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Rosmarinic Acid Inhibits Cell Growth and Migration in Head and Neck Squamous Cell Carcinoma Cell Lines by Attenuating Epidermal Growth Factor Receptor Signaling

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Abstract

Objective: Active components of natural foods are increasingly receiving attention for their chemopreventive and chemotherapeutic potential in a wide variety of cancers. Rosmarinic acid (RA), a phenolic compound in various herbal plants, is well-recognized for its anti-oxidant, anti-inflammatory, anti-proliferative properties in a variety of cell types. In this report, we describe a novel role for RA as an inhibitor of epidermal growth factor (EGF) stimulated signaling in HNSCC and propose a cellular reactive oxygen species (ROS)-mediated mechanism for the observed effects.

Methods: Cellular growth, migration and reactive oxygen species (ROS) profiles were examined in RA-treated and untreated HNSCC cell lines (UM- SCC-6 and UM-SCC-10B) using the WST-1 viability assay, established in vitro migration assays, and the CM-H2DCFDA ROS assay, respectively. The influence of RA on EGF-stimulated phosphorylation and its downstream pathways was evaluated using Western-blotting techniques.

Results: RA inhibited cell viability, migration and cellular production of ROS in HNSCC cell lines. Furthermore, RA inhibited EGF-induced phosphorylation of the EGFR at tyrosine residues 992 and 845, which led to down-regulation of the phosphatidylinositol 3-kinase Akt (PI3K/Akt) and mitogen-activated protein kinase ERK (MAPK/ERK) pathways.

Conclusions: This is the first report describing both growth- and motility-inhibitory roles for RA in HNSCC cells. Additionally, our study is the first to demonstrate that treatment with RA can reduce EGF-induced activation of PI3K/Akt in tumor cells. The present data suggests that RA holds promise as a chemotherapeutic agent against HNSCC.

Keywords: Rosmarinic acid; Epidermal growth factor receptor (EGFR); Epidermal growth factor (EGF); PI3K/Akt; MAPK/ERK; Reactive oxygen species; Signal transduction; Head and neck squamous cell carcinoma

Background

Head and neck squamous cell carcinoma (HNSCC) comprises approximately 90% of all head and neck cancers and represents the sixth most common type of cancer worldwide [1]. Historically, the treatment of HNSCC has been surgery and radiotherapy, but in the last several years the integration of surgery, radiotherapy and chemotherapy has become the standard of care. Unfortunately, despite advancements in these treatment approaches, the survival rates have not improved for decades. Furthermore, aggressive surgery, radiation, and chemotherapy treatments are physically and emotionally debilitating and are selected on the basis of tumor size and spread rather than on the biology of individual tumors, underscoring the need for new approaches to treat this malignancy [2].

Since epidermal growth factor receptor (EGFR) abnormality is found in up to 90% of HNSCC cases, overexpression of EGFR and resultant overactive downstream signaling through tyrosine kinases is thought to be causative for carcinogenesis, metastasis, and poor prognosis [3,4]. Indeed, recent studies in our laboratory have further characterized the role EGFR plays in HNSCC invasion through activation of RhoC GTPase and the phosphatidylinositol 3-Kinase/Akt pathway (PI3K/Akt), which leads to loss of the cell-to-cell adhesion molecule, epithelial cadherin (E-cadherin), and increased invasive potential [5]. The mitogen-activated protein kinase/ERK pathway (MAPK/ERK) is also activated by EGFR in HNSCC, which leads to increased tumor cell proliferation, migration and survival [6]. Monoclonal antibodies against EGFR (cetuximab) and tyrosine kinase inhibitors (gefitinib and erlotinib) have been developed to abrogate the deleterious effects of EGFR overexpression and signaling, but so far the full promise of these treatment approaches has not been fully realized due to intrinsic or acquired drug resistances [7,8]. The exact mechanisms responsible for these resistances are unclear, but a number of potential acquired mechanisms have been suggested [9]: (1) constitutive up-regulation of downstream effectors of EGFR (PI3K-Akt in particular), (2) activation of AXL receptor tyrosine kinase, (3) compensatory up-regulation of other receptor tyrosine kinases (RTKs), (4) Amplification of MET receptor tyrosine kinase, and (5) Epithelial to mesenchymal transition leading to E-cadherin loss. It is apparent that strategies to optimize EGFR-targeted therapy in head and neck cancer must include combination strategies to override resistance and more effectively abrogate co-activated pathways. In this study we describe

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a novel strategy to down-regulate EGFR signaling using the phenolic compound, rosmarinic acid.

Active components of natural foods are increasingly receiving attention for their chemopreventive and chemotherapeutic potential in a wide variety of cancers. One such food component, rosmarinic acid (RA), is a widely distributed phenolic compound in various herbal plants such as rosemary, sweet basil, sage, mint, and perilla (Figure 1) [10,11]. RA is regarded as a daily-consumed safe ingredient, due to its extensive use in the food industry for flavoring, but studies have also elucidated a medicinal role for RA, especially in regard to its anti-oxidant, and anti-inflammatory activities [12,13]. Furthermore, an emerging body of literature has also described the growth inhibitory and anti-invasion properties of RA in colon, skin, breast and ovarian cancers in vitro and/or in vivo [14-19], but the mechanisms underlying these effects are poorly understood. A chemopreventive role for RA in HNSCC has been described in 7,12- dimethylbenz (a) anthracene-induced hamster buccal pouch carcinogenesis [20], but a chemotherapeutic role for RA in the treatment of HNSCC has not yet been reported.

Given the anti-oxidant properties attributed to RA, it is possible that RA's anti-tumor effects stem from its ability to combat oxidative stress in HNSCC. Oxidative stress is a major contributor in the pathogenesis oral cancer. Enhanced nitrosative and oxidative stress with decreased total antioxidant capacity has been reported in oral precancer and oral squamous cell carcinoma patients [21]. Patients with oral cavity cancer exhibited lower total antioxidant capacity, uric acid concentration, salivary peroxidase and superoxide dismutase (SOD) activity in their saliva than did healthy subjects [22,23]. Studies have shown that lipid peroxidation, total antioxidant status, and total thiol levels predict overall survival of oral squamous cell carcinoma patients [24]. Reactive oxygen species (ROS) cause oxidative damage to DNA, protein, and lipids, contributing to mutagenesis and neoplasm formation [25]. Activation of the EGFR pathway under oxidative stress has been well recognized [26,27] and EGFR activation itself also increases ROS production [28,29]. Importantly, RA displays a strong scavenger activity for free radicals, such as peroxynitrite (ONOO−) and hydrogen peroxide (H_2O_2). Of the ROS generated by tumor cells, H_2O_2 deserves special mention here given that its production it increased when epidermal growth factor (EGF) binds to EGFR [28]. The newly generated H_2O_2 can then go on to help maintain EGFR phosphorylation through modification at a critical cysteine (Cys 797) in the active site [28].

In this study, we describe a novel role for RA as an inhibitor of EGFR-stimulated cell growth and migration in HNSCC, and propose a cellular ROS-mediated mechanism for the observed effects. We hypothesized that RA would reduce HNSCC viability and migration through inhibition of EGFR phosphorylation. We show that RA inhibited cell viability and migration in HNSCC in a dose-dependent manner and decreased cellular production of ROS. Furthermore, RA inhibited EGF-induced phosphorylation of EGFR at tyrosine residues 992 and 845, which led to down-regulation of the PI3K/Akt and MAPK pathways. To our knowledge, this is the first report describing both growth- and motility-inhibitory roles for RA in HNSCC cells, suggesting that RA holds promise as a chemotherapeutic agent against HNSCC.

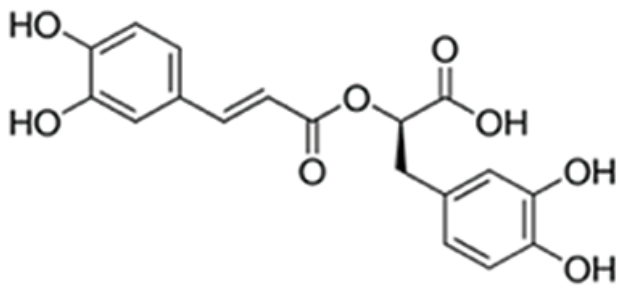

Figure 1: Chemical structure of RA.

Materials and Methods

Reagents

Rosmarinic acid and EGF were purchased from sigma Aldrich, USA. RA was solubalized in DMEM / F-12 medium, then filtered through a 0.22 μm membrane and diluted to concentrations ranging from 10-80 ug/ml.

Cell culture

HNSCC cell lines UM-SCC-6 and UM-SCC-10B were grown in Dulbecco's modified Eagle's medium / F-12 (DMEM/F-12, Thermo scientific) containing 2.5 mM L-Glutamine, 15 mM HEPES buffer, 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomysin.

Cell viability

Cells were incubated in 96-well plates (5×10^3 cells/well) in 10% FBS DMEM/F-12 medium for overnight, and cells were starved with FBS free medium for 6 h. Then cells were treated with different concentrations of RA for 24 h. After that, cells were incubated with WST-1 solution (10 μl/well) for another 3 h. Absorbance was measured using a microplate reader at 450 nm.

Cell migration assay

Cell migration was examined by wound healing assay and the QCM™ 24-Well Colorimetric Cell Migration assay kit (Millipore Corporation, Billerica, MA, USA). Cells were cultured in 6-well plates until confluence followed by a change to FBS free medium for overnight starvation. Cells were then wounded by using a sterile 200 ul pipet tip and washed twice with PBS. After washing, cells were incubated with or without 80 μg / ml RA for two to three days. The migration was observed under the microscope (NiKON Eclipse TS100, Japan). The images were taken using cyberlink Power Director 10 software at 4X magnification. For the migration assay kit, cells were treated according to manufacturer's instruction. Cells were stimulated with EGF with or without RA for 48 h and the results were read by using microplate reader at 562 nM.

Measurement of ROS production

To measure the production of ROS, CM-H2DCFDA (Invitrogen), a ROS-sensitive fluorescent dye, was used as a ROS probe. Cells were incubated in 96-well plates (104 cells/well) in 10% FBS DMEM/F-12 medium for overnight, and cells were starved with FBS free medium for 6 h. Then the cells were treated with different concentrations of RA for 24 h. After removal of the RA medium, the cells were incubated with 5 μM CM-H2DCFDA and washed twice with PBS. H_2O_2 release from cells was determined using Aplex® Red hydrogen peroxide/peroxidase assay kit according to the manufacture's protocol. Fluorescence intensity was measured at excitation 530/25 nm and emission 590/35 nm using a fluorescence microplate reader.

Western blot analysis

Cells were washed with ice-cold phosphate - buffered saline and lysed in RIPA buffer (25 mM Tris.HCl pH 7.6, 150 mM NaCl, 1% NP-

40, 1% sodium deoxycholate, 0.1% SDS) containing Halt™ protease inhibitor cocktail (thermo scientific). For Phospho – antibodies the lysis buffer was supplemented with Halt™ phosphotase inhibitor cocktail (thermo scientific). Cell lysates were scraped into Microfuge tubes and left for 20 min with occasional vortexing, and was pelleted by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was collected, and protein content was measured by the Coomassie (Bradford) Protein Assay Kit (Thermo scientific). Equal amount of protein (25 µg) were electrophoresed on 8 or 12% SDS-PAGE gels and transferred to PVDF membranes (BioRad). The membranes were blocked with 5% nonfat milk or with 3% BSA in Tris - buffered saline containing 0.1% Tween -20 (TBS-T) (Fisher) for 1 h at room temperature to block nonspecific binding. Membranes were incubated with the primary antibody for 2h at room temperature or overnight at 4°C. primary antibody concentrations were as follows: EGF receptor (D38B1) XPTM rabbit monoclonal antibody (1:1000), phospho-EGF receptor (Tyr-992) antibody (1:500), phospho-EGF receptor (Tyr-845) antibody (1:1000), phospho-GSK-3β (ser9) (5B3) rabbit monoclonal antibody (1:2000), GSK-3β (27C10) rabbit monoclonal antibody (1:2000), phospho-p44/42 MAPK (ERK1/2) (Thr-202/Tyr-204) (D13.14.4E) XPTM rabbit monoclonal antibody (1:2000), p44/42 MAPK (ERK1/2) (Thr-202/Tyr-204) (137F5) rabbit monoclonal antibody (1:2000), phospho-Akt (Ser473) (D9E) XPTM rabbit monoclonal antibody (1:2000), Akt (pan) (C67E7) rabbit monoclonal antibody (1:2000). Membranes were washed in TBS-T. Anti-rabbit IgG, HRP-linked antibody was used as a secondary antibody to detect primary antibodies. Visualization of immunoreactive proteins was accomplished by superSignal® West Pico chemiluminescent substrate (Thermo Scientific) and exposure to film.

Statistical analysis

Data were expressed as mean ± SD. To compare the values between the groups, student t-test or one way ANOVA test were performed. Results were considered statistically significant when the p value was less than 0.05.

Results

RA inhibits HNSCC cell viability

Rosmarinic acid's cytotoxic and tumor preventative properties continue to come into focus in the literature. For example, a recent study by Karthik kumar et al., showed that RA supplementation reduced cell proliferation markers in 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis [30] and other studies in the last five years have described cytotoxic and anti-proliferative roles for RA in a number of other cancer cell types [19,31-33]. In this study, initial experiments examined whether treatment with RA at concentrations ranging from 10-80 µg/ml, could reduce HNSCC cell numbers at 24 hrs. The RA concentrations were selected based on previous studies where RA doses in the 10-80 µg/ml range inhibited proliferation in ovarian cancer cell lines [19] and reduced the invasive capability of colon cancer cells [14]. UM-SCC-6 (originating from the ventral tongue) and UM-SCC-10B cells (derived from lymph node metastasis) were chosen for experiments based on the observation that total EGFR was highly expressed in these cells and endogenous EGFR phosphorylation was relatively low in the absence of exogenous epidermal growth factor stimulation. RA reduced cell viability in both cell lines in a dose dependent matter, however cell-specific differences were observed: RA significantly inhibited cell growth of UM-SCC-6 cells at concentrations as low as 10 µg/ml (Figure 2A) whereas UM-SCC-10B cell growth was significantly inhibited at concentrations of 40 µg/ml and higher (Figure 2B). The 80 µg/ml dose was chosen for subsequent experiments based on the following considerations:1) this dose produced the most significant degree of inhibition of proliferation in all cell lines; and 2) use of the 80 µg/ml dose has been supported in the literature for other tumor cell types [14].

MAPK cascades function downstream of cell surface receptors and other cytoplasmic signaling proteins whose functions are deregulated in cancer and other human pathologic disorders [34]. MAPKs transduce a vast array of extracellular signals into intracellular responses controlling proliferation, differentiation, cell motility and apoptosis. To date, three major MAPK subfamilies have been described in detail: ERK1 and 2 (extracellular signal regulated kinase 1 and 2), p38-MAPK and JNK/SAPK (c-Jun amino-terminal kinase/stress activated protein kinase). There is robust evidence that MAPK/ERK signaling promotes HNSCC cell proliferation, cell survival, and metastasis, along with the overwhelming frequency in which this pathway is aberrantly activated by upstream phosphorylation of EGFR [6]. In the current study, treatment of UM-SCC-6 cells with 80 µg/ml of RA inhibited ERK phosphorylation (Figure 2C), paralleling the observed reduction in viability and migration in these cells at the same RA concentration.

RA reduces cell migration

It is generally accepted that the high mortality and poor prognosis associated with HNSCC is best predicted by the presence of cervical lymph node metastases, a common and adverse event in patients with HNSCC, reducing survival by approximately 50% [35,36]. To evaluate the effect of RA on cell migration, HNSCC cells were treated with 80 µg/ml RA from 24 h to 72 h. RA significantly reduced migration of UM- SCC-6 cells up to 48 hrs and reduced UM-SCC-10B cell migration as late 72 hours as shown in Figures 3A and 3B, respectively. This difference in cellular response is consistent with other treatment agents that display distinct cellular effects at a given dose, depending on cell type.

RA serves as a potent antioxidant in HNSCC

Oxidative stress is elevated and antioxidant defenses are often overwhelmed in patients with HNSCC [21,37]. Specifically, generation of ROS has been implicated in HNSCC development in tobacco users

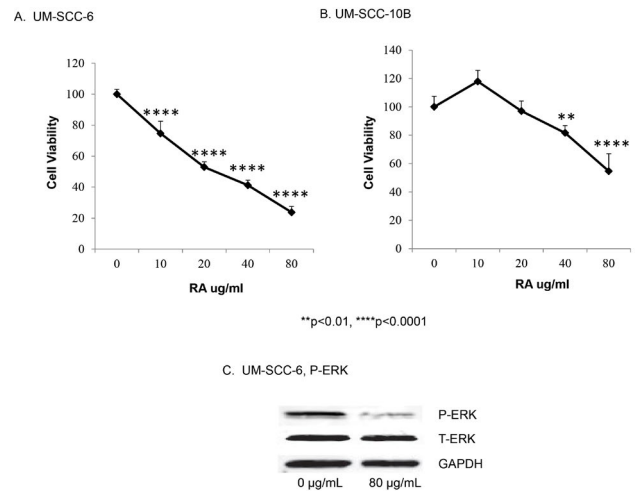

Figure 2: Effect of RA on cell growth. Cells were treated at concentrations ranging from 10 µg/ml to 80 µg/ml of RA for 24 hrs. and then treated with WST-1 for 3 h. UM-SCC-6 (A) and UM-SCC-10B (B) cell viability was determined by measuring absorbance at 460 nm. Data are expressed mean ± SD (n = 5). (C) UM-SCC-6 cells were treated with 80 ug/ml RA and expression of P-ERK was measured by western-blotting. **p<0.01, ****p<0.0001 *vs* control.

where elevated oxidative stress increases lipid peroxidation and oxidative DNA damage [37]. ROS can also serve as signaling agents themselves by activating a number of key signaling cascades that are important to tumor development and progression, such as the PI3K-Akt and MAPK pathways. Therefore, we hypothesized that perhaps RA exerts its effects on cell growth and migration through its antioxidant capacity. To determine antioxidant capacity we measured cellular ROS levels in UM-SCC-6 and UM-SCC-10B cells following 24 hrs of treatment with increasing concentrations of RA. RA treatment significantly reduced ROS levels in UM-SCC-6 cells at concentrations as low as 20 µg/ml RA (Figure 4A) whereas statistically significant decreases in ROS were seen at the lower 10 µg/ml dose in UM-SCC-10B cells (Figure 4B). Not surprisingly, the highest degree of ROS inhibition for both cell lines was observed at the 80 µg/ml concentration.

RA inhibits EGFR phosphorylation and abrogates PI3K and MAPK signaling

The importance of EGFR expression, phosphorylation, and activation of downstream effectors has been well characterized in a variety of tumor types [38-41]. Upon activation by its growth factor ligands, EGFR undergoes a transition from an inactive monomeric form to active homodimer and heterodimers. EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity and autophosphorylation of several tyrosine residues in the C-terminal domain of EGFR occurs [42]. In the current study, EGFR phosphotyrosine residues Tyr-992 and Tyr-845 were selected for investigation based on their known associations with tumor progression. In the presence of 100 ng/ml of EGF, EGFR was strongly phosphorylated at residues Tyr-992 (Figure 5A) and Tyr-845 (Figure 5B) in UM-SCC-6 cells. However, when the cells were pre-incubated with 80 µg/ml RA for 30 minutes prior to EGF stimulation, phosphorylation at both the Tyr-992 and Tyr-845 residues was significantly reduced (Figures 5A and 5B, respectively).

EGFR autophosphorylation elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. The PI3K/Akt/GSK3β pathway represents one such downstream signaling cascade following EGFR phosphorylation in HNSCC, where it is involved in tumor development and progression and likely plays a role in tumor resistance to radiotherapy and chemotherapy [5,43,44]. As seen in Figures 5C and 5E, pre-treatment with 80 µg/ml of RA in UM-SCC-6 cells decreased EGF-induced phosphorylation of Akt and GSK-3β ser9 by 56% and 54%, respectively. Similarly, RA pre-treatment abrogated EGF-stimulated phosphorylation of the MAPK family member, ERK, by 46% (Figure 5D).

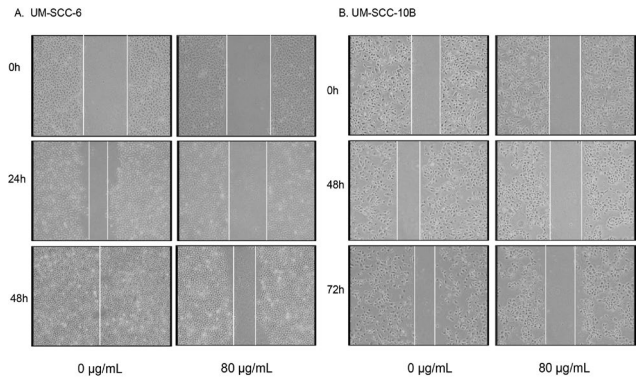

Figure 3: Effect of RA on cell migration. UM-SCC-6 (A) and UM-SCC-10B (B) cells were treated with 80 µg/ml of RA for 24 to 72 hrs and cell migration was determined by wound healing assay.

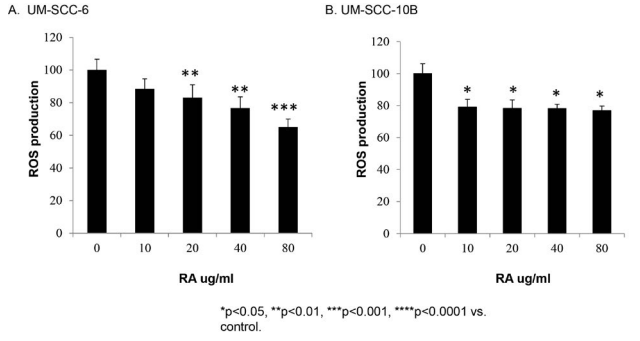

Figure 4: Effect of RA on ROS production. UM-SCC-6 (A) and UM-SCC-10B (B) cells were treated with RA at concentrations ranging from 10 µg/ml to 80 µg/ml for 24 hrs, followed by incubation with CM-H_2DCFDA for 30 mins. ROS production was determined by measuring fluorescence at excitation 485/20 and emission at 528/20. Data are expressed mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.0001 *vs* control.

RA reduces EGF-induced cell growth, migration and ROS generation

We next evaluated the inhibitory effect of RA on EGF- induced cell growth, migration, and ROS generation in HNSCC cells. As shown in Figure 6, 80 µg/ml RA inhibited EGF-induced cell viability and migration in UM-SCC-6 (Figures 6A and 6C, respectively) and UM-SCC-10B (Figures 6B and 6D, respectively) cells as well as reduced both EGF-stimulated total ROS and H202 production in UM-SCC-6 (Figures 7A and 7C, respectively) and in UM-SCC-10B cells (Figures 7B and 7D, respectively). H_2O_2, one of the ROS most implicated in tumor cell signaling and progression, deserves special mention here because of the role it plays in PI3K and MAPK signaling [28].

Discussion

In this study, we describe the anti-cancer effects of RA in HNSCC cell lines. To our knowledge, this is the first report describing a potential chemotherapeutic role (rather than a chemopreventive role)

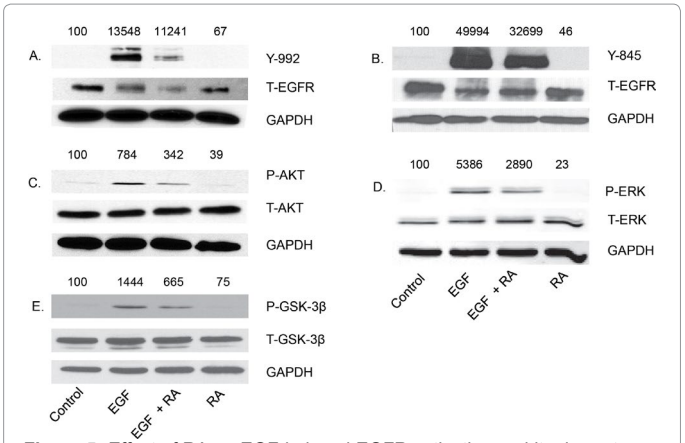

Figure 5: Effect of RA on EGF-induced EGFR activation and its downstream pathway. Cells were pretreated with RA for 30 minutes and then stimulated with EGF for 5 minutes. Phosphorylation of EGFR at tyrosine 992 (A) and tyrosine 845 (B) and its downstream effectors, Akt (C), ERK (D) and GSK-3βser9 (E) and were analyzed by western blotting. The membrane was subsequently blotted with total Akt, GSK-3βser9, ERK and GAPDH antibodies as loading controls.

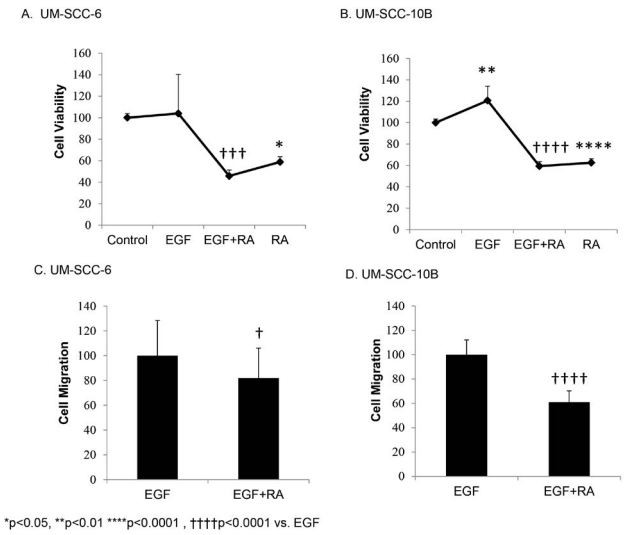

Figure 6: Effect of RA on EGF-induced cell growth and migration**.** Cells were pretreated with RA for 30 minutes and then stimulated with EGF for 24 hrs. UM-SCC-6 (A) and UM-SCC-10B (B) cell growth was determined by measuring absorbance at 460 nm. Data are expressed as mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001 *vs.* control. †††p<0.001, ††††p<0.001 *vs* EGF. To determine the effect of RA on migration, cells were pretreated with RA for 30 minutes, stimulated with EGF and migration was analyzed at 48 hrs. for UM-SCC-6 (C) and 72 hrs. for UM-SCC-10B cells (D). Data are expressed mean ± SD (n = 6). †p<0.05, ††††p<0.001 *vs* EGF.

for RA in HNSCC. In UM-SCC-6 cell lines, RA significantly inhibited cell viability at 10 µg/ml RA, but in UM-SCC-10B cells it significantly blunted growth at the higher dose of 40µg/ml (Figures 2A and 2B, respectively). The maximal inhibitory effect of RA was calculated for each cell line and the IC50 value for UM-SCC-6 cells was 20 µg/ml and for UM-SCC-10B cells it was 40 µg/ml. Similarly, RA at a fixed dose of 80 µg/ml maximally inhibited migration at different time points between the two cell lines. In UM-SCC-10B cells, RA restrained migration at 48 hours to a degree that was indistinguishable from baseline, whereas in UM-SCC-6 cells migration was inhibited to a lesser degree at 48 hours compared to baseline (Figures 3A and 3B respectively). This outcome parallels that of other emerging natural compounds being investigated in the fight against HNSCC. For example, Masuda et al. found that the YCU-N 861 cell line, derived from squamous cell carcinoma of the hypopharynx, was more sensitive to lower concentrations of a green tea extract called epigallocatechin-3-gallate (EGCG) than the nasopharynx-derived YCU-H891 cell line in that 1.0 µg/ml of EGCG caused ~50% inhibition of YCU-N861 cells but had no effect on YCU-H891 cells [45]. The difference in the current study might be explained by the different growth profiles of the two cells; namely, UM-SCC-6 cells in our experience double at a faster rate and migrate more readily as compared to UM-SCC-10B cells. Another possible explanation might be that the two different cell lines preferentially up regulate different signaling cascades governing their aberrant growth and migratory properties, and that RA favorably targets one or more of these cascades more robustly than others. Whatever the explanation, these findings suggest that RA possesses anti-proliferative and anti-migratory properties across different HNSCC cell lines, but the dose and time course required to significantly reduce these hallmark tumor progression processes, and the degree of the effect at a given dose or time point, will likely be different based on cell type. Further studies will be necessary across several more cell lines to more comprehensively characterize these differences.

A number of studies have shown that RA's anti-cancer effects stem from modulation of key signaling cascades known to be implicated in tumor development and progression. For example, Xavier et al. showed that RA reduced cell growth and inhibited the MAPK/ERK pathway in the colon carcinoma-derived cell line, CO115 [46]. Consistent with this finding, we observed inhibition of ERK phosphorylation in HNSCC following treatment with 80 µg/ml RA (Figure 2C). However, it is important to note that there are a number of signaling pathways, in addition to the MAPK/ERK pathway, that are responsible for cell proliferation and survival in HNSCC, such as Wnt [47] and JAK/STAT [48,49] signaling. Given the relative paucity of information in the literature describing the effects of RA on tumor cell signaling, next steps in our laboratory will focus on elucidating parallel signaling cascades and upstream receptors that might also be targeted by RA in HNSCC.

In this report, we show that RA inhibits both EGF-induced and non-EGF treated HNSCC cell migration (Figures 7 and 4, respectively). It is likely that this inhibition of migration is at least in part explained by RA's ability to attenuate EGFR phosphorylation (Figures 5A and 5B) given EGFR's established role in HNSCC invasion and metastasis [50]. Upon EGF binding, EGFR dimerizes and stimulates its intrinsic intracellular protein-tyrosine kinase activity and autophosphorylation of several tyrosine residues in the C-terminal domain [42]. We examined two important EGFR tyrosine kinase domains, Tyr-992 and Tyr-845, based to their known associations with tumor progression. Specifically, Tyr-992 has been shown to increase the duration of interaction with effector molecules, link EGFR to a diversity of downstream signaling pathways, including the PI3K pathway, and affect cytoskeletal remodeling leading to increased cell migration [5,51,52]. Phosphorylation of EGFR at Tyr-845, on the other hand, plays important roles in EGFR nuclear translocation, a cause of drug resistance in HNSCC chemotherapy, and this tyrosine residue has been described as a prognostic biomarker in oral squamous cell carcinoma [53,54]. Importantly, we demonstrated

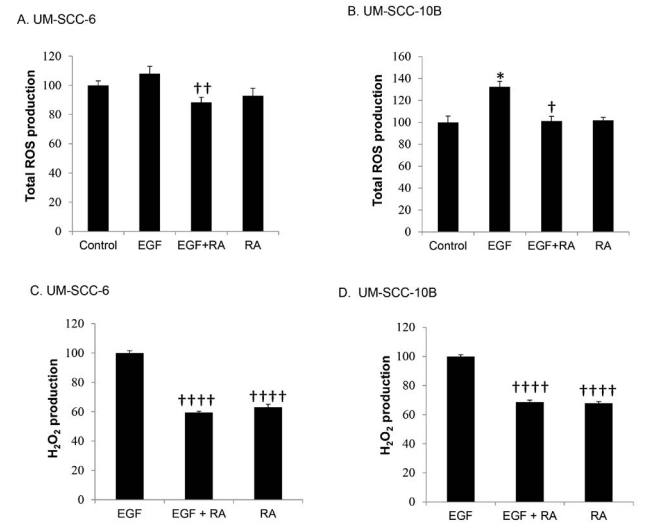

Figure 7: Effect of RA on EGF–induced total ROS and H_2O_2 production. Cells were pretreated with RA for 30 minutes followed by stimulation with EGF. Then, cells were incubated with CM-H_2DCFDA for 40 min. Total ROS production was determined in UM-SCC-6 (A) and UM-SCC-10B (B) by measuring fluorescence at excitation 485/20 and emission at 528/20. Data are expressed as mean ± SD (n = 5). *p<0.05 *vs* control. †p<0.05, ††p<0.01 *vs* EGF. To measure H_2O_2 production, cells were treated with RA for 2 h and then stimulated with EGF for 5 minutes. The amount of H_2O_2 was detected in UM-SCC-6 (C) and UM-SCC-10B (D) using the Amplex™ peroxide assay. Data are expressed as mean ± SD (n = 5). ††††p<0.001 *vs* EGF.

that RA is capable of significantly down-regulating EGF-induced EGFR phosphorylation at Tyr-992 and Tyr-845, and this attenuation of EGFR signaling correlates with reductions in HNSCC cellular viability and migration. In future experiments it will be important to explore RA's effects on additional tyrosine kinase domains.

It is well established that PI3K/AKT and GSK-3β are major downstream signaling effectors of EGFR and that they play key roles in cancer cell proliferation, cell survival, cell migration, and drug resistance [8]. GSK-3β plays a major role in epithelial cell homeostasis [55,56], and its activity is regulated by site-specific phosphorylation of Tyr-216/Ser9 residues. Phosphorylation at ser9 can cause various pathological conditions, including epithelial cancers, and GSK-3βser-9 is increasingly becoming a target for oral cancer treatment development [57]. Activation of the PI3K/Akt signaling cascade leads to inhibition of GSK-3β [58] and loss of cell-to-cell contact through negative regulation of the cell adhesion protein, E-cadherin [38,40,41]. This results in enhanced migratory and invasive capacity in HNSCC [5]. Our study is the first to demonstrate that treatment with RA can reduce EGF-induced phosphorylation of Akt and GSK-3βser-9 in tumor cells (Figure 5C and 5E, respectively), an intriguing outcome given constitutive up-regulation of downstream effectors of EGFR (PI3K-Akt in particular) and E-cadherin loss have both been implicated in the development of drug resistance to EGFR-target therapies [9].

Activation of EGFR increases ROS generation and release, which serves an up-stream messenger for the PI3K and MAPK pathways [28]. Elevated ROS is a main trigger of cell proliferation and migration [59,60]. Here, we show that cells pretreated with RA reduced EGF-induced total ROS production and H_2O_2, specifically, in HNSCC cell lines (Figure 7). These results suggest that, through its ROS scavenger activity, RA can block down-stream signaling of EGFR that regulates cell proliferation and migration. This result is not surprising given RA's well-established ability to protect against ROS-mediated effects in a number of tissues and disease states, including protection of the liver from acute oxidative damage [61] and resistance to memory loss in Alzheimer's disease [62]. Exploring RA's effects on H_2O_2 release is important in this context, because recent discoveries have shown that EGFR signaling results in H_2O_2 production and oxidation of downstream proteins, and that the direct modification of EGFR by H_2O_2 at a critical active site cysteine (Cys797), enhances its tyrosine kinase activity [28]. Even though RA's status as an ROS scavenger represents a probable explanation for the observed effects on EGFR signaling in HNSCC cells, it is important to point out that other possible mechanisms may be at play (Figure 8). For example, it is possible that RA acts directly on EGFR tyrosine kinase domains to inhibit the receptor and its downstream signaling cascades. Other possibilities include influences on EGF binding and direct inhibition of downstream effectors.

Conclusions

Taken together, the above data support the continued exploration of RA as a promising cancer chemotherapeutic agent in HNSCC. In vivo use of RA alone or with other chemotherapeutic reagents in HNSCC animal models will be the logical next step in determining RA's potential clinical efficacy. Considering RA is recognized for its role in liver and kidney protection and drug resistance, in addition to its anti-tumor effects, this natural compound holds immense potential in the combat against HNSCC.

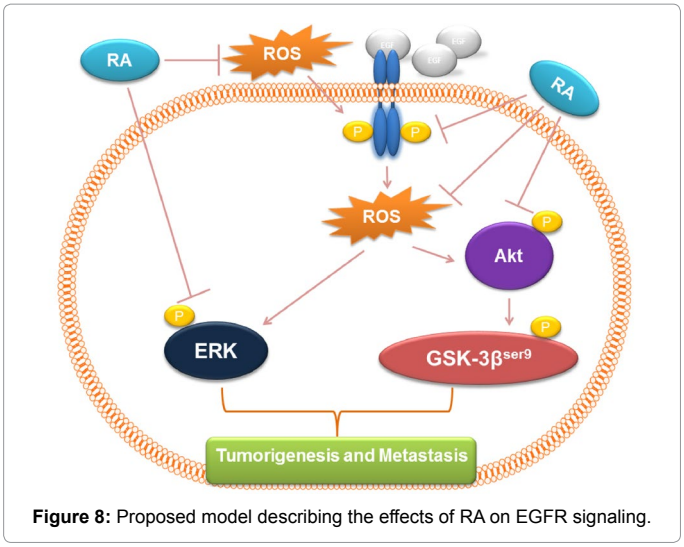

Figure 8: Proposed model describing the effects of RA on EGFR signaling.

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