Targeting the Expression of the Angiogenic Phenotype in Head and Neck Cancer

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Introduction

With over 600,000 new cases annually, squamous cell carcinoma of the head and neck (HNSCC) is the 6th most common malignancy in the world today (1). Despite therapeutic advances, long-term survival remains modest with several factors contributing to poor outcome. First, HNSCC is often diagnosed at an advanced stage. Second, as a result of "field cancerization", the development of multiple primary tumors has a major impact on survival. For patients with early stage disease, second primary tumors are the most common cause of treatment failure and death (2). Therefore, to improve outcomes and quality of life, comprehensive treatment must include effective screening and prevention strategies.

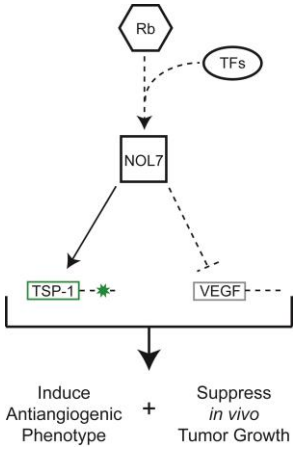
Chemoprevention can be defined as the use of natural or synthetic agents to halt or reverse the progression of premalignant lesions. Chemopreventive therapies for HNSCC are currently being tested in both the preclinical and clinical settings. However, initial promising results have been hampered by significant toxicities and the development of resistance. The challenges of toxicity and resistance are particularly important in the context of chemoprevention because prolonged therapy is typically required.

Angiogenesis is an essential phenotype in both physiologic and pathologic settings. Because of its critical role in cancer biology, the inhibition of angiogenesis is thought to be an attractive therapeutic target. Importantly, because angiogenesis is one of the first recognizable phenotypic changes observed in both experimental models and human HNSCC (3-6), inhibitors of angiogenesis hold promise in the field of chemoprevention. However, the diversity and redundancy of angiogenic factors, combined with our limited knowledge of their higher level regulation, hamper our ability to therapeutically target this phenotype.

We are pursuing a number of basic and translational projects focused on the angiogenic phenotype and its potential to target blood vessel growth. Our specific areas of inquiry are: a) defining the mechanism by which NOL7 acts as a master regulator of angiogenesis; b) testing the hypothesis that angiogenesis inhibitors are efficacious chemopreventive agents; c) identifying mechanisms of resistance to angiogenesis inhibitors in the chemopreventive setting; d) testing the hypothesis that local delivery of chitosan nanoparticles is an efficacious chemopreventive strategy that will result in reduced systemic toxicities.

NOL7 is a Novel Master Regulator of Angiogenesis

My lab cloned NOL7, a novel gene that induces an anti-angiogenic phenotype and suppresses *in vivo* tumor growth by 95% (7,8). NOL7 acts as a master regulator of angiogenesis by modulating the expression of angiogenesis-associated mRNAs via both steady-state downregulation and posttranscriptional upregulation (9). NOL7 itself is positively regulated by the retinoblastoma (Rb) gene, supporting the paradigm shift that Rb can act as a positive regulator of gene transcription (10,11). Our long-term goal is to understand how transcriptional and posttranscriptional regulation of angiogenesis-related mRNAs contributes to the expression of the angiogenic phenotype and to leverage this knowledge to develop novel therapeutic approaches. Our central hypothesis is that Rb positively regulates NOL7 expression and that loss of NOL7 protein expression results in decreased regulation of NOL7 target mRNA transcripts, the gain of expression of the pro-angiogenic phenotype and the loss of tumor growth inhibition (Figure 1). We further hypothesize that reactivation of NOL7, particularly in HPV-associated mucosal lesions where Rb is inactivated, is an



effective therapeutic approach to inhibit the angiogenic phenotype. This research is innovative because it will further define the mechanisms by which NOL7 acts as a master regulator of angiogenesis. It will also provide fundamental insights into the poorly understood area of posttranscriptional regulation of angiogenesis. This research is expected to have a positive impact on human health because it may provide new therapeutic avenues for targeting angiogenesis in both physiologic and pathologic conditions.

Targeting the Angiogenic Phenotype in HNSCC Chemoprevention

The expression of the angiogenic phenotype is both an early and an essential step in the development of HNSCC (3-6), making it an attractive target for cancer prevention. The long-term goal of this work is to develop novel, nontoxic chemopreventive strategies for HNSCC that are based in part upon the inhibition of angiogenesis. Using the 4-Nitroquinoline 1-Oxide (4-NQO) mouse model, we have demonstrated that ABT-510 (global inhibitor of angiogenesis), Erlotinib (tyrosine kinase inhibitor (TKI) against EGFR), ZD6474 (Vandetanib, a dual TKI against EGFR and VEGFR-2), and Crizotinib (dual TKI against c-Met and ALK) significantly decreased the incidence of dysplasia and HNSCC (6,12,13, unpublished data). Furthermore, two clinical trials have been initiated to evaluate the efficacies of Erlotinib and ZD6474 as chemopreventive agents. Erlotinib Prevention of Oral Cancer (EPOC) was a Phase III placebo-controlled, randomized study that investigated the ability of Erlotinib to decrease the incidence of HNSCC in subjects with high risk premalignant lesions. With 135 subjects enrolled, the study is now closed, the database has been locked and analysis for the primary endpoint is proceeding. We anticipate that the data will be released in the spring of 2014. ZD6474 Prevention of Oral Cancer (ZPOC), is an ongoing Phase II, placebo controlled, randomized trial that is assessing the pharmacodynamic and clinical effects of ZD6474, in high-risk patients. With a targeted enrollment of 55, we have recruited 20 subjects to date and anticipate reaching accrual by the end of the 2014 calendar year. This research is innovative because it represents the first HNSCC chemoprevention trials using agents that function in part by inhibiting angiogenesis. It is also novel because we are using tissues derived from the 4-NQO model and the EPOC and ZPOC trials to investigate mechanisms of acquired resistance in the chemopreventive setting.

Defining Mechanisms of Angiogenic Resistance in the Chemopreventive Setting

Because anti-angiogenic therapies target genetically stable endothelial cells, it was originally hypothesized that resistance to anti-angiogenic therapies would not occur (14). We now appreciate that tumors can develop resistance to anti-angiogenic therapies via a number of different mechanisms (15-18). However, data regarding resistance in the chemopreventive setting are limited. In the 4-NQO model, we found that ~10-15% of mice developed acquired resistance (6,12,13, unpublished data). Our central hypothesis is that acquired resistance is mediated in part by the expression of alternative angiogenic signaling pathways. Using the 4-NQO model and RNA expression profiling, we are currently identifying the alternative angiogenic factors produced by resistant lesions. Our preliminary data suggest that the fibroblast growth factor receptor (FGFR) and the platelet-derived growth factor receptor (PDGFR) signaling pathways may be important in the expression of the resistant phenotype (unpublished data). We are now functionally validating these findings by determining if prolonged chemopreventive efficacy in the 4-NQO model can be achieved by targeting the alternative angiogenesis pathways once their expression has been detected (Figure 2). To achieve this, per our standard protocol, mice are treated with 4-NQO in the drinking water for 8 weeks and then randomized to placebo or drug. The mice are then evaluated weekly for the presence of mucosal alterations. When lesions are identified, a full thickness collection of cells is obtained via brush-based liquid cytology, cytospun and interrogated via IHC for the expression of the candidate alternative angiogenic factors. Mice with lesions expressing the alternative factors are then randomized: ZD6474 alone or ZD6474 + New Agent (Figure 2).

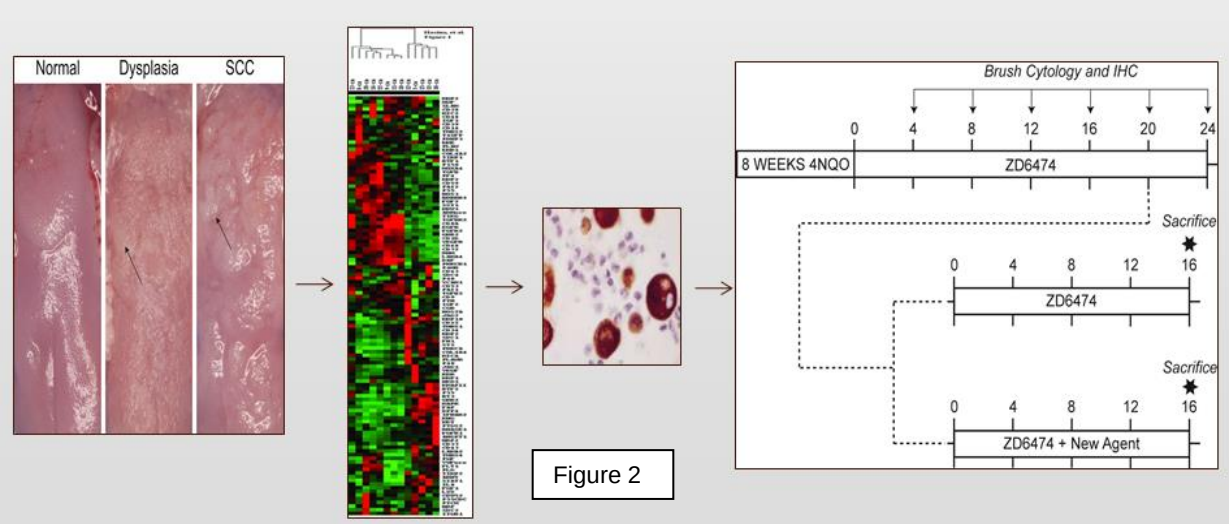


Figure 2

Animal models of carcinogenesis are useful as they enable the development and testing of new approaches to prevention and treatment, identification of early diagnostic markers and an understanding of the biology and genetics of cancer biology. However, as with any model system, their findings require correlation/validation with the human disease under investigation. Therefore, using the sequential biopsies obtained during the course of the EPOC and ZPOC prevention trials, we will determine if a similar pattern of alternative angiogenic factor expression is observed. Confirmation of similar changes may lead to subsequent chemoprevention trials in which the expression of alternative/resistance pathways are actively monitored and treated in order to prolong the chemopreventive effect. This research is innovative because it represents the first attempts to define mechanisms of angiogenesis resistance in the HNSCC chemoprevention setting. It is likely to have a positive impact on human health because it may provide insights into the biology of resistance as well as define therapeutic models for prolonging efficacious chemopreventive strategies for HNSCC.

Decreasing Systemic Toxicities With Nanoparticle-based Delivery Systems

Because current chemoprevention strategies require prolonged systemic administration lasting months to years, there is considerable risk for the development of acute and chronic toxicities. Conversely, directed local delivery has demonstrated limited success as directed topical application to focal mucosal areas will insufficiently treat the entire area of genetically altered mucosa. Molecular characterization of fields from carcinogen-based HNSCC has demonstrated that these areas can be as large as 6 cm in diameter. Furthermore, in contrast to carcinogen-associated mucosa, which often appears as identifiable leukoplakias or erythroplakias, HPV-associated premalignancies/cancers are not easily observed clinically as they typically occur in difficult to examine locations such as the tonsil and the base of tongue. Our long term goal is to develop mouth wash-based platforms for HNSCC chemoprevention that are efficacious with limited systemic toxicities. We hypothesize that chitosan-based nanoparticles, containing siRNAs targeting

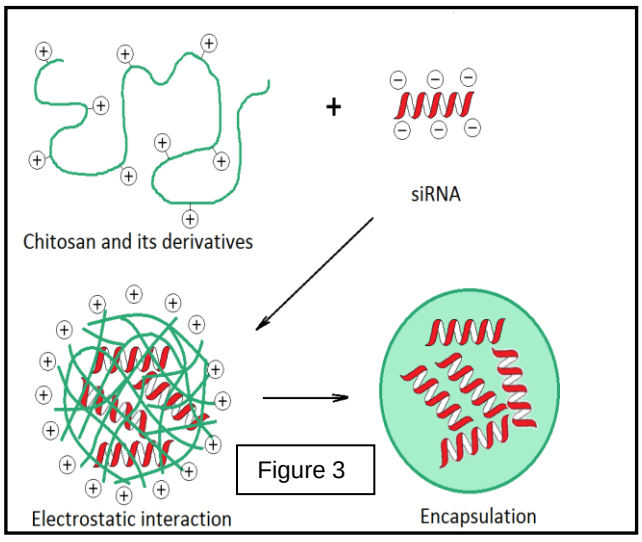


Figure 3

angiogenesis signaling pathways, will be superior to treatments involving systemically administered TKIs. Similarly, we hypothesize that chitosan nanoparticles containing siRNAs for HPV-associated oncoproteins (E6 and/or E7) will provide a novel mechanism of chemoprevention for patients harboring high risk HPV infections of the upper aerodigestive tract.

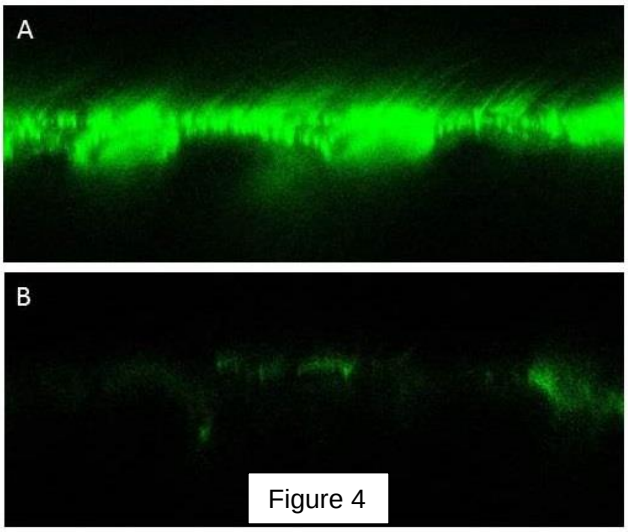


Figure 4

Chitosans are linear polysaccharides derived from crustacean shells. Because they are biocompatible, nontoxic and biodegradable, there is considerable interest in chitosan nanoparticles as therapeutic delivery vehicles. Chitosans have attractive because of their natural mucoadhesive properties, which may facilitate the complete coverage/treatment of the mucosa when delivered as a mouthwash. To test the feasibility of chitosan-based nanoparticle delivery to the oral mucosa *in vivo*, we fabricated chitosan nanoparticles containing fluorescently labeled control siRNAs. Briefly, mice were anesthetized and the dorsal aspect of their tongues painted with the chitosan emulsion. After 30 minutes, the animals were sacrificed, tongues harvested, and evaluated. Incorporation of the fluorescent control siRNA was clearly observed (Figure 4), supporting the feasibility of a nanoparticle-based delivery system for chemoprevention protocols for the oral mucosa (unpublished data). We are currently pursuing several avenues of research using this platform. First, using the 4-NQO model, we are determining the optimal formulation, delivery, dosage, timing, and treatment duration required for *in vivo* delivery. In addition, we are determining the therapeutic efficacy of VEGF and E6/E7 siRNA nanoparticle delivery in the chemopreventive setting. Similarly, using the 4-NQO model, we are determining if the delivery of FGF-2 and PDGF siRNA nanoparticles can prolong efficacy following the development of ZD6474 acquired resistance. Finally, using human tissue explants, we are refining the nanoparticle delivery for future clinical applications.

Summary

HNSCC chemoprevention holds promise to improve long-term survival. However, current strategies have met with limited success. Our research will aid in defining higher level regulation of the angiogenic phenotype and strategies to target this phenotype. In addition, our work will provide novel insights into the development of acquired angiogenesis resistance. Finally, our research is innovative because we are seeking novel local delivery platforms that will both treat the entire mucosal field and decrease systemic toxicities.

References

1. Leemans CR, Braakhuis BJ, Brakenhoff RH. 2011. The molecular biology of head and neck cancer. Nature Reviews Cancer, 11:9-22.
2. Kelloff GJ, Lippman SM, Dannenberg AJ, et al. Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer--a plan to move forward. Clin Cancer Res 2006; 12:3661-97.
3. Lingen MW, DiPietro LA, Solt DB, Bouck NP, Polverini PJ. The angiogenic switch in hamster buccal pouch keratinocytes is dependent on TGFbeta-1 and is unaffected by ras activation. Carcinogenesis 1997; 18:329-38.
4. Jin Y, Tipoe GL, White FH, Yang L. A quantitative investigation of immunocytochemically stained blood vessels in normal, benign, premalignant and malignant human oral cheek epithelium. Virchows Arch 1995; 427:145-51.
5. Carlile J, Harada K, Baillie R, et al. Vascular endothelial growth factor (VEGF) expression in oral tissues: possible relevance to angiogenesis, tumour progression and field cancerisation. J Oral Pathol Med 2001; 30:449-57.
6. Hasina R, Martin LE, Jones C, Jalil A, Lingen MW. 2009. ABT-510 is an Effective Chemopreventive Agent in the 4NQO Mouse Model of Oral Carcinogenesis. Cancer Prevention Research, 2:385-393.
7. Hasina R, Fekete MJ, Martin L, Qi X-M, Brigaudeau C, Pramanik R, Edith I. Cline, Coignet L, Lingen MW. 2006. NOL7 is a Nucleolar Candidate Tumor Suppressor Gene in Cervical Cancer that Modulates the Angiogenic Phenotype. Oncogene, 25:588-598.
8. Zhou G, Jones C, Lingen MW. 2010. Identification and Functional Characterization of the Nuclear and Nucleolar Localization Signals in NOL7. BMC Cell Biology, 11:74-93.
9. Doci CL, Zhou G, Lingen MW. 2013. The Novel Tumor Suppressor NOL7 Post-transcriptionally Regulates Thrombospondin-1 Expression. Oncogene, 32:4377-4388.
10. Mankame TP, Zhou G, Lingen MW. 2010. Identification and Characterization of the Human NOL7 Gene Promoter. Gene, 456:36-44.
11. Mankame TP, Lingen MW. 2012. The Rb tumor suppressor positively regulates transcription of the anti-angiogenic protein NOL7. Neoplasia, 14:1213-1222
12. Zhou G, Hasina R, Wroblewski K, Mankame TP, Doci CL, Lingen MW. 2010. Dual Inhibition of VEGFR and EGFR is an Effective Chemopreventive Strategy in the Mouse 4NQO Model of Oral Carcinogenesis. Cancer Prevention Research, 3:1493-1502.
13. Leeman-Neill RL, Seethala RR, Singh SV, Freilino ML, Bednash JS, Thomas SM, Panahandeh MC, Gooding WE, Joyce SC, Lingen MW, Neill DB, Grandis JR. 2011. Inhibition of the EGFR-STAT3 signaling axis with erlotinib, but not guggulsterone, prevents transformation in a carcinogen-induced mouse model of oral squamous cell carcinoma. Cancer Prevention Research, 4:230-237.
14. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature, 390:404-407, 1997.
15. Kerbel, R S. Yu, J. Tran, J. Man, S. Viloria-Petit, A. Klement, G. Coomber, B L. Rak, J. Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. Cancer Metastasis Rev. 20:79-86, 2001.
16. Casanovas, O. Hicklin, DJ. Bergers, G. Hanahan, D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell. 8:299-309, 2005.
17. Bergers, G. Hanahan, D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 8:592-603, 2008.