THE DETECTION OF METHYLATED MICRORNAS IN THE SALIVA OF HEAD AND NECK CANCER PATIENTS FOR TUMOR DIAGNOSIS, PROGNOSIS, AND SURVEILLANCE.

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Introduction: We have previously demonstrated that certain microRNAs (miRNAs) can be silenced epigenetically through DNA methylation in the cell lines of head and neck squamous cell carcinoma (HNSCC) patients. Head and neck squamous cell carcinoma desquamates from the primary tumor. We therefore hypothesized that tumor-specific miRNAs can be detected within the tissue and saliva of HNSCC patients, and that this can lead to a novel non-invasive method for tumor diagnosis, prognosis, and surveillance.

Methods: Saliva and tissue was collected from 42 patients enrolled in the study. Patients that were undergoing tonsillectomy or sleep apnea were enlisted as controls. Patients with a known diagnosis of squamous cell carcinoma (n=30) were enrolled as subjects. Both tumor and adjacent normal tissue was harvested from the subject group. Genomic DNA isolated from tissue and saliva samples from both groups underwent bisulfite conversion. Quantitative methylation specific PCR was then used to quantify the methylated level of five microRNAs: 9-1, 124-1, 124-2, 124-3, and 137. All experiments were normalized to one specific control patient.

Results: All five microRNA studied demonstrated a higher level of methylation in both tissue and saliva samples from HNSCC patients vs. normal controls. Using a defined methylation level as a cut-off for diagnosis, all five microRNAs tested demonstrated an increased in methylation with a very high sensitivity and specificity (>90%) in both the tissue and saliva samples.

Discussion: Our results confirm that increased methylation of certain specific microRNAs are found in the tissue and saliva of HNSCC patients. Furthermore, these miRNAs can be detected with a high degree of sensitivity and specificity even in the saliva of HNSCC patients. Therefore, salivary testing may represent a novel non-invasive diagnostic assay for HNSCC. Multi-center clinical trials of this assay is currently being planned.
The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates many normal cellular processes, such as cell proliferation, survival, apoptosis and glucose metabolism. Deregulation of this pathway and aberrant changes to its genetic components have been associated with cancer development in a wide variety of cancers, including in head and neck squamous cell carcinoma (HNSCC). Notably, mutations of the PIK3CA gene, encoding for the catalytic subunit of PI3K, have been reported in up to 20% of head and neck tumors. Currently, a major limitation in HNSCC has been the lack of animal models to test current genetic paradigms and explore the effectiveness of new treatment modalities and chemopreventative strategies. Therefore, to better understand the role of mutant PIK3CA in tumor initiation and progression, a novel PI3K mutant knock-in mouse was generated carrying a gain-of-function allele (LSL-PIK3CA\textsuperscript{H1047R}). Conditional expression of the mutant PI3K in the squamous epithelium of the upper digestive tract was driven by Keratin14-Cre (K14-Cre). These mice were crossbred with heterozygous p53\textsuperscript{H172R} mutants to yield PIK3CA\textsuperscript{H1047R};p53\textsuperscript{+/H172R};K14-Cre double-mutants. Upon tumor induction with 4-nitroquinoline-1-oxide (4NQO) and follow-up for 8 and 16 weeks, PIK3CA\textsuperscript{H1047R};p53\textsuperscript{+/H172R};K14-Cre mice developed tumors which histologically mimic human HNSCC. Furthermore, PIK3CA\textsuperscript{H1047R};p53\textsuperscript{+/H172R};K14-Cre mice presented significant gross and histological differences compared to single-mutants and wild-type control mice. Moreover, molecular analysis of primary cell lines derived from tumors revealed activation of the PI3K pathway only in cancer cells harboring the H1047R mutation. Our data showed that an activating PIK3CA mutation could synergize with mutant p53 to enhance susceptibility to carcinogen-induced oral tumorigenesis. These results underscore the importance of PIK3CA in oral neoplastic development and provide a model to evaluate effectiveness of novel molecular-targeted therapies targeting the PI3K pathway as well as to explore the cross-talk between the PI3K pathway and p53. To the best of our knowledge, this is the first time this PIK3CA mutant has been applied to HNSCC.
MULTI-INSTITUTIONAL VALIDATION OF INTRA-TUMOR GENETIC HETEROGENEITY AS AN OUTCOME BIOMARKER IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Purpose: To determine whether mutant-allele tumor heterogeneity (MATH), a previously developed biomarker of intra-tumor genetic heterogeneity, was related to outcome in an independent group of head and neck squamous cell carcinoma (HNSCC) patients from a large multi-institutional study.

Patients and Methods: Clinical and corresponding exome sequencing data were obtained from The Cancer Genome Atlas (TCGA) on 305 HNSCC patients from 14 institutions. MATH values were calculated for each tumor, with methods identical to those reported previously in a smaller study of 74 patients. Relations of MATH to clinical variables were determined with linear analyses that also took human papillomavirus (HPV) status into account. Relations of clinical variables, MATH, and HPV status to overall survival were determined with Cox proportional hazards analysis. M and C molecular tumor classifications based on frequently occurring DNA disruptions among 3000 TCGA tumors were as described by Ciriello et al Nature Genetics 2013. M-class tumors tend to have predominantly point mutations or small indels, while C-class tend to have substantial copy number alterations (CNA).

Results: Higher MATH was strongly related to shorter overall survival among all patients (hazards ratio 2.2, 95% CI 1.4 to 3.3) and specifically among those treated with chemoradiation (hazard ratio 5.2, 95% CI 1.2 to 23). The association of MATH with outcome was not due to its relations to other clinical or molecular characteristics. In particular, although HPV positive tumors demonstrate lower MATH values, MATH maintained a significant relation to overall survival in analyses incorporating MATH and HPV status with each of the other clinical and molecular characteristics examined. Of clinical importance, stepwise Cox proportional hazards analysis identified MATH as significantly related to outcome in multivariate analysis in the TCGA data set.

Of the tumors considered here that were molecularly classified as either M or C by Ciriello, HPV-positive tumors were predominantly in the M-class, while HPV-negative tumors were predominantly in the C-class (p =0.0003, Fisher exact test). MATH in these M-class tumors was significantly lower than in C-class tumors even when HPV status was taken into account, suggesting that significant CNA may be associated with higher tumor MATH scores and poor outcome.

Conclusion: The ability of tumor MATH values to identify low-risk and high-risk patients strongly supports its role as an important prognostic biomarker. The especially strong association of high MATH scores with poor survival after chemoradiation suggests that tumor MATH values may also serve as a predictive biomarker. In addition, MATH tumor values can be included as continuous measures of intra-tumor heterogeneity in models designed for general prognostication or for identifying patients at particularly high or low risk of succumbing to disease for clinical trial design or treatment de-intensification or selection. Further, the ready availability of next generation sequence data from additional TCGA efforts supports the evaluation of MATH as a biomarker in other types of cancer.
DIFFERENTIAL GENE EXPRESSION IN RECURRENT HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Despite recent improvements in the prognosis of head and neck squamous cell carcinoma (HNSCC), due in part to the increasing prevalence of HPV-associated neoplasia, a significant number of patients still develop local recurrence after chemoradiation therapy. Following recurrence, treatment options can be limited due to tumor location or comorbidities. The purpose of this study was to examine gene expression differences between tumor biopsies obtained prior to chemoradiation treatment and samples obtained from the subsequent post-treatment recurrence. Knowledge of the post-treatment gene expression profile will increase our understanding of how the biology of the recurrent tumor differs from the original tumor as well as indicate potential opportunities for alternative targeted therapy in recurrent HNSCC. Fourteen patients with recurrent HNSCC following chemoradiation treatment at William Beaumont Hospital were identified and met the inclusion criteria of having available archival formalin-fixed paraffin-embedded samples from both a pre-treatment diagnostic biopsy as well as from the recurrent tumor. Tumor sites consisted of 7 larynx, 3 tonsil, 3 tongue and 1 hypopharynx cancers. Areas of invasive carcinoma were identified by a pathologist and then isolated from surrounding stroma via laser capture microdissection. RNA was isolated, labeled, and then hybridized to Affymetrix Exon microarrays, enabling analysis of more than 17,600 genes. Analysis using Partek Genomics Suite revealed 60 differentially expressed genes (p<=0.05 and 2-fold cutoff) between the untreated biopsy and the recurrent tumor. Utilizing Ariadne Pathway Studio and subnetwork enrichment analysis, the untreated biopsy and the recurrent tumors both demonstrated high regulation of genes involved with biological processes such as cell invasion, cell motility, and epithelial to mesenchymal transition. Genes involved with regulating keratinization were predominantly downregulated in the recurrent tumors. Signaling pathways centered around platelet-derived growth factor, fibroblast growth factor 1, and connective tissue growth factor were also highly regulated. Specific genes that were upregulated in the recurrent tumors include apolipoprotein D, secreted phosphoprotein 1, TIMP metalloproteinase inhibitor 1, and matrix metalloproteinase 2; genes downregulated in the recurrent tumors include lipocalin 2, SERPINB2, and transglutaminase 1. The results of this study indicate that there are substantial gene expression differences between the initial tumor and the subsequent recurrence following chemoradiation. We are currently investigating these differences in a larger clinical population, focusing on genes and pathways that could lead to improved, targeted therapy for recurrent HNSCC, ultimately resulting in improved patient outcomes.
**Objective:** Afatinib, a dual EGFR/Her2 TKI, is currently being investigated in several clinical trials for head and neck squamous cell carcinoma (HNSCC). Since afatinib preferentially inhibits EGFR/Her2 tyrosine kinase activity, it is assumed that EGFR-negative HNSCC will not respond to this treatment modality. However, there is no experimental data to support this supposition. In this study, we determined the efficacy of afatinib in EGFR-positive and EGFR-negative HNSCC cells.

**Methods:** The effect of afatinib on cell proliferation and clonogenic survival was assessed in EGFR-positive CAL27 and EGFR-negative UMSCC74A HNSCC cells. In addition, clonogenic survival was performed in CAL27 and UMSCC74A using a sequential treatment protocol of neoadjuvant afatinib followed by RT, RT followed by adjuvant afatinib or concurrent afatinib + RT.

**Results:** Single-agent afatinib inhibited the proliferation and clonogenic survival of EGFR-positive CAL27 and EGFR-negative UMSCC74A HNSCC cells. Afatinib suppressed the proliferation (24 hours) of CAL27 cells with an IC50 of 3.2 µM and UMSCC74A cells with an IC50 of 10.3 µM. Interestingly, using the long-term clonogenic survival assay (8 days), the efficacy of afatinib was similar in CAL27 and UMSCC74A cells; IC50 of 1.9 µM and 3.3 µM, respectively. In CAL27 and UMSCC74A cells, combined afatinib + RT was more active (p=0.01) than afatinib or RT monotherapy. Moreover, neoadjuvant afatinib followed by RT was more potent than RT followed by adjuvant afatinib (p<0.01). In both cell lines, concurrent afatinib + RT was the most active regimen.

**Conclusion:** Afatinib is an active anti-cancer therapeutic in HNSCC regardless of EGFR levels. In addition, pre- and concurrent treatment with afatinib sensitizes EGFR-positive and EGFR-negative HNSCC cells to RT. The clinical implication of our result is that afatinib may have therapeutic efficacy in EGFR-negative HNSCC patients.
Chromatin remodeling is a critical epigenetic process that regulates gene expression. SWI/SNF is an ATP-dependent chromatin remodeling complex involved in cellular differentiation, growth control, and DNA repair. Recently, silencing of Brahma (BRM), a key catalytic subunit of SWI/SNF, was observed in a significant proportion of head and neck squamous cell carcinoma (HNSCC) tumors and cell lines, suggesting that epigenetic dysregulation via alterations in chromatin structure likely plays an important role in a subset of HNSCC. Moreover, two novel insertion variants, BRM -741 (rs34480940) and BRM -1321 (rs3832613) located within the BRM gene promoter have been shown to correlate with loss of BRM expression in HNSCC cell lines and elevated HNSCC risk in patients, demonstrating their potential to serve as functional biomarkers representative of this aberrant chromatin remodeling process. As such, the aim of this study was to further evaluate the insertion variants’ utility as biomarkers in HNSCC by assessing their ability to identify the subset of tumors with BRM loss as well as predict prognosis in patients affected by this oncogenic process. Utilizing a tissue microarray (TMA), BRM expression was examined in 79 surgically resected primary HNSCC tumors via immunohistochemistry. Genotyping of polymorphisms was performed on DNA extracted from peripheral blood lymphocytes. The association between BRM promoter polymorphisms and survival was first examined in 609 patients with primary HNSCC (oral cavity, oropharynx, larynx, and hypopharynx) treated at the Princess Margaret Hospital in Toronto, Canada. A validation study was then performed using an independent cohort of 502 patients with stage I and II HNSCC enrolled in a randomized trial. In TMA expression studies, presence of insertion variants was significantly associated with decreased BRM expression (p<0.001). Compared to the wild type genotype without insertion variants, the double homozygous genotype was associated with the lowest average BRM expression. In both univariate and multivariate analyses, BRM -741 and BRM -1321 were highly associated with overall survival (OS) and cancer specific survival (CSS) (p<0.001 for both). Individuals who were homozygous for both polymorphisms were at the highest risk for death (OS hazard ratio: 12.6, 95% CI: 3.8-41.7, p<0.001; CSS hazard ratio: 14.5; 95% CI: 3.4-62.5, p<0.001). In the validation cohort, BRM -741 and BRM -1321 remained highly associated with OS (double homozygote hazard ratio: 4.1, 95% CI: 2.1-7.9, p<0.001). In this study, we provided the first evidence of BRM polymorphisms as prognostic biomarkers in two large independent cohorts of HNSCC patients. These polymorphisms have the potential to identify a subset of patients that have an elevated risk of HNSCC and develop tumors that harbor a poor prognosis. In addition to their utility in screening and prognostication, the polymorphisms also have potential application in individualized treatment planning as this subset of HNSCC patients may benefit from newer classes of cancer therapeutic agents such as histone deacetylase inhibitors that specifically target chromatin remodeling.
Metastasis is the final step leading to patient death in most solid tumors, including head and neck squamous cell carcinoma (HNSCC). For metastasis to proceed, tumor cells must become mobile and invasive, losing their communal behavior. A goal of our work is to delineate the downstream effectors of growth factor signaling pathways that contribute to invasion and ultimately metastasis. Using a phosphotyrosine proteomics screen we previously identified neural precursor cell expressed, developmentally down-regulated 9 (NEDD9; CasL) as a mediator of VEGF and IGF-1 signaling to invasion in HNSCC cell lines. NEDD9 tyrosine phosphorylation led to matrix metalloproteinase9 (MMP9) expression and secretion, invadopodia formation and enhanced invasion. To determine the molecular details of NEDD9 substrate domain protein:protein interactions leading to invasion, we have generated a series of NEDD9 substrate domain tyrosine mutants (Y to F, YXXP) and SH3 domain mutants. Mutation of all 13 YXXP motifs to FXXP blocked NEDD9 tyrosine phosphorylation, resulting in loss of MMP9 expression/secretion and invadopodia formation. Cells expressing the Y12F SH3 domain mutant exhibited NEDD9 hyperphosphorylation, loss of MMP9 expression/secretion, with cells being spread out with distinct actin filaments and focal adhesions. The Y12E mutant (phosphomimetic) exhibited loss of MMP9 expression, but lacked focal adhesions and actin filaments, having a more mesenchymal phenotype. Cells expressing either of these SH3 domain mutations lacked invadopodia formation. As NEDD9 has been reported to interact with Molecule Interacting with CasL 1 (MICAL1) via its SH3 domain, we tested this possibility. To this end, we demonstrated that NEDD9 co-immunoprecipitated with MICAL1. To further define this interaction and its downstream impact, we silenced NEDD9 or MICAL1 expression by generating stable NEDD9 or MICAL1 HNSCC cell lines using lentiviral shRNAs. Knockdown of either NEDD9 or MICAL1 resulted in reduced migration and invasion, consistent with their roles in invasive signaling. These studies show, for the first time, that MICAL1 may play a role in invasive signaling leading to invadopodia formation. We are currently determining the role of reactive oxygen species (ROS; H2O2) formation resulting from MICAL1's monooxygenase domain in contributing to MMP9 secretion and invadopodia formation. A better understanding of this pathway will undoubtedly lead to identification of novel therapeutics for the prevention of metastasis. Supported by NIH R01CA134845 (SAR), P30 CA138313 (Hollings Cancer Center) and NIH/NCRR UL1 TR000062/TL1 TR000061 from the South Carolina Clinical & Translational Research Institute (COH).
IDENTIFICATION OF A NOVEL TUMOR SUPPRESSOR GENE USING HIGH-THROUGHPUT CDNA RESEQUENCING OF A HUMAN HEAD AND NECK CANCER CELL LINE

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Objective: Characterization of the molecular mechanisms of head and neck squamous cell carcinoma (HNSCC) oncogenesis is expected to provide important information for the development of novel anticancer agents and the identification of biomarkers.

Methods: cDNAs for cancer-related genes were purified from a human HNSCC cell line and analyzed with a next generation sequencer (NGS). Clinical relevance of the nonsynonymous mutations thus identified was evaluated with various functional assays including luciferase-based pathway analyses. Regarding a candidate for a tumor-suppressive transcriptional factor, chromatin immunoprecipitation coupled with sequencing (ChIP-seq) was conducted to elucidate its in vivo roles.

Results: Screening for missense mutations, indels, and gene fusions with our in-house computational pipeline for the NGS dataset resulted in the identification of ~20 nonsynonymous mutations, including a novel missense mutation mapped within the conserved DNA-binding domain of a transcriptional factor. This mutation weakened its transcriptional activity, and forced expression of the wild-type protein markedly suppressed the growth of the cell line, revealing a tumor-suppressive role of the transcriptional factor. Furthermore, the wild-type factor was shown to exert such effects through suppression of G0/G1 to S phase transition in cell cycle and through the activation of Caspase 3/7. Interestingly, loss-of-function mutations for this transcriptional factor are present in a wide array of human tumors. ChIP-seq revealed that this factor selectively binds to loci adjacent to genes, products of which relate to cell cycle regulation and apoptosis. Through genome-wide gene expression profiling and shRNA analyses, we further pinpointed two pro-apoptotic proteins that are the essential mediators for the tumor-suppressive function of the transcriptional factor.

Conclusion: Our data reveal that a subset of cancer in common carries loss-of-function mutations for the tumor-suppressive transcriptional factor, which likely constitutes a novel subfamily among human cancer including HNSCC.
Introduction: Head and neck squamous cell carcinoma (HNSCC) is a highly invasive cancer, with a five-year survival rate of around 50%. Our research group identified miR-375 as the most consistently down-regulated miRNA in tumor samples when compared to paired normal samples. Patients in the lowest quartile of miR-375 expression had significantly decreased disease-specific survival with increased incidence of loco-regional recurrence and distant metastasis. We have published observed that increased miR-375 expression in HNSCC cells resulted in diminished cell invasion in vitro. One major determining feature of the ability of cells to invade is their capability to degrade extracellular matrix barriers. Invadopodia are cellular structures containing colocalized cortactin and Tks5, which can mediate degradation of extracellular matrix barriers. We evaluated the impact of miR-375 expression on extracellular matrix degradation and invadopodial maturation.

Methods: For evaluation of the matrix degradation properties of the transductant lines, a fluorescent matrix degradation assay was used. For the detection of invadopodia, the fluorescent matrix degradation assay was conducted in combination with immunostaining of cortactin and Tks5. Invadopodium precursors were defined as puncta of colocalized Tks5 and cortactin staining which were not associated with gelatin degradation; mature invadopodia were defined as Tks5 and cortactin colocalized puncta that were associated with gelatin degradation holes. Cellular levels of cortactin, Tks5, MMP-2 and MMP-9 in the transductant lines were assessed by western blot analysis. Immunoprecipitation experiments were conducted to assess whether there were changes in tyrosine phosphorylation of cortactin as a result of miR-375 expression. Secreted levels of MMP-2 and MMP-9 in the transductant lines were assessed by western blot, ELISA and Proteome Profiler™ Human Protease Array analyses.

Results: We found that UMSCC1 and UMSCC47 Pre375 cells showed a significant reduction in the matrix degradation area per cell compared to their empty vector control cells. In parallel, UMSCC1 and UMSCC47 Pre375 cells showed a significant reduction in the number of mature invadopodia per cell. MiR-375 over-expression in either UMSCC1 or UMSCC47 cells did not significantly change the numbers of invadopodium precursors per cell. We determined that miR-375 expression in HNSCC cell lines does not reduce cellular levels of cortactin and Tks5. We observed that the levels of tyrosine phosphorylation of cortactin measured were not significantly changed in UMSCC1 Pre375 cells compared to the empty vector control. Likewise, we determined that the level of phosphocortactin (pY421) was not consistently changed in UMSCC1 Pre375 cells compared to the empty vector control. However, cellular and secreted levels of MMP-9 are diminished in miR-375 over-expressing cells compared to their respective control cell lines. We are currently testing candidate target proteins of miR-375 for possible involvement in the diminished matrix degradation and invadopodial maturation of HNSCC cells.

Conclusion: Increased miR-375 expression may suppress the invasive properties of HNSCC through diminished invadopodia activity. Improvements in HNSCC patient outcome may be obtained by clearly understanding the mechanism by which miR-375 expression diminishes HNSCC invasion.