**Feasibility Analysis of Using Patient-Derived Tumour Organoids for Treatment Decision Guidance in Locally Advanced Head and Neck Squamous Cell Carcinoma**

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**Supplementary information**

## *Tissue processing*

Tissue samples were immediately transferred into a tube containing RPMI1640 medium (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) complemented with 2.5% penicillin/streptomycin (Sigma-Aldrich, St.Louis, MO, USA) and 1 % gentamicin (Thermo Fisher Scientific), and stored in the refrigerator until transport to the laboratory for further processing. Processing was performed on ice. Tissue was cut into small pieces (~1 mm3), and enzymatically digested in tissue dissociation mix, consisting of Advanced DMEM/F12 medium (Gibco­™) complemented with DNAse I (100 µg/ml, VWR International, Radnor, PA, USA), Dispase (100 µg/ml, Corning®, Corning, NY, USA) and Y-27632 dihydrochloride (10 µmol/L, Abmole, Houston, TX, USA). After incubation at 37°C for 30 min, enzymatic dissociation was stopped on ice by adding basal medium, consisting of Advanced DMEM/F12 (1X), HEPES (1mol/L, Sigma-Aldrich), GlutaMax (1 %, Gibco­™), N-Acetyl-L-cystein (1 mmol/L, Sigma-Aldrich), penicillin/streptomycin (1 %), gastrin (10 nmol/L, Sigma-Aldrich) and Y-27632 dihydrochloride (5 µmol/L). Suspension was strained over 500-µm and 40-µm filters (pluriSelect®), and centrifuged at 300 g for 5 min at 4°C. Fragments ranging from >40 µm to <500 µm in size were employed for organoid cultures.

## *Organoid cultures*

Tissue fragments were plated in cell culture plates (Corning®) and covered with an organoid medium (herein referred to PDO complete+2 medium) initially formulated by Driehuis *et al.*[1] and further optimised during the pilot phase of this project. PDO complete+2 medium consisted of basal medium supplemented with serum-free B-27 (1X, Gibco­™), N2 (1X, Gibco­™), recombinant human FGF basic (20 ng/ ml, PeproTech, Cranbury, NJ, USA), recombinant human EGF (50 ng/ml Invitrogen, Carlsbad, CA, USA), Y-27632 dihydrochloride (10 µmol/L), TGF-ß RI kinase-inhibitor VI, SB431542 (0.5 µmol/L*,* Sigma-Aldrich) and conditioned medium from the cell line CRL-2376™ (1:100, ATCC, Manassas, VA, USA) containing Wnt-3A, R-spondin and Noggin. Amphotericin B (250 µg/ml, Sigma-Aldrich) was added only for the first three days of culture. Cultures were kept at 37 °C and 5 % pCO2 in a humidified incubator. At 80% confluence, cells were passaged with TrypLE™ Express Enzyme Phenol Red (1x, Thermo Fisher Scientific). Two-thousand cells were seeded in 15 µl of Matrigel (Corning®). Drops solidified at 37 °C for 30 min with the plate upside down before prewarmed PDO complete+2 medium was added. Medium was exchanged every 3-4 days. For passaging, Matrigel was scratched from the plate, transferred to a microcentrifuge tube and washed with cold Dulbecco‘s Phosphate Buffered Saline (Thermo Fisher Scientific). Organoids were then incubated for 2-5 min in TrypLE™ before reseeding. Alternatively, cells were resuspended in Recovery™ cell culture freezing medium (Thermo Fisher Scientific) for cryopreservation in liquid nitrogen.

## *Histopathological characterisation*

At a minimum size of 100 µm, organoids were harvested by dissolving Matrigel on ice using Cell Recovery Solution (Corning®). Organoids were washed with PBS and transferred to Eppendorf tubes that have been coated with 1% Bovine Serum Albumin (BSA, Thermo Fisher Scientific) in PBS. After fixation with 4 % paraformaldehyde (Carl Roth GmbH) for 1 h at room temperature or overnight at 4 °C, organoids were stained with haematoxylin in PBS (1:200) for 15 min. Washing steps were performed with PBS complemented with 0.1 % BSA. The organoid pellet was overlayed with Histogel (Thermo Fisher Scientific), and stored at ‑20 °C until further processing. Samples were dehydrated and embedded in paraffin. Sections of paraffin-embedded organoids were stained with haematoxylin-eosin. Additionally, immunohistochemical staining for the squamous cell markers cytokeratin 5/6 (CK5/6, clone EP24/EP67, Epitomics Inc., CA 94010, USA) and p40 (clone BC28, Ventana in Ventana Benchmark Ultra) were performed. Proliferating cells were detected by Ki67 staining (clone Mib1, all Dako Denmark A/S, Glostrup, Denmark). Two experienced head and neck cancer pathologists (S.S., I.P.) performed the histopathological analyses.

## *Molecular characterisation*

Genomic DNA from organoids and germline controls (whole blood or peripheral mononuclear cells) was isolated with DNeasy blood and tissue kit (Qiagen, Hilden, Germany, cat no. 69504) according to manufacturers’ instruction. DNA from formalin-fixed paraffin-embedded (FFPE) samples was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was determined by the Qubit 4 Fluorometer (Thermo Fisher Scientific), and purity verified with the Nanodrop spectrophotometer. If necessary, DNA was purified and concentrated using the DNA Clean and ConcentratorTM -25 kit (Zymo Research, Irvine, CA, USA). DNA libraries for whole exome sequencing (WES) were prepared using SureSelect XT HS2 DNA System in combination with the SureSelect XT HS Human All Exon V8 probes (Agilent Technologies, Santa Clara, CA, USA) according to manufacturers’ instruction.

WES was conducted on an Illumina NovaSeq 6000 platform (Illumina Inc. through Novogene Bioinformatics Technology Co., Ltd). Mean coverages of 330x and 400x were achieved for organoids and FFPE tumour samples, respectively. Germline samples (whole blood, PBMCs or normal tissue) were sequenced with a mean coverage of 150.

The data was mapped using bwa-mem2 [2] against the GRCh38.d1.vd1 version of the human genome, which includes decoys & common viral sequences. Molecular barcodes were processed with the AGeNT software [3], using the trimmer and creak modules prior to mapping and for de-duplication, respectively. Finally, the base quality scores were re-calibrated using BaseRecalibrator & ApplyBQSR from GATK (version 4.4) [4]. Somatic variants were called with Mutect2 (GATK version 4.3) [5], using a panel of normal controls built from 43 normal samples collected from fresh-frozen material. The variants passing the Mutect2 filters were annotated with vep version 102 [6], and then filtered in the following way: variants with less than 6 reads supporting the alternate allele; or with a coverage inferior to 51 reads; or with a variant allele fraction less than or equal to 5 % (10 % for C>T & G>A SNVs in tutor samples) were excluded from the analysis. Somatic variants found in locations overlapping with HotSpots locii [7] were preserved from filtration, to avoid rejecting potential drivers. Somatic copy number alterations were called with PureCN [8].

## *Radiosensitivity assays*

One hundred cells per 9 µl Matrigel were plated in six replicates per dose in white 96-well plates. Upon reaching 30-40 µm, organoids were subjected to X-ray irradiation using single doses of up to 8 Gy delivered by YXLON Maxishot (YXLON International GmbH, Hamburg, Germany) at 0.89 Gy / min. After seven days, the medium was replaced with 100 µl of Cell-Titer-Glo® luminescence cell viability reagent per well (Promega, Fitchburg, WS, USA). The plates were shaken orbitally at 300 rpm for 30 min to facilitate Matrigel dissolution. Luminescence was measured using a plate reader (VICTOR Nivo multimode, PerkinElmer, Waltham, MA, USA).

In the 24-well assay, organoids were dissociated into single cells using TrypLE™ for 15-20 min. Subsequently, 1000 cells per 15 µl Matrigel were re-seeded in triplicates per dose in a new 24-well plate. After 9 to 10 days, organoids were counted per well, and survival fractions were calculated according to Nagle and coworkers [9]. Clonogenic survival curves, fitted by the linear-quadratic model, were generated using mean survival fractions derived from three independent experiments. Calculations were conducted with GraphPad Prism (version 8, GraphPad Software, Boston, Massachusetts, USA).

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