

Epigallocatechin-3-gallate suppresses cell proliferation and promotes apoptosis and autophagy in oral cancer SSC-4 cells

Alexandra Iulia Irimie¹
 Cornelia Braicu²
 Oana Zanoaga²
 Valentina Pileczki^{2,3}
 Claudia Gherman^{2,4}
 Ioana Berindan-Neagoe^{2,4-6}
 Radu Septimiu Campian⁷

¹Department of Prosthodontics and Dental Materials, Faculty of Dental Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; ²Research Center for Functional Genomics, Biomedicine and Translational Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; ³Department of Analytical Chemistry, Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; ⁴Department of Functional Genomics and Experimental Pathology, The Oncology Institute "Prof Dr. Ion Chiricuta", Cluj-Napoca, Romania; ⁵Department of Immunology, Faculty of Medicine, University of Medicine and Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; ⁶Department of Experimental Therapeutics MD Anderson Cancer Center Houston, TX, USA; ⁷Department of Oral Rehabilitation, Faculty of Dental Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania

Correspondence: Ioana Berindan-Neagoe
 Research Center for Functional Genomics and Translational Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, 23 Marinescu Street, 40015, Romania
 Tel +40 264 450 749
 Fax +40 264 598 88
 Email ioana.neagoe@umfcluj.ro

Abstract: Epigallocatechin-3-gallate (EGCG) is the major bioactive component of green tea. Our experimental data indicated that EGCG treatment suppresses cell proliferation of SSC-4 human oral squamous cell carcinoma (OSCC), the effect being dose- and time-dependent. In parallel was observed the activation of apoptosis and autophagy, in response to EGCG exposure in SSC-4 cells. Treatment with EGCG activates the expression of the *BAD*, *BAK*, *FAS*, *IGF1R*, *WNT11*, and *ZEB1* genes and inhibits *CASP8*, *MYC*, and *TP53*. All of these results suggest that EGCG has an excellent potential to become a therapeutic compound for patients with OSCC, by inducing tumor cell death via apoptosis and autophagy.

Keywords: oral squamous carcinoma, time dependent cell proliferation, gene expression

Introduction

Oral cancer is a highly common malignant tumor of the oral cavity^{1,2} and the tenth most frequent common cancer in the world.³⁻⁵ The most frequent malignancy is oral squamous cell carcinoma (OSCC), which constitutes more than 90% of the oral malignancies^{6,7} and which has poor prognosis due to therapy-resistant locoregional recurrences and distant metastases.⁸ OSCC is defined as a neoplastic disorder in the oral cavity and is a complex malignancy, where environmental factors, viral infections, and genetic alterations most likely interact and thus give rise to the malignant condition.² Development of oral cancer proceeds through epigenetic alteration and discrete molecular genetic changes that are acquired from the loss of genomic integrity after continued exposure to environmental or dietary risk factors.^{9,10}

OSCC is characterized by serial epigenetic and genetic alteration. The accumulations of this alteration leads to uncontrolled cell proliferation of the mutated human oral squamous cells, and the accumulation of damaged genetic material leads to uncontrolled division of mutant oral keratinocytes cells.¹¹ Multiple gene alterations result in oral carcinogenesis and cause aberrant expression and function of proteins in a number of cellular processes, including apoptosis and angiogenesis.¹²

Natural phytochemicals have received a significant interest for chemoprevention and treatment for a wide range of diseases, including OSCC.¹³ Phytochemicals, such as polyphenols, are known for their antioxidant capacity and free-radical scavenging properties. In the last years, phytochemicals have received significant attention and been proved to interfere in key cellular pathways.¹⁴ Epigallocatechin-3-gallate (EGCG) is the most abundant and most active phenolic constituent of green tea and has strong antioxidant properties, possessing chemotherapeutic and chemopreventive roles.^{15,16}

Recently, the involvements of EGCG in apoptotic or autophagy-induced cell death have been increasingly appreciated.^{3,10,13,14} Consequently, our main interest was to understand the role of EGCG-mediated cell death, comprising both apoptotic and nonapoptotic cell death, but also, the impact on the gene expression pattern for the main genes involved in apoptosis, in SSC-4 cells, a relevant model for oral cancer. The studies were performed in a noncytotoxic dose in order to avoid prooxidant mechanisms of action that we observed previously (Irimie, unpublished data, 2014).

Materials and methods

Materials

EGCG was purchased from Sigma-Aldrich Corp (St Louis, MO, USA).

Cell culture

SCC-4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham F12 (1:1), supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), and 0.5 µg/mL sodium hydrocortisone succinate (all procured from Sigma-Aldrich Corp). Cells were maintained at 37°C and 5% CO₂, in a humidified incubator.

Evaluation of cell proliferation in real time, using the E-Plate 16

Cell proliferation evaluation in the presence of different EGCG doses (0, 10, 20, 50, 100, and 200 µM) was evaluated using a real-time cell analyzer (RTCA) DP instrument (F Hoffman-La Roche Ltd, Basel, Switzerland), based on basic assay protocol. Background measurement was done using 100 µL of cell culture medium incubated for 30 minutes in CO₂ incubator at 37°C. Once the E-Plate 16 was equilibrated, it was placed in the RTCA DP station, and the background cell index values were measured. Afterwards, the E-Plate 16 was removed from the RTCA DP station, and 15,000 cells/well were added in each well. Cell proliferation kinetics was registered by the RTCA DP instrument for over 49 hours.

Apoptosis evaluation

SSC-4 cells were seeded at a density of 0.5×10⁶ cells in six-well plates in 2 mL culture media and treated for 24 and 48 hours with a single dose of 20 µM. The cells were harvested, then washed once with phosphate-buffered saline (PBS) and centrifuged at 500 RPM for 5 minutes. Then, the SSC-4 cells were resuspended and stained with Annexin-V-FITC solution (BD Biosciences, Franklin Lakes, NJ, USA)

and incubated for 15 minutes at room temperature. Just before analysis, it was stained with propidium iodide (PI) in order to quantify the necrotic cells. Apoptotic and necrotic cells were evaluated on a BD FACSCanto™ II flow cytometer.

Autophagy test

The autophagy was evaluated using an Autophagy/Cytotoxicity Dual Staining Kit (Cayman Chemical Co, Ann Arbor, MI, USA), in accordance with the producer recommendation for inverted fluorescence microscopy, in a 96-well plate seeded with 20,000 cells/well. Microscopic evaluation of the autophagic vacuole staining intensity was confirmed by fluorescence evaluation, with an excitation wavelength of 335 nm and emission wavelength at 512 nm, using a BioTek Microplate Reader.

Gene expression evaluation

For gene expression evaluation, cells were plated in six-well plates at 0.5×10⁶ cells/well and treated with a single dose of 20 µM EGCG. At 24 hours posttreatment cells were lysed using Tri Reagent® (Sigma-Aldrich Corp), and total RNA was extracted. RNA quantification was done using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and quality control was done using an Agilent Bioanalyzer 2100. For complementary DNA (cDNA) synthesis, we used 500 µg of total RNA and a Transcriptor First Strand cDNA Synthesis Kit (F Hoffman-La Roche Ltd), and for gene expression evaluation, we used the LightCycler® TaqMan® Master kit, on a LightCycler 480 instrument (F Hoffman-La Roche Ltd).

For gene expression normalization, we used *β-actin* (AGG AAT GGA AGC TTC CGG TA3'/AAT TTT CAT GGT GGA TGG TGC). Specific primers sequences for each gene of interest are presented below: *AKT3*: GGGAG-GCCAAGGTAGATGA/TCA CAC CTA TAA TCC CAC ATG C; *BAD*: ACC AGC AGC AGC CAT CAT/GGT AGG AGC TGT GGC GAC T; *BAK*: CCT GCC CTC TGC TTC TGA/CTG CTG ATG GCG GTA AAA A; *CASP8*: TAG GGG ACT CGG AGA CTG C/TTT CTG CTG AAG TCC ATC TTT TT; *FADD*: CCG AGC TCA AGT TCC TAT GC/ AGG TCT AGG CCG CTC TGC; *FAS*: GGC CAA GTT GCT GAA TCA AT/GAG ACG AGC TCA CGA AAA GC; *MYC*: GCC GTA GGA AAT GAG CGA TA/AAC CTC GGT TGC TCT TCG T; *NOD1*: GAA TGC AAA GGC CTC ACG/GGC GAG ATA CTT CCC TCC TT; *SMAD3*: CAC CAC GCA GAA CGT CAA/GAT GGG ACA CCT GCA ACC; *SMAD4*: CCT GTT CAC AAT GAG CTT GC/GCA ATG GAA CAC CAA TAC TCA G; *SMAD7*: ACC CGA

TGG ATT TTC TCA AA/AGG GGC CAG ATA ATT CGT TC; *TNF- α* : CAG CCT CTT CTC CTT CCT GAT/TGG GGA ACT CTT CCC TCT G; *TP53*: AGG CCT TGG AAC TCA AGG AT/CCC TTT TTG GAC TTC AGG TG; *WNT11*: AGC TCG CCC CCA ACT ATT/ATA CAC GAA GGC CGA CTC C; *WNT3*: CTC GCT GGC TAC CCA ATT T/GAG CCC AGA GAT GTG TAC TGC; *XIAP*: GCA AGA GCT CAA GGA GAC CA/AAG GGT ATT AGG ATG GGA GTT CA; and *ZEB1*: TGA CTA TCA AAA GGA AGT CAA TGG/GTG CAG GAG GGA CCT CTT TA. For each target gene, we used the $\Delta\Delta C_t$ method for quantification of the relative gene expression.¹⁷

Genes having a fold change ≤ -1.25 or ≥ 1.25 and P -value < 0.05 were considered to be genes of interest. The significant statistical genes were analyzed with Ingenuity® Pathway Analysis (IPA®) software (Ingenuity Systems, Redwood City, CA, USA).

Immunoblotting for protein validation

SSC-4 cells were treated with the same dose as for qRT-PCR evaluation, in order to validate gene expression data at the protein level. At 48 hours posttreatment, the cell culture medium was removed, and cells were washed twice with PBS and 150 μ L cell lyse solution added, followed by a sonication step for 30 seconds; then cells were centrifuged at 16,000 g for clarification of the cellular lysate.

Cells were treated with 20 microM EGCG and seeded at a confluence of half a million/well in a six-well plate. After 48 hours from treatment, the medium was removed, and the cells were removed by scraping, in 200 μ L of cell lyses solution (containing 2.5 mM tris(hydroxymethyl)aminomethane [Tris]-HCl [pH 6.8 at

25°C], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol [DTT], and bromophenol blue) and sonicated for 30 seconds and then, centrifuged at 16,000 g for 5 minutes; then, the samples were transferred to a new tube. The protein determination was done using a BCA™ Protein Assay Kit (Thermo Fisher Scientific) in order to normalize the protein quantity. The protein separation was done using 7% acrylamide electrophoresis gel, based on a standard electrophoresis and nitrocellulose transfer. The immunoblotting was done for β -actin (1:1,000), p53 (1:1,000) (both antibodies from R&D Systems, Minneapolis, MN, USA) and FAS (1:500) from Cell Signaling Technology (Danvers, MA, USA). Membranes were washed and then incubated for 2 hours, with anti-mouse secondary antibody (1:1,000) (DakoCytomation, Glostrup, Denmark) for p53, and with anti-rabbit secondary antibody for β -actin. The staining was done by a colorimetric method using a Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific).

Results

The RTCA DP instrument is an innovative method for the monitoring of cell proliferation in real time, by using culture plates coated with gold microelectrodes at the base. These electrodes are used to measure the impedance from the electrical circuit, this being proportional with the cell proliferation index. Based on this device, we were able to register treatment response curves for the interval of time between 0–49 hours (Figure 1). As expected, we found a reduced proliferation rate in EGCG-treated SSC-4 cells.

The inhibitory effect was dose-dependent and accumulated over time, as displayed by the xCELLigence measurements in the 0–49 hours interval. Based on the

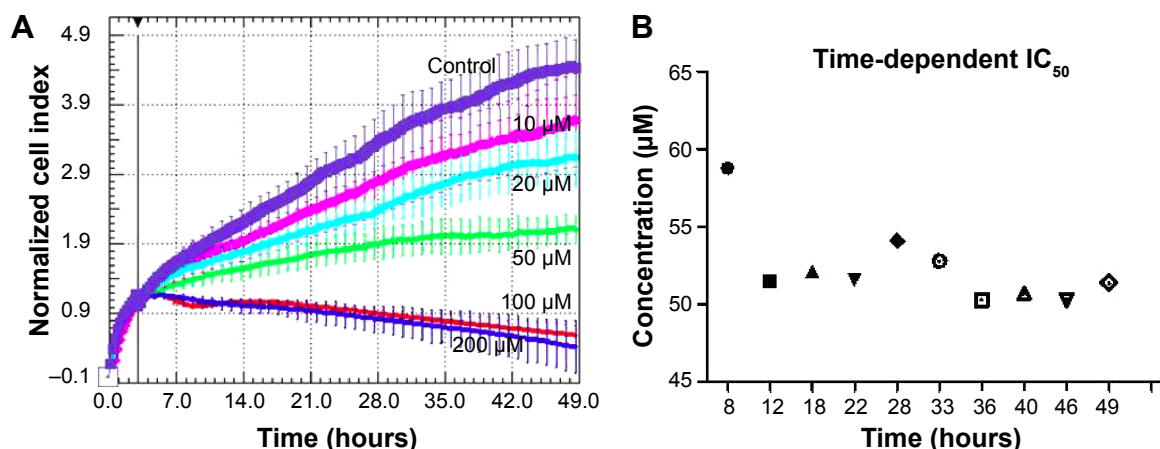


Figure 1 Time-dependent cell proliferation evaluation for EGCG treatment at different EGCG doses (0, 10, 20, 50, 100, and 200 μ M), in SSC-4 cells, using the xCELLigence system.

Notes: (A) Data are displayed as the normalized cell index, over 49 hours. (B) Time-dependent IC₅₀ evaluation using RTCA software.

Abbreviations: EGCG, Epigallocatechin-3-gallate; IC₅₀, half maximal inhibitory concentration; RTCA, real-time cell analysis.

cell proliferation data, the time-dependent half maximal inhibitory concentration (IC_{50}) of EGCG was determined, and the average of was $52.3 \mu\text{M}$ in the case of SSC-4.

Apoptosis/necrosis evaluation in the SSC-4 cells treated with EGCG

The assessment of cellular apoptosis after 24 and 48 hours was done by comparing annexin V-fluorescein isothiocyanate (Annexin V-FITC) binding and PI staining of EGCG-treated cells ($20 \mu\text{M}$) versus that of a control (Figure 2). The results were expressed as % of apoptotic cells, and an increase of 28.6% of apoptotic cells was observed at 48 hours posttreatment. EGCG administration revealed that at 24 hours and 48 hours, 16.9% and 28.6% cells, respectively, underwent apoptosis. A relatively low percent of necrotic cells was observed for the same treatment (the values being 0.8% and 2.1%, respectively, at 24 hours and 48 hours posttreatment). This evaluation allowed differentiation among the different apoptosis stages and necrosis.

Autophagy evaluation in the SSC-4 cells treated with EGCG

Autophagy was proved to be related to the modulation of specific mechanisms of cell death. Using inverted fluorescence microscopy, we assessed whether EGCG treatment ($20 \mu\text{M}$) led to the modulation of the autophagy in SSC-4

human OSCC cells. Evaluation of the autophagy was done using a protocol based on monodansylcadaverine, a fluorescent product that is incorporated by the multilamellar bodies. Although SSC-4 is a cell line that manifests autophagy spontaneously, EGCG treatment intensified the level of autophagy (Figure 3B).

EGCG treatment caused increase fluorescence intensity (Figure 3C), indicating that EGCG caused autophagy in SSC-4 cells.

Gene expression quantification as response to EGCG

To evaluate the relative gene expression in response to EGCG exposure, TaqMan technology was used for 20 genes. β -actin served as a housekeeping gene. Data were analyzed using the $\Delta\Delta C_t$ method. Genes with a fold change of ≤ -1.25 or ≥ 1.25 and P -value < 0.05 were considered statistically significant. The fold change data are presented in Figure 4. Therefore, among the analyzed genes, we had six genes upregulated (*BAD*, *BAK*, *FAS*, *IGF1R*, *WNT11*, and *ZEB1*) and three genes downregulated (*CASP8*, *MYC*, and *TP53*).

IPA analysis is a very useful program, integrating the altered genes as response to EGCG, in order to predict the altered biological processes and cellular functions (Figures 5 and 6). The color indicates altered genes (red for the upregulated and green downregulated genes) involved

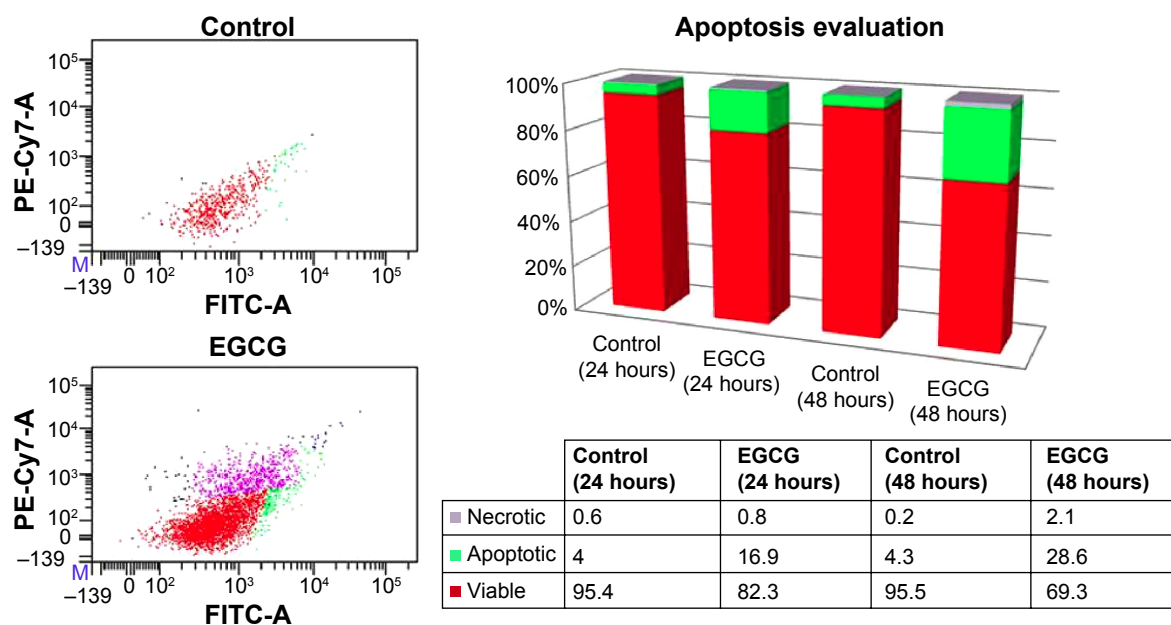


Figure 2 Flow cytometry evaluation using annexin V FITC/PI, showing the effects of $20 \mu\text{M}$ EGCG treatment for 24 hours and 48 hours on SSC-4 cells.

Notes: Viable cells are located in the lower left quadrant, and early apoptotic cells are positioned in the lower right quadrant of the two flow cytometric displays, while late apoptotic cells are located in the upper right quadrant, and necrotic cells are displayed in the upper left quadrant (24 hours). The image at the right presents the % apoptotic, necrotic, and viable cells, from total numbers of cells.

Abbreviations: EGCG, epigallocatechin-3-gallate; FITC, fluorescein isothiocyanate; PI, propidium iodide.

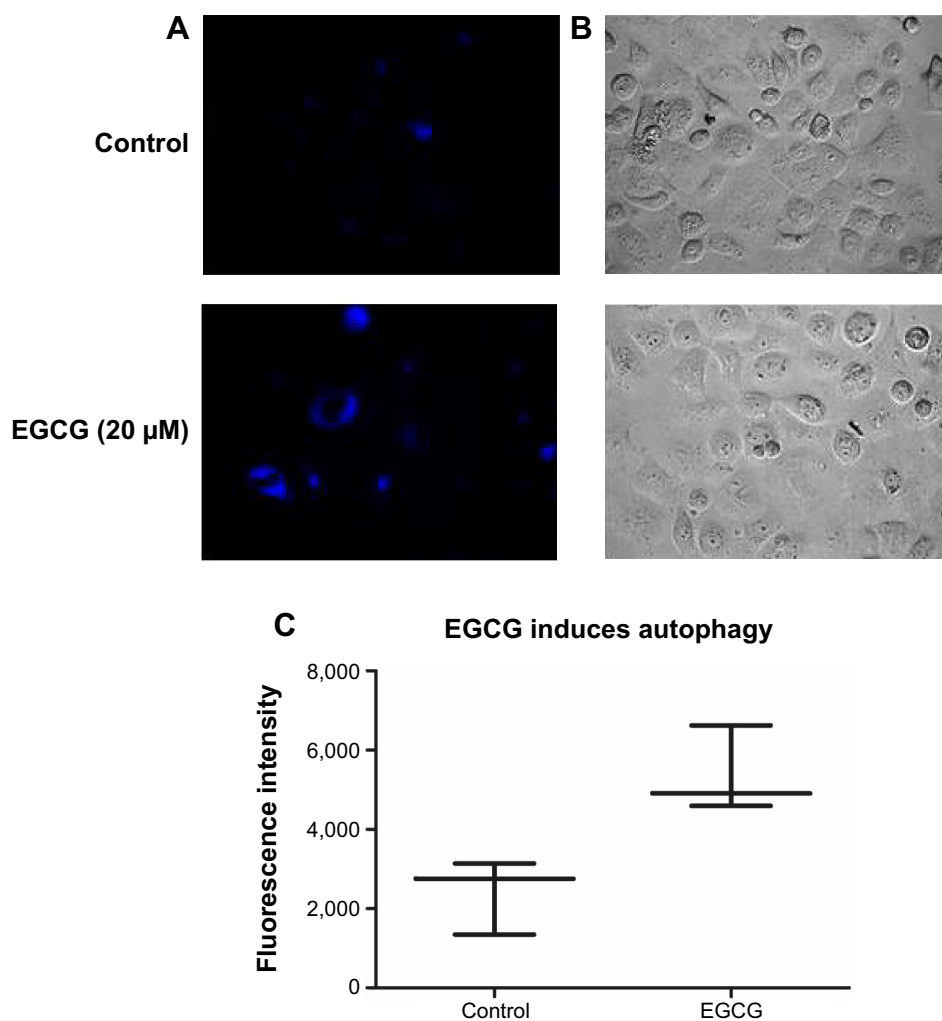


Figure 3 EGCG-induced autophagy in SSC-4 cells, using fluorescence microscopy evaluation after MDC staining.

Notes: (A) The control shows a low level of basal autophagy, indicated by blue "dot" staining of the autophagic vacuole, while MDC staining of SSC-4 cells treated with 20 μ M EGCG led to an increase in fluorescence intensity and number of autophagic vacuoles compared with the untreated cells. (B) Bright-field microscopy. (C) Evaluation of the MDC fluorescence intensity, using a BioTek Microplate Reader.

Abbreviations: EGCG, epigallocatechin-3-gallate; MDC, monodansylcadaverine.

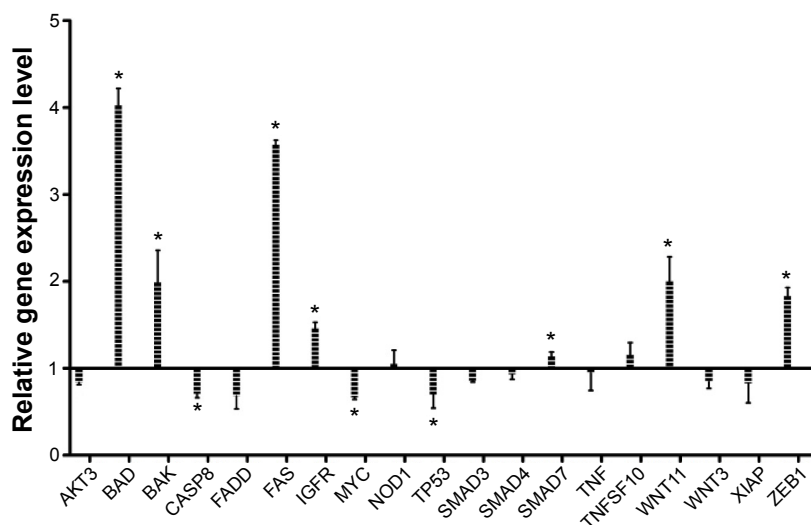


Figure 4 Relative gene expression level showing the effect of treatment with a single dose of EGCG (20 μ M), at 24 hours posttreatment.

Abbreviation: EGCG, epigallocatechin-3-gallate.

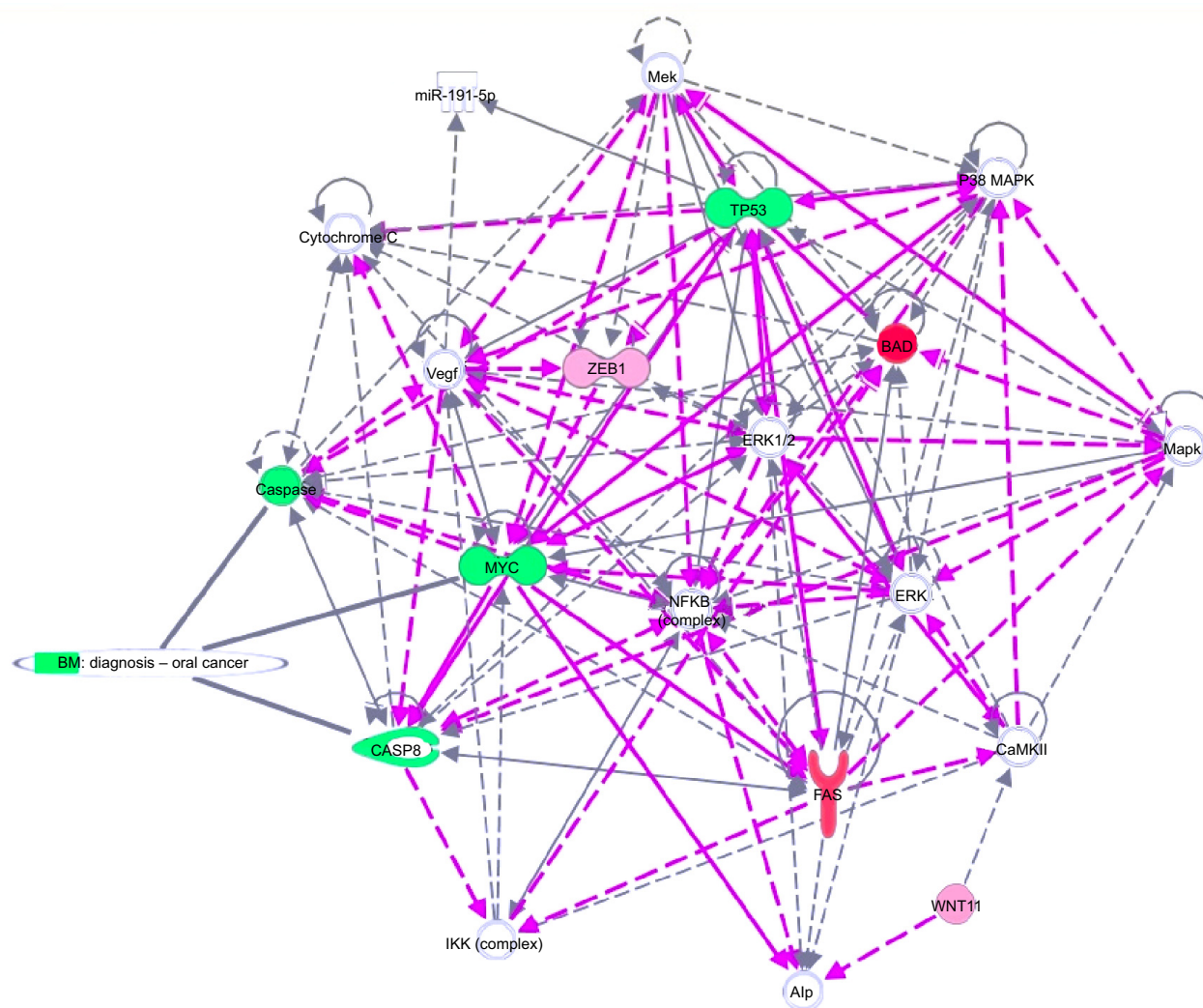


Figure 5 Gene network, created using IPA® software, reflecting EGCG treatment.

Notes: The overexpressed genes are labeled in red, while those downregulated are marked in green.

Abbreviations: EGCG, epigallocatechin-3-gallate; IPA, Ingenuity® Pathway Analysis.

in disease progression or with prognostic value, all related to the alteration of cellular functions. EGCG treatment was activate proapoptotic genes (*BAD*, *BAK* or *FAS*) or cell cycle checkpoints (p53), all genes recognized as markers involved diagnostic or prognostic as displays IPA. It was observed downregulation of genes that decrease transmembrane potential of mitochondria (*CASP8*, *MYC*, *TP53*). The downregulation of genes that decrease the transmembrane potential of mitochondria (*CASP8*, *MYC*, and *TP53*) and that increase the damage by mitochondria (*FAS*) was observed, confirming the microscopic observation.

Immunoblotting for gene expression validation of the data

EGCG treatment led to *p53* downregulation and *FAS* overexpression, not only at the gene expression level but also, at

protein level. The protein evaluation at 48 hours posttreatment is presented in Figure 7.

Discussion

EGCG reduces cell proliferation via apoptosis in a dose-dependent manner, as was confirmed in multiple cell lines. Autophagy is another important mechanism adding to apoptotic processes to reduce cell proliferation.^{2,18,19} In a previous study, the reduction of cell proliferation rate was shown to be related to the activation of the autophagy and induced reactive oxygen species (ROS).^{20,21} One of our previous studies demonstrated an essential function in ROS having the EGCG metabolic product.^{22,23} An important role in exerting biological active properties are the EGCG oxidation products, such as quinones and semiquinones.²⁴ Consequently, our investigation presents the fact that the

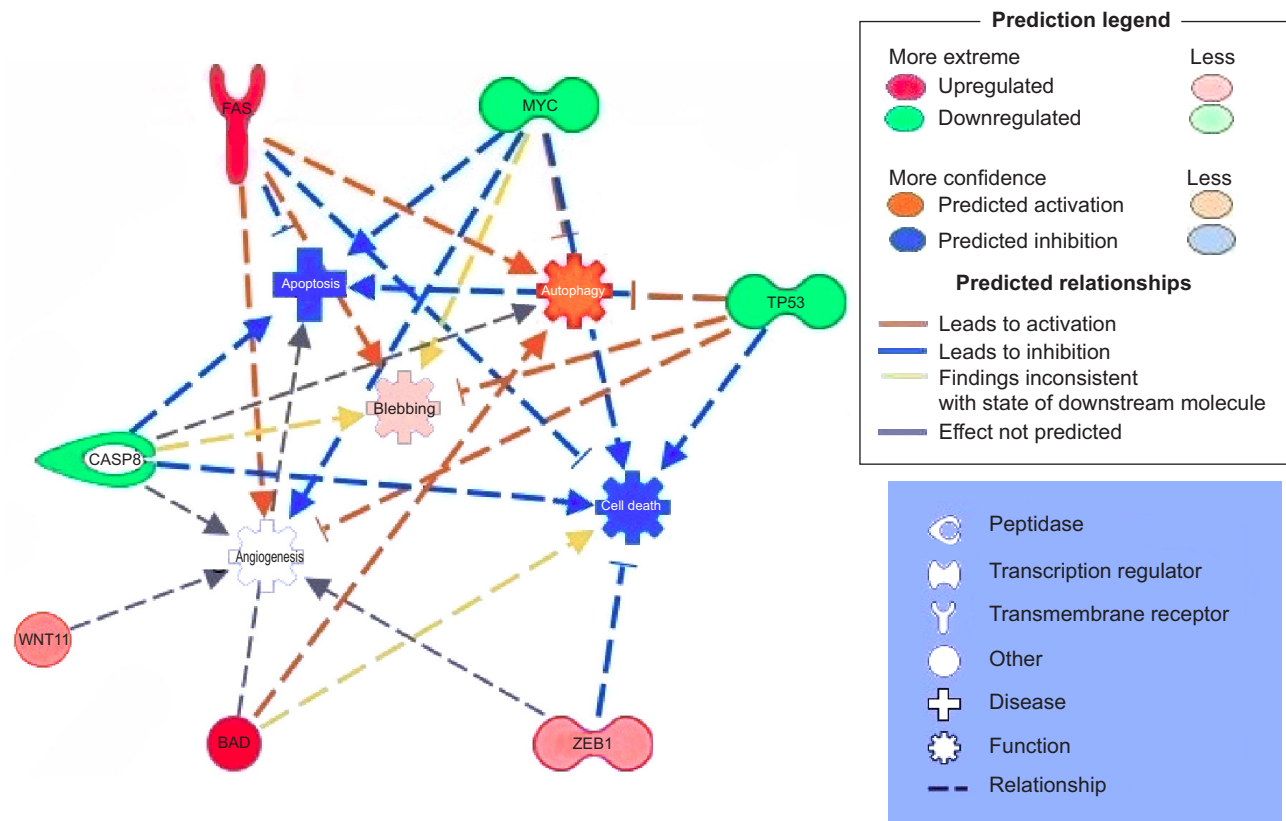


Figure 6 Putative model of multiple antitumoral mechanisms activated by EGCG, in SSC-4 human oral cancer cells.

Notes: Overexpressed genes are labeled in red, and downregulated in green.

Abbreviation: EGCG, epigallocatechin-3-gallate.

therapeutic actions of EGCG on the human squamous tumor cells involves, not only apoptosis but also, autophagy. This fact might have significant importance for chemoresistance mechanisms, based on the fact that EGCG is able to target multiple death pathways. It was proved that the autophagy enhances EGCG-induced cell death – this suggests the utility of autophagy inhibitors in enlarging the therapeutic response or in preventing activation of resistance to therapy.¹⁷

The Ingenuity software allowed identification of the biological processes and functions related to the affected up- and downregulated genes. As can be observed in Figures 5 and 6, EGCG treatment was able to target multiple genes involved in apoptosis, autophagy, and angiogenesis. This sustains the idea of developing novel therapeutic strategies based on EGCG. Gene expression data confirmed our

previous findings concerning the activation of apoptosis and autophagy.^{22,25,26} The reduction of cell proliferation is related to these processes. We summarize the effect of EGCG treatment on different signaling genes and the potential effect on cell survival, apoptosis, and autophagy in SSC-4 cells, in Figure 6: Pharmacological activation of autophagy, by EGCG treatment, led to activation of apoptosis.

TP53, an essential regulator of tumor cell growth and proliferation, controlling cell cycle progression, cellular senescence, apoptosis, or autophagy of tumor cells, was downregulated by EGCG treatment.²⁷ *TP53* downregulation was associated with an increased response to chemotherapy.²⁸

An interesting finding was the inhibition of *CASP8*, a key upstream mediator in death receptor-mediated apoptosis

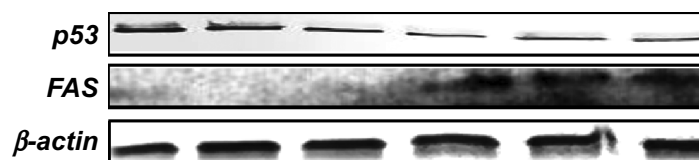


Figure 7 *p53*, *FAS*, and β -actin immunoblotting on SSC-4 cells, showing the effects of treatment with a single dose of EGCG (20 μ M), at 48 hours posttreatment.

Abbreviation: EGCG, epigallocatechin-3-gallate.

that participates in mitochondria-mediated apoptosis via cleavage of proapoptotic factors.^{29–32} In concert with the *CASP8* activation by EGCG, an enhanced expression in the functional *FAS/CD95* pathway has also been identified,³³ which was confirmed in the present study on SSC-4 cells. The *FAS* gene plays important roles in oral cancer cell apoptotic mechanisms.³⁴ Apoptosis in response to local *FAS* activation in vivo has also been demonstrated in epithelial cells.^{35,36} The *MYC* gene has been found to mediate apoptosis of oral squamous cell carcinoma in certain conditions, and this might be related to upregulation of *FAS* expression.³⁷ *MYC* has two outputs in normal cells, the induction of apoptosis and proliferation.^{38–40}

BAD and *BAK* are proapoptotic *BCL-2* family member proteins that regulate the intrinsic apoptosis pathway.⁴¹ *BAK* is an essential effector of the intrinsic pathway of apoptosis,⁴² specifically activated by EGCG in SSC-4 cells. In OSCC, compared with oral epithelium, there is a decreased *BCL-2* expression.⁴³

ZEB1 overexpression, using lentiviral based delivery system for *ZEB1* (lenti-siRNA/*ZEB1*) in lung adenocarcinoma cells, was previously found to be related to the inhibition of cell proliferation both in vitro and in vivo, and induces cell apoptosis.³¹ This has significance for the development of novel treatment strategies, particularly in context of the capacity for multitargeted therapy.

IGF1R expression increases with tumor progression,⁴⁴ and downregulation of *IGF1R* expression causes apoptosis.^{45,46} The *IGF1R* gene also activates alternative pathways for protection from apoptosis and in some cases, is involved in cell proliferation and differentiation. The multiplicity of signaling pathways used by *IGF1R* may explain why its receptor has such a powerful and widespread antiapoptotic activity.^{47–49} Expression of *IGF1R* was shown to be significantly increased in the carcinogenesis of OSCC, with metabolically active regions of OSCC being strongly correlated to proliferating cancer cells without detection of apoptosis.⁵⁰ *IGF1R* overexpression is related to familial breast cancer,⁵¹ and this factor might be related to resistance to therapy in the case of OSCC cells.⁵²

The literature, to date, has presented *WNT11* as overexpressed in OSCC.^{53–55} This might represent a therapeutic target for OSCC,⁵³ based on the fact that WNT-pathway genes were related to the activation of tumorigenesis mechanisms,⁵⁴ caused by epigenetic alteration.⁵⁵

Our results showed that EGCG induces activation of cell death receptors, leading to activation of intrinsic apoptotic pathways. Therefore, data from this study have identified

an important role of autophagy in the occurrence of a cell death mechanism induced by EGCG, in SSC-4 cells. All of these results suggest that EGCG has an excellent potential for treatment or as adjuvant therapy for patients with OSCC, by inducing cell death via apoptosis and autophagy.

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Disclosure

The authors report no conflicts of interest in this work.

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