Evaluation of the antineoplastic activity of gallic acid in oral squamous cell carcinoma under hypoxic conditions

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The purpose of the current study was to develop and test a theoretical model that could explain the mechanism of action of gallic acid (GA) in the oral squamous cell carcinoma context for the first time. The theoretical model was developed using bioinformatics and interaction network analysis to evaluate the effect of GA on oral squamous cell carcinoma. In a second step to confirm theoretical results, migration, invasion, proliferation, and gene expression (Col1A1, E-cadherin, HIF-1α, and caspase-3) were performed under normoxic and hypoxic conditions. Our study indicated that treatment with GA resulted in the inhibition of cell proliferation, migration, and invasion in neoplastic cells. Observation of the molecular mechanism showed that GA upregulates E-cadherin expression and downregulates Col1A1 and HIF-1α expression, suggesting that GA might be a potential anticancer compound. In conclusion, the present study demonstrated that GA significantly reduces cell proliferation, invasion, and migration by increasing E-cadherin and repressing Col1A1. *Anti-Cancer Drugs* 27:407–416 Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Oral cancer is identified as a significant public health threat worldwide. In addition, its treatment often produces dysfunction and changes in speech, mastication, and social interaction, and even death [1,2]. Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm that occurs in the mouth [3]; however, over the past several decades, the survival of patients with OSCC has not significantly improved [4]. Metastasis to the lymph nodes and distant organs is responsible for 90% of deaths [5]. OSCC is a type of malignant tumor with a potent capacity to metastasize locally and distantly [6]. Studies have shown the effects of various genes that exhibit the metastatic potential of OSCC and unravel the molecular mechanism of OSCC pathogenesis [5,7,8]. Neoplastic cells at the invasive front frequently lose epithelial cell phenotypes and acquire mesenchymal cell-like phenotypes, referring to the epithelial–mesenchymal transition (EMT) [9–11]. The degradation of Collagen type1A1 (Col1A1) and reduction or loss of the E-cadherin expression is considered a hallmark of EMT [12].

Malignant neoplasms generally demonstrate a fast growth rate under hypoxia [13]. Characteristically, OSCC is a locally aggressive malignant neoplasm with rapid progression and reduced oxygen concentration [14]. The adaptability of cancer cells to hypoxia is critical for tumor survival [15]. It was suggested that the invasive and metastatic nature of OSCCs is a consequence of its adaptation to the hypoxic microenvironment [16,17]. An important mechanism for adaptation to reduced oxygen concentrations is the regulation of hypoxia-inducible factor-1α (HIF-1α) [18]. HIF-1α mediates adaptive responses at cellular and systemic levels for the maintenance of homeostasis, and is the main mechanism whereby tumor cells respond to acidosis and hypoxic stress [19]. Overexpression of the HIF-1α has been observed in many human cancers, and increased levels of HIF-1α protein correlate with advanced disease stages and poor prognosis [7,20–22], including epidermal carcinogenesis [23]. Recent evidence suggests that EMT and hypoxia pathways might be associated with metastasis development [24,25]. Gallic acid (GA) has been suggested to induce apoptosis [26–29] and play a primordial role in cancer prevention [30]. However, the molecular mechanism related to the effect of GA in metastasis remains unclear. Considering these facts, the purpose of

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the current study was to develop and test a theoretical model that could explain the mechanism of action of GA in OSCC for the first time.

Materials and methods

Study design

The current study mixed two study designs [31]. First, the theoretical model was developed using bioinformatics and interaction network analysis. In the second step, to test the theoretical model, applied cell study was performed using hypoxia to simulate the OSCC tumor microenvironment.

Bioinformatics and interaction network analysis

The leader gene approach was used as it was described previously [32,33] and modified [34]. Briefly, using OSCC, Gallic acid, and Hypoxia as keywords a search was carried out in PubMed, Gene-Bank, and Genecards. Only experimental studies with a high degree of confidence (0.9–0.99) were considered. After candidates' genes relation to OSCC and GA were established, the list was then expanded using the Web-available software STRING (version 9.05) [35,36]. According to the weighted number of links, genes were clustered, using K-means. Genes with no interactions were orphan genes [32]. Topological analysis was carried out with Cytoscape [32] and FANMOD [37], whereas ontological analysis was performed with BinGO [38–40].

Cell culture

SCC-9 and SCC-4 cells (1×10^5) were maintained in Dulbecco's modified Eagle's medium (DMEM/F12; Gibco, Billings, Montana, USA) containing 10% fetal bovine serum (FBS) (Gibco), 400 ng/ml hydrocortisone, and antibiotic/antimycotic solution (Invitrogen, Carlsbad, California, USA). HaCaT cells were cultured in DMEM (Invitrogen) supplemented with 10% of FBS at 37°C with 5% CO_2 in a humidified air atmosphere.

Drug sensitivity assay

SCC-9, SCC-4, and HaCat cell proliferation analysis was performed in the presence of increasing concentrations of GA with trypan blue. Various concentrations of GA (1, 5, and 10 μg/ml) were incubated for 24, 48, and 72 h. The concentration and time chosen was 10 μg/ml for 24 h. The results were derived from triplicate experiments. GA was dissolved in ethanol, and five groups were used: control, control + ethanol (vehicle), $CoCl_2$, GA 10 μg/ml, and $CoCl_2$ + GA 10 μg/ml.

Cell culture and hypoxia

To simulate hypoxic conditions, SCC9, SCC4, and HaCat cells were cultured in media with the addition of 100 μmol/l cloret cobalt ($CoCl_2$). HIF-1α expression was evaluated to determine whether the environment was hypoxic. When the tumor grows, it develops extensive regions of poor oxygenation due to the discrepancy between the rapid rate of tumor growth and the capacity of existing blood vessels to supply oxygen [41]. At a cellular level, a hypoxic stress generates an adaptive response that is mediated by HIF-1α [42]. Thus, a positive correlation between HIF-1α overexpression and poor prognosis has been reported [41]. The $CoCl_2$ stock solutions were filter-sterilized (0.22 μm). The resultant solutions were kept at 4°C and used within 24 h for the assay.

RNA isolation and real-time PCR

RNA was isolated from cells using the Trizol reagent (Gibco; Invitrogen), according to the manufacturer's instructions.

Total RNA was reverse transcribed and the subsequent cDNA was heated at 95°C to terminate the reaction. For real-time PCR, 2 μl of the cDNA was added to SYBR GREEN reagent (Life Technologies, Carlsbad, California, USA) with the HIF-1α [43], Col1A1 [44], E-cadherin [45], and caspase-3 [46] specific primer/probe set as described in Supplementary, Table SI, Supplemental digital content 1, *http://links.lww.com/ACD/A149* (Life Technologies); amplification was performed on an StepOne Real-Time PCR System (Life Technologies). All reactions were carried out in triplicate, and β-actin (Life Technologies) served as an internal control. The results were quantified as C_t values, where C_t was defined as the threshold cycle of PCR at which the amplified product is first detected and defined as relative gene expression (the ratio of target/control).

Western blot analysis

Proteins were extracted from SCC-9 cells, separated using SDS-PAGE gels (10%), and then transferred onto nitrocellulose membranes and blocked with Odyssey blocking buffer 1× (LICOR Biosciences, Lincoln, Nebraska, USA). The primary antibodies were HIF-1α 115 kDa (1 : 1000, NB100–479; Novus Biologicals, Minneapolis, Minnesota, USA) and internal control β-actin 45 kDa (1 : 1000, number 4967L; Cell Signaling Technology, Danvers, Massachusetts, USA). The secondary antibody was goat anti-rabbit (1 : 15 000, 926–32211 IgG IRDye 800; LICOR Biosciences). The blots were visualized and analyzed using the Odyssey Infrared Imaging System (LICOR Biosciences) [47].

Proliferation assay

SCC-9, SCC-4, and HaCaT cells (1×10^5) were seeded in a 16-well plate supplemented with 10% FBS, and after 24 h the medium was replaced with a fresh medium supplemented with 2% FBS. After 24 h the medium was replaced with a fresh medium without FBS. Subsequently, GA (Sigma, St Louis, Missouri, USA) and $CoCl_2$ (Sigma) were added to the culture medium. After 24 h, the cells of the five groups were trypsinized and counted using a Newbauer chamber. The experiment was performed in triplicate. Cell viability was assessed

using trypan blue in all assays. SCC-4 demonstrated a low proliferative index in 24 h and was excluded from the migration, invasion, and gene expression experiments.

Wound-scratch assay

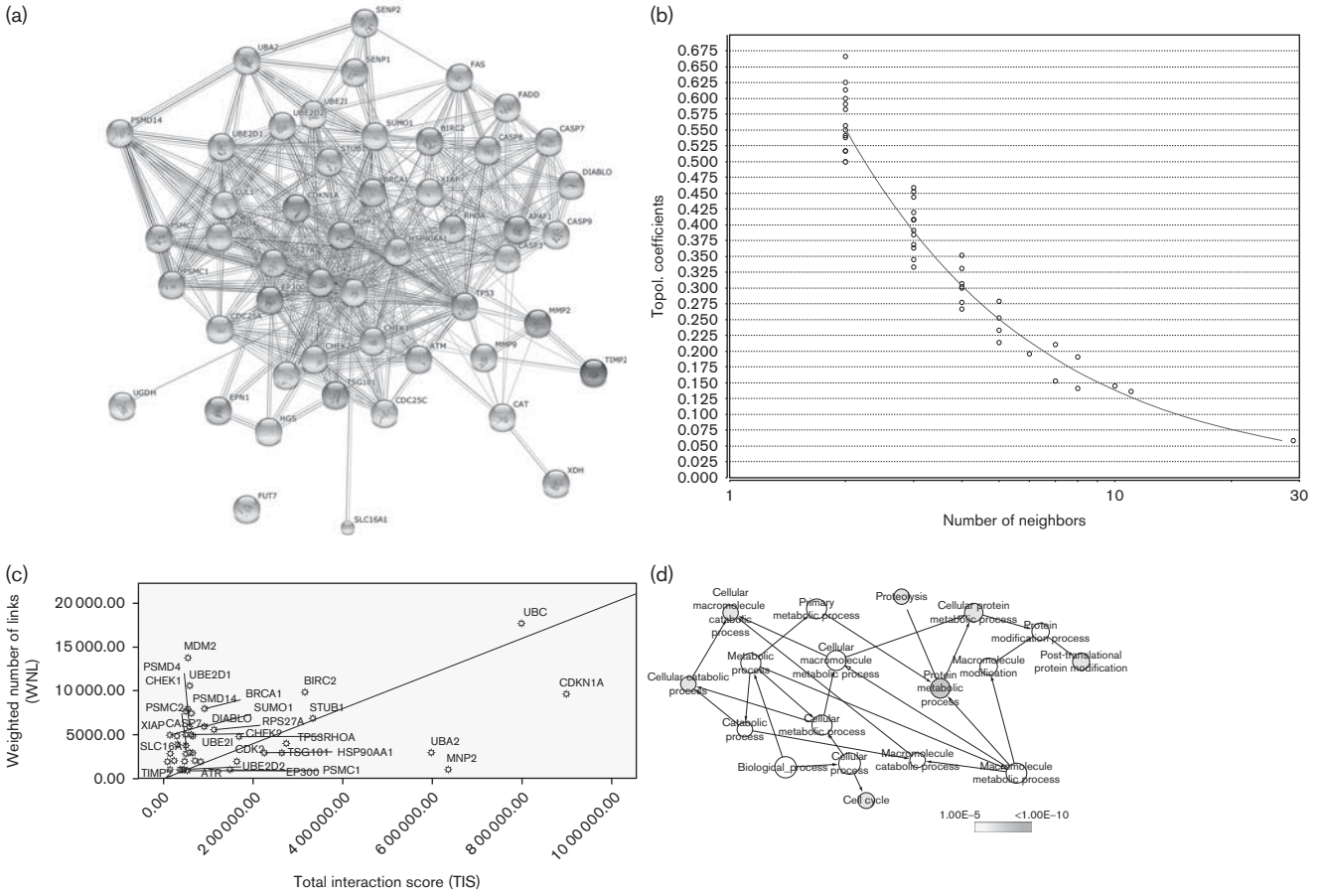
Cell migration was monitored in a wound-scratch assay as described previously [48]. Briefly, a scratch was made with a sterile pipette tip in a confluent cell layer, washed twice in PBS, and then 10 μg/ml GA and 100 μmol/l/ml $CoCl_2$ were added in serum-free medium. The wells were photographed at the beginning of the experiment and after 24 h (SCC-9/HaCaT cells). Pictures were obtained using camera SC30 (Olympus, Center Valley, Pennsylvania, USA) in an IX81 inverted microscope (Olympus). ImageJ software was used for analysis. The experiment was performed in triplicate.

Invasion assay

For the transwell invasion assay, a polycarbonate membrane with pores of 8 μm in diameter was used for the upper chamber covered with 50 μl of Matrigel. SCC-9 cells were trypsinized and resuspended from 3×10^5 cells/ml serum-free DMEM to each well. These cells were placed in the upper chamber of migration. Treatments were performed only in the lower chamber of migration, diluted in DMEM without serum. To control this experiment, the lower chamber was filled with DMEM without serum. The multiwell plate was covered and incubated for 24 h at 37°C in CO_2 incubator. After the incubation period, the upper chamber was inverted to remove both cells that had not invaded and culture medium from the upper chamber.

Only the cells that had migrated remained, being located on the underside of the membrane. These cells were fixed in 4% paraformaldehyde in PBS, stained with Crystal Violet solution of 2% methanol, and images of fields of each membrane were acquired at a final magnification of ×500 camera SC30 (Olympus) in an IX81 inverted microscope (Olympus). The number of cells that invaded was determined through counting. The experiment was performed in triplicate.

Fig. 1



Bioinformatics analyses. (a) STRING results for OSCC, (b) power law behavior, (c) leader genes, and (d) GA ontological analyses. GA, gallic acid; OSCC, oral squamous cell carcinoma.

Statistical analysis

Analyses were performed using SPSS (version 18.0; IBM, Armonk, New York, USA) and GraphPad Prism Software (version 5.0; GraphPad Software Inc., San Diego, California, USA). Data were evaluated using one-way analysis of variance, followed by the Tukey post-test. All data are given as mean ± SD. Statistical significance was accepted at P value less than 0.05.

Results

Bioinformatics and interaction networks

For evaluation of the possible role of GA in cancer cells under hypoxic condition, a bioinformatics approach was used considering human protein interactions. Using GA, hypoxia, and cancer as the preliminary keywords, genecards suggested 12 genes that scored higher than 0.70. The 12 genes suggested in genecards were expanded on STRING software. The network had 45 genes (Fig. 1a). The network exhibits a power law behavior (correlation: 0.919; R^2: 0.867 Fig. 1b) in agreement with the scale-free theory of bionetwork. The leader genes were ubiquitin C and cyclin-dependent kinase inhibitor 1A (CDKN1A) (Fig. 1c). The ontological analysis demonstrated that cell cycling, proteolysis, and protein metabolism are the main biological processes associated with GA treatment. (Fig. 1d). These data suggest that GA might reduce human cancer cell proliferation under hypoxic conditions.

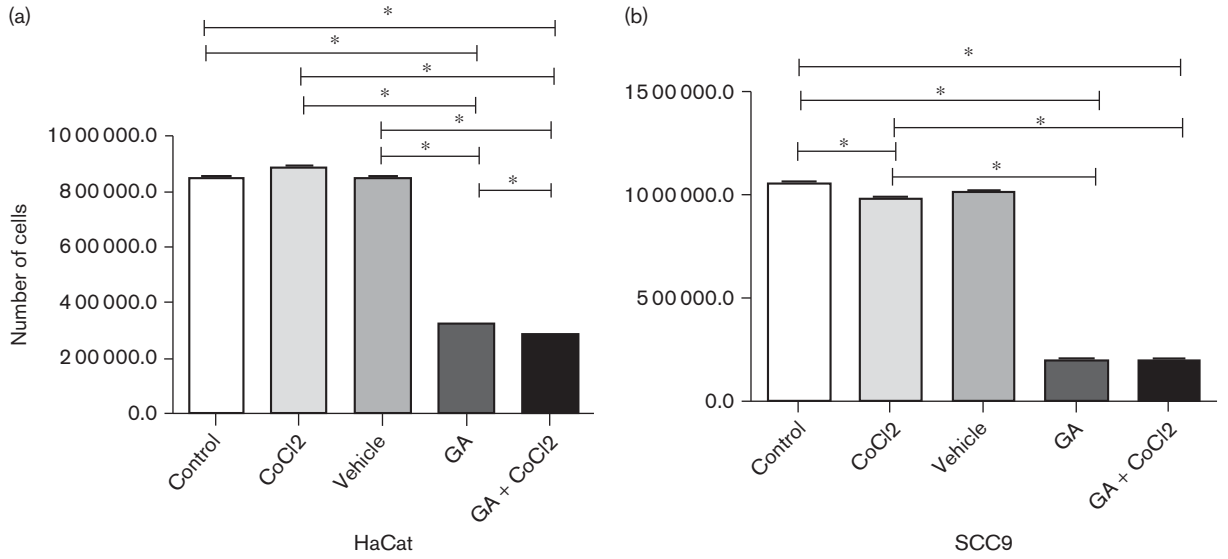
GA reduces cell proliferation

To elucidate whether GA contributes to the inhibition of cell cycling, proliferation assay was performed on the HaCaT and SCC-9/SCC-4 cells treated with 1, 5, and 10 μg/ml of GA for 24, 48, and 72 h (Supplementary Fig. S1, Supplemental digital content 2, *http://links.lww.com/ACD/A150*). GA significantly decreased the percentage of treated cells. The concentration of 10 μg/ml for 24 h was chosen. These results indicate that GA elicits an antiproliferative effect in OSCC cells. After treatment with GA for 24 h, proliferation was dramatically decreased in both SCC-9 and SCC-4 cells in concentrations of 10 μg/ml (Supplementary Fig. S1 A and B, Supplemental digital content 2, *http://links.lww.com/ACD/A150*). GA also dramatically decreased HaCaT cells in concentrations of 10 μg/ml after 24 h (Supplementary Fig. S1C, Supplemental digital content 2, *http://links.lww.com/ACD/A150*).

GA reduces migration and invasion of oral squamous carcinoma cells under hypoxic conditions

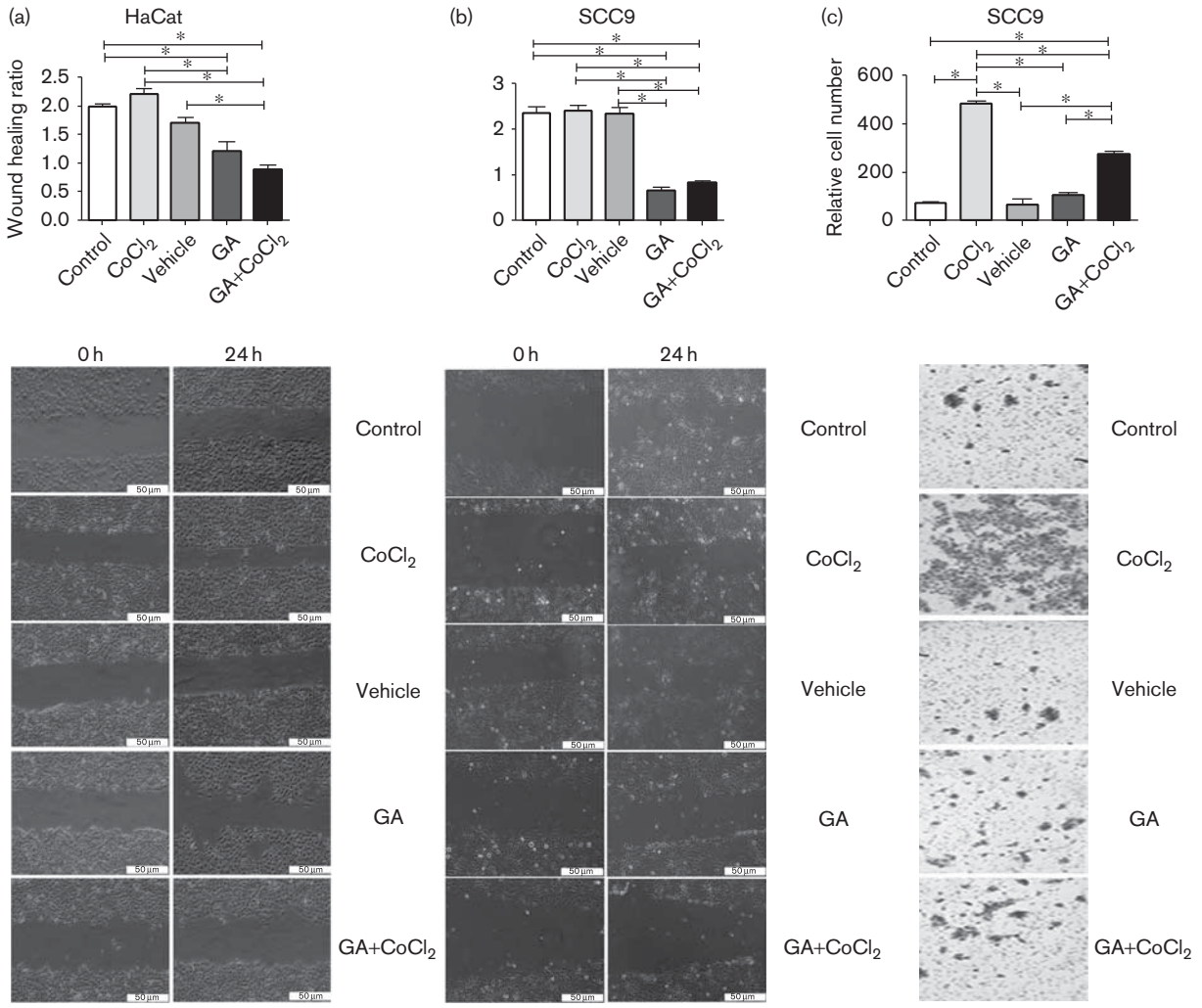
To evaluate the effect of GA on hypoxic tumor microenvironment, $CoCl_2$ assay was performed. GA decreased the number of HaCaT (Fig. 2a) and SCC-9 (Fig. 2b) cells under hypoxic conditions. Wound-scratch and Matrigel invasion assays were performed on the OSCC cells. In comparison with the untreated group, in the SCC-9 cells (Fig. 3), the closure of the gap distance was inhibited significantly by GA at 10 μg/ml in both HaCaT and SCC-9 cells (Fig. 3a and b). Invasiveness is an important characteristic of oral squamous carcinoma cells and a target for the development of anticancer agents [14]. As shown in Fig. 3c, GA significantly reduced invasiveness and migration in $CoCl_2$ + GA groups in SCC-9 cells (Fig. 3c). The proliferation index (Supplementary Fig. S2A, Supplemental digital content 3, *http://links.lww.com/ACD/A151*) for HaCaT, SCC-4, and SCC-9 was determined.

Fig. 2



Effect of GA on the number of cells under normoxic condition and hypoxic conditions in carcinoma cells. (a) Effect of GA on the number of HaCaT cells. (b) Effect of GA on the number of SCC-9 cells. *Statistical significance. GA, gallic acid.

Fig. 3



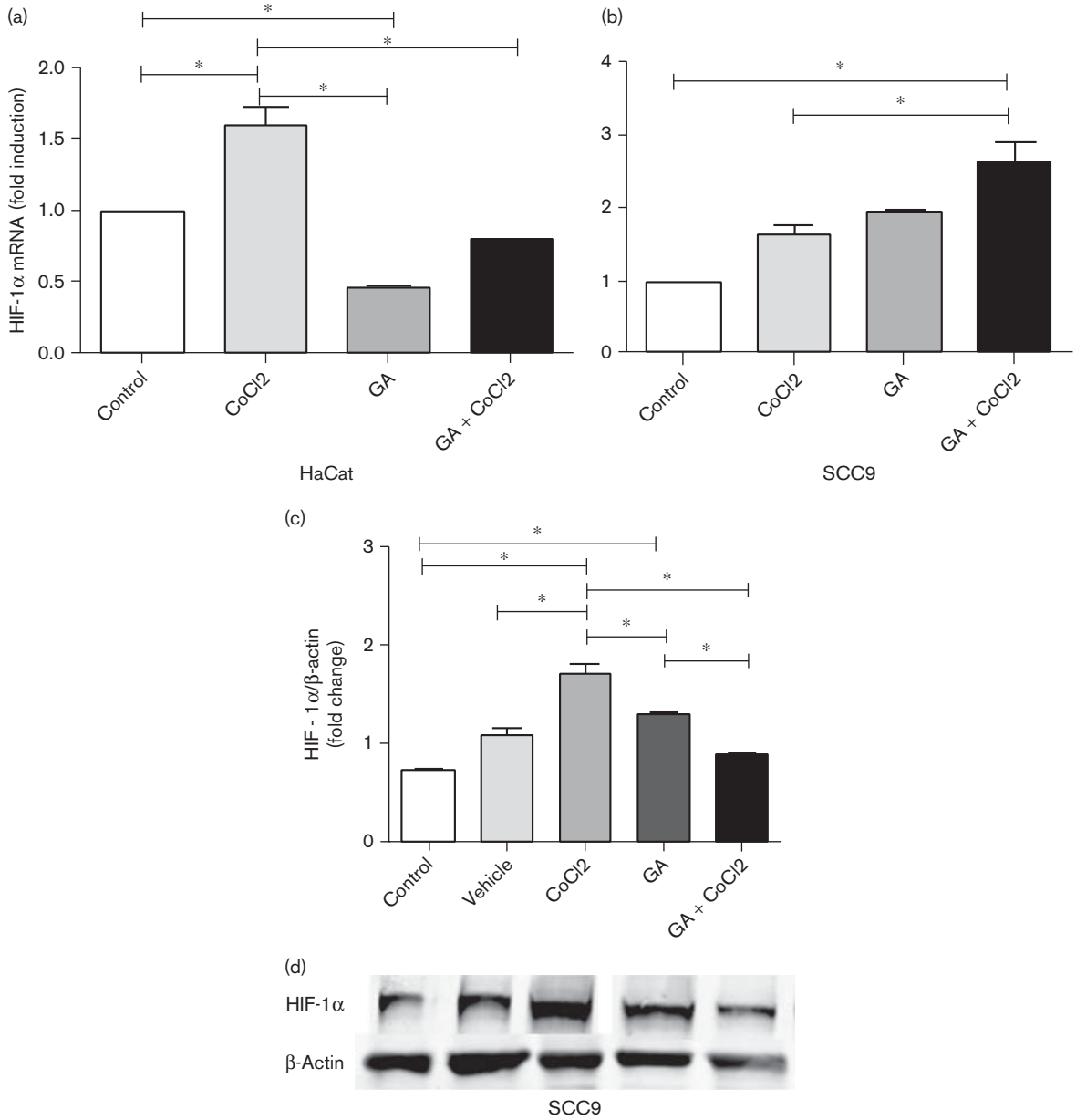
Effect of GA on the migration and invasion under hypoxia in carcinoma cells. (a) Effect of GA wound-scratch assay of HaCaT cells. (b) Effect of GA wound-scratch assay of SCC-9 cells, and (c) effect of GA in Matrigel assay of SCC-9 cells. *Statistical significance. GA, gallic acid.

Effect of GA on HIF-1α, caspase-3, E-cadherin, and Col1A1 expression

The hypoxic condition is clearly observed by the higher levels of HIF-1α with the addition of $CoCl_2$ in comparison with the control in HaCaT cells (Fig. 4a). Interestingly, GA decreased the levels of HIF-1α in HaCaT cells under hypoxic conditions (Fig. 4a). In contrast, the levels of HIF-1α were increased in SCC-9 cells treated with GA and $CoCl_2$ in comparison with GA alone and the control group (Fig. 4b). In addition, we also checked the HIF-1α protein levels in SCC-9 cells and observed that GA + $CoCl_2$ reduced HIF-1α levels in hypoxia, but it increased HIF-1α levels in normoxia (Fig. 4c and d). It is important to highlight that increasing HIF-1α levels were also observed in immortalized neoplastic cells with the use of *n*-propyl gallate [49].

To check if apoptosis could be activated as a consequence of GA + $CoCl_2$ treatment, we performed reverse transcription-PCR for caspase-3. GA + $CoCl_2$ increases caspase-3 transcription when compared with controls in HaCaT cells, but GA did not demonstrate effect in caspase-3 mRNA levels in cells under hypoxic conditions (Fig. 5a). In SCC-9 cells, GA had increased caspase-3 mRNA levels under hypoxic conditions, but did not alter caspase-3 mRNA levels in normoxia (Fig. 5b). We also used quantitative real-time PCR to evaluate the mRNA expression levels of EMT-related proteins (E-cadherin, Col1A1). The groups GA and $CoCl_2$ + GA induced upregulation of E-cadherin and suppression of Col1A1 in the SCC-9 cells (Fig. 5d and e). In HaCaT cells, GA and $CoCl_2$ + GA also induced an increase in RNA levels of E-cadherin (Fig. 5c). Col1A1

Fig. 4



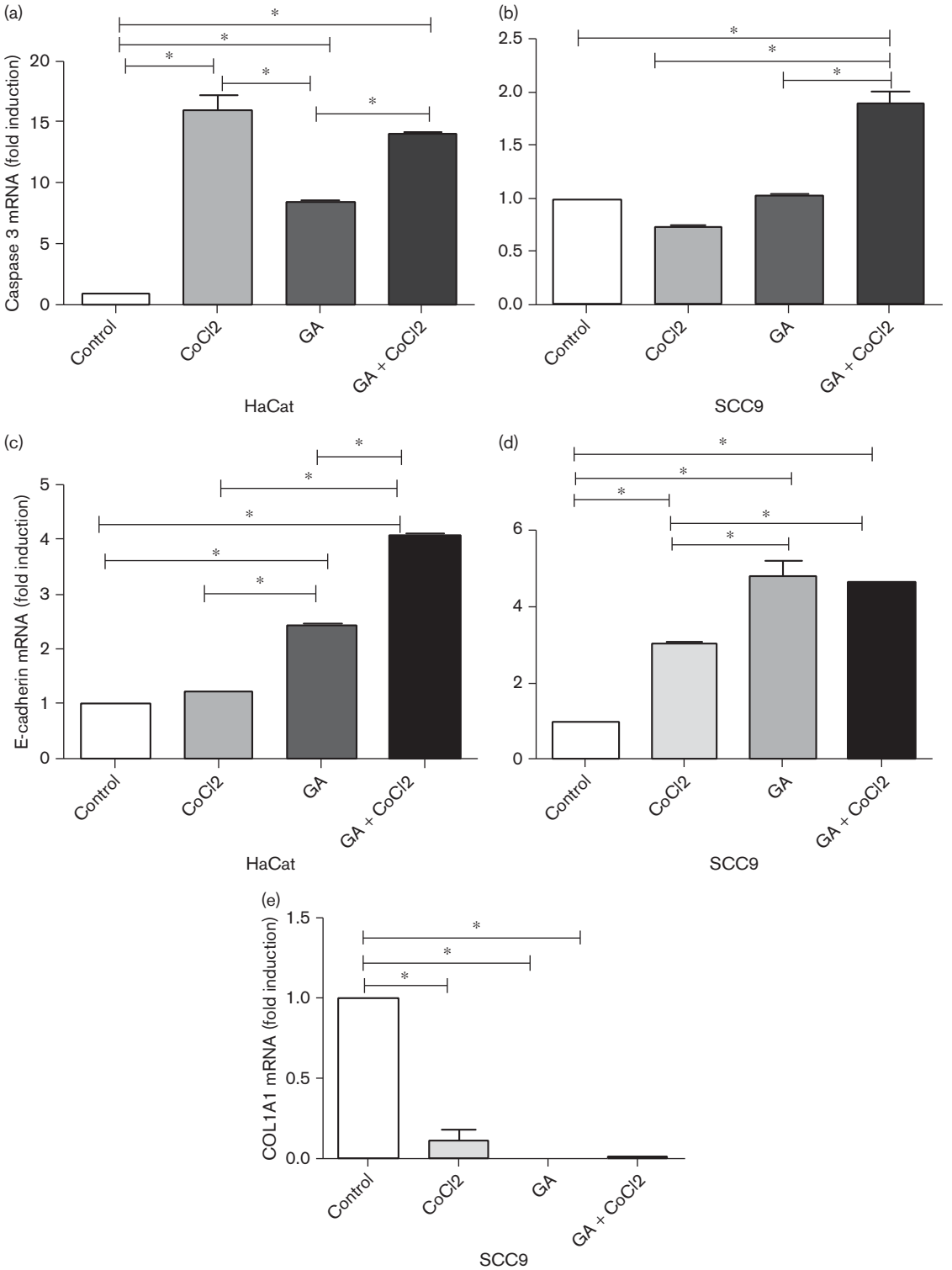
Effect of GA on HIF-1α under hypoxia. (a) Effect of GA on HIF-1α mRNA levels in HaCaT cells. (b) Effect of GA on HIF-1α mRNA levels in SCC-9 cells. (c) Quantification of the effect of GA on HIF-1α protein levels in SCC-9 cells, and (d) western blot of the effect of GA on HIF-1α protein levels in SCC-9 cells. *Statistical significance. GA, gallic acid; HIF-1α, hypoxia-inducible factor-1α.

was not detected in the HaCaT cells, as expected according to The Human Protein Atlas (*http://www.proteinatlas.org/ENSG00000108821-Col1A1/cell*) [50].

Discussion

Metastasis is a cause of death among the majority of cancer patients and is dependent on several steps, including vessel formation, cell adhesion, migration, invasion, and cell proliferation. These events are regulated by complex mechanisms [7,51]. A low oxygen microenvironment is one of the factors responsible for the induction of metastasis in several solid tumors [7]. In hypoxia, cells can alter their protein expression to maintain cell survival [52]. Under normal oxygen tension, HIF-1α is degraded by means of ubiquitin-dependent proteolysis, whereas HIF-1β is stable [7]. HIF-1 is a transcription factor complex, which is a basic helix–loop–helix transcription factor composed of subunits: HIF-1α and HIF-1β [18]. Angiogenesis, glycolytic metabolism, cell survival, and invasion are regulated by

Fig. 5



Effect of GA on caspase-3, E-cadherin, and Col1A1 mRNA levels under hypoxia. Effect of GA on caspase-3 (a, b) and E-cadherin (c, d) mRNA levels were detected in HaCaT and SCC-9 cells. Col1A1 (e) was detected only in SCC-9 cells. *Statistical significance. Col1A1, collagen type1A1; GA, gallic acid.

HIF-1α; this protein acts as an accelerating factor in tumor progression and metastasis [7,53]. In the current study, human information about the effect of GA under hypoxic conditions was assessed using bioinformatics. Bioinformatics suggested that cell cycling, proteolysis, and protein metabolisms are the main biological processes associated with GA treatment. Genetic [54] and epigenetic [55] factors associated with CDKN1A, which was one of the leader genes, has been associated with CDKN1A levels of several neoplasias. Evidence has demonstrated that p53 inactivation correlates with low E-cadherin expression [56,57].

Great importance is being placed on developing agents or drugs that can inhibit metastasis; however, antimetastatic agents are still lacking [58]. Numerous studies have established that GA inhibits cell growth, induces apoptosis, and suppresses migration and invasion in many cancer cell lines [53,59,60]. This present study showed that GA possesses strong anticancer properties; we investigated the effects of GA on invasion/migration/proliferation of OSCC cells. The results indicated that GA is able to inhibit in-vitro invasive and migratory abilities as well as inhibition of cell proliferation. In addition, GA also changes expression of genes associated with metastasis, HIF-1α, Col1A1, and E-cadherin. These effects might lead to the inhibition of metastatic ability. Thus, GA might have the potential to be used in oral cancer chemoprevention.

GA is one of the major components of natural products such as green tea, grapes, strawberries, and many other fruits [61]. Previous studies have reported that GA plays an important role in the prevention of malignant transformation and cancer development [18,62]. It has been shown that the loss of E-cadherin expression/function is associated with the development [57] and progression [63] of SCC. The loss of E-cadherin is frequently associated with EMT and the acquisition of a more invasive and metastatic phenotype [12,64]. In the current study GA recovered the levels E-cadherin in cancer cells under both normoxic and hypoxic conditions. In addition, our data demonstrate that GA, under hypoxic conditions, is associated with decreased HIF-1α protein expression as observed before [49]. The divergence between RNA and protein HIF-1α levels are explained by translational regulation of HIF-1α [65]. HIF-1α may control the expression of vascular endothelial growth factor and erythropoietin genes, promoting tumor angiogenesis, as both stimulate endothelial cell proliferation and migration. Erythropoietin alone promotes an increase in the proliferation and growth of various human neoplasms.

It is well-known that apoptosis can be stimulated through two main pathways, such as the mitochondria-dependent pathway (intrinsic pathway) and the death receptor-dependent pathway (extrinsic pathway) [26,66]. Fas, its receptor Fas ligand, and caspase-8 are part of an important cellular pathway regulating the induction of apoptosis in diverse cell types and tissues [66]. Here, our data indicated that treatment of GA markedly triggers caspase-3 transcription.

The novelty and significance of the current study is due to the anti-EMP effect of GA in OSCC context. These data indicate that dietary polyphenol could be a promising coadjutant agent for cancer treatment. On the other hand, in-vitro studies had important limitations by its characteristics.

In conclusion, the present study demonstrated that GA significantly reduces cell proliferation, invasion, and migration by increasing E-cadherin and repressing Col1A1.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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