Human papillomavirus (HPV) appears to be responsible for dramatic increases in the incidence of oropharyngeal squamous cell carcinoma (OPSCC) in the United States in the last two decades, in spite of a decreased incidence of smoking. HPV integration into the host genome occurs in most cervical cancers and the effect of this is increased genome instability which causes additional downstream alterations that leads to invasive cancer. The common fragile sites (CFSs) are highly unstable chromosomal regions found in all individuals that are particularly sensitive to genomic instability and also hot-spots for viral integrations. The CFSs span several megabases and many of them also span extremely large genes. The three most frequently expressed CFSs: FRA3B (3p14.2); FRA16D (16q23.2) and FRA6E (6q26) span FHIT (1.5 Mbs), WWOX (1.1 Mbs) and PARK2 (1.3 Mbs), respectively, and these three large genes have been demonstrated to function as important tumor suppressors involved in the development of a number of different cancers. Many of the other large CFS genes have also been suggested to function as tumor suppressors. We analyzed RNAseq data that we had previously generated on 11 OPSCCs and matched normal oropharyngeal tissue and focused our analysis on the expression of the largest human genes. The two large genes with the greatest decreases in expression in the tumors as compared to matched normals were PARK2 and the putative tumor suppressor DLG2, but three other large CFS genes, CTNNA3, LRP1B and DMD, and one large gene not yet precisely localized within a CFS region, PDE4D, had much more decreased expression than any other of the large genes. Real-time RT-PCR was used to measure the expression of these six genes in a much larger number of OPSCC tumor-normal pairs. The expression of these six genes was concordant in most OPSCCs; an individual tumor had either decreased expression of all six genes, no change in the expression of all genes, or slight increased expression of all six genes, relative to matched normal oropharyngeal tissue. There was no correlation between changes in the expression of these genes and the patient’s previous history of smoking. However, when the tumors were grouped with respect to their HPV status it was observed that 40% of the HPV-positive OPSCCs had over a 100-fold decrease in the expression of all six genes. Half of the HPV-minus OPSCCs also had decreased expression, but it was much more modest (4-8 fold). Most striking, however was that ten of the 18 tumors that had decreased expression of the large genes had recurrence. In contrast, only a single of the 16 tumors that did not have decreased expression of these genes had recurrence. Thus, decreases in the expression of large CFS genes could be a valuable diagnostic marker for tumor recurrence.
A COMPUTATIONAL APPROACH TO THE IDENTIFICATION OF PROGNOSTIC BIOMARKERS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Background.

Advances in the treatment of patients with head and neck squamous carcinoma (HNSC) have resulted in marginal improvement in overall survival. Much of the survival change is attributed to the rapidly increasing prevalence of prognostically favorable patients with HPV-associated HNSC. Therefore, a comprehensive analysis of all HNSC tumor genomes is needed to elucidate new prognostic biomarkers and potential novel therapeutic agents. Current applications in molecular characterizations have provided inconsistent results from different platforms. We sought to interrogate the head and neck cancer dataset from the multi-institutional Cancer Genome Atlas (TCGA) for miRNAs and mRNAs associated with cancer pathology and predictive for survival.

Methods.

A large data set was obtained from TCGA data coordination center using TCGA-Assembler [Zhu et al., 2014]. The data set consists of matched miRNA and mRNA expression profiles for 301 patients, with matched tumor and solid normal tissue samples for some of the patients. For each patient, expressions of 1046 miRNAs and 20531 mRNAs were recorded. In addition, survival data were available as well for all the patients. T-tests were performed to identify miRNAs and mRNAs that were differentially expressed between tumor and normal samples and Cox regression was performed to find miRNAs and mRNAs that are associated with survival. Multiple-test correction was based on the false-discovery-rate procedure.

Results.

A total of 156 miRNAs (Figure 1) and 3,481 mRNAs was differentially expressed between tumor and normal, with a false discovery rate controlled at 0.001 and a fold change either >= 2 or <= 0.5. Survival analysis yielded 38 miRNAs and 139 mRNAs that were associated with patient survival, with a false discovery rate controlled at 0.01. Combining the results from both analyses on multivariate platform, 17 miRNAs and 25 mRNAs demonstrated differential expression compared to normal and had a significant impact on survival.

Conclusion.

Our integrative analysis of patients with HNSC using TCGA database has provided specific miRNAs and mRNAs that are associated with poor prognosis. This data provides the foundation for future studies validating the identified miRNAs and mRNAs demonstrating mechanistic effects in HNSC carcinogenesis and disease progression. These works should lead to the potential discovery of novel therapeutics in patients affected with this disease.
GAINS ON 2Q24.2-Q24.3 AND LOSSES ON 5Q13.1-Q13.2 AND 10Q21.3 REVEAL CANDIDATE GENES ASSOCIATED WITH POOR SURVIVAL IN LARYNGEAL SQUAMOUS CELL CARCINOMA PATIENTS

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Background: Laryngeal squamous cell carcinoma (LSCC), one of the most common head and neck carcinomas worldwide, presents with an aggressive clinical phenotype conferring to the patients a high rate of recurrence and risk of developing second primary tumors. In recent years, several molecular studies have been conducted to better understand laryngeal carcinogenesis and identify prognostic markers and therapeutic targets that can be utilized in the clinical practice. In this study our main aim was to identify driver genes associated with LSCC using an integrative molecular approach of large-scale genomic, transcriptomic and miRNAs analysis.

Methods: Nucleic acids (DNA and RNA) were obtained from 37 LSCC samples from untreated patients at the time of the diagnosis, with a clinical follow up of 10 years. The cases were evaluated using array-CGH, mRNA and miRNA large-scale oligoarrays platforms (Agilent Technologies). Copy number alterations (CNA) were analyzed using the Nexus Copy Number v.7.0 software, and differentially expressed miRNAs and transcripts were obtained using the TMeV software. Cancer-specific survival (CSS) was evaluated by Kaplan-Meier and Log-Rank tests. The data obtained from the above analyses was integrated to identify chromosomal regions presenting concomitantly CNAs and differentially expressed transcripts (both mRNA and miRNA) that may be associated with a worse outcome (r>0).

Results: Genomic analysis revealed three chromosome regions associated with shorter CSS: gains on 2q24.2-q24.3, and losses on 5q13.1-q13.2 and 10q21.3. The integration of this data with the ones obtained from the transcriptomic analysis, revealed that overexpression of the DPP4, GCA and KCNH7 genes were associated with gains on 2q24.2-q24.3. Similarly, reduced expression of the CCNB, CDK7 and MRP36 genes were associated with loss on 5q13.1-q13.2 and of the DNA2, SLC25A16 and TET1 genes with 10q21.3 loss. microRNA analysis performed in the same samples revealed that these genes can be targeted by five differentially expressed microRNAs, indicating that these transcripts can be regulated epigenetically.

Conclusions: The data described in this study highlight specific genomic regions with combined CNAs and gene expression changes in association with a poor survival rate. These alterations were not previously described in LCCC and therefore can be considered novel molecular drivers with prognostic and therapeutic potential for the clinical management of these tumors.
A COMPREHENSIVE EXPRESSION ANALYSIS OF CANCER TESTIS ANTIGENS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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INTRODUCTION: Although many strategies have been used in the treatment of head and neck squamous cell carcinoma (HNSCC), the overall survival rate is still close to 50%. So, the development of novel therapeutic approaches is essential to improve this dismal outcome. Cancer-testis antigens (CTA) comprise families of tumor-associated antigens that are immunogenic in different cancers. Their restricted expression makes them attractive targets for immunotherapy. The aim of this study was to profile the CTA gene expression in a large number of HNSCC to assess their potential as targets for immunotherapy and to evaluate their prognostic significance in HNSCC patients.

METHODS: Using an in silico approach, we selected, among 139 CTA genes previously described, those potentially expressed in HNSCC. Then, the expression pattern of 36 CTA genes was evaluated by RT-PCR in a series of 89 HNSCC and 20 normal mucosa samples.

RESULTS: Five CTAs highly associated with HNSCC cases were identified and, at least one of these CTAs, was expressed in 85% of the tumors examined. Co-expression of two or more genes occurred in 65.4% of cases. Furthermore, the mRNA positivity of CXORF48, MAGEB6, and CRISP2 showed a significant association with the presence of perineural invasion, extracapsular spread of metastatic lymph nodes and lymphovascular invasion, respectively.

CONCLUSIONS: HNSCC expresses different CTAs and some of them could be useful as targets for immunotherapy approaches. Moreover, the mRNA positivity of MAGEB6, CRISP2 and CXORF48 is significantly associated with recognized poor outcome clinical features.

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EFFECTS OF PERITUMORAL NANOCONJUGATED CISPLATIN ON CANCER STEM CELLS
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BACKGROUND: Cancer stem cells (CSCs) are considered by many to be primary mediators of treatment failure in head and neck squamous cell carcinomas (HNSCC). Systemic platinum-based chemotherapy, a current standard, is limited by poor lymphatic penetration, dose-limiting toxicity, resistance, and a lack of specificity in targeting CSCs. Hyaluronan (HA) nanoparticle conjugated peritumoral chemotherapy is a unique strategy that has shown higher efficacy than systemic therapy in locally-advanced HNSCC xenograft models. Since HA is a ligand for CD44 receptors, a potential marker of CSCs, part of the increased efficacy of HA-cisplatin may be explained by CSC-targeting via this CD44 interaction.

OBJECTIVES: 1) To evaluate whether peritumoral HA-cisplatin therapy has higher efficacy and less toxicity than standard systemic cisplatin in vivo. 2) To evaluate the effect of HA-cisplatin on the CD44+ HNSCC population ex vivo.

METHODS: An in vivo xenograft murine model was used by inoculating 33 athymic nude mice with UMSCC-12 laryngeal cancer cells, high in CD44+ expression. Once tumor volumes reached 50 mm³, treatment was rendered in a randomized, controlled manner, with 11 mice in each of the three arms: control, systemic cisplatin, and peritumoral HA-cisplatin. Subjects were treated at 50% maximum tolerated dose for 3 weeks. Three mice from each arm were then euthanized and tumors were harvested. Tumors were processed into single-cell suspensions and stained with APC anti-CD44 antibody to evaluate ex vivo the early post-treatment effect on CD44+ cell proportion via flow cytometry. Animals were then monitored for 9 weeks post-treatment for tumor size and body weight. At end-of-study, 4 mice from each arm were analyzed for CD44+ cell proportion in the tumors ex vivo as well as for renal toxicity histologically.

RESULTS: HA-cisplatin showed superior antitumor efficacy compared to cisplatin as shown by tumor volumes at 3 weeks post-treatment (41.1mm³ vs. 59.9mm³, p = 0.009) and at end-of-study (68.4mm³ vs. 105.6mm³, p = 0.05), with one study subject in the HA-cisplatin arm with a complete response (CR) and one with a sustained partial response (PR) whereas none of the standard cisplatin mice had CR or PR. In evaluating toxicity, animal weights were similar between the two treatment arms, but no nephrotoxic effects were histologically seen with the 4 sampled HA-cisplatin mice while 2 of 4 of the cisplatin mice showed signs of nephrotoxicity. In the early post-treatment group, CD44 reduction was seen in both treatment groups, but to a greater degree with HA-cisplatin. Long-term durability of this effect was seen end-of-study in the HA-cisplatin group compared to cisplatin (8.1%CD44+ vs. 23.9%CD44+, p = 0.02) which was similar to control (23.9%CD44+ vs. 24.8%CD44+, p = 0.86).

CONCLUSIONS: Peritumoral nanoconjugated HA-cisplatin provides superior antitumor efficacy with less toxicity compared to standard cisplatin therapy in an in vivo laryngeal cancer xenograft model. Moreover, it may potentially target CSCs as evidenced by its selective effect on CD44+ cells within a heterogeneous tumor population. These results provide support for further translational investigation of this treatment modality as a potential CSC-targeting agent for future clinical application to patients with locally advanced HNSCC.
Treatment Effect on CD44% Proportion

Treatment Groups

- Control
- Cisplatin
- FA-Cisplatin
Perineural invasion (PNI) is an insidious form of cancer progression in which cancer cells invade and extend along nerves. PNI is a significant clinicopathologic feature in head and neck cancers, heralding decreased survival, increased locoregional recurrence and metastasis rates, and a shorter time to recurrence. To identify novel ligand-receptor interactions that may be involved in the process of PNI, a chemokine array was used to screen nerve factors secreted from explants of murine dorsal root ganglion (DRG). CCL2, a chemokine that regulates immune cell recruitment, was identified as a candidate factor. The expression of CCR2, the transmembrane receptor to CCL2, by different cancer cell lines correlates with migration towards CCL2. CCL2 activates the MAPK and Akt pathways in cancer cells. In vitro nerve invasion assays using co-cultures of cancer cells and DRG demonstrate that cancer cell expression of CCR2 facilitates PNI. PNI was significantly diminished in this assay when using DRG harvested from CCL2-/- knockout mice as compared with CCL2+/+ wild type mice. An in vivo murine sciatic nerve model of PNI demonstrates that cancer cell expression of CCR2 supports PNI. We demonstrate that nerve-released CCL2 significantly enhances PNI of cancer cells though CCR2-mediated signaling. These results advance our understanding of the mechanisms involved in the process of PNI, and identify a novel therapeutic target.
**S273  H-4073, A POTENT CURCUMIN ANALOG, REVERSES CHEMO-RESISTANCE IN HEAD AND NECK CANCER**

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**Objectives:** Chemotherapy constitutes the standard modality of treatment for localized head and neck squamous cell carcinomas (HNSCC). However, many patients fail to respond and relapse after such treatments due to the acquisition of chemo-resistance. Therefore, there is an urgent need to develop novel drugs that could reverse the resistant phenotype. Curcumin, the constituent of the spice turmeric has been shown to have anti-inflammatory, anti-oxidant and anti-proliferative properties in several tumor types. However, use of curcumin has been limited due to its poor bio-absorption. Recently, a novel class of curcumin analogs, based on diarylidenylpiperidones (DAP), has been developed by incorporating a piperidone link to the beta-diketone structure and fluoro substitutions on the phenyl groups. We have observed that these DAP compounds, in general are more effective than curcumin in inhibiting the proliferation of a variety of cancer cell lines and xenograft tumors. In this study, we evaluated the effectiveness of H-4073, a parafluorinated variant of DAP, using both in vitro and in vivo head and neck cancer models.

**Methodology:** The study was performed using a panel of established human head & neck cancer cell lines. Cell-viability was assessed by MTT assay, cell migration was measured using the xCELLigence system. Apoptosis was quantified by Annexin V staining. Cleaved caspase-3 and JAK/STAT3 signaling proteins were measured by Western blot. A SCID mouse xenograft model was used to access the in vivo efficacy of H-4073.

**Results:** Our results showed that H-4073 is a potent anti-tumor agent and it significantly inhibited cell proliferation in all the head and neck cancer cell lines tested in a dose- and time-dependent manner, irrespective of p53 mutation status or human papillomavirus positivity. In addition, pretreatment of a highly chemo-resistant HNSCC cell line (UM-SCC-74A) with H-4073 significantly reversed the chemoresistance as observed by cell viability assay (MTT); colony formation assay; apoptosis assay (Annexin V binding) and cleaved caspase-3 (Western blot). H-4073 induced chemosensitivity via the inhibition of JAK/STAT3 pathway. To further examine the effect of H-4073 on chemoresistance, we established a cisplatin-resistant cell line (CAL 27-CisR) in the laboratory by growing a tongue cancer cell line CAL 27 in increasing concentrations of cisplatin. CAL 27-CisR is highly resistant to cisplatin (IC50 20 μM) as compared to the parental cell line (IC50 2 μM). Treatment with H-4073 resulted in a 4-fold increase in apoptosis in the cisplatin resistant CAL 27-CisR as compared to untreated cells. In the SCID mouse xenograft model, H-4073 significantly enhanced the anti-tumor and anti-angiogenesis effects of cisplatin, with no added systemic toxicity.

**Conclusion:** Taken together, our results suggest that H-4073 is a potent anti-tumor agent and it can be used to overcome chemo-resistance in head and neck cancer cells.
CD44 VARIANT 9 EXPRESSING CANCER STEM-LIKE CELLS ATTENUATE THE EFFICACY OF CHEMORADIOSELECTION IN ADVANCED HEAD AND NECK CANCER

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Background: In our institute, chemoradioselection strategy has been used as a tool to measure the biological aggressiveness of individual tumor. In brief, patients who demonstrate favorable response to the 40Gy of CCRT, i.e., chemoradioselected (CRS), proceed to further CCRT up to 60-70Gy, while the remaining unfavorable responders, i.e., non-chemoradioselected (N-CRS), undergo radical surgery. Intriguingly, CRS patients demonstrate significantly better survival and organ preservation irrespective of their clinical stages. Thus, if we could potentate the efficacy of chemoradioselection, more improved survival and organ preservation might be feasible. Through a series of elegant studies by the group of Prof. Saya at Keio University (Nat Commun 2012;3:883, Cancer Cell 2011;19(3):387-400, Cancer Res 2013;73(6):1855-66, Br J Cancer 2013;109(2):379-86, Oncogene 2013;32(44):5191-8), CD44 variant 9 (CD44v9), a splicing variant of CD44, has emerged as a novel marker of cancer stemness expressed in several solid tumors including HNSCC. Functionally, CD44v9, coupling with xCT, increases the intracellular levels of reduced glutathione and thereby protect cells from ROS and oxidative stress. This is supposed to be a major mechanism by which CD44v9 expressing cancer stem-like cells (CSC) survive chemo/radiation. Collectively, the aim of this study is to analyze the clinic roles of CD44v9 in the chemoradioselection strategy.

Materials and Methods: Through the search of medical charts, 102 patients with advanced HNSCC treated with chemoradioselection from 1997 to 2008 were enrolled to the present study. According to the algorithm, 30 patients were CRC, while 72 patients were N-CRS; CRS demonstrated significantly better outcomes, consistent with our previous findings. Using the conventional immunohistochemical technique, 60 biopsy specimens (30 from CRC and 30 from N-CRS) obtained prior to the treatment, and 72 surgically removed tumor specimens obtained form the N-CRS patients were immuno-stained with the anti-CD44v9 specific antibody (generated by Prof. Saya). According to the staining score ranging from -1 to 5, the samples were defined as CD44v9-negative (score, -1~1) and CD44v9-positive (score, 2~5).

Results: The expression levels of CD44v9 in the biopsy specimens (n=60) didn't correlates with the chemoradioselection and patients' survival. However, among the N-CRS patients, CD44v9-positive group (n=31) demonstrated significantly (p=0.008) worse prognosis compared to CD44v9-negative group (n=41). Multivariate analyses demonstrated that among 5 candidate factors CD44v9 positivity alone was significantly correlated with the prognosis (HR: 3.140, 95% CI: 1.230-8.017, p=0.0167). We further compared the CD44v9 expression levels in the 30 paired biopsy and surgically removed specimens obtained from the identical patient in the N-CRS group. The CD44v9-induced group (N=12) demonstrated significantly (p=0.04) worse overall survival than that of CD44v9-non-induced group (n=18). These results strongly indicate that not the intrinsic but the CCRT-induced CD44v9 expression is a therapeutic hurdle to chemoradioselection.

Conclusions: CCRT-induced CD44v9 expressing CSC appear to be a novel molecular target for improved chemoradioselection and consequent organ preservation and survival. The addition of xCT inhibitor
(e.g., sulfasalazine) to chemoradioselection might open up a new avenue for clinically feasible CSC targeted therapy in HNSCC.
Background: A critical problem in the treatment of Head and Neck Squamous cell carcinoma (HNSCC) is radioresistance. Recently, Gold Nanoparticles (GNP) has demonstrated great potential for cancer therapy as drug carriers, imaging contrast agents and radiosensitizers. It is well known that radiation can be enhanced by the presence of high atomic number materials within the tumor by causing significant increase in the absorption of photons and deposition of energy. However, the energy used in previous studies was not in a clinically relevant range, and the gold concentrations in tumors were not sufficient.

Objective: In this study we investigated the possibility to directly connect GNP to HNSCC EGFR receptors tumors through Cetuximab coated GNP (CTXGNP) and evaluate the effect of GNP on radiosensitivity.

Methods: Eighteen nude mice with HNSCC were divided into six equal groups according to treatment modality: control, radiation, radiation and Cetuximab (RTCTX), Cetuximab alone, non specific GNP with radiation, and CTXGNP with radiation. The mice and tumors were followed for six weeks. The change in size of tumors was measured weekly and CT scans of all tumors were performed. Blood tests were performed to evaluate toxicity. Tunel assay was performed on tumor histological sections of the various treatment groups in order to determine apoptosis rate. PCNA and Ki67 staining were employed to assess repair and proliferation rates.

Results: Liver and kidney function and blood count tests of all groups taken after one week and five weeks detected no toxicity. As far as tumor growth comparison between all groups showed that all radiation groups had a stronger effect than Cetuximab alone; CTXGNP had the strongest impact on tumor growth, much stronger impact than the standard RTCTX or the Radiation+IgG-coated GNP. The disease remained stable and all mice survived. The results were nearly statistically significant (P=0.07). All treatments exhibited enhanced apoptosis rate compared with control. At six weeks post treatment, the apoptosis signal was reduced in the GNP- group indicating earlier apoptosis than in other groups. The CD34 was significantly lower in the GNP group indicating lower level of angiogenesis.

Conclusions: This study has demonstrated a novel method to improve radiosensitivity and therefore survival in HNSCC. Intravenous administration of targeted GNP, improved the radiation therapy outcome by increasing the radiation absorbed in the tumor, without major adverse events.