

## Research Article

# Dual Inhibition of Vascular Endothelial Growth Factor Receptor and Epidermal Growth Factor Receptor is an Effective Chemopreventive Strategy in the Mouse 4-NQO Model of Oral Carcinogenesis

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## Abstract

Despite recent therapeutic advances, several factors, including field cancerization, have limited improvements in long-term survival for oral squamous cell carcinoma (OSCC). Therefore, comprehensive treatment plans must include improved chemopreventive strategies. Using the 4-nitroquinoline 1-oxide (4-NQO) mouse model, we tested the hypothesis that ZD6474 (Vandetanib, ZACTIMA) is an effective chemopreventive agent. CBA mice were fed 4-NQO (100 µg/mL) in their drinking water for 8 weeks and then randomized to no treatment or oral ZD6474 (25 mg/kg/d) for 24 weeks. The percentage of animals with OSCC was significantly different between the two groups (71% in control and 12% in the ZD6474 group;  $P \leq 0.001$ ). The percentage of mice with dysplasia or OSCC was significantly different (96% in the control and 28% in the ZD6474 group;  $P \leq 0.001$ ). Proliferation and microvessel density scores were significantly decreased in the ZD6474 group ( $P \leq 0.001$  for both). Although proliferation and microvessel density increased with histologic progression in control and treatment cohorts, epidermal growth factor receptor and vascular endothelial growth factor receptor-2 phosphorylation was decreased in the treatment group for each histologic diagnosis, including mice harboring tumors. OSCC from ZD6474-treated mice exhibited features of epithelial to mesenchymal transition, as shown by loss E-cadherin and gain of vimentin protein expression. These data suggest that ZD6474 holds promise as an OSCC chemopreventive agent. They further suggest that acquired resistance to ZD6474 may be mediated by the expression of an epithelial to mesenchymal transition phenotype. Finally, the data suggests that this model is a useful preclinical platform to investigate the mechanisms of acquired resistance in the chemopreventive setting. *Cancer Prev Res*; 3(11); 1493–502. ©2010 AACR.

## Introduction

With an annual incidence of nearly 600,000 cases, oral and pharyngeal squamous cell carcinoma is the sixth most common malignancy in the world today (1). There will be over 35,000 new cases in the United States in 2010 with nearly 8,000 deaths from the disease (2). When focusing specifically on the oral cavity squamous cell carcinoma (OSCC), it is estimated that there will be over 23,000 new cases and more than 5,300 deaths (3). Despite advances in diagnosis and treatment, improved long-term survival for OSCC patients has remained modest. Several factors contribute to this relatively poor outcome. First, OSCC is often diagnosed at an advanced stage. The 5-year

survival rate of early stage disease is approximately 80%, although the survival drops to approximately 20% for late stage disease (2). Second, as a result of field cancerization, the development of multiple primary tumors has a major effect on survival. For patients with early stage disease, second primary tumors are their most common cause of treatment failure and death (4, 5). Therefore, to improve outcomes, a comprehensive treatment plan must include both improved early detection and secondary prevention.

Chemoprevention can be defined as the use of natural or synthetic agents to reverse or halt the progression of premalignant lesions. Chemopreventive agents are currently being tested for their efficacy in preclinical and clinical settings for many malignancies including OSCC (6, 7). However, initial promising results for OSCC chemoprevention have not been consistently reproduced and toxicity has often been a significant complication. The issue of toxicity is particularly important in the realm of chemoprevention as it is conceivable that patients may require therapy for prolonged periods of time.

Angiogenesis is an essential phenotype in both physiologic and pathologic settings including growth and development,

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wound healing, reproduction, arthritis, and tumor formation (8). Because of its critical role in cancer biology, the inhibition of tumor angiogenesis is an attractive target for cancer therapy. The induction of the angiogenic phenotype in OSCC is mediated by the direct and indirect production of various factors capable of inducing blood vessel growth (9). Among these, the vascular endothelial growth factor (VEGF) family is thought to play an important role. The biological effects of the VEGF ligands are mediated through their binding to members of the VEGF receptor family (VEGFR-1, VEGFR-2, and VEGFR-3). This interaction leads to the autophosphorylation of specific tyrosine residues and subsequent downstream activation of intracellular signaling pathways, such as the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways. Importantly, the expression of the angiogenic phenotype is one of the first recognizable phenotypic changes observed in both experimental models as well as in human OSCC (10–13), suggesting that inhibitors of angiogenesis may also hold promise in the field of chemoprevention.

The development, growth, and survival of OSCC are also highly dependent on the epidermal growth factor receptor (EGFR) signaling pathway. EGFR is a transmembrane glycoprotein that is a member of the ErbB/HER receptor tyrosine kinase family. Upon ligand binding, EGFR signaling is mediated by the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways. Increased expression of EGFR and its ligand transforming growth factor- $\alpha$  (TGF- $\alpha$ ) are observed in most OSCC and premalignant oral lesions, and this expression correlates with poor prognosis (14). In addition to directly influencing tumor cell growth, members of the EGFR pathway can contribute to the expression of the angiogenic phenotype. For example, the expression of either TGF- $\alpha$  or EGFR results in increased expression of VEGF (15, 16). Because of its importance in epithelial malignancies, there is considerable interest in targeting the EGFR pathway in the realm of chemoprevention.

ZD6474 (Vandetanib, ZACTIMA) is an orally available tyrosine kinase inhibitor with direct activity against multiple signal transduction pathways including VEGFR-2 and EGFR (17–19). ZD6474 has an  $IC_{50}$  of  $\sim 0.04$   $\mu\text{mol/L}$  for VEGFR-2 and an  $IC_{50}$  of  $0.5$   $\mu\text{mol/L}$  for EGFR (18, 20). In preclinical studies, ZD6474 was found to be a potent inhibitor of tumor angiogenesis and the proliferation of a number of different tumor cell types including OSCC xenografts (21–30). Furthermore, it is currently under active investigation in clinical trials for the treatment of various malignant neoplasms (31). To date, it has been found to have greatest activity in non-small cell lung cancer and recurrent medullary thyroid cancer (32–34). However, the clinical utility of this agent in the realm of chemoprevention, particularly for OSCC, is unknown. Because it has the potential to inhibit two pathways that are essential for the development of OSCC, we tested the hypothesis that ZD6474 is an effective chemopreventive agent in the 4-NQO model.

## Materials and Methods

### Administration of 4-NQO and treatment with ZD6474

CBA mice, 6 to 8 weeks of age, were purchased from The Jackson Laboratory and housed in the Animal Resource Facility under controlled conditions and fed normal diet and autoclaved water. All animal procedures were carried out in accordance with Institutional Animal Care and Use Committee-approved protocols. Mice were given 4-NQO in their drinking water on a continuous basis at the required dose for the required duration as previously described (35). Briefly, 4-NQO powder (Sigma) was first dissolved in DMSO at 50 mg/mL as a stock solution and stored at  $-20^{\circ}\text{C}$  until used. On the days of 4-NQO administration, the stock solution was dissolved in propylene glycol (Sigma) and added to the drinking water bottles containing autoclaved tap water to obtain a final concentration of 100  $\mu\text{g/mL}$ . A fresh batch of water was prepared every week for each of the 8 weeks of carcinogenic treatment. Normal autoclaved drinking water was resumed at the end of this period. Control mice not receiving 4-NQO were given water containing vehicle only. ZD6474 was provided by Astra Zeneca and dissolved in Tween 80 solution (P8192-5X10ML, Sigma). Mice receiving ZD6474 treatment were given a daily dosage of 25 mg/kg/d for 24 weeks via oral gavage.

### Histologic examination

Mice were sacrificed in accordance with Institutional Animal Care and Use Committee recommendations. Specifically, cervical dislocation was done subsequent to anesthesia by i.p. injection of xylazine and ketamine. Immediately following death, the tongues were excised, longitudinally bisected, and processed in 10% buffered formalin and embedded in paraffin. Fifty 5- $\mu\text{m}$  sections from each specimen were then cut and the 1st, 10th, 20th, 30th, 40th, and 50th slides were stained with H&E for histopathologic analysis. Histologic diagnoses were rendered as previously described (35). Briefly, hyperkeratoses were characterized by a thickened keratinized layer, with or without a thickened spinous layer (acanthosis), and an absence of nuclear or cellular atypia. Dysplasias were characterized as lesions that showed various histopathologic alterations including enlarged nuclei and cells, large and/or prominent nucleoli, increased nuclear to cytoplasmic ratio, hyperchromatic nuclei, dyskeratosis, increased and/or abnormal mitotic figures, bulbous or teardrop-shaped rete ridges, loss of polarity, and loss of typical epithelial cell cohesiveness. Because of the subjective nature of grading of epithelial dysplasia and its limited ability to predict biological progression (36, 37), we chose not to assign descriptive adjectives of “severity” to the dysplastic lesions. Rather, we grouped all lesions demonstrating cytologic atypia but lacking evidence of invasion into the single category of dysplasia. HNSCC were characterized by lesions that showed frank invasion into the underlying connective tissue stroma.

### Immunohistochemistry

For detection of phosphorylated EGFR (pEGFR) and phosphorylated VEGFR-2 (pVEGFR-2), antigen retrieval was achieved on deparaffinized 5  $\mu$ m sections using Immuno/DNA retriever with citrate (Bio SB). Endogenous peroxidase activity was quenched with mouse/rabbit ImmunoDetector Peroxidase Block Kit. Sections were incubated using primary antibody to pVEGFR-2 1:300 (Abcam) or pEGFR 1:250 (Cell Signaling) for 1 hour at room temperature. Antibody binding was visualized by using mouse/rabbit ImmunoDetector HRP/DAB Detection System (Bio SB).

For detection of CD31 and vimentin, antigen retrieval was achieved by using 10 mmol/L of citrate buffer (pH 6.0) on 5  $\mu$ m deparaffinized sections. Endogenous peroxidase activity was quenched with 1% hydrogen/methanol. The primary antibody for vimentin (Epitomics) was applied at 1:250 dilution for a 1-hour incubation at room temperature. For CD31 (Abcam), a 1:50 dilution was applied, followed by anti-rabbit polymer-labeled horseradish peroxidase (HRP)-bound secondary reagent (DAKO EnVision+ System, HRP).

For detection of E-cadherin and Ki67, antigen retrieval was achieved on 5- $\mu$ m deparaffinized sections using 10 mmol/L of Tris-base and 1 mmol/L of EDTA (pH 9.0). Endogenous peroxidase activity was quenched with 1% hydrogen peroxide/methanol. The primary antibody for E-cadherin (Zymed) was applied at a 1:25 dilution for 1 hour at room temperature. This was followed by anti-rabbit polymer-labeled HRP-bound secondary reagent (DAKO, EnVision+ System, HRP). For Ki67 (NeoMarkers), sections were incubated at a 1:300 dilution at room temperature for 1 hour followed by anti-rabbit polymer-labeled HRP-bound secondary reagent (EnVision+ System, HRP).

All immunohistochemistry stains were developed with DAB chromogen and counterstained with hematoxylin. Corresponding negative control experiments were done by omitting the incubation step with the primary antibody.

### Scoring of immunohistochemistry

Scoring of immunohistochemical staining was done using the Automated Cellular Imaging System (Chroma Vision). Stained sections were scanned and acquired using Automated Cellular Imaging System. Proliferation was measured by calculating the average labeling percentage of the epithelial compartment for Ki67 for each specimen. For determination of microvessel density (MVD), the total number of CD31-stained clusters or single cells, with or without a lumen, was quantified for each specimen. For pVEGFR-2 and pEGFR, quantification was done as previously described (38, 39). Briefly, an index of staining was calculated and expressed as the percentage of staining multiplied by staining intensity after subtracting the index staining of corresponding negative controls.

### Data analysis

Fisher's exact test was done for the comparison of cancer and cancer + dysplasia rates between groups. Two-sample *t* tests, assuming unequal variances, were used for com-

parison of MVD, Ki67, pEGFR, and pVEGFR-2 levels between groups. The nonparametric Wilcoxon rank-sum test was also done to confirm the results from the *t* tests. For pEGFR and pVEGFR-2, the average of five measurements for each mouse was first calculated, and this summary measure was used in the analyses.  $P \leq 0.05$  was considered statistically significant. All analyses were done using Stata version 11 (Stata Corp.).

## Results

### Effects of ZD6474 administration on the development of dysplasia and OSCC

Mice were given 4-NQO (100  $\mu$ g/mL) in their drinking water for a period of 8 weeks, returned to normal water, and then randomized to observation or daily oral gavage of ZD6474 (25 mg/kg/d) for 24 weeks. We have previously shown that following the 8 weeks of 4-NQO administration, mice developed histologically identifiable hyperkeratotic and/or dysplastic lesions (35). Therefore, initiation of ZD6474 treatment at this time point was chosen because it closely mimics the clinical setting in which one would consider initiating chemopreventive therapy in patients. During the 24-week chemoprevention regimen, no significant differences in food and fluid consumption or activity were observed between the groups. At the completion of the 32-week study, there was a significant difference in the incidence of dysplasia and OSCC in the ZD6474 treatment group compared with the control group (Table 1). Overall, 71% (17 of 24) of the control mice and 12% (3 of 25) of the ZD6474-treated mice showed histologic evidence of OSCC ( $P \leq 0.001$ ). Similarly, the proportion of mice with dysplasia or OSCC was significantly different between the two treatment groups. In the control group, 96% (23 of 24) of the animals showed dysplasia or OSCC, whereas 28% (7 of 25) of the ZD6474 treatment group had dysplasia or OSCC ( $P \leq 0.001$ ). In total, this represented a 71% decrease in OSCC or dysplasia and an 83% decrease in OSCC.

### Effects of ZD6474 administration on proliferation and MVD

ZD6474 has been shown to inhibit both tumor cell proliferation and angiogenesis via its dual activity against EGFR and VEGFR-2 (17–19). Therefore, we performed

**Table 1.** Effect of ZD6474 treatment on the development of OSCC in the mouse 4-NQO model

	Control	ZD6474	Total
Hyperkeratosis	1	18	19
Dysplasia	6	4	10
OSCC	17	3	20
Total	24	25	49

immunohistochemistry for Ki67 and CD31 as surrogate markers for cell proliferation and angiogenesis, respectively. Overall, the Ki67 proliferative index (PI) for the ZD6474-treated animals was significantly decreased when compared with the control mice (Table 2). The control group had a PI of  $46 \pm 10$ , whereas the ZD6474 treatment group had a PI of  $29 \pm 10$  ( $P \leq 0.001$ ). Proliferation increased with histologic progression in both control and treatment cohorts (Fig. 1). Of note, the OSCC that arose in the ZD6474 treatment group ( $n = 3$ ) had a mean PI (54.3) that was similar to the PI of the control animals ( $n = 17$ ) who developed OSCC (51.6), suggesting that the ZD6474-associated tumors were still actively proliferating.

Overall, there was a significant decrease in MVD in the ZD6474-treated mice when compared with controls. The control group showed a MVD score of  $265 \pm 60$ , although the ZD6474 treatment group had a MVD score of  $106 \pm 73$  ( $P \leq 0.001$ ). However, there was no difference in vascularity when comparing the MVD between similar histologic diagnoses (hyperkeratosis or dysplasia or OSCC) from different treatment groups (control versus ZD6474-treated; Fig. 1). The OSCC that arose in the ZD6474 treatment group had a mean MVD (253.7) that was similar to the MVD of the OSCC control group (300.2) suggesting that the tumor was still actively inducing angiogenesis.

#### Effects of ZD6474 administration on EGFR and VEGFR-2 activation

In an effort to identify the potential mechanism(s) of acquired resistance to ZD6474 treatment, immunohistochemistry for pEGFR and pVEGFR-2 was done to determine if ZD6474 was still inhibiting the activation of these receptors. Overall, tissue from the control cohort of mice showed significantly stronger cytoplasmic membrane staining for pEGFR when compared with ZD6474-treated mice. The control group had a mean intensity score of  $97 \pm 5$ , whereas the mean intensity score was  $33 \pm 3$  ( $P \leq 0.001$ ) for the ZD6474-treated cohort (Table 2). When comparing pEGFR expression between histologic groups within the same treatment scheme (control or

ZD6474) there was no difference in the intensity scores between the hyperkeratotic, dysplastic, or OSCC specimens (Fig. 2). Interestingly, the pEGFR intensity scores for the OSCC from the ZD6474 treatment group were much lower than the intensity scores for the control OSCC cohort (Fig. 2).

Overall, tissue from control mice had a significantly higher expression of pVEGFR-2 when compared with the ZD6474-treated mice (Table 2; Fig. 3). The combined mean intensity score for the control tissue was  $106 \pm 11$ , whereas the combined intensity score of the tissue from the ZD6474-treated animals was  $32 \pm 3$  ( $P \leq 0.001$ ). When comparing expression between histologic groups within the same experimental group (control or ZD6474), the intensity scores between hyperkeratotic, dysplastic or OSCC specimens were very similar (Fig. 3). Like the pEGFR findings, expression of pVEGFR-2 in the OSCC from the ZD6474-treated group was much lower in intensity when compared with the OSCC from the control group (Fig. 3). These data show that ZD6474 was pharmacologically active in the 4-NQO model. In addition, the data suggests the OSCC that arose in the ZD6474-treated group may have developed acquired drug resistance, as ZD6474 was still actively inhibiting the phosphorylation of both EGFR and VEGFR-2.

#### ZD6474-resistant OSCC express epithelial to mesenchymal markers

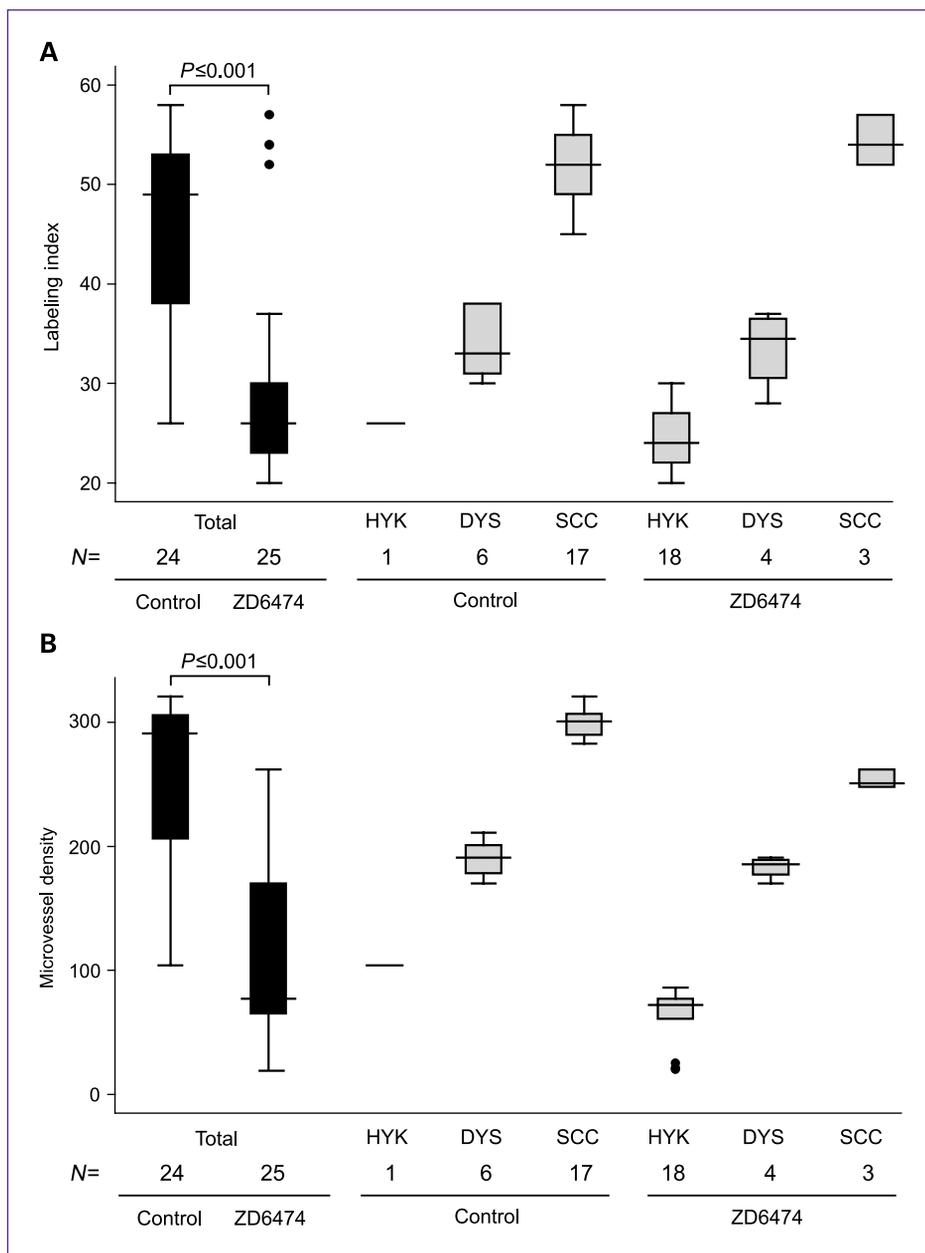
ZD6474 was able to significantly reduce the incidence of OSCC when compared with the control group (Table 1). Although the inhibition of tumor development was statistically significant, 12% of the animals in the ZD6474 group developed OSCC. Furthermore, our data suggests that ZD6474 was still pharmacologically active because low levels of both pEGFR and pVEGFR-2 were still observed after 24 weeks of treatment (Figs. 2 and 3). In addition, the PI and MVD data from the OSCC arising in ZD6474-treated mice were similar to the PI and MVD data in the control animals harboring OSCC (Fig. 1). Overall, these data suggest that the OSCC in the ZD6474-treated mice had developed a form of acquired drug resistance. Similar acquired resistance has been associated with the expression of an epithelial to mesenchymal (EMT) phenotype, an intricate process that can be both physiologic and pathologic in nature (39–43). For example, the induction of EMT may be a novel mechanism of acquired resistance to chemotherapy and radiation in cancer therapy (40, 44). To address the possibility that the development of resistance to ZD6474 treatment in the mouse 4-NQO model of OSCC was driven by the expression of an EMT phenotype, we did immunohistochemistry for the EMT markers E-cadherin and vimentin (45). Each of the tumors from the control group expressed high levels of E-cadherin and undetectable levels of vimentin protein (Fig. 4). Conversely, the tumors from ZD6474-treated mice lost expression of E-cadherin and expressed high levels of vimentin protein (Fig. 4). Although the sample size is small ( $n = 3$ ), these data show a correlation between resistance to ZD6474

**Table 2.** Modulation of surrogate biomarkers for angiogenesis, proliferation, and activation of the EGFR and VEGFR-2 pathways by ZD6474

	Control, N = 24	ZD6474, N = 25
MVD	$265 \pm 60$	$106 \pm 73^*$
Ki67	$46 \pm 10$	$29 \pm 10^*$
pEGFR	$97 \pm 5$	$33 \pm 3^*$
pVEGFR-2	$106 \pm 11$	$32 \pm 3^*$

\* $P \leq 0.001$  for comparison with the control group. All numbers indicate mean  $\pm$  SD.

**Fig. 1.** ZD6474-treated animals show lower proliferative indices and microvessel densities compared with control animals. A, tissue sections were immunohistochemically stained for Ki67 and labeling indices were quantified. The overall labeling indices of the ZD6474 specimens were all significantly lower when compared with the control specimens ( $P \leq 0.001$ ). B, tissue sections were immunohistochemically stained for CD31 and MVD quantified. MVD of the ZD6474 specimens were significantly lower when compared with the control specimens ( $P \leq 0.001$ ). The total group was used for all statistical analysis.



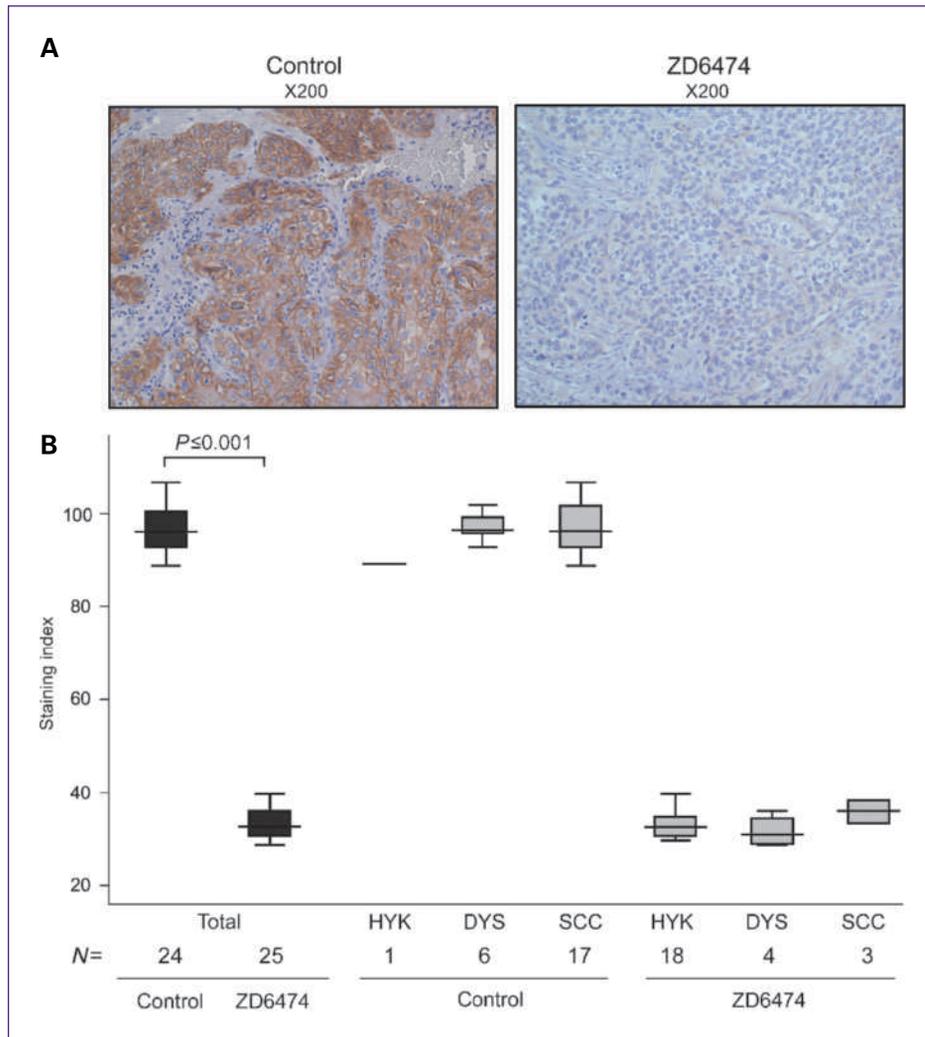
and the expression of EMT markers. They further suggest that the expression of an EMT phenotype may be a novel mechanism of acquired resistance to chemoprevention therapy for OSCC.

## Discussion

The induction of cell proliferation and blood vessel growth are two critical phenotypes that are necessary for the development of malignant neoplasms. Aberrant EGFR tyrosine kinase activity plays an important role in a number of different tumor phenotypes including proliferation, apoptosis, angiogenesis, and metastasis. Furthermore, because

EGFR has such a critical role in the development of OSCC, this signaling pathway has considerable therapeutic potential in the areas of cancer therapy and chemoprevention. Similarly, the activation of the VEGFR-2 pathway by VEGF is a critical component for the induction of angiogenesis in both physiologic and pathologic settings including OSCC. Therefore, because ZD6474 has the ability to inhibit both EGFR and VEGFR-2 activation, it has the potential to inhibit two critical signal transduction pathways and phenotypes involved in the development of OSCC.

In this study, we show that ZD6474 was pharmacologically active in the 4-NQO model of OSCC. Animals treated with 25 mg/kg/d had significantly lower expression



**Fig. 2.** ZD6474 inhibits the phosphorylation of EGFR in the 4-NQO model of OSCC. A, tissue sections from control and ZD6474-treated animals were immunohistochemically stained for pEGFR and quantified. B, expression of pEGFR was significantly lower in the ZD6474-treated specimens when compared with the control specimens ( $P \leq 0.001$ ).

levels of pEGFR and pVEGFR-2 when compared with controls (Figs. 2 and 3). We also report for the first time that daily treatment with ZD6474 decreased the incidence of dysplasias and carcinomas in the mouse 4-NQO model of OSCC (Table 1). The rationale for the 24-week treatment schedule was based on our previous work, which showed that the majority of control animals harbor OSCC by week 24, while dysplasia was the predominant histologic diagnosis at weeks 16 and 20 (35). Because we were testing the hypothesis that ZD6474 would reduce the incidence of OSCC, we believe that it is most appropriate to carry out the prevention study to a time point where the predominant histologic diagnosis in the control group would be expected to be OSCC. Overall, we observed an 83% reduction in the incidence of OSCC when comparing the control and treatment groups (71% versus 12%,  $P \leq 0.001$ ). We also observed a 71% decrease in the incidence of both dysplasia and OSCC when comparing the control and ZD6474 treatment groups (96% versus 28%,  $P \leq 0.001$ ). These data strongly support the

hypothesis that ZD6474 may be an effective chemopreventive agent for OSCC.

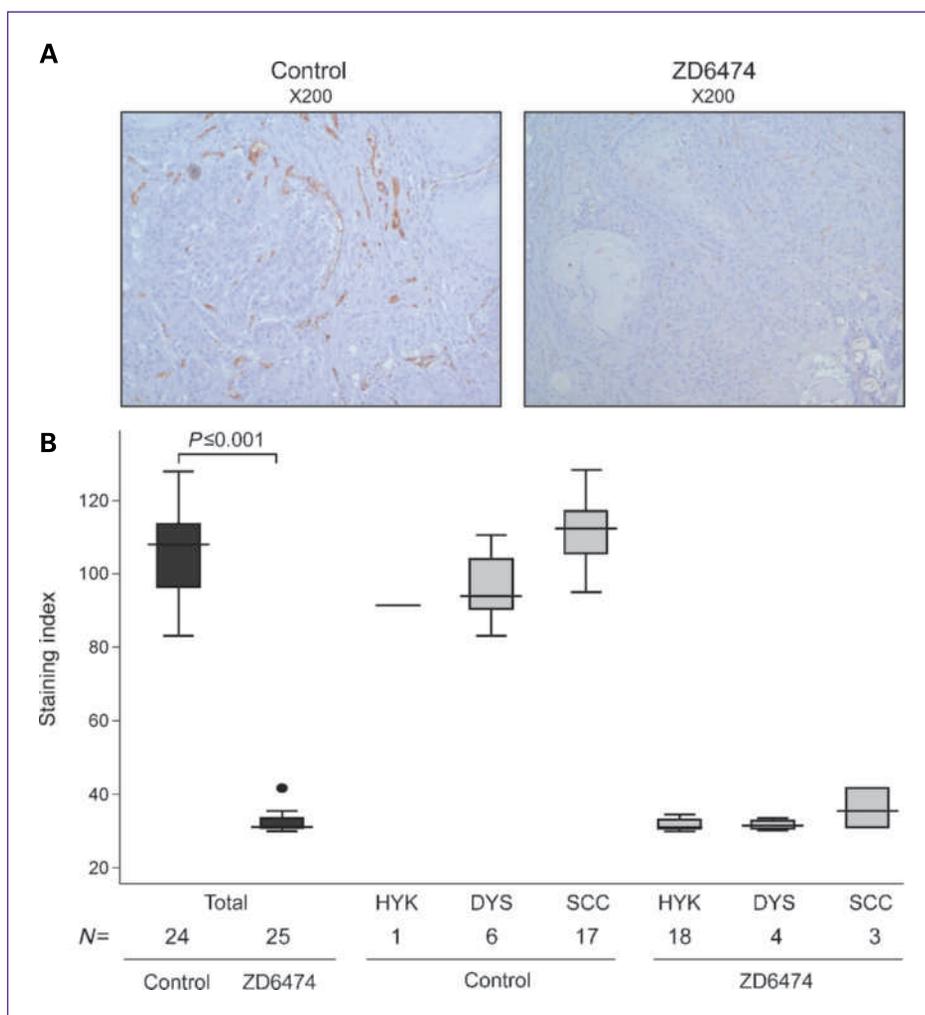
In preclinical studies, ZD6474 has been shown to be a potent inhibitor of tumor angiogenesis and proliferation for several different tumor cell types (21–30). It is also under active investigation in clinical trials for the treatment of various malignant neoplasms (31), with the greatest activity observed in non-small cell lung cancer and medullary thyroid carcinoma (32–34). However, published data regarding the potential utility of ZD6474 in the realm of chemoprevention is limited. In one study, ZD6474 markedly reduced the number and the size of intestinal polyps, resulting in a 75% decrease in tumor burden in a mouse model of colon cancer (46). The data from the colonic polyp study and our current work suggests that further studies are warranted to evaluate the potential utility of ZD6474 as a chemopreventive agent.

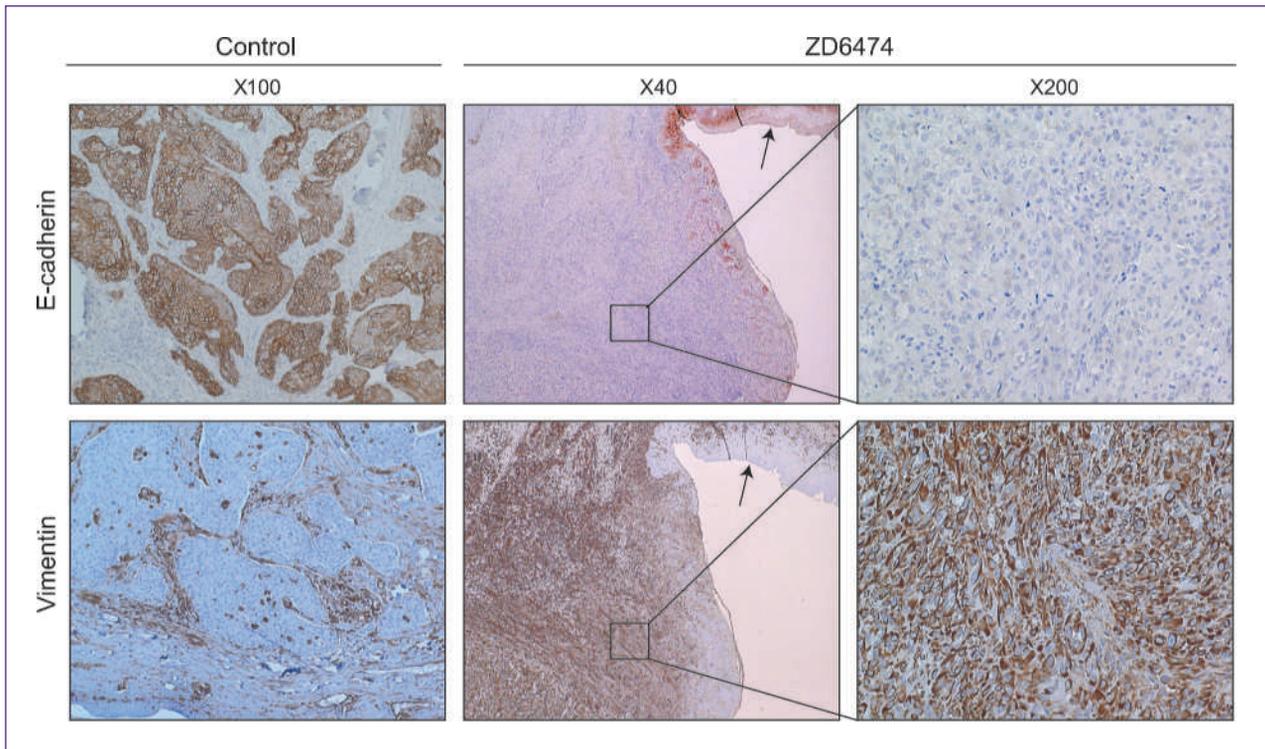
One of the long-term goals of chemoprevention must be the development of treatments that can be easily taken by at-risk individuals for prolonged periods of time with

minimal toxicities to achieve widespread acceptance and long-term compliance. This would be particularly important in the case of high-risk patients who have not yet developed their first OSCC. We have previously shown that ABT-510, a mimetic peptide of thrombospondin-1, significantly decreased the incidence of dysplasia and OSCC in the 4-NQO model (35). However, because there is no oral formulation of the drug, the translation of this agent into clinical trials for prevention seems unlikely. Conversely, because ZD6474 is an orally available drug, it is potentially more feasible for prolonged use in human prevention studies. The maximum tolerated dose as well as toxicity profile of ZD6474 when used in cancer therapy is well described. ZD6474 has been well-tolerated at doses of 100 to 300 mg/d, with the most common adverse events being rash, diarrhea, fatigue, and asymptomatic QTc prolongation (31). However, because the drug may be initiated at a lower dose range in a chemoprevention setting, one might anticipate a lesser degree of side effects. Furthermore, treatment with higher doses of ZD6474 (50 and 100 mg/kg) than the current study (25 mg/kg/d) resulted

in only a modest delay, but not inhibition, of cutaneous wound healing in a mouse model (47). Taken together, these data suggest that the toxicity profile of ZD6474, when used as a chemopreventive agent, might be acceptable when lower doses of the agent are used. This might be particularly true in the context of OSCC, in which the modest long-term survival is due in part to the frequent development of multiple additional primary tumors in individuals with a previous SCC. The rate of second primary tumors in these patients has been reported to be 3% to 7% per year, which is higher than for any other malignancy (48). This observation led Slaughter et al. to propose the concept of "field cancerization." This theory suggests that multiple individual primary tumors develop independently in the upper aerodigestive tract as a result of years of chronic exposure of the mucosa to carcinogens (49). As a result of field cancerization, an individual who is fortunate to live 5 years after the initial primary tumor has up to a 35% chance of developing at least one new primary tumor within that time period. The occurrence of new primary tumors can be particularly devastating for individuals whose

**Fig. 3.** ZD6474 inhibits the phosphorylation of VEGFR-2 in the 4-NQO model of OSCC. A, tissue sections from control and ZD6474-treated animals were immunohistochemically stained for pVEGFR-2 and quantified. B, expression of pVEGFR-2 was significantly lower in the ZD6474-treated specimens when compared with the control specimens ( $P \leq 0.001$ ).





**Fig. 4.** OSCC arising in ZD6474-treated mice express EMT markers. Tumor samples from control mice show strong epithelial expression of E-cadherin and stromal expression vimentin. Histologically normal epithelium from the ZD6474-treated animals show strong expression of E-cadherin and no expression of vimentin (arrows). Conversely, tumor cells from ZD6474 mice show loss of expression of E-cadherin and strong expression of vimentin.

initial lesions are small. Their 5-year survival rate for the first primary tumor is considerably better than late stage disease, but second primary tumors are their most common cause of treatment failure and death (4, 5).

Resistance to cytotoxic chemotherapy and radiation therapy is well appreciated in the context of cancer therapy. In addition, mechanisms of resistance in response to targeted therapies have also been described. For example, several types of intrinsic and acquired resistance to inhibitors of angiogenesis have been postulated (50, 51). Similarly, several mechanisms of resistance related to anti-EGFR therapy have been reported for non-small cell lung cancer, although the mechanisms for EGFR resistance in the context of OSCC seem to be different and remain unclear (52). Conversely, there are limited data concerning the potential mechanisms of acquired resistance in response to long-term chemoprevention therapy using targeted agents (53–55). In the 4-NQO model of OSCC, 12% of the mice chronically treated with ZD6474 developed a form of acquired resistance to the drug. This resistance correlated with a loss of E-cadherin and a gain in vimentin protein expression, suggesting that these tumors began to express an EMT phenotype (44). Conversely, none of the control group OSCC expressed EMT markers. This correlation between resistance to ZD6474 and the expression of an EMT phenotype suggests a novel mechanism of acquired resistance in the chemopreventive

setting. The expression of EMT transitions is well appreciated in embryology and various types of pathophysiology (40). Recently, there has been an increased interest in the role of EMT in areas of cancer progression as well as resistance to chemotherapy and radiation therapy (40, 44). At this time, we do not know if there is a causal link between the expression of EMT markers and resistance to ZD6474. However, the fact that EMT markers were not expressed in control OSCC provides compelling preliminary evidence worthy of further investigation. In addition, we do not know the timing of the gain of expression of the EMT phenotype. As designed, this prevention study harvested all tissues after 24 weeks of ZD6474 therapy. Therefore, to investigate the dynamics of EMT marker expression, one could sacrifice subsets of mice at specified intervals after the initiation of ZD6474 treatment to determine the incidence and timing of EMT marker expression at the stages of hyperkeratosis, dysplasia, and OSCC. It is also important to determine if one or both receptor pathways are mediating the expression of the EMT phenotype. Resistance to EGFR inhibitors erlotinib, gefitinib and cetuximab has been reported to induce an EMT transition (41–43). Similarly, the induction of hypoxia has also been shown to induce EMT (40). Therefore, it is possible that ZD6474 may drive the expression of EMT via both pathways. In addition, the downstream mechanisms of the expression of the EMT phenotype are unknown. The transcription

factors Twist, Snail, and Slug are major mediators of EMT and have been shown to repress E-cadherin expression (40). Further investigation into the altered expression of these and other EMT-related regulatory factors may aid in our understanding of how the expression of an EMT phenotype occurs in the setting of chemoprevention. In addition, the biological and clinical implications of EMT expression in ZD6474-resistant tumors requires further investigation, as the expression of the EMT phenotype can lead to resistance to multiple drugs and potentially lead to the progression of tumors (30). However, the potential for altered clinical behavior following a tyrosine kinase inhibitor-based chemopreventive treatment is not limited to this class of drugs, as resistance towards other types of chemopreventive agents has also been described (39–41). Finally, if the pattern of EMT development can be modeled, one could envision using EMT markers as diagnostic beacons to herald the expression of acquired resistance. Such beacons may be useful as they could be used as indicators for when it would be most efficacious to switch to an alternative chemopreventive agent. For example, the expression of EMT markers might dictate a switch to a histone deacetylase inhibitor, as these have been shown to reverse the EMT phenotype (56). By doing so, one might hypothesize that the histone deacetylase could thereby restore sensitivity to ZD6474s and prolong

its chemopreventive activity. We believe that the mouse 4-NQO model is an excellent model system to pursue each of these important preclinical questions.

In conclusion, our data provides novel evidence that ZD6474, a combined inhibitor of the EGFR and VEGFR-2 pathways, holds promise as a chemopreventive agent for OSCC. They further suggest that the development of acquired resistance to ZD6474 may be mediated by the expression of an EMT phenotype. Finally, the data suggests that the 4-NQO model of OSCC is a useful preclinical platform to investigate the mechanisms of acquired resistance in the chemopreventive setting.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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