Study Title: Predictive Biomarkers for Response to Nivolumab in Head and Neck Squamous Cell Carcinoma (HNSCC)

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Study rationale

Nivolumab is FDA-approved for the treatment of patients with recurrent/metastatic HNSCC whose disease has progressed within 6 months after platinum-based chemotherapy. The development of predictive biomarkers is needed to optimize patient benefit, minimize risk of toxicities and guide combination strategies. The greatest focus has been on tumor-cell Programmed Death Ligand 1 (PD-L1) expression. Although PD-L1 positivity enriches for populations with clinical benefit, PD-L1 testing alone is insufficient for patient selection in most malignancies. PD-L1 expression can be transient, and intrapatient and even intratumor heterogeneity in PD- L1 tumor expression can exist. Therefore, tumor sampling at one time point might not accurately reflect the state of PD1 axis in a patient. Another important aspect is that PD-L1 immunohistochemistry (IHC) alone does not take into account factors that could impede the anti-PD1 therapy response such as whether or not active immune cell engagement of the PD1 axis occurs in the tumor microenvironment or other concurrent immune suppressive pathways are present. Assessment of biomarkers at baseline may not predict benefit from immunotherapy. In a phase II study of ipilimumab in patients with metastatic melanoma baseline tumor- infiltrating lymphocyte

status was not associated with clinical activity. However, increases in tumor infiltrating lymphocyte density in tumor biopsy samples collected after the second dose of ipilimumab was associated with significantly greater clinical activity with ipilimumab compared to samples without increases in lymphocyte density. For a better understanding of the mechanisms of resistance to nivolumab in HNSCC, The investigators propose to study a cohort of longitudinal HNSCC samples from recurrent/metastatic HNSCC patients (pts) treated with nivolumab and identify biomarkers of response and resistance. The investigators will specifically focus on modulation of immune phenotype (ImmR) following one cycle of nivolumab as surrogate biomarker for response to nivolumab. Mutational landscape and gene expression data will also be correlated with response to shed light in potential molecular pathways that should be co-targeted to reverse resistance to nivolumab. The investigators hypothesize that DNA Damage repair (DDR/R) pathways are strongly correlated with immune response.

Primary Objective

The primary objective of the current study is to prospectively explore candidate biomarkers from the tumor and tumor microenvironment for associations with clinical response to nivolumab in patients with recurrent/metastatic HNSCC treated with nivolumab.

Primary Endpoint

The primary endpoint will be the change in the percentage of immune cells that is caused by nivolumab treatment. Investigator assessment of best overall response (BOR), determined between the date of first dose and the last tumor assessment (TA), will be image-based and scored using RECIST 1.1. BOR will be defined as categorical variable with 3 levels { Benefit (CR, PR, SD lasting 6 months from the first nivolumab dose), non-benefit (PD or SD lasting less than 6 months from the first nivolumab dose), and unkown}

Hypothesis

The investigators hypothesize that modulation of immune phenotype following one cycle of nivolumab can be used as surrogate biomarker for response to nivolumab. In addition, alterations in the DDR/R network and the ImmR system contribute to the outcome of immunotherapy thus providing unique tools for discovery of biomarkers, therapeutic targets and their validation.

Study Assessments

Materials and Methods: Longitudinal tissue biopsies will be collected from HNSCC patients treated with nivolumab . Biopsies will be taken at baseline, 24-72 hours after the second cycle of nivolumab and at progression. The investigators will generate multidimensional profiling data (WES, nanostring, multiplex tissue biomarker imaging data). Multidimensional profiling data will be analyzed to identify genomic and immune correlates of treatment response and resistance mechanisms of immune checkpoint blockade.

Patient cohort and tumor samples: The investigators will include recurrent/metastatic HNSCC patients who progressed after cisplatin-based chemotherapy and are to be treated with nivolumab. Tumor samples will be collected with appropriate written informed consent and analyzed. All tumor measurements will be performed by a physician formally trained in RECIST 1.1.

Sample processing

Diagnostic tumor FFPE block(s) will be retrieved for all consenting patients.

1. Archival tumor tissue from primary debulking surgery or baseline biopsies will be used for Whole Exome Sequencing, Nanostring gene expression profiling and immunohistochemistry

for the analysis of the immune landscape and tumor microenvironment.

2. Paired core needle biopsies from post-cycle 1 and / or progression will be used for Nanostring gene expression profiling and immunohistochemistry for the analysis of the immune landscape and tumor microenvironment.

Whole exome sequencing and targeted gene sequencing

WES to identify gene expression associated with DNA repair and immune response. Targeted gene sequencing will identify the burden of somatic non-synonymous mutations.

Nanostring gene expression profiling

Multiple tumour, inflammatory- and immune related genes will be analyzed by multiplex Nanostring technology by using the nCounter PanCancer Immune Profiling 770-plex gene expression Panel. This platform will be used to assess expression of genes related to immune signaling and attempt to define a gene set critical for clinical benefit from nivolumab. Data will be correlated with the immune landscape analysis (next section).

Immune landscape

The assessment of the tumor-associated immune microenvironment will be performed in formalin-fixed, paraffin-

embedded tumor samples, at baseline and in cases in which post-therapy needle biopsy material is available. In these cases, the spatiotemporal dynamics of immune cell subsets will be quantified in both tumor nests and stroma, in order to highlight any increased homing, functional efficacy and clonal expansion in post-therapy tissues. The investigators hypothesize that the outcome would be determined by the spatial co-localization and close proximity of PD-1+ and PD-L1+ cells. Furthermore, other immune cell populations will be quantified by using lineage specific markers combined with functional markers, to assess the activation and differentiation status, by using multiplexed immunohistochemistry (IHC).

The immune landscape in the tumor microenvironment will be correlated with the expression of PD-L1, PD-L2 and with the expression of molecules of the antigen presenting machinery in neoplastic epithelial cells, the gene expression profile (Nanostring) and the type and intensity of the peripheral blood immune responses.

More specifically:

1. **Neoplastic epithelial cells will be examined for:**
    a. The expression of PD-L1 and PD-L2 at the protein (IHC) level.

b. The expression of HLA class I and HLA class II molecules, to evaluate the integrity of the antigen presenting machinery

2. The immune microenvironment will be examined for:

a. The presence of adaptive immunity cell populations e.g. CD3, CD8, CD20, Foxp3.

b. The expression of PD-1 on different T cell subpopulations (multiplexed IHC for co-localization assessment).

c. The expression of PD-L1 on stromal cells e.g. M1 and M2 macrophages, Dendritic cells.

d. Markers of tumor vasculature e.g. CD34 (endothelial cells) and D2-40 (lymphatics).

<u>Blood analyses</u>

1. PBMC (baseline, post-cycle 1 and at progression) will be used for:

a. Quantification of T cell subsets by flow cytometry

T cell subpopulations will be enumerated using a volumetric flow cytometric method and monoclonal antibody combinations for

characterization of CD4, CD8, CD25, CD45RA, CD45RO, CD122, CD127, CD62L, CCR7, CTLA-4 and expression levels of PD-1. The main focus will be identification of shifts in effector and memory cells and finally populations of T cells that have been exhausted.

b. Quantification of tumor-specific T cell responses.

Assessment of peripheral blood T cell responses against known tumor-associated antigens (TAAs) expressed in most Head and Neck cancers, including HER-2, p53, HPV-related or Cancer testis antigens (or non TAA-specific, e.g. flu and tetanus, T cell responses as control) at baseline and after treatment.

2. Serum (from baseline, post-cycle 1 and at progression) will be used for:

In addition to T-cell responses, B-cell responses will be characterized. Serum obtained from patients at pre- and post-treatment will be screened for the presence of antibodies directed against a panel of cancer testis antigens, and a panel of >9000 purified human proteins coated onto microarray slides (using the 'seromics' ProtoArray protein microarray assay from Invitrogen/Life Technologies). This protein microarray technology offers an unprecedented platform to simultaneously assay the

serological response of patients to multiple antigens in a comprehensive fashion.

3. **PD-L1 expression on circulating tumor cells:** The investigators will capture CTCs using the Parsotrix system and The investigators will then analyze PD-L1 expression using RT-PCR. The investigators expect decrease in number of PD-L1 expressing

CTCs following PD1 blockade (Strati…Psyrri: Prognostic significance of PD-L1 expressing CTCs in HNSCC Ann Onc Sept 2017)).

Secondary Objective

The secondary objective of the current study is to assess candidate biomarkers for acquired resistance to nivolumab comparing baseline and post-progression specimens in patients with recurrent/metastatic HNSCC treated with nivolumab.

Secondary Endpoints

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1. Safety of performing a biopsy after second nivolumab dose
2. Best overall response rate (BOR) according to RECIST 1.1 criteria

3. Number of participants with tolerability to the treatment
4. The burden of somatic non-synonymous mutations in association with BOR and survival.
5. The interferon-gamma gene signature in association with BOR and survival
6. The expression of PD-L1 in association with BOR and survival
7. The expression of human leukocyte antigens, HLA class I and HLA class II molecules in association with BOR and survival
8. The presence of adaptive immunity cell populations
9. The expression of PD-L2 in association with BOR and survival
10. The expression of PD-L1 will be assessed in circulating tumor cells (CTCs)

Statistical analysis

Sample size calculation: Primary endpoint: Using a t-test at one-sided significance level 2.5% for a paired design, assuming the mean ratio of post- number of immune cells ETC will increase by 50% compared to the pre- number (ratio post/pre equal to 1.5; assumed standard deviation equal to 1), with the planned sample size of 50 cases, a power of 80% is achieved.

References

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