DIRECT GENETIC ANALYSIS OF SINGLE HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) CELLS ISOLATED FROM PRIMARY TUMORS AND MATCHED NODAL METASTASES

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Background Genetic heterogeneity is a hallmark of cancer and significant genetic and phenotypic evolution occurs locally and at metastatic sites with disease progression. Heterogeneity can exist between tumors (inter-tumor heterogeneity) as well as within individual tumors (intra-tumor heterogeneity) and might significantly contribute to resistance to systemic therapy. Since systematic in-depth analyses are lacking in head and neck squamous cell carcinomas (HNSCC), we established and tested a protocol that enables comprehensive single cell analysis of cancer cells. We present the first systematic analysis of somatic copy number alterations (SCNA) in primary tumors and matched lymph node metastases of head and neck squamous cell carcinomas (HNSCC) on a single cell level.

Material & Methods From matched primary tumors and lymph node metastases of HNSCC patients (n=5) a single cell suspension was generated by enzymatic digestion and mechanical disaggregation. Epithelial tumor cells were identified with double-immunofluorescence staining for CD44v6 and CK5/14. The genome of isolated single cells was amplified with an adapter-linker PCR. Afterwards Array Comparative Genomic Hybridization (aCGH, 4 x 180 k) was performed on single cell genome amplification products. aCGH-results were confirmed via Fluorescence in situ Hybridization (FISH) of different genes (CCND1, MYC, CDKN2A, EGFR and TP53).

Results Totally we performed aCGH of 103 single tumor cells derived from 5 matched primary tumor and metastasis samples. The summarized profiles showed typical alterations for HNSCC. On the single cell level we observed a lesser degree of heterogeneity in 80% (four in five) of the matched metastases. This was further validated by the analysis of shared and private individual SCNA (iSCNA) present in each investigated tissue revealing an increase of the shared alterations in most lymphatic metastases. Interestingly, chromosomal gains were more frequently enriched than chromosomal losses among cells isolated from metastases. The most frequent SCNA gains were enriched in the metastasis at chromosome 8 and could be detected in every metastasis but not in every investigated cell of one metastasis.

Conclusion Our comprehensive single cell screening of primary tumors and matched metastases on a single-cell level revealed a selection towards a certain genotype during metastasis. In the context of effective targeted therapies, this observed genetic variability among cancer cells might be of high clinical importance.
Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide and five-year survival rates (<50%) are among the lowest of the major cancers. The high mortality associated with advanced HNSCC is in large part due to the metastatic spread to vital organs. Although our understanding of the molecular and biological events that contribute to tumor progression has increased considerably over the last decade, we still know very little about the role of tumor-accessory cells in promoting tumor metastasis. Previously, we have shown that tumor samples from HNSCC patients contained significantly higher Bcl-2 positive tumor blood vessels and this enhanced Bcl-2 expression in tumor-associated endothelial cells directly correlated with metastatic status of these patients. It is estimated that from a 1 cm primary tumor as many as 2 million tumor cells can be shed each day into the circulation, though very few of these tumor cells ever form metastases. Most of the cancer cells, particularly squamous cancer cells, have very low survival rates in circulation and undergo rapid anoikis. In addition to circulating tumor cells, increased levels of circulating endothelial cells are also observed in cancer patients with progressive disease. Interestingly, we also observed a marked increase in Bcl-2 positive circulating endothelial cells in the blood samples of head and neck cancer patients. These results raise an intriguing question about the biological significance of these circulating endothelial cells. In this study, we performed a series of experiments to examine if tumor-associated endothelial cells could promote tumor cell metastasis by binding to circulating tumor cells and chaperoning these tumor cells to distal sites. Our results demonstrate that endothelial cells overexpressing Bcl-2 (EC-Bcl-2) show significantly higher E-selectin expression and exhibit enhanced tumor cell binding. In addition, tumor cells showed a significant decrease in anoikis when co-cultured with EC-Bcl-2 in non-adherent conditions. This EC-Bcl-2-tumor cell binding and anoikis resistance was mediated by the E-selectin adhesion molecule and Src-FAK survival signaling. We further examined the role of Bcl-2 in tumor cell metastasis using two SCID mice models. In the first model, tumor cells were co-implanted with EC-Bcl-2 or EC-VC (GFP labeled) in the flanks of SCID mice. Tumor cells when co-implanted with EC-Bcl-2 showed significantly higher lung metastasis as compared to EC-VC group. Interestingly, blood and lung samples from these animals showed the presence of EC-Bcl-2 cells (GFP-labeled, primary tumor origin) thereby demonstrating that tumor-associated endothelial are released from the primary tumors. In the second in vivo model, we examined the chaperone function of EC-Bcl-2 by co-injecting tumor cells and endothelial cells via the tail vein. Animals co-injected with tumor cells and EC-Bcl-2 showed significantly higher lung metastasis as compared to tumor cell and EC-VC or tumor cell alone group. Taken together, these results suggest a novel role for tumor-associated endothelial cells in binding to tumor cells, protecting them from anoikis and chaperoning them to distal sites.
PREDICTING CERVICAL METASTASIS IN EARLY STAGE ORAL TONGUE CANCER: A META-ANALYSIS
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Introduction: Early stage oral tongue squamous cell cancer carries a risk of cervical metastasis posing a clinical dilemma for head and neck surgeons. On one hand it is not high enough (<20%) to warrant routine neck dissection yet not low enough to go without consideration (>10%) thus creating the debate of how to manage the neck in early stage oral tongue cancer.

Objective: The objective of this investigation was to study the clinical utility of individual tumor markers previously reported as potentially predictive of cervical lymph node metastases in specimens of squamous cell carcinomas of the oral tongue.

Design: A meta-analysis of tumor markers associated with cervical metastasis in oral tongue cancer over the last 25 years.

Results: Forty five articles were identified meeting inclusion criteria of which thirty one papers provided adequate data for analysis. A total of 52 unique markers were reported with 39 of those reporting adequate data for analysis. Eight markers were studied in two or more papers. Fourteen markers had sensitivity (sens) over 75%. Four markers achieved this in two or more papers, namely mmp-9 (sens 0.80), vegf (sens 0.94), E cadherin (sens 0.90), cyclin d1 (sens 0.85), with a combined sens of 0.87. A total of eight markers had specificity (spec) over 75%. p52 was the only marker achieving this in more than one study (combined spec 0.86). Two markers had both sensitivity and specificity over 75%, namely, E cad (0.89 and 0.89) and cd 105 (0.82 and 0.94). Seventeen markers had a negative predictive value (NPV) over 75%. Seven markers achieved a NPV of 90% or better. Three markers achieved this in two or more papers, namely E cadherin, VEGF, and cyclin d1 with combined NPV of 0.91.

Conclusion: Based on these results, a panel of tumor markers most apt to predict nodal metastases in early tongue carcinomas is proposed for prospective validation.
MATTED NODES AS AN INDICATOR OF POOR PROGNOSIS IN OROPHARYNGEAL SQUAMOUS CELL CARCINOMA: A VALIDATION STUDY

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IMPORTANCE: Matted nodes in patients with oropharyngeal squamous cell carcinoma (OPSCC) has previously been shown to be an independent prognostic factor and predictor of distant metastasis and survival in a cohort of patients treated with a single protocol of weekly carboplatin, taxotere and IMRT. The importance of this finding needs to be evaluated in patients who have undergone differing treatment approaches.

OBJECTIVE: This study seeks to evaluate if matted nodes are a predictor of poor prognosis in a group of patients who have undergone different treatment approaches.

DESIGN: This data is from a convenience sample of patients whose data was collected prospectively as the part of the population core of a Head and Neck SPORE. The patients in this cohort are patients who presented with previously untreated patients with OPSCC. This database was cross-sectioned retrospectively.

SETTINGS: Academic Tertiary Care Center

PARTICIPANTS: 114 previously untreated patients with OPSCC. All patients received treatment with curative intent in the form of chemoradiation (CRT), radiation, or surgery with adjuvant treatment. All patients had computed tomography (CT) scans within 30 days of beginning treatment. Overall 75% of patient received CRT, 5% received radiation alone, 5% received surgery alone and 10% were treated with surgery and adjuvant radiation with or without chemotherapy. Of those that received chemotherapy, 63% had carboplatin and taxotere, 14% received cisplatin, 11% received cetuximab. Chemotherapeutic regimen was unknown for 10% of patients. All patient treated with chemotherapy also received radiation.

INTERVENTIONS OR EXPOSURES FOR OBSERVATIONAL STUDIES: A panel of 3 clinicians assessed the presence of matted nodes on pre-treatment CT scans. Patient’s lymph nodes were designated as either "matted" or "non-matted" based on the CT scans. If there was disagreement or ambiguity, then scans were reviewed by a neuroradiologist who made the final determination of matted versus non-matted.

MAIN OUTCOME AND MEASURES: Primary endpoints were overall survival and disease free survival.

RESULTS: Overall 23 patients had matted nodes (20.2%). Human Papillomavirus (HPV) was detected in 97 (85.1%) of tumors with 17 (14.9%) being HPV (-). Matted nodes were present in 18 (18.6%) of HPV (+) tumors and 5 (29.4%) of HPV (-) tumors. Overall survival at 48 months was 51% in patients with matted nodes as compared with 92% in patients without matted nodes (p=0.0016) Figure 1.
Disease free survival was also statistically significantly worse for patients with matted nodes. The disease free survival at 48 months was 50% versus 85% for patients without matted nodes (p=0.0132).
Figure 2. Kaplan-Meier curve of disease free survival stratified by matted nodes.
CONCLUSIONS AND RELEVANCE: Matted nodes remains a predictor of poor prognosis in patients with OPSCC who have undergone a variety of treatment approaches. This finding should be
ROUTINE HISTOLOGICAL EVALUATION FOLLOWED BY SERIAL STEP SECTIONING ON LYMPH NODES FROM CN0 ORAL AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

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Introduction. The presence of lymph node metastases is well established as the major determinant for prognosis in head and neck squamous cell carcinoma (HNSCC). It's related to a higher risk of distant metastases and to a lower disease-free survival rate. The assessment of the node negative neck (N0) is currently done by imaging techniques and, in some institutions, by sentinel node biopsy (SNB). The presence of occult metastases can be missed by routine histological examination (H&E).

Methods. We analyzed 400 lymph nodes from 18 patients who had oral or oropharyngeal squamous cell carcinoma and clinically a radiologically N0 necks, treated with excision of the primary tumor and elective neck dissection, plus adjuvant treatment if necessary. The specimens were initially submitted to conventional (H&E) histological examination and then to a serial step sectioning (5 um).

Results. Eighteen patients were initially selected, being 15 males (84%) and three female. The mean age was 54 years old (41 - 87 yo) and the majority of patients were caucasian (84%). Six of the selected patients were found to be node positive (pN1) at the initial analysis, despite no clinical or radiological evidence, and 12 patients were node negatives (pN0). After the step section analysis, 25% (3/12) of the initially pN0 patients were found to harbor occult metastases.

Discussion. The presence of occult metastases in HNSCC is not an unusual phenomenon, and it has been shown in other types of cancer, such as melanoma, breast cancer and esophageal cancer, to be prognostically important. Van der Brekel et al performed thorough serial sectioning using H&E in specimens from elective neck dissection and found a 25% incidence of occult metastases. Resemblant data were reported by Woolgar et al, who found a 28% incidence using a similar technique.

Conclusion. The presence of occult metastases of HNSCC to the neck can be better assessed with a meticulous analysis of the lymph node. The high incidence of occult metastases shown by literature indicates that conventional H&E assessment does not accurately assess the neck in early stage disease. The role of this occult metastases at the patient's prognosis is yet to be determined.
Introduction: Dysregulated signaling through the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways is often the result of genetic alterations in critical components in these pathways or upstream Erb B activators; elevated levels of activated components of these pro-survival pathways are associated with unrestricted cellular proliferation and consequently invasion capacity of the tumor.

Objective: To map immunohistochemical expression of ErbB signaling pathway as marker of tumor invasivity in locally advanced laryngeal squamous cell carcinoma.

Material and methods: In our study, immunohistochemical expression of ten biomarkers (EGFR, Erb B2, Erb B3, Erb B4, RAS, MEK, ERK, mTOR, pTEN, p53) was explored to map Erb B signaling pathway in 94 cases of locally advanced laryngeal squamous cell carcinoma (SCC), organised in a tissue microarray for 74 cases and traditional whole-slide for 18 cases.

Results: In the present case series, three Erb B receptors were present in more than 70% of the tumours (EGFR in 92.5%, Erb B3 in 72.8%, Erb B4 in 81.8%); mTOR expression was observed in 73.6% tumors, RAS in 92.4%. MEK was absent in 74.4% and ERK in 100% of the samples. pTEN was absent in almost 75% of the cases, p53 present in 57.6%. The presences of ErbB3 and ErbB4 and one biomarker of MAPKs and one of AKT-PI3K pathways (MEK and mTOR) were statistically associated each other and with lymphatic spread of the tumor (p<0.05).

Conclusions: A pattern of ErbB signaling pathway showed a correlation with a pathological characteristic of tumor invasivity as lymphatic spread. Expression of three upstream Erb B activators (EGFR, Erb B3, Erb B4) suggested up-regulation of MAPKs and AKT-PI3K pathway: in literature combined inhibition of both pathways is an effective anti-tumor strategy; the activated components of these two pathways were mTOR and MEK. This pattern resulted to be related to a characteristic of invasion capacity of the tumor as lymphatic invasion, associated with poor prognosis. If confirmed the predictive power of ErbB signaling pathway map, the immunohistochemical evaluation of Erb B activators and MAPKs and AKT-PI3K pathway could guide the choice of treatment and possibly targeted therapy.
**S325** EXPRESSION OF CD44 CORRELATES WITH PATTERN OF INVASION AND IS PREDICTIVE OF POOR OUTCOME IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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**Objective:** The objective of the study was to evaluate the association of cancer stem cell marker CD44 and the pattern of invasion and their role in the prognosis of Head and Neck Squamous cell carcinoma.

**Methods:** Pattern of invasion was scored as invasive pattern grading system (IPGS). Its association with cancer stem cell marker CD44 was analysed in a cohort of 173 patients by immunohistochemistry. The immuno-reactivity was scored according to the percentage of cells and the intensity [1 (weak), 2 (moderate) and 3 (strong)] [maximum score of 300]. IPGS and CD44 were independently correlated with all the other clinic-pathological variables by chi square analysis. The predictive risk factors for recurrence/mortality were determined by the logistic regression model while the overall survival rate was determined by the log rank test. Results were considered significant with p<=0.05 (95% CI).

**Results:** Analysis revealed that increased expression of CD44 (score>100) correlated with IPG score >4 (p=0.051). IPGS was significantly associated with peri-neural invasion, lymphocytic infiltration (p=0.020) and differentiation (p=0.004). Multivariate analysis indicated that the high CD44 (HR=3.30, 95%CI=1.24-8.79, p=0.017) and mild LPI (HR=3.11, 95% CI=1.13-8.63, p=0.029) were predictive of recurrence. Analysis also indicated recurrence (HR=12.64, 95% CI=4.33-36.92, p=<0.002), habits (p=0.057) and low CD44 expression (HR=0.34, 95% CI=0.13-0.94, p=0.034) as predictive of overall survival. LPI (P=0.026), PNI (p=0.001), recurrence (p<0.001), differentiation (p<0.001), IPG (p=0.07) were independent prognostic factors for predicting the overall survival.

**Conclusions:** Our findings show a correlation between increasing CD44 expression and the invasive patterns of invasions, both being a predictor of poor prognosis in the patients. This suggests a role for these stem cells at the invasive front of the tumour, the exact correlation needs to be further investigated.
Importance: Head and neck cancer survival rates have been relatively stable for several decades. Individualized prognostic indicators such as biomarkers are needed to identify patients at risk for poorer outcomes who may need enhanced surveillance or treatment.

Objective: To determine whether surgical drain fluid cytokine levels in head and neck cancer patients are associated with poor cancer outcomes.

Design: A prospective, cohort study was performed from April, 2011 to the present.

Setting: Tertiary care center.

Participants: Patients with advanced stage head and neck squamous cell carcinoma who required surgical treatment were recruited from our academic practice. Thirteen Stage IV patients (8 male, 5 female) were enrolled and had complete specimen collection.

Intervention(s): Patients underwent surgical treatment as dictated by the standard of care. Standard surgical drains were placed as indicated. Drain fluid was collected every 8 hours post-operatively until drains were removed because of clinical criteria. Cytokines and matrix metalloproteinases (MMPs) were measured using electrochemiluminescence, patterned array, multiplex technology via Meso Scale Discovery, Spectro 6000. Two sample, two-sided t-tests evaluated differences in cytokine and MMP levels by disease outcomes.

Main Outcome(s) and Measures: The primary clinical outcome measures were disease status (dead of disease / alive with disease versus no evidence of disease / dead of other causes) and recurrence. The biomarkers measured included interleukins (IL) 1 beta, 6, and 8; tumor necrosis factor alpha (TNF-a); basic Fibroblastic Growth Factor (bFGF); vascular endothelial growth factor (VEGF); soluble fms-like tyrosine kinase-1 (sFLT-1); Transforming Growth Factor beta (TGF-b); Epidermal Growth Factor (EGF), Placental Growth Factor (PIGF), and Matrix Metalloproteinases (MMPs) 1, 2, 3, 9, and 10. Other clinical and pathological cancer characteristics were recorded.

Results: In this set of 13 stage IV patients, 7 were alive with disease or dead of disease and 6 were disease free. Eight patients experienced recurrence while 5 were recurrence free. MMP-9 levels were associated with recurrence status (p = 0.08), and Placental Growth Factor levels were associated with disease status (p = 0.06).

Conclusions and Relevance: In this pilot sample of Stage IV head and neck, squamous cell carcinoma patients, MMP-9 and Placental Growth Factor levels in wound fluid were associated with poor clinical cancer outcomes in the form of survival and recurrence. This is consistent with the tumor microenvironment literature in saliva, serum, and tumor tissue biomarkers. To our knowledge, this is the first report of such findings in surgical drain fluid, an easily accessible means of cytokine measurement. Measurement of these biomarkers in surgical fluid potentially represents a novel means of assessing cancer prognosis in this population.
A ROLE FOR APOLIPOPROTEIN E IN INVASION IN HNSCC
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Introduction:

Head and neck squamous cell carcinomas (HNSCC) have a poor patient prognosis, which is attributed to their invasive nature. Our goal was to identify novel genes that are important in HNSCC invasion. We utilized genome-wide expression data generated from HNSCC patient tumor samples that each exhibited a different "pattern of invasion"- a histological parameter, which in a previous study, correlated significantly with the appearance of local recurrence and decreased overall survival of the patients after surgery.

Methods:

RNA from flash frozen tumor samples was analyzed using the Illumina® HumanHT-12 v4Expression BeadChip. From FFPE tumor-cell enriched cores from the same patient tumors, RNA was analyzed using the Illumina® WG-DASL array. We selected genes from the two microarray platforms that were overexpressed at least 1.5-fold in the more invasive tumors. Ingenuity Pathway Analysis was used to prioritize which genes that were overexpressed in the microarrays that should be assayed for their effects on invasion in vitro. Candidate genes were transiently knocked down with siRNA in the cell line UMSCC1. Ability to invade was determined by using in vitro transwell invasion assays.

Results:

Analysis of the microarray data identified 104 genes that were overexpressed in the more invasive tumors compared to the less invasive tumors. Ingenuity pathway analysis identified 51 genes out of the 104 that fell into the top five functional categories of cell death, cell to cell signaling and interaction, cellular assembly and organization, cellular movement, and cell morphology. Invasion ability of UMSCC1 cells was impaired significantly by knockdown of 16 out of the 51 genes. The gene with the most significant effect on invasion from this screen was APOE (apolipoprotein E).

Conclusion:

This initial screen of global gene expression data in combination with pattern of invasion has revealed APOE as a novel gene that may play a critical role in HNSCC invasion. Downstream signaling of APOE receptors and how this may interact with invasion are currently being assessed.