence (Nishimura et al. 2012).

Human glioma stem-like cells designated U87 GSLCs express increased levels of the mRNAs and proteins of the stem cell markers ALDH1 and CD133, decreased levels of the astrocyte differentiation marker glial fibrillary acidic protein (GFAP) (Pang et al. 2017) and cell cycle protein cyclin D1, compared with the parental cells U87 (Table 1) (Zhang et al. 2015). ALDH1 is a marker of normal and malignant human mammary stem cells (Ginestier et al. 2007), and GFAP is an intermediate filament protein that is expressed by numerous cell types of the central nervous system, but may represent an autoantigen candidate for type 1 diabetes mellitus (Pang et al. 2017). These results indicate that U87 GSLCs comprise neural stem cells with drug-resistant properties (Zhang et al. 2015).

EGCG inhibits the expression of mRNAs and proteins that serve as stemness markers of human CSCs

EGCG and green tea extract inhibit the growth of human cancer cell lines in culture and in rodents (Suganuma et al. 1998; Fujiki et al. 2002, 2012). Therefore, we next studied whether EGCG possesses anticancer activity against drug-resistant human CSCs. The results of studies on 20 human CSCs collected from nine different cancer tissues reveal that EGCG inhibits the expression of mRNAs and proteins that serve as stemness markers, such as Oct4 and Nanog, whereas the expression of a few markers such as Bax and caspase 8 is increased, indicating that EGCG exerts inhibitory effects on human CSCs (Table 2).

Breast CSCs: EGCG (40 μg/ml, 87.3 μM) inhibits the expression of genes that promote growth and contribute to the transformed phenotype and survival of SUM-190 spheres. In SUM-149 and SUM-190 cells, EGCG decreases the levels of mRNAs of the proliferation markers cyclin D1 (CCND1); ras homolog family member C (RHOC); and B-cell lymphoma-extra large (BCL-XL), which is a major antiapoptotic protein of the Bcl2 family, as well as decreasing ATP levels. In contrast, the levels of fibronectin 1 (FN1), E-cadherin (CDH1), and vimentin were decreased only in SUM-149 cells (Table 2). These results indicate that tumoursphere formation was inhibited by EGCG (Mineva et al. 2013).

ALDH-positive SUM-149 cells that are functionally active CSCs were implanted into the inguinal mammary fat pad of female non-obese mice with diabetic/severe-combined immunodeficiency. When palpable tumors were detected, mice were administered 0.1 ml intraperitoneal injection of 16.5 mg/kg EGCG or control PBS five times weekly for 5 weeks. Tumor weight significantly decreases by 28.6 ± 6.5% in EGCG-treated mice (Mineva et al. 2013). The phenotypes of human estrogen receptor (ER)-negative MDA-MB-231 and MDA-MB-436 cells reflect tumors with a poor prognosis. In ER-negative breast cancer cell lines, ER-α36 is overexpressed and is associated with malignant growth (Zhang et al. 2011). EGCG (10–40 μM) inhibits tumoursphere formation and down-regulates ER-α36 expression by 24 h, which is consistent with down-regulation of the epidermal growth factor receptor (EGFR). We found it interesting that the inhibitory effects of EGCG are weak in ER-α36 knockdown cell lines compared with control cells. However, EGCG inhibits the growth of ER-negative human breast CSCs through down-regulation of ER-α36 expression, indicating that EGCG treatment will predict longer survival of patients with mammary cancers (Pan et al. 2016) (Table 2). The longer survival of patients with green tea was reported by Nakachi et al. (1998), as noted in the Introduction.

Lung CSCs: EGCG (0–100 μM) reduce the mRNA levels of the lung CSC markers CD133, CD44, ALDH1A1, Nanog, and Oct4 in CSC-A549 and CSC-H1299 cells; the protein levels of proliferation markers proliferating cell nuclear antigen (PCNA) and cyclin D1 as well as that of B-cell lymphoma 2 (Bcl2). In contrast, EGCG reduces the protein levels of β-catenin and c-Myc. However, EGCG increases the levels of Bax, caspase 8, and cleaved caspases-3 and -9 (Table 2). These results show that EGCG inhibits proliferation and induces apoptosis of lung CSCs (Zhu et al. 2017). The Wnt/β-catenin pathway is implicated in self-renewal and maintenance of CSCs, and it is dysregulated in human cancers (Jang et al. 2015). EGCG (0–100 μM) inhibited the phosphorylation of glycogen synthase kinase 3β (GSK3β) at Ser 9, which significantly increases the expression of GSK3β, and decreases the expression of β-catenin and its downstream target gene c-Myc. Thus, EGCG down-regulates the activation of the Wnt/β-catenin pathway in human lung CSCs (Zhu et al. 2017; Oh et al. 2014) (Table 2).

Prostate CSCs: EGCG (0–60 μM) dose-dependently inhibits the growth of tumor spheroids and the self-renewal of prostate CSCs derived from PC-3 and LNCaP cell lines. Nanog is a key regulator of embryonic stem cell self-renewal and pluripotency (Jeter et al. 2009), and the knockdown of Nanog by a short hairpin RNA enhances the antiproliferative effects of EGCG on prostate CSCs (Tang et al. 2010). EGCG (30–60 μM) inhibits the expression of X-linked inhibitor of apoptosis protein (XIAP), Bcl2, and survivin as well as that of the EMT markers vimentin, Slug, Snail, and nuclear β-catenin. These results indicate that EGCG will serve as a useful compound that targets prostate CSCs (Tang et al. 2010) (Table 2).

Colorectal CSCs: 5-Fluorouracil (5FU)-resistant (5FUR) CRC cells exhibit an increased ability to form spheroids compared with parental cells, indicating the presence of
a larger CSC population. EGCG (50 μM) inhibits tumorspheroid formation and the expression of the mRNAs of the stem cell markers Oct4 and Nanog. The expression of self-renewal markers that are components of the Notch signaling pathway, Notch1, cleaved-Notch1, and c-Myc as well as that of the polycomb repressive complex subunits Bmi-1, polycomb protein SUZ (Suz12), and enhancer of zeste homologue (Ezh2) is inhibited in 5FUR CRCs treated with EGCG (Toden et al. 2016). EGCG (50 μM) down-regulates the expression of the ATP-binding cassette transporter genes ATP-binding cassette subfamily C member 1 (ABCC1) and ABCG2 and decreases the protein levels of never in mitosis gene related kinase 2 (Nek2), which is involved in mitotic regulation, and phosphorylated Akt, a proto-oncoprotein, in HCT116-SDCSCs (Wubetu et al. 2016).

Liver CSCs: EGCG (50 μM) down-regulates the expression of CD133, Nanog, ABC1, ABCG2 mRNAs as well as that of the Nek2 and p-Akt proteins in human hepatoma HepG2-CSCs to an extent similar to that of HCT116-SDCSCs (Wubetu et al. 2016).

Pancreatic CSCs: Human pancreatic CSCs (CD133+/CD44+/CD24+/epidermal surface antigen ESA+) represent the bulk of the pancreatic CSC population. EGCG (0–60 μM) inhibits the growth of tumorspheroids. EGCG inhibits the expression by human pancreatic CSCs of the mRNAs of the pluripotency maintaining transcription factors Nanog, c-Myc, and Oct4; the apoptosis-related proteins

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**Table 2** EGCG decreases or increases the expression of stemness marker mRNAs and proteins by human CSCs

| Cancers and names of CSCs | Inhibited expression of stemness markers (mRNAs and proteins) | References |
|--------------------------|-------------------------------------------------------------|------------|
| Breast CSCs              |                                                             |            |
| SUM-149 & SUM-190        | mRNAs: CCND1, RHOC, BCL-XL                                  | Mineva et al. (2013) |
| SUM-149                  | mRNAs: FN1, CDH1, vimentin                                   |            |
| MDA-MB-231 & MDA-MB-436  | Proteins: ER-α36, EGFR, p-ERK1/2, p-AKT                     | Pan et al. (2016) |
| Lung CSCs                |                                                             |            |
| A549 & H1299             | mRNAs: CD133, CD44, ALDH1A1, Nanog, Oct4                    | Zhu et al. (2017) |
|                         | Proteins: CD133, CD44, ALDH1A1, Nanog, Oct4, PCNA, cyclin D1, Bcl2, p-GSK3β, β-catenin, c-Myc |            |
|                         | Increased Bax, caspase 8, cleaved caspase-3 and -9           |            |
| Prostate CSCs            |                                                             |            |
| PC-3 & LNCaP             | Proteins: XIAP, Bcl2, survivin, vimentin, Slug, β-catenin    | Tang et al. (2010) |
| Colorectal CSCs          |                                                             |            |
| HCT116-5FUR & SW480-5FUR | mRNAs: Oct4, Nanog                                         | Toden et al. (2016) |
|                         | Proteins: Notch 1, cleaved-Notch 1, c-Myc, Bmi-1, Suz12, Ezh2 |            |
| HCT116-SDCSCs            | mRNAs: CD133, Nanog, ABC1, ABCG2                            | Wubetu et al. (2016) |
|                         | Proteins: Nek2, p-Akt                                      |            |
| Liver CSCs               |                                                             |            |
| HepG2-SDCSCs             | mRNAs: CD133, Nanog, ABCC1, ABCG2                           | Wubetu et al. (2016) |
|                         | Proteins: Nek2, p-Akt                                      |            |
| Pancreatic CSCs          |                                                             |            |
| Pancreatic CSCs          | mRNAs: Nanog, c-Myc, Oct4, Bcl2, survivin, XIAP, SMO, PTCH1, PTCH2, Gli1, Gli2, Snail, ZEB1, Slug | Tang et al. (2012) |
|                         | mRNA: K-ras (by green tea extract)                          | Appari et al. (2014) |
| Head and neck CSCs       |                                                             |            |
| HNSC (K3, K4, K5)        | mRNAs: Oct4, Sox2, Nanog, Notch1, Hey1, Hes1, ABC2, ABCG2   | Lee et al. (2013) |
|                         | Proteins: Oct4, Sox2, Notch1, Hey1, Hes1, ABC2, ABCG2       |            |
|                         | Increased involucrin                                        |            |
| Nasopharyngeal CSCs      |                                                             |            |
| TW01 & TW06 spheres      | mRNAs: Oct4, Klf-4, vimentin, Snail                         | Lin et al. (2012) |
|                         | Increased E-cadherin                                        |            |
| Glioma CSCs              |                                                             |            |
| U87 GSLCs                | mRNAs: ABCB1, ABCG2, MGMT                                   | Zhang et al. (2015) |
|                         | Proteins: p-Akt, CD133, ALDH1, Bcl2, P-gp                  |            |
|                         | Increased Bax, c-PARP                                        |            |
Bcl2, survivin, and X-linked inhibitor of apoptosis protein (XIAP); the Sonic hedgehog (Shh) receptors smoothed (SMO), Patched-1 (PTCH1), and Patched-2 (PTCH2); the transcription factors Gli1 and Gli2; and the EMT factors Snail, ZEB1, and Slug (Table 2). In contrast, EGCG does not inhibit the expression of Sox2 (Tang et al. 2012).

Numerous Sox factors act redundantly in the maintenance of stem cells (Sarkar and Hochedlinger 2013). The inhibition of Nanog expression by EGCG is important for cancer prevention. The human pancreatic ductal adenocarcinoma (PDA) cell line MIA-PaCa2 has characteristic CSC features. EGCG (40 μM), (−)-epicatechin gallate (ECG, 40 μM), or (−)-catechin gallate (CG, 40 μM) decrease colony-forming activity, and ECG or CG exert stronger effects compared with those of EGCG. Treatment of Mia-PaCa2, BxPc-3, and CSC-enriched PDA cells (PacaDD-183) combined with 40 μM green tea extract enhances the expression of the tumor suppressor microRNA (miR-let-7a), which suppresses the expression of genes encoding stemness markers by binding to the 3′-untranslated region of target mRNAs (Yu et al. 2008, 2016). Further, miR-let-7a inhibits the expression of K-ras (Appari et al. 2014). These results indicate that green-tea catechins inhibit colony formation.

Head and neck CSCs: Treatment of three head and neck squamous carcinoma (HNSC) CSCs (K3, K4, and K5) with EGCG (5 and 10 μM) significantly inhibit sphere formation, suggesting that EGCG effectively inhibits the self-renewal of HNSC CSCs. EGCG inhibits the expression of the stem cell markers Oct4, Sox2, and Nanog and increases the expression of the early differentiation marker involucrin. EGCG decreases the expression of the mRNAs of the ABC transporters ABCC2 and ABCG2 and ABCC2 and ABCG2 proteins, suggesting that suppressing the transcription of ABCC2 and ABCG2 transporter genes with EGCG augments cisplatin-mediated chemosensitivity. Further, EGCG inhibits the expression of mRNAs of Notch signaling-pathway target genes Notch1, Hey1, and Hes1 and the expression of the proteins Notch1, Hey1, and Hes1 by K3 CSCs (Lee et al. 2013). In contrast, the Notch pathway ligands Delta1, Jag1, and Jag2 are not affected in cochlear explant culture by EGCG (Gu et al. 2015).

Nasopharyngeal CSCs: EGCG (20–40 μM) inhibits colony formation by TW01 and TW06 sphere-derived cells. EGCG inhibits the expression of the mRNAs of the stemness markers Oct4 and Krüppel-like factor (KLF-4) but not that of the mRNAs of Sox2 and the EMT related proteins vimentin and Snail, but increases that of E-cadherin in TW01 sphere-derived cells (Lin et al. 2012). KLF-8 plays a key role in oncogenic transformation, induces the EMT, and enhances motility and invasiveness (Wang et al. 2007).

Glioma CSCs: EGCG (0–200 μM) inhibits cell viability, neurosphere formation, and the migration of human glioma stem-like cells (U87 GSLCs). Although EGCG inhibits the expression of the mRNAs of the proteins that confer drug resistance, such as P-glycoprotein (P-gp, ABCB1), ABCG2, and O-6-methylguanine-DNA methyltransferase (MGMT) in U87 GSLCs, it does not change the protein level of ABCG2. Further, EGCG down-regulates p-Akt as well as the levels of the glioma stem cell markers CD133, ALDH1, Bcl2, and P-gp. In contrast, EGCG increases the levels of Bax and cleaved poly-ADP-ribose polymerase (c-PARP) proteins in U87 GSLCs (Zhang et al. 2015).

EGCG inhibits the expression of EMT phenotypes of CSCs

Tumor progression is frequently associated with up-regulation of vimentin and transcription factors such as Snail, Twist, and Slug as well as down-regulation of E-cadherin (Polyak and Weinberg 2009; Thiery et al. 2009). Functional loss of the cell–cell adhesion molecule E-cadherin is required for the EMT, which induces migration, invasion, and stem cell features (Sobrado et al. 2009). E-cadherin repressors are classified into two groups depending on their effects on the E-cadherin promoter as follows: Inducers of the EMT and repressors of E-cadherin, such as Snail, zinc finger E-box-binding homeobox (Zeb), E2A immunoglobulin enhancer-binding factor (E47), and Klf-8 that binds to and represses the activity of the E-cadherin promoter (Peinado et al. 2007; Wang et al. 2007). In contrast, Twist, homeobox protein goosecoid (Goosecoid), basic helix-loop-helix protein (E2.2), and Forkhead box C1 (FoxC1) indirectly repress E-cadherin transcription (Yang and Weinberg 2008).

We found that EGCG (50 μM) inhibits high expression of vimentin and Slug in H1299 human non-small cell lung cancer cells at a leading edge of a scratch and that EGCG increases the stiffness (Young’s modulus) of H1299 and Lu99 cells, suggesting that EGCG inhibits EMT phenotypes through the alterations of membrane organization (Takahashi et al. 2014; Suganuma et al. 2016). The combination of EGCG and cisplatin markedly down-regulate the expression of NF-κB p65, Bmi-1, Twist1, and vimentin and up-regulates E-cadherin in human nasopharyngeal CNE2-SCs from the tumor nodules of nude mice, which is associated with the inhibition of tumorigenesis (Li et al. 2015). We found that EGCG in drinking water inhibits the formation of hematogenous and lymphogenous (spontaneous) metastases by two different B16 melanoma variants in the lungs of C56BL/6 mice (Tanguchi et al. 1992). EGCG induces a twofold increase in the stiffness of highly motile B16-F10 melanoma cells and inhibits their migration (Watanabe et al. 2012).
Inhibitory effects of EGCG on CSCs are weaker than on parental cells

The acquisition of drug resistance by cancer cells presents a formidable clinical challenge, particularly because drug resistance enables tumor recurrence. When EGCG is administered for cancer prevention, it is necessary to consider the different potencies of EGCG against CSCs and parental cells. Two investigations of nasopharyngeal CSCs and neuroblastoma CSCs found that the effects of EGCG on the inhibition of cell proliferation and the induction of apoptosis differed slightly between sphere-derived and parental cells (Table 3). Specifically, the ID$_{50}$ (dose “μM” required to achieve 50% inhibition) of EGCG was calculated from the curves of MTT assays (Fig. 1a, b). The ID$_{50}$ values of EGCG treatment of TW01 sphere-derived cells and parental cells are approximately 150 and 60 μM, respectively, and approximately 115 μM EGCG and 38 μM EGCG for TW06 sphere-derived and parental cells, respectively (Fig. 1a, b; Table 3) (Lin et al. 2012). Treatment of BE(2)-C sphere and BE(2)-C parental cells with 50 μM EGCG inhibits cell proliferation by 25.2 and 93.8%, respectively, compared with non-treated cells (Nishimura et al. 2012). Induction of apoptosis using EGCG was determined in sphere-derived and parental TW01 and BE(2)-C cells (Table 3). The results indicate that EGCG is a less effective inhibitor of the sphere-derived cells compared with parental cells, although EGCG inhibits the growth of both.

| Table 3 | Inhibitory effects of EGCG on CSCs are weaker than those on parental cells |
|----------|---------------------------------|
| **Nasopharyngeal cancer** | TW01 sphere | TW01 parental | Lin et al. (2012) |
| ID$_{50}$ μM | 150 | 60 | |
| | TW06 sphere | TW06 parental | Lin et al. (2012) |
| ID$_{50}$ μM | 115 | 38 | |
| **Neuroblastoma** | BE(2)-C sphere | BE(2)-C parental | Nishimura et al. (2012) |
| EGCG 50 μM | 25.2 (% of inhibition) | 93.8 (%) |
| **Nasopharyngeal cancer** | TW01 sphere | TW01 parental | Lin et al. (2012) |
| EGCG 40 μM | 12.7 (%) | 63.6 (%) |
| **Nasopharyngeal cancer** | BE(2)-C sphere | BE(2)-C parental | Nishimura et al. (2012) |
| EGCG 50 μM | 9.1 (%) | 91.7 (%) |

Fig. 1 EGCG inhibits the proliferation of nasopharyngeal CSCs and parental cells. MTT assays of (a) TW01 sphere-derived cells (square) and TW01 parental cells (filled square) and (b) TW06 sphere-derived cells (filled triangle) and TW06 parental cells (filled circle) (Lin et al. 2012, Reproduced with the permission of Dr. Yann-Jang Chen)
investigators extended these studies by combining EGCG with other green-tea catechins and anticancer drugs in 42 in vitro studies and 13 studies of mouse xenograft models (Fujiki et al. 2015). The combination of EGCG with anticancer drugs frequently induced synergistic anticancer activity against human cancer cell lines.

Considering the evidence indicating that the effects of EGCG on human CSCs are slightly weaker compared with the parental cells, a new strategy is required to increase efficacy. We conducted a literature search that identified studies of five human CSCs treated with the combinations (Table 4). Docetaxel inhibits mitosis, and the combination of EGCG, quercetin, and docetaxel reduces colony formation by two prostate CSC lines compared with controls (Wang et al. 2015) (Table 4). 5FU damages cancer cells during S phase, and cisplatin damages DNA, leading to the induction of apoptosis (Fujiki et al. 2015). The combination of EGCG and 5FU or EGCG and cisplatin reduces the weights of tumors formed by three CSCs derived from the colorectum, head and neck, and nasopharynx (Toden et al. 2016; Lee et al. 2013; Li et al. 2015). The combination of EGCG and anticancer drugs is a promising therapeutic strategy for treating human CSCs.

### Discussion

It is necessary to briefly discuss the effects of EGCG on normal cells. The antiangiogenic effects of EGCG are not observed in normal endothelial cells (NECs), compared with tumor-associated endothelial cells (TECs) and endothelial progenitor cells (EPCs). The reason why EGCG is inactive on NECs is explained by evidence indicating that the stem cell marker stem cell antigen-1 (Sca-1) is expressed at a low level in NECs, although it is overexpressed by TECs and EPCs (Ohga et al. 2009). Sca-1 was originally identified as an 18-kDa glycoprotein 1-binding surface protein and is a member of the lymphocyte activation protein-6 family.

The difference between cancer cells and normal cells is a vital subject: Treatment with 87.3 μM EGCG for 48 h resulted in apoptosis in human epidermoid carcinoma cell line (A431), human carcinoma keratinocyte (HaCaT), and human prostate carcinoma cell line (DU145), but 349.1 μM EGCG was not effective on normal human epidermal keratinocytes (NHEK) (Ahmad et al. 1997). EGCG (10–80 μM) for 24 h results in lowering of NF-κB levels in both the cytoplasm and nucleus in a dose-dependent manner in both A431 and NHEK cells. However, EGCG-mediated inhibition of NF-κB constitutive expression and activation, only occurred at a higher dose of EGCG (40–80 μM) in NHEK, compared with A431 cells (Ahmad et al. 2000).

As reported in Introduction, a single topical application of 5 mg was applied to mouse skin. The specific binding of 3H-TPA and that of 3H-okadaic acid to the receptors in a membrane fraction of normal mouse skin were then determined. Both specific bindings decreased immediately, reaching minimum in 5–10 min. The levels gradually returned to normal, suggesting that EGCG induces the sealing of the membrane on mouse skin because of EGCG-protein interaction (The sealing effects of EGCG), resulting in inhibition of tumor promotion and tumor development associated with internalization of 3H-EGCG (Yoshizawa et al. 1992; Okabe et al. 1997).

| Human prostate CSCs | Treatments and reduction of colony formation (%) | Reference |
|---------------------|-----------------------------------------------|-----------|
| CSCs                | Control                                       | EGCG (40 μM) + quercetin (5 μM) | Docetaxel (5 nM) | EGCG + Q + Doc |
| LAPC-4-AI           | 0                                             | 36        | 27        | 86           | Wang et al. (2015) |
| PC-3                | 0                                             | 66        | 32        | 77           |           |

| Human colorectal CSCs | Treatments and reduction of tumor volume (%) | Reference |
|-----------------------|----------------------------------------------|-----------|
| CSCs                  | Control                                       | 5FU (10 μM) | EGCG (100 μM) | EGCG + 5FU |
| HCT116-SCSCS          | 0                                             | 27.3      | 59.1       | 86.4        | Toden et al. (2016) |

| Human head and neck CSCs | Treatments and reduction of tumor weight (%) | Reference |
|--------------------------|----------------------------------------------|-----------|
| CSCs                     | Control                                       | cisplatin (10 μM) | EGCG (5 μM) + cisplatin (10 μM) |
| HNSC CSCs                | 0                                             | 16.8      | 79.8       |             | Lee et al. (2013) |

| Human nasopharyngeal CSCs | Treatments and reduction of tumor weight (%) | Reference |
|---------------------------|----------------------------------------------|-----------|
| CSCs                      | Control                                       | Cisplatin (10 μM) | EGCG (20 μM) + cisplatin (10 μM) |
| CNE2-CSCs                 | 0                                             | 24.0      | 80.0       |             | Li et al. (2015) |
In contrast to our initial work, recent investigation on the interaction of EGCG and cellular molecules, along with receptor activation and gene expression, have shown outstanding progress in the elucidation of therapeutic mechanisms for green tea catechin: Excessive concentrations of vascular endothelial growth factor (VEGF) increase growth of tumors. VEGF stimulates cell responses by binding to VEGF receptor-2 (type III receptor tyrosine kinases) on the cell surface, resulting in phosphorylation of VEGFR-2. Treatment of human umbilical vein endothelial cells (HUVECs) with 1 μM EGCG resulted in complete inhibition of VEGF-induced VEGFR-2 activation, indicating that direct interaction of EGCG with VEGF is a significant mechanism. In these circumstances, endothelial nitric oxide synthase is still activated through the PI3K/Akt signaling pathway that is downstream of VEGFR-2 (Moyle et al. 2015). Matrix metalloproteinases (MMPs) are involved in tumor progression, and treatment with 10.9–87.3 μM EGCG inhibited fibroblast conditioned medium (FCM)-induced phosphorylation of ERK1/2 and/or p38 concomitant reduction in MMP-2 and -9 in human prostate carcinoma DU-145 cells. It also inhibited androgen-induced pro-MMP-2 expression in androgen-sensitive human prostate adenocarcinoma LNCaP cells (Vayalil and Katiyar 2004).

Summary

EGCG and green tea extracts effectively prevent the early stages of cancer, and the combination of EGCG and anticancer drugs is more effective for inhibiting the viability of human CSCs.

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Compliance with ethical standards

Conflict of interest We declare no potential conflicts of interest.

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Research involving human participants and/or animals This review does not contain studies of humans, and the results of animal studies are published.

Informed consent Informed consent was obtained from all participants included in this study.

Glossary

ABCB1 ATP-binding cassette subfamily B member 1
ABCG2 ATP-binding cassette subfamily G member 2
Akt RAC-alpha serine/threonine-protein kinase
Bcl2 B-cell lymphoma 2
Bax Bcl-2-associated X protein
Bmi-1 B-lymphoma Moloney murine leukemia virus integration site 1 homologue
ERK1/2 Extracellular-signal regulated kinase 1/2
GP1 Glycoprotein 1
Gli1 Zinc finger protein GLI1
Hey1 Hairy/enhancer-of-split related with YRPW motif protein 1
K-ras KRAS oncogene
KLf Krüppel-like factor
Myc v-Myc avian myelocytomatosis viral oncogene homolog
Nanog Nanog homeobox protein
Notch1 Notch homolog 1
Oct4 Octamer-binding transcription factor 4
Patched-1 Protein patched homolog 1
SMO A class Frizzled (class F) G protein-coupled receptor
Slug SNAI2
Snail Snail family transcriptional repressor
Sox2 SRY (sex determining region Y)-box 2
Twist Twist family BHLH transcription factor
Wnt Mammalian homologs of Drosophila ‘wingless’ signaling

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