



## SESSION 3 - Translational science

### 1. IDENTIFICATION AND VALIDATION OF 48-GENE EXPRESSION MODEL PROGNOSTIC MODEL IN ORAL SQUAMOUS CELL CARCINOMA

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### 2. THE ONCOGENIC ROLE OF MIR-96-5P IN THE MUTANT P53 CONTEXT OF HEAD AND NECK CANCER CELLS

**Manciocco Valentina**

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### 3. TELOMERE LENGTH AND HTERT C250T PROMOTER MUTATION ASSESSMENT IN PERIPHERAL BLOOD LEUKOCYTES PRESENTS POTENTIAL DIAGNOSTIC VALUE IN HEAD AND NECK CANCER PATIENTS

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### 4. USING STATISTICAL MODELS TO PREDICT OUTCOMES IN PATIENTS WITH OROPHARYNGEAL CANCER

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## 5. PERFORMANCE OF AN ORAL RINSE POINT-OF-CARE ASSAY TO AID IN THE DIAGNOSIS OF HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) IN A HIGH RISK DANISH EAR-NOSE THROAT (ENT) CLINIC

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## 6. FUNCTIONAL IMAGING OF HETEROGENEITY IN HEAD AND NECK TUMORS - VALIDATION FROM SURGICAL SPECIMENS

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## 7. DISCOVERY OF NOVEL CANDIDATE GENES OBSERVED LOSS OF METHYLATION-BASED EXPRESSION IN ORAL PREMALIGNANT LESIONS BY METHYLATION AND EXPRESSION ARRAYS

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## 8. DECODING OROPHARYNGEAL CARCINOMA (OPCC) PHENOTYPE BY NONINVASIVE IMAGING USING A QUANTITATIVE RADIOMICS MAGNETIC RESONANCE (MR) IMAGES-BASED APPROACH

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## 1. Identification and validation of 48-gene expression model prognostic model in oral squamous cell carcinoma

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**Rationale:** The 5-years overall survival for oral cavity squamous cell cancer (OCSCC) mainly depends on the extent of the tumour at diagnosis, as defined by the TNM stage. However, TNM staging classification, which is employed for treatment planning, it is group-based and meets limitations for personalizing treatment of the individual patient.

**Materials and methods:** To identify a prognostic gene signature in OCSCC patients, a survey of the gene-expression data publicly available at 30th September 2017 was accomplished. Following the Omics-based test development process (CM Micheel, 2012), the samples entering into our discovery phase were divided in a training and test set and should fulfill the following eligibility criteria: i) primary tumour; ii) oral cavity anatomical subsites that includes buccal mucosa, hard palate, tongue, floor of mouth, alveolar ridge; iii) HPV annotation; iv) overall survival data, as clinical endpoint to be reported. TCGA containing 266 HPV-negative OCSCC cases was used as training set; four microarray gene expression datasets reporting survival data of a total of 148 samples were retrieved from publicly repositories and used as test set. Three publicly available datasets were selected as validation set along with an in-house gene-expression dataset for a total of 344 cases.

**Results:** The training set led us to identify a 48-gene signature able to stratify patients in low or high risk based on 10-fold cross validation (log-rank,  $p=2.14e-05$ ). The prognostic model was tested on a meta-analysis of four datasets and its prognostic validity was confirmed (log-rank,  $p=0.0247$ ). The performance of the model was challenged in other independent datasets, including: i) 168 cases from GSE41613 and GSE42743; ii) 66 cases from GSE85446; iii) 110 cases from a in-house OSCC dataset. When our model was challenged against available demographic and clinical parameters, it retained a significant independent association in multivariate Cox regression analysis.

**Conclusions:** We have identified and validated a prognostic model based on the expression of 48-genes, able to improve assessment of patient's risk based on overall survival. In order to transpose our model into a useful clinical grade assay, additional work is needed following the framework established by the Institute of Medicine and REMARK guidelines.

## 2. The oncogenic role of miR-96-5p in the mutant p53 context of head and neck cancer cells

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**Rationale:** HNSCC are typically characterized by a high incidence of local recurrences, which takes place in 60% of cases. The TNM staging system, used to classify HNSCC patients, does not adequately address the molecular heterogeneity of HNSCC tumors. This indicates the need to obtain a more detailed molecular characterization in order to stratify patients better. TP53 is the most mutated gene in HNSCC and patients carrying TP53 mutations are associated with a higher probability to develop local recurrence. Among the mediators of the oncogenic activity of mtp53 protein there are miRNAs, which are emerging as an appealing tool for screening, diagnosis and prognosis of cancer. In particular, by the previous characterization of miRNAs expression in our IRE cohort of 121 HNSCC and TCGA cohort, miR-96-5p is emerging among the best promising miRNAs working as biomarkers in HNSCC.

**Materials and methods:** In order to evaluate the oncogenic role of miR-96-5p in a mtp53 tumoral context, we performed a colony assay, cell migration and cell viability in two HNSCC cell lines carrying mt-p53 protein, transfected for miR-96-5p mimic or inhibitor and treated with or without radio/chemotherapy. In addition, to identify genes positively and negatively correlated to miR-96-5p expression in HNSCC, we analyzed the correlation between gene expression and miR-96-5p level in the subset of TCGA HNSCC tumors carrying missense TP53 mutations by Spearman and Pearson correlation.

**Results:** Data show that the overexpression of miR-96-5p leads to cell migration and radio-resistance increasing in HNSCC cells carrying mutant p53. In agreement with these results, among the most statistically significant pathways in which miR-96-5p is involved are extracellular matrix organization, focal adhesion and PI3K-Akt signalling pathways. In addition, as putative target of miR-96-5p and negatively correlated with its expression in TCGA cohort, we identified PTEN, already linked with the prognosis of HNSCC patients and involved PI3K-Akt signalling pathway.

**Conclusions:** This results support the possibility that miR-96-5p expression could be used ad as a novel promising biomarker to predict radiotherapy response and local recurrence development in HNSCC patients. In addition, the identification of pathways in which miR-96-5p is involved could be lead to develop new therapeutic strategies able to overcame radio-resistance.

### 3. Telomere length and hTERT C250T promoter mutation assessment in peripheral blood leukocytes presents potential diagnostic value in head and neck cancer patients

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**Rationale:** The head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide. Cancer cells, including HNSCC, are characterized by an increased telomerase activity. This enzymatic complex is active approximately in 80-90% of all cancers, and it is responsible for lengthening of telomeres. Recently, highly recurrent point mutations in hTERT promoter have been reported in multiple human malignancies. The aim of this study was to analyze the frequency of the hTERT promoter C250T mutation, and the telomere length in blood leukocytes of 61 HNSCC patients and 49 healthy individuals.

**Materials and methods:** DNA was extracted from PBL (Peripheral Blood Leukocytes) of 61 patients with histologically diagnosed HNSCC and 49 healthy volunteers. Telomere length was assessed using quantitative PCR-based technique with two pairs of primers (telomere-specific and a single copy gene-specific). To identify C250T hTERT promoter mutation, the High Resolution Melting analysis was performed. Statistical analysis of the results was performed using the Student's, ANOVA, Chi-square, and Fisher's exact tests.

**Results:** The average relative telomere length in the studied and control groups was evaluated, and no significant difference was observed ( $P=0.787$ ). Telomeres in leukocytes from individuals with T2 HNSCC cancer were significantly shorter when comparing with telomere length in leukocytes of healthy individuals ( $6.329\pm 1.864$  and  $19.06\pm 1.801$ , respectively;  $P=0.0001$ ). There was also significant difference of telomeres length between T2 and T3 patients ( $6.329\pm 1.864$  and  $16.94\pm 3.301$ , respectively  $P=0.0063$ ), and T2 and T4 ( $6.329\pm 1.864$  and  $26.3\pm 7.615$ , respectively  $P=0.0028$ ). hTERT promoter mutation was identified in 36% of HNSCC patients and in 27% of healthy individuals. There was significant correlation between frequency of mutation and grade of tumor (T1=27%; T2=36%; T3=35% T4=46%;  $P\leq 0.0001$ ). Opposite trend was found in case of wild allele.

**Conclusions:** C250T hTERT promoter mutation represent common event during cancerogenesis in HNSCC patients, and together with telomere length assessment may be one of the molecular markers of HNSCC progression. The finding of long or short telomeres in PBL of HNSCC patient does not necessarily indicate the presence or absence of hTERT promoter mutation, and both parameters should be consider to characterize patients status.

#### 4. Using statistical models to predict outcomes in patients with oropharyngeal cancer

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**Rationale:** The ability of a model to predict which patient, disease and treatment factors impact on the outcomes of a patient is of great importance to clinicians. Unfortunately, there is a paucity of literature relating to statistical models in head and neck cancer as most studies use logistic regression to predict patient outcomes. Previously, we evaluated the performance of logistic regression and decision tree in the prediction of local control and cancer-specific survival (CSS) in glottic and hypopharyngeal cancer patients. Despite no difference in model accuracy, decision tree identified more predictors. Thus, we investigated the performance of logistic regression and decision tree, in predicting these two outcomes in oropharyngeal cancer patients.

**Materials and methods:** Patients treated with definite radiotherapy ± adjuvant treatment at the Prince of Wales Hospital between 2005 and 2015 were analysed. Univariate analysis was conducted on potential predictors of local control and CSS using Kaplan-Meier in SPSS Statistics v24. Significant patient, disease and treatment factors ( $p < 0.05$ ) were subsequently analysed using logistic regression and decision tree in SPSS Modeler v18. Model performance was evaluated by receiver operating characteristic curves, based on accuracy, sensitivity and specificity.

**Results:** One hundred and forty-eight patients were analysed. Decision tree yielded 80% accuracy, 75% sensitivity, and 79% specificity, compared to 72% accuracy, 50% sensitivity, and 94% specificity achieved by logistic regression in predicting local control. Both models achieved 81% accuracy in predicting CSS, with 80% and 81% sensitivity and specificity using logistic regression, and 90% and 69% sensitivity and specificity with decision tree. Cancer operability was identified by each model as a significant predictor for local control and CSS, and fitness for surgery for local control, however, decision tree also identified ECOG status (0-1) and length of radiotherapy treatment  $\leq 42$  days as significant predictors of local control, and T-stage (T1 and T2) disease as a significant predictor for CSS.

**Conclusions:** The decision tree algorithm enables identification of predictors which may not be recognised by logistic regression. Therefore, decision tree should be considered as a model to predict outcomes in head and neck cancer patients.

## 5. Performance of an Oral Rinse Point-Of-Care Assay to aid in the diagnosis of Head and Neck Squamous Cell Carcinoma (HNSCC) in a High Risk Danish Ear-Nose Throat (ENT) Clinic

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**Rationale:** Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cause of cancer mortality. The ability to detect HNSCC an earlier stage could have significant impact on overall outcome. Previous studies demonstrated that a point-of-care (POC) lateral flow assay and an ELISA LAB test measuring CD44 and total protein (TP) aid in the diagnostic process for HNSCC. We sought to better understand the prospective performance of the POC assay in a hospital-based high risk oral clinic in Denmark.

**Materials and methods:** Oral rinses were obtained from 150 consecutive patients presenting for physical exam and biopsy in a high risk ENT clinic (Rigshospitalet, Copenhagen, Denmark). Operators were provided POC visual tools to record assay results. A positive POC test is a visible CD44 band or level of TP (i.e. color-graded scale from 0-5, recommended cut-off  $\geq 2$  or adjusted  $\geq 3$ ), with Sensitivity (Se), Specificity (Sp), NPV to evaluate correlation with biopsy.

**Results:** 131 biopsy evaluable patients, 30% with HNSCC. Average age: 57 years, 43% male, 100% white and 59% smokers. Using POC levels of CD44 or a TP cut-off of 3, the assay achieved a Se of 73%, and Sp 50%. Applying a TP of 2 for non-smokers and 4 for smokers further improved assay performance: Se 80% and Sp 43%. Normal non-smoking, female controls (n=18) had a Sp 78%. With a prevalence of 9.27%, the NPV was  $>90\%$ .

**Conclusions:** POC assay performed well for discriminating HNSCC. Additional studies are underway to further confirm these results and compare with the LAB test.

## 6. Functional imaging of heterogeneity in head and neck tumors - validation from surgical specimens

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**Rationale:** Precision medicine has brought increased awareness to inter- and intra-tumor heterogeneity; however single tumor biopsies cannot detect all heterogeneity. If a correlation between the functional imaging and molecular biology can be established, functional imaging becomes the ideal support to the single tumor biopsies to access intra- and inter-tumor heterogeneity. The purpose of this prospective study is to investigate if heterogeneity in multiparametric functional imaging with FDG PET/MRI correlates with heterogeneity in histology and immunohistochemical (IHC) biomarkers in the surgical specimen.

**Materials and methods:** Patients with HNSCC referred for surgery will be included in this prospective imaging/biomarker expression study.

All patients are scanned with multiparametric imaging (MPI) prior to surgery with an integrated PET/MR scanner (Siemens Biograph mMR). The scan protocol includes diffusion weighted MRI (DWI), dynamic contrast enhanced perfusion MRI and FDG-PET. The PET scan is performed 60 minutes and again 90 minutes after FDG injection. Anatomical landmarks are marked per-operatively with the surgeon and the specimen is scanned morphometric with a 3D T2 weighted sequence (voxel size 0.5 mm isotropic) in order to co-register the specimen scan with the patient scan. The whole surgical specimen is sectioned contiguously for histological processing to access the correlation between histology and imaging. All steps in the work flow are photographically documented.

**Results:** Currently 18 patients with 21 lesions have been included. The median SUVmax=8.3 (4.3-26.5) and 9.1 (5.3-34.2) after 60 and 90 minutes, respectively. The difference was statistically significant ( $p<0.001$ ) tested with Wilcoxon signed rank test. There was no statistically difference in tumor volume assessed on PET and on anatomical MRI ( $p=0.40$ ), however these delineations was not performed blinded. There was no significant correlation between SUVmax and ADCmin, suggestion that these measurements are too crude to detect an actual correlation between FDG PET and DWI. Currently the histology and IHC staining is processed and will be correlated to FDG uptake, DWI and perfusion from different parts of each tumor lesion.

**Conclusions:** A setup for histological verification of MPI in patients with HNSCC is presented. The potential for tumor tissue phenotyping based on MPI and histological biomarkers will be pursued in this ongoing study.



## 7. Discovery of novel candidate genes observed loss of methylation-based expression in oral premalignant lesions by methylation and expression arrays

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**Rationale:** DNA methylation plays an important role in tumor initiation and progression. Gene silencing, which stems from abnormal methylation in the promoter regions of tumor suppressor genes, is a promising epigenetic mechanism to identify new methylation biomarkers for early diagnosis and screening. Oral premalignant/ precancerous lesions (OPML) are one of the most important etiological factors for oral squamous cell carcinoma. The silencing by hypermethylation of tumor suppressor genes play a key role in the transformation of the oral cavity lesions to the malignancy. OPML are detected in people over the age of 40 and those with similar risk factors for oral cavity cancer, such as smoking and alcohol consumption. Our major aim is to determine the candidate genes with loss of methylation-dependent expression in OPML by methylation and expression arrays.

**Materials and methods:** Tumor and corresponding normal tissues from 12 Turkish patients with OPML were collected. After DNA extraction and bisulfite modification, methylation patterns of the samples were investigated by using the Illumina Infinium Human Methylation 450 (450K) Bead Chip array for methylation profiling. After RNA extraction and cDNA synthesis, expression profiling of the samples were investigated by using the Illumina iScan, and then bioinformatics analysis was performed by using the Illumina Genome Studio program and sorted by chip bar code numbers. Then, we integrated the data obtained from methylation and expression arrays.

**Results:** CpGs located in 4 candidate tumor suppressor genes' promoter regions were methylated and had downregulated expression levels ( $p < 0.05$ ).

**Conclusions:** We concluded these four genes may be potential biomarker candidate genes playing role in the OPML development. These genes will be validated in the larger cohort of patients.

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## 8. Decoding oropharyngeal carcinoma (OPCC) phenotype by noninvasive imaging using a quantitative Radiomics Magnetic Resonance (MR) Images-based approach

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**Rationale:** We propose a radiomic approach using Magnetic Resonance (MR) images to decode tumor phenotype characteristic and treatment response by mean of quantitative features.

**Materials and methods:** Clinical characteristics: We retrospectively reviewed 22 patients (pts) (19 male, median age 61 yrs, range 45-89 yrs). Most pts (64%) had locally advanced stage (III and IV) tumors. The treatment strategy was surgery (+/- adjuvant radiotherapy or chemoradiotherapy) or radiotherapy (+/- concurrent chemotherapy) in 13 and 9 cases, respectively. After a median follow up 32 months (range 4-90 months), 19 pts were alive without disease, 1 patient was alive with disease and 2 pts dead for tumor. MR Features extraction: IBEX (Imaging Biomarker Explorer) software was used for the purpose. Radiomic features were automatically extracted, quantifying parameters related to the lesion Shape, the frequency Histogram of the voxel values, and the Texture. IBEX provided 1766 Radiomic features for each patient. Hierarchical clustering was performed in order to group the features in 10 clusters exhibiting high intra-cluster correlation and low inter-cluster correlation. HPV status, local recurrence and clinical stage were considered as clinical endpoints for the statistical analysis.

**Results:** Patients were divided into two groups according to the value of the clinical parameter: HPV status positive/negative; local recurrence yes/no; stage initial =1,2/advanced=3,4. The rank-sum Wilcoxon test was performed for each clinical endpoint within each cluster and a statistically significant correlation ( $p= 0.02$  to  $p= 0.04$ ) between all clinical endpoints and features of the category F2-GraylevelcooccurrenceMatrix 3 was found.

**Conclusions:** Despite preliminary, the above results indicate that Radiomic features calculated from Gray Level Cooccurrence Matrix (quantifying the spatial relationship of voxels and their local correlation) may have the potential to differentiate patients according to HPV status, presence of local recurrence and stage. More sophisticated statistical analysis will be performed on a wider patient sample, as well as deeper investigation on features stability, robustness and image processing.