Objective: Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer worldwide with a 50% patient survival rate 5 years post-diagnosis despite current therapeutic strategies. A direct comprehensive genome wide analysis of epigenetic and transcriptional alteration in primary HNSCC that integrates single gene epigenetic and transcriptional data into specific, common, pathway-defined alterations has not been reported. Using such approach we sought to identify novel drivers of HNSCC carcinogenesis.

Experimental Design: 44 primary HNSCC and 25 normal mucosal samples were used in the Affymetrix GeneChip Human Exon 1.0 array and for the Illumina Infinium Human Methylation 27 array. To analyze the data we employed a novel genome wide integrative screening approach based on Cancer Outlier Profile Analysis (COPA). 81 significant differentially expressed genes with both oncogenic and tumor suppressing properties.

Results: With use of the employed approach we have identified 81 candidate proto-oncogenes and tumor suppressor genes with significantly changed expression in tumors as compared to normal tissues. We have also discovered that out of total 81 genes, 11 tumor suppressor genes have the expression changed in the methylation-dependant manner due to the strong correlation between hypermethylation and decreased expression. Using an independent validation cohort of primary 63 tumor and 31 normal tissues we were able to confirm that several of these genes are differentially methylated in tumor samples. Top scoring candidates from them were DTX1 (positive NOTCH-pathway regulator) and BANK1 (a scaffold protein). Thus, we have demonstrated that DTX1 and BANK1 are significantly downregulated and hypermetylated in HNSCC (p-values: 0.02 (DTX1 differential methylation), 0.0001 (BANK1 differential methylation), 0.00023 (DTX1 differential expression) and 0.000087 (BANK1 differential expression)). The functional contribution of DTX1 and BANK1 to HNSCC development will be discussed.

Conclusions: Our new screening approach allowed identification of 11 candidate tumor suppressor genes aberrantly methylated and transcriptionally suppressed in HNSCC. Validation of gene expression and methylation allowed the detection of DTX1 and BANK1, the prospective drivers of HNSCC carcinogenesis.
**MIR-27A* TARGETS THE EGFR SIGNALING AXIS IN HNSCC**

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**Background:** The epidermal growth factor receptor (EGFR) has been implicated as a critical driver in multiple solid tumor types, including head and neck squamous cell carcinoma (HNSCC). Overexpression of EGFR has been associated with increased tumorigenicity and poor prognosis in HNSCC. However, clinical response to EGFR-specific treatment has not been as dramatic as preclinical studies promised. This discordance suggests that resistance pathways are present that abrogate the effects of EGFR-specific therapies and alternative approaches to inhibiting EGFR and its downstream mediators may be more effective. The current study examines the role of microRNAs (miRNAs) in the regulation of the EGFR signaling axis.

**Methods:** Candidate miRNAs that bind the EGFR gene were identified using in silico analysis. Expression levels of the miRNAs were assessed by quantitative real-time polymerase chain reaction (RT-PCR). The effects of the specific miRNAs on EGFR and its downstream mediators were quantified by immunoblotting and assays for cell viability and apoptosis. Direct interaction of the miRNAs with target genes was assessed using a reporter vector containing the luciferase gene fused to the 3'-untranslated region (UTR) of those genes. Candidate miRNAs were also used in combination with erlotinib. For in vivo analysis, orthotopic xenografts were established using cell lines stably transfected with a constitutive or inducible vector expressing the candidate miRNAs. The effects of direct intratumoral injection of the candidate miRNAs were also assessed.

**Results:** We identified multiple miRNA sequences that have putative binding sites within the EGFR sequence. Among these, miR-27a (miR-27a-3p) and miR-27a* (miR-27a-5p) are novel miRNAs targeting EGFR, which were significantly down-regulated in multiple HNSCC cell lines. Analysis of human specimens also showed that miR-27a* was significantly underexpressed in HNSCC as compared to normal mucosa. Re-introduction of miR-27a* into HNSCC cells produced a profound cytotoxic effect, which was not seen with miR-27a. Further analysis for potential targets of miR-27a* led to the identification of AKT1 (protein kinase B) and mTOR (mammalian target of rapamycin) within the EGFR signaling axis. Treatment with miR-27a* resulted in the coordinated downregulation of EGFR, AKT1 and mTOR. A direct interaction between miR-27a* and EGFR, AKT1, and mTOR was confirmed using a luciferase reporter containing the 3'-UTR of these target genes with site-directed mutagenesis of the candidate binding sites. The addition of miR-27a* to erlotinib could overcome treatment resistance in certain cell lines and tumors. Constitutive and inducible expression of miR-27a* in a murine orthotopic model of oral cavity cancer led to decreased tumor growth compared to controls. We also found direct injection of miR-27a* into HNSCC tumors decreased growth in vivo.

**Conclusions:** Our findings demonstrate the coordinated regulation of EGFR-mediated signal transduction at multiple levels by miR-27a*. MiR-27a* significantly altered HNSCC viability in vitro and in vivo. The functional effects of miR-27a* expression in HNSCC were related to decreased expression of not only EGFR, but also AKT1 and mTOR. MiR-27a* also enhanced the effectiveness of erlotinib. Thus, miR-27a* may represent a novel targeted therapy for HNSCC.
SCCRO REGULATES THE CONTRIBUTION OF BONE MARROW-DERIVED CELLS TO THE TUMOR MICROENVIRONMENT

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Background: The tumor microenvironment (TME) is a critical regulator of tumor development, progression, metastasis, and resistance to treatment. Given its essential role, homogeneity in composition, and stable genetic makeup, it is an attractive therapeutic target. Our preliminary studies suggest that the novel oncogene SCCRO is a major regulator of TME activity. The goal of this work is to define the specific contributions of SCCRO to TME activity and tumorigenesis. In an effort to understand the role of SCCRO in tumorogenesis, we assessed oral cavity squamous cell carcinomas induced in SCCRO-/- mice and found a reduction in tumor incidence and growth, with changes attributable not only to cancer cell autonomous activity but also to defects within the TME. Thus, we sought to elucidate the role of SCCRO in the TME.

Methods: To assess the effects of SCCRO on spontaneous development of oral cancer, we fed SCCRO-/- (n=6) and SCCRO+/+ (n=6) mice with 4-Nitroquinoline 1-oxide (4NQO) and monitored them periodically. To assess the specific contributions of the TME, we developed a syngeneic xenograft mouse model by injecting B16F1 melanoma cells and Lewis Lung Cancer cells into the flanks of SCCRO-/- and SCCRO+/+ mice. To determine whether the contribution of SCCRO involved bone marrow elements, we performed syngeneic tumor experiments described above after reciprocal transplantation of bone marrow (BMT) between different groups of SCCRO+/- and SCCRO-/- mice (n=6 in each group). To determine the requirements of SCCRO, we transplanted hematopoietic stem cells (HSCs) (excluding the mesenchymal stem cell [MSC] niche) from SCCRO+/+ mice into SCCRO-/- mice. Rates of tumor growth were analyzed, and TME changes, including angiogenesis, immune infiltrate, extent of desmoplasia, patterns of invasion, and development of metastasis, were assessed by gross, histological, and immunohistochemical analysis, as well as by flow cytometry. Skin wound healing, ischemia-reperfusion injury to liver, and liver regeneration were also assessed in SCCRO+/- and SCCRO-/- mice to determine the effects of SCCRO on related normal physiological processes.

Results: Broad abnormalities in growth, invasion, and metastasis, as well as angiogenesis, desmoplasia, and immune cell infiltration, were seen in tumors from SCCRO-/- mice, compared with those from SCCRO+/- mice, in all models, confirming the requirement for SCCRO in TME function. Tumor growth was completely rescued in SCCRO-/- mice by transplantation with SCCRO+/- whole BMT and was lost in reciprocally transplanted SCCRO+/- mice, suggesting that the effects of SCCRO were on the contribution of the bone marrow to the TME. Transplantation of HSCs from SCCRO+/- into SCCRO-/- mice failed to rescue tumor characteristics, suggesting that MSCs are dependent on SCCRO within the TME. Moreover, we found no defects in parallel physiological processes in SCCRO-/- mice, suggesting that SCCRO has activity that is essential and unique to the TME.

Conclusions: SCCRO plays an essential role in the contributions of MSCs to the TME without affecting related physiological processes. The selective effect on the TME is unique and makes SCCRO an attractive therapeutic target for prevention and treatment of a broad range of human cancers.
RHOC REGULATES CANCER STEM CELLS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA BY OVER-EXPRESSING IL-6 AND PHOSPHORYLATION OF STAT3

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Introduction: Important molecular and genetic research in the past decade has revealed that RhoC, a pro-metastatic oncogene is over-expressed in wide range of invasive carcinomas. Significantly these studies have found a strong correlation between elevated RhoC expression and tumor progression, invasion and metastasis to distant body regions. Moreover, RhoC has been shown to act as a transforming oncogene for human epithelial cells converting them into a highly motile and invasive phenotype. The increased expression levels of RhoC in a wide range of invasive carcinomas suggest that it is regulating more than one aspect of metastasis. To test our hypothesis, we analyzed for the presence of cancer stem cells (CSCs) in two head and neck cancer cell lines (UM-SCC-1 and -47) in which RhoC is highly expressed and in their RhoC knockdown counterparts. In this study we investigated the correlation between RhoC expression and CSCs formation in head and neck squamous cell carcinoma (HNSCC).

Methods: Tumorspheres formations were performed in ultralow attachment plates in absence of serum. ALDH and CD44 positive cells were sorted by FACS analysis. Expression of transcription factors were analyzed by Real time RT-PCR. Western blot analysis and ELISA were performed to elucidate the signaling mechanism involve in cancer stem cell progression.

Results: The inhibition of RhoC function was achieved using shRNA. The expression of stem cell surface markers, ALDH and CD44 were significantly low in two RhoC depleted HNSCC lines. Expressions of stem cell markers; ALDH and CD44 in scrambled control and RhoC knockdown lines were investigated by FACS analysis. In the control cell lines, 18% and 10% ALDH positive cells were detected. In contrast, only 13% and 4% ALDH positive cells were observed in the corresponding RhoC knockdown clones. A similar pattern was observed using CD44, interestingly, the scrambled sequence controls showed about 98% CD44 positive cells while in the corresponding RhoC knockdown lines it was 60% and 41%. Furthermore, a striking reduction in tumorsphere formation was observed in RhoC knockdown lines. The mRNA expression of RhoC in RhoC knockdown adherent and tumorspheres are dramatically down regulated as compared with the scrambled control. The mRNA expression of stem cell transcription factors; nanog, oct3/4 (Pouf1), and sox2 were significantly depleted in RhoC knockdown clones. Further, the phosphorylation of STAT3ser727, and STAT3tyr705 were significantly down regulated in RhoC knockdown clones. The overexpression of STAT3 in RhoC knockdown did not show any change in expression patterns of either-STAT3tyr705 or stem cell transcription factors, signifying the role of RhoC in STAT3 activation and thus the expression of nanog, oct3/4 and sox2 in HNSCC. The expression of IL-6 in RhoC knockdown HNSCC cell lines was dramatically low as compared to the scrambled control. Further, we have shown a rescue in STAT3 phosphorylation by IL-6 stimulation in RhoC knockdown lines.

Conclusion: This study is the first of its kind to establish the involvement of RhoC in STAT3 phosphorylation and hence in promoting the activation of core CSCs transcription factors. These findings suggest that RhoC may be a novel target for HNSCC therapy.
MICRORNAS PROFILE IN PATIENTS WITH FAMILY HISTORY OF HEAD AND NECK CANCER

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INTRODUCTION: Although most cases of head and neck cancer (HNSCC) occur sporadically and generally are related to chronic exposure of alcohol and tobacco, family history and genetic susceptibility have earned special interest in the last two decades.

OBJECTIVE: The aims of this study were to characterize the clinical and epidemiological profile of patients with head and neck cancer and family history of cancer, evaluate the expression profile of miRNAs in peripheral blood samples from patients with HNSCC and familial history of cancer with their relatives and check, by using miRNAs databases, the main regulated genes and their possible relationship to cancer.

MATERIALS AND METHODS: A total of 74 cases were selected through pre-existing databases of the Department of Head and Neck Surgery and Otorhinolaryngology, AC Camargo Hospital, Sao Paulo from 2003 to 2011. The criteria used to characterize the familial cases of HNSCC were: 1) two or more first degree relatives affected by HNSCC or related tumors, 2) Age of onset of HNSCC less than 45 years in at least one of the family members, 3) Appearance of HNSCC at any age if no prior exposure to tobacco and alcohol or any other known etiologic factor. Tumors considered related HNSCC were those related to tobacco consumption (lung, esophagus, stomach, pancreas, liver, kidney, bladder, uterus and bone marrow) or other epithelial tumors such as colorectal carcinoma, breast and melanoma. Then, peripheral blood samples of these patients were collected and, when possible, of one of them relatives affected by cancer. Subsequently, the evaluation of miRNAs expression was did by RT-qPCR.

RESULTS: The most common tumor sites of probands were oral cavity, with 31 cases (42%), followed by the larynx, 24 cases (31.5%). Among the 74 families, the number of affected relatives was 171, with 121 of first-degree and 50 of second and third degree. In this group there were 19 different tumor sites and the most common were: head and neck (18.6%), breast (16%), colon (13%), stomach (11%) and, finally, uterus (11%). Among first-degree relatives, the most common tumors in descending order were: breast (17.4%) and head and neck (15.7%). By analyzing only the first-degree relatives, the prevalence was found in siblings with 54 (46%) cases. Utilizing scores that could clarify differences between the selected sample and the pool of controls, 6 main miRNAs were identified: hsa-miR-582-3p, hsa-miR-597, hsa-miR-135b, hsa-miR-496, hsa-miR-431 and hsa-miR-517a. The cases with high scores were compared with microRNAs databases and those that had relations with important cancer-related genes were selected for confirmation of the findings. Hsa-miR-496, hsa-miR-582-3p e hsa-miR-597 are candidates to regulate genes as TGFBR1, PTEN, CDH1, TNF, FGFR4 e EGFR.

CONCLUSION: In conclusion, the data suggest that there may be an specific and peculiar expression profile of microRNAs in patients with a family history of cancer and that these microRNAs cited, in particular, hsa-miR-597 and hsa-miR-496 should be investigated in subsequent studies once there are few validated data in our literature.
We have recently synthesized a peptide called Disruptin, which is comprised of the SVDNPHVC segment of the EGFR and which inhibits binding of Hsp90 to the EGFR and EGF-dependent EGFR dimerization to cause EGFR degradation. The effect is specific for EGFR versus other Hsp90 client proteins. Here we show that Disruptin decreases the clonogenicity of a variety of EGFR-dependent cancer cells in culture but not of EGFR independent cancer cells or non-cancerous cells. The selectivity of Disruptin towards EGFR driven cancer cells is due to the production of EGF and the resulting high level of EGF stimulation of EGFR in EGFR-dependent tumor cells relative to normal cells. When administered by intraperitoneal injection into nude mice bearing EGFR driven human tumor xenografts, Disruptin causes extensive degradation of EGFR in the tumor but not in adjacent host tissue. Disruptin markedly inhibits the growth of EGFR driven tumors without producing the major toxicities caused by the Hsp90 inhibitor geldanamycin or by cisplatin. These findings provide proof of concept for development of a new Disruptin-like class of anti-tumor drugs that are directed specifically against EGFR driven tumors.
A TRANSCRIPTION FACTOR SIGNATURE OF HNSCC IMPLICATES STAT AND NFKB ACTIVATION IN HPV NEGATIVE TUMORS

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Background: Head and Neck Squamous Cell Carcinoma (HNSCC) results in significant mortality and morbidity despite current therapeutic strategies. The molecular biology of HNSCC is related to abnormal transcriptional regulation. A direct comprehensive genome wide analysis of deregulation of key transcription factors (TF) in primary HNSCC has not been performed. In this study we sought to analyze the differences in transcription factor signatures in subtypes of cancer patients and the normal population.

Methods: To evaluate the TF signatures of 44 HNSCC samples and 25 healthy oral mucosa samples we used the Affymetrix GeneChip Human Exon 1.0 ST Array data and estimated transcription levels of all genes. The TF activity signature of each of 2,600 human transcription factors was characterized by the expression of its target genes, as reflected in TRANSFAC, and corrected for methylation and CNV status. The significance of each TF based on the expression levels of its targets was compared for HPV positive (HPV+) and HPV negative (HPV-) samples. Both the expression of the subset of target genes of NFKB, STATs and AP1 pathways was confirmed by qRT-PCR and co-activation of NFKB and STAT3 in HPV-tumors was confirmed by immunohistochemical (IHC) analysis in separate, larger validation clinical cohorts, including a separately collected single institution cohort, as well as the TCGA cohort.

Results: Of the top ranked TFs analyzed AP1, NFKB and STATs exhibited the greatest differences in TF activity in HPV+ and HPV- HNSCC tumor tissue. The changes of activity of these factors do not depend on DNA methylation or copy loss for their targets. We confirmed coordinated activation of STAT3 and NFKB pathways in tumor samples, and showed that these pathways are the most activated in an HPV-population of HNSCC patients.

Conclusions: We have discovered that HPV+ and HPV- HNSCC differ significantly based on the level of activity of key TF, such as AP1, STATs and NFKB. These data have implications for therapeutic targeting of tumors, as well as potential insight into biologic variability of behavior and treatment response for HPV+ and HPV- HNSCC patients.
EVIDENCE OF HER3/4 RECEPTOR MEDIATED RESISTANCE TO EPIDERMAL GROWTH FACTOR INHIBITION

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The only FDA-approved targeted treatment for HNSCC is cituximab, an epidermal growth factor receptor (EGFR) antagonist, which is effective in only a subset of squamous cell cancers. Prior lab studies suggest a "rescue" phenomenon in which some cells sensitive to EGFR inhibition become resistant in the presence of insulin-like growth factor 1 (IGF-1), a naturally occurring cellular stimulant. This is likely mediated by the common pathways mediated by the EGF and IGF-1 receptors. The synergistic effect of inhibiting both receptors in the presence of IGF-1 suggest a more complex intracellular interaction. While IGF-1R inhibitors exist clinically, they are not currently used in HNSCC.

A proxy for measurement of pathway activation is phospho-species within the treated cell. Reverse-phase protein microarray (RPPA) allows assessment of hundreds of subspecies of proteins from multiple specimens at once with very small quantities of protein required. Thus we were able, in multiple HNSCC cell lines, to compare the response of cell lines to EGFR and/or IGF-1R inhibition with or without des-IGF stimulation. For EGFR inhibition, we used one of lapatinib, gefitinib, or erlotinib, either alone or in combination with an IGF-1R inhibitor (BMS754 or OSI).

See Table 1 for a highlight of our final data analysis. Cell lines were divided into two groups based on their survival response to EGFR inhibition with and without IGF stimulation. The "rescue" group were those cell lines with significant (18.9-50.7%, mean 32.7%) improvement in survival when adding des-IGF to EGFR blockade. The "nonrescue" group were those with minimal (0-15.2%, mean 7.9%) improvement with des-IGF. Quantity of protein was determined by intensity of fluorescence, and these values were scaled to a control value within each cell line to account for inter-experiment variation and the basal impact of IGF stimulation (unstimulated cells were scaled to an unstimulated control and IGF stimulated cells to an IGF stimulated control).

Of primary interest, we found those cell lines in the "rescue" group have significantly elevated quantities of ErbB3/Her3 and ErbB4/Her4 receptors when compared to those in the "nonrescue" group, as shown by the ratio presented in Table 1. These receptors may play a role in the ability of cells to gain resistance in the presence of IGF. From these data, we can conclude that RPPA provides important data to broadly analyze pathway activation in HNSCC and determine cellular mechanisms of resistance that are not currently targeted.

| Table 1. ErbB3/HER3 and ErbB4/HER4 Rescue/Nonrescue Ratio |
|----------------|----------------|----------------|
|                | -IGF           | +IGF           |
| ErbB3/HER3     | 11522569.86    | 2583990.99     |
| ErbB3/HER3 (Y1289) | 8303421.26 | 1.38           |
| ErbB4/HER4     | 13569568.09    | 0.82           |
| ErbB4/HER4 (Y1284) | 12708118.35 | 8816911.68     |
| ErbB4/HER4 (Y984) | 2.90           | 1.44           |
THE GROWTH OF EXPERIMENTAL ORAL SQUAMOUS CELL CARCINOMA IS STIMULATED BY AN OBESOGENIC DIET

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Objective/Background: Obesity is a growing problem in the United States and the world. It is estimated that obesity rates in many states will exceed 65% by 2030. Obesity has been associated with increased mortality for numerous malignancies in men and women such as colon, lymphoma, prostate and breast. Recently, we reported a previously unrecognized association between obesity and worse prognosis in patients with T1 and T2 squamous cell carcinoma of the oral tongue. Specifically, body mass index (BMI) of 30 or greater conferred a five-fold worsening of disease specific survival compared to normal BMI (less than 25). The biologic mechanisms underlying this association, however, are unknown. In the current study, we evaluated the effect of diet induced obesity on the growth of murine oral cavity squamous cell carcinoma xenografts.

Study Methods: Male C57BL/6J mice were fed either a low fat (10 kcal% fat) or a high fat diet (60 kcal% fat) for 12 weeks to induce lean and obese mice, respectively. Subsequently, indolent (MOC1) or aggressive (MOC2) mouse oral squamous cell carcinoma cell lines were implanted in the flanks. The mice were kept on their predetermined diets for the remainder of the study. Weekly measurements of tumor volume were taken to determine the rate of tumor growth in the obese vs. lean mice. When tumor growth reached a predetermined size, the mice were sacrificed.

Results: At the time of tumor cell implantation, the mice fed the high fat diet weighed approximately 40% more than the mice fed the low fat diet. The growth rate of the MOC2 xenografts was greater in the obese than the lean mice. At sacrifice, tumor size was greater in obese vs. lean mice (668.06mm³ vs. 281.75mm³, p=0.02). The development of MOC1 xenografts was faster in obese than in lean mice. Tumors were found in the majority of obese mice by 14d post tumor cell implantation. In contrast, tumors did not appear in the lean mice until 35d post implantation. Due to tumor burden, the obese mice were sacrificed by day 28; however, the lean mice were not sacrificed until day 51, at which point tumors had reached the same size as the tumors formed in the obese mice at day 28.

Conclusions: Diet induced obesity is associated with enhanced experimental tumor growth. This finding fits with the recent clinical observation that obesity is associated with poor prognosis for patients with early stage oral tongue cancer. The development of a mouse model provides the opportunity to develop targeted therapies to inhibit obesity-related tumor growth.