PATIENT DERIVED ORGANOIDS TO TEST CHEMOSENSITIVITY IN GASTRIC CARCINOMA

Research Report

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1. Abstract

Esophagogastric Cancer is the third leading cause of cancer related deaths worldwide. Lack of early onset symptoms often result in diagnosis at an advanced stage of the disease with poor prognosis for patients. Currently advanced stages of esophagogastric cancer are considered non-curable. Recent clinical trials indicate promising results for patients administered a combination therapy of HER2 targeted drugs and immunotherapy.

To effectively analyze the efficacy of this therapy approach, we aimed to establish a concise co culture platform of patient derived esophagogastric cancer organoids and T cells.

For the purpose of the study 62 tissue samples were obtained, establishing a thorough esophagogastric biobank. 8 organoid lines were selected for in depth analysis. After HER2 characterization, the co culture platform was optimized. Tumor killing was investigated by viability assays, flow cytometric analysis and live imaging.

Results indicate that the co-culture model provides a robust model to investigate tumor killing *ex vivo* and correlate it with patient response.

2. Introduction

2.1 Esophagogastric cancer

Esophagogastric cancers constitute a major global health problem. It accounts for more than 13,2% of deaths worldwide and is the fifth most common cause of malignancy. In recent decades gastric cancer incidences decreased due to advances in modern medicine. Nevertheless, it is still the third leading cause of cancer related deaths. Furthermore, malignancies in the upper gastrointestinal region originating in the esophagus and esophagogastric junction (EGJ) junction are one of the least diagnosed cancers within the United States with rising numbers.[1]

Gastric and esophagogastric junction malignancies present a significant overlap on a histopathological and molecular level. Thus, are referred to as esophagogastric cancers (EGC) in literature. The vast majority of EGC is histologically classified as adenocarcinoma with high incidences in the western hemisphere. EGC is a multifactorial disease with a variety of risk factors, the most reported thereof are Tobacco, alcohol consumption and obesity. [1,2,3]

The lack of early onset symptoms often leads to a late diagnosis at advanced stages resulting in a high mortality rate and overall poor prognosis for patients. Nearly 50% of patients have cancers that extend beyond the primary tumor region at time of diagnosis, making this disease vastly non-curable. First line treatment often includes endoscopic or surgical resection in combination with perioperative

chemotherapies which is currently the only curative option available to patients [4,5].

2.2 Staging

While the World Health Organization (WHO) classified gastric cancer into four main subtypes, the widely used Lauren classification divides gastric cancer into the intestinal, diffuse and intermediate subtype [6]. The intestinal subtype shows a glandular morphology of tumor cells and often metastasizes to the liver and lung. In contrast, the diffuse subtype is characterized by a non-coherent diffuse growth of cancer cells, characteristic signet-ring cells and a frequent metastatic spread to the peritoneum [7].

The Cancer Genome Atlas (TCGA) study classified according to molecular alterations. The subgroups include Epstein Barr Virus (EBV) , microsatellite instability (MSI-H), chromosomal instability (CIS), and genomically stable (GS) subtypes [8,9].
EBV infected cancers often show phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and AT-rich interactive domain-containing protein 1 A (ARID1A) mutations, cyclin-dependent kinase inhibitor 2 A (CDKN2A) silencing and a widespread hypermethylation of promotor regions [8,9,10].

The characteristics of MSI-H subtypes are a very high mutational burden with common MutL homolog (MLH1) silencing. Mutations in the DNA repair system are also very common [8,9,11].

Tumor cells that exhibit the intestinal glandular structure are often defined genetically as CIN subtype. Frequent mutation of tumor

protein 53 (TP53), receptor tyrosine kinase (RTK)-RAS pathway activation by receptor amplifications and a high amount of somatic copy number alterations (SCNA) were found in these cancer cells [8,9,12].

The GS subtype has frequent mutations of the cell adhesion molecule cadherin 1 (CDH1) resulting in a diffuse morphology. Additionally, Ras homolog family member A (RHOA) and ARID1A are mutated often [8,9,13].

2.3 Principles of Biomarker Testing

To improve overall survival in patients with locally advanced, unresectable, and metastatic EGC malignancies, molecular testing of promising new drug targets became standard of therapy. Presently, patients are tested for growth receptor HER2 expression, programmed death-ligand PD-L1 expression and for microsatellite instability status.

2.3.1 Assessment of HER2 Positivity

HER2 gene overexpression or protein amplification was observed in a variety of cancer types including EGC. Some studies correlated HER2 positivity with tumor invasion and lymph node metastasis leading to poor prognosis. Further studies are needed to prove the prognostic significance of HER2 as biomarker. Although promising FDA approved treatment regimens suggest the HER2 targeted therapy as viable treatment option for patients with HER2-positive metastatic disease. Reported rates of HER2 amplification varies widely in EGC from 2%–45%. [14,15]

The evaluation of the ToGA trial that evaluated the addition of trastuzumab to chemotherapy in patients with HER2-positive advanced EGJ or gastric cancers, indicates higher rates of HER2-positivity (around 33%). Thus, HER2 amplification might serve as a stable indicator [16]

Currently, HER2 testing is recommended for all patients with advanced stage EGC. Recommendations from the College of American Pathologists (CAP), American Society for Clinical Pathology (ASCP), and ASCO, the NCCN Guidelines advise using immunohistochemistry (IHC) for analysis of HER2 status. This technique evaluates the immunostaining of the outer layer of tumor cells. The intensity and extent of staining gives a score with s range from 0 (negative) to 3+ (positive) [14, 15, 16, 17].

Additional FISH analysis is advised if the score is 2+ (weak to moderate membranous reactivity in ≥10% of cancer cells). Calculation of the score obtained by FISH results is expressed as the ratio between the number of copies of the *HER2* gene and the number of chromosome 17 centromeres (CEP17) within the nucleus counted in at least 20 tumor cells. No further testing with FISH is required if the score is positive (3+) or negative (0 or 1+) [16,17]

2.3.2 Assessment of Microsatellite Instability and PD-L1 Expression

Due to its first-ever site-agnostic approval by the FDA, pembrolizumab is common standard care treatment for MSI-H cancer patients. Thus, the tumor mutational burden and MSI-H status needs to be assessed

[8,11]. Especially patients with metastatic disease have high incidences for DNA mismatch repair mutations. MSI-H status is assessed by IHC staining to measure MLH1, MSH2 and other mutation correlated to the transcription machinery. Testing is performed on formalin-fixed, paraffin-embedded tissues in accordance to CAP DNA Mismatch Repair Biomarker Guidelines [18].

Another FDA approved treatment as subsequent treatment in EGC patients was pembrolizumab. Patients with locally advanced metastatic disease in EGC can order a FDA approved diagnostic test. As with the other biomarkers this test is performed by IHC assays. PD-L1 protein levels are detected in formalin-fixed, paraffin embedded tissue. The score CPS is determined by the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells evaluated, multiplied by 100. Recommendation for a CPS of ≥10 are definite immunotherapy treatment. Thus, PD-L1 testing proved to be an important biomarker to assess if the patient is indicative of metastatic disease [19].

2.4 Esophagogastric cancer treatment

2.3.3 First-Line Therapy

First line systemic therapy in patients with locally advanced or metastatic esophagogastric adenocarcinomas often undergo surgical resection. To enhance the quality of life and improve survival, a 2 or 3 drug therapy regimen of cytotoxic drugs is used in these patients.

[2,3,4]. Chemotherapeutic agents regulary administered include the FOLFOX and FOLFIRI regimen. FOLFOX is a combination of folinic acid (leucovorin, FOL), fluorouracil (5-FU, F), and oxaliplatin (Eloxatin, OX) and is standard of care in clinics. Whereas FOLFIRI consists of of folinic acid (leucovorin, FOL), fluorouracil (5-FU, F) and irinotecan (IRI) [20].

The results of the ToGA trial suggest the addition of trastuzumab to first-line chemotherapy for patients with HER2-positive metastatic adenocarcinoma. A phase III trial conducted by the German Study Group compared treatment with fluorouracil and cisplatin to FOLFOX in patients was associated with higher overall survival with a median PFS (5.8 vs 3.9 months; *P*=.77) [14,15,16,21].

Irinotecan-based first-line treatments were analyzed in several randomized phase III studies in untreated advanced stage EGC cancer patients. It compared FOLFIRI (fluorouracil and irinotecan) to 5-fluorouracil + cisplatin (CF) (n=337). The results showed that FOLFIRI was at the same level of standard of the widely used standard CF treatment regimen in terms of PFS (PFS at 6 and 9 months were 38% and 20%, respectively, for FOLFIRI compared with 31% and 12%, respectively, for CF). On the other hand overall survival was not affected. Thus, FOLFIRI was accepted as acceptable option for the first line treatment of EGC patients. In addition to the standard chemotherapy regimens, CF and the before mentioned FOLFOX.[22]

2.3.4 Second-Line and Subsequent Therapy

Second-Line treatments depends on the prior therapy and overall medical status of the patient. Approved therapies from the FDA

include ramucirumab as single agent or on combination with paclitaxel.

For MSI-High patients, pembrolizumab has been included as a viable option for EG cancers with PD-L1 expression levels by CPS of ≥10 or as third-line treatment if CPS is of ≥1. [8,11,19].

The cytotoxic regimen FOLFIRI has also been approved as second-line therapy. Efficacy and toxicity was proven in several phase II trials with metastatic disease. or EGJ cancers refractory to docetaxel-based chemotherapy [20,22].

2.3.5 Targeted Therapies

At present, 3 targeted therapeutic agents, trastuzumab, ramucirumab, and pembrolizumab, have been approved by the FDA for use in esophageal and EGJ cancers. Treatment with trastuzumab is based on testing for HER2 status. Treatment with pembrolizumab is based on testing for microsatellite instability and/or PD-L1 expression.[23]

Trastuzumab

The ToGA trial was the first randomized, prospective, multicenter, phase III trial conducted to evaluate the effect of trastuzumab in HER2-positive advanced EGC. The majority of patients with HER2 positive cases in this trial (n=594) administered with trastuzumab and chemotherapy showed significant improvement in overall survival compared to chemotherapy alone. This study established a new standard treatment in these patients. The benefit was showed for

cases with a tumor score of IHC 3+ or IHC 2+ and FISH positivity for HER2. A retrospective study analyzed the additional effects of trastuzumab in combination with FOLFOX in untreated patients. The results suggest it to be a very effective and safe treatment for HER2 positve EGC patients with an improvement of 41% in median PFS and overall survival increase from 9.0 months to 17.3 months [19,21,23].

Pembrolizumab

Since 2017 the FDA has approved Pembrolizumab, a monoclonal PD-1 antibody to disrupt the PD-1/PD-L1 tumor suppression pathway as treatment for MSI-HIGH cases. The KEYNOTE-016 clinical trial, a multicenter phase II trial that evaluated the activity of pembrolizumab in 41 patients with a variety of solid cancer types showed tremendous improvement in overall survival [19].

Combination therapy with Trastuzumab was recently added to a further Keynote trial in progressed EGC cases.

Overall, these data suggest that pembrolizumab may be an effective second-line therapy for patients with advanced EGC with a PD-L1 CPS ≥10.

1.1 Organoids in cancer research

The upcoming ex vivo 3D organoid technology is supposed to mimic organ structure and the tumor microenvironment. Recent advances suggested the advantages over 2D cell culture as a more viable physiological human cancer model. Wild-type organoids can be grown

from pluripotent or embryonic stem cells or be established from patient derived tissue samples. They display phenocopying aspects, morphological similarity and self-organizing capacities as the organs they were derived from. A variety of different organoids have been established over the last decades. Gastric organoids from Clevers et al proved the concept of the feasibility of EGC organoid structures.

The potential of patient-specific drug testing and individualized treatment regimens arose from these recent advances.

3. Methods and Materials

3.1 Establishment of tumor organoid culture from biopsies and tissue resection

The surgical resected esophagogastric tissue and endoscopic biopsy samples were obtained from 62 patients under Memorial Sloan Kettering Institutional Review Board biospecimen research protocol 14-244. The enrolled patients all provided pre-procedure informed consent. The processing of tissue was generally done within 1 hour of the procedure.

The collected tissue was first washed with cold PBS containing antibiotics Penicillin and Streptomycin. It was then further processed by cutting it into 5mm pieces in a petri dish with razor blades. The chopped-up tissue was then transferred into a 20-30ml digestion buffer (Adv. DMEM, 10% FBS, 500mg/ml collagenase, 1mM dispase and freshly added DNase 1:1000). The total incubation time can vary depending on specimen size and tumor type. Generally, it can range from 15 to 30 min for biopsies and small tissue resections. After digestion of 30-40 minute at 37°C in a rotating manner, the suspension was filtered through a 40μM filter. Cells were pelleted by centrifugation for 5min, 900xg at 4°C Celsius. If the cell pellet is red, it indicates the abundance of erythrocytes, in which case the RBCs will be lysed by incubating them in 3ml ACK lysis buffer for 5min at RT. Otherwise proceed with washing the cells in ADF wash buffer. After washing, count the cells under a hematocytometer. Half of the cells will be frozen down in freezing media (Gastric medium, 10% FBS, 10% DMSO). The other half of the cells are resuspended in ice-cold gastric organoid medium (1-2 x104 cells per 10μL) and the same volume of ice-cold BME in a 12-

well plate (30-40µL/ drop). Place the plate in a 37 °C incubator for 15-30min to let the BME solidify. Add gastric medium containing ROCK-inhibitor Y and Noggin to the wells. Media change was done twice a week.

3.2. Thawing and freezing of organoids

Organoid aliquots were stored at -80°C or liquid nitrogen tank. To ensure viability of the organoids over the course of freeze and thaw cycles, organoids are frozen as intact structures instead of dissociated single cells. It is important to thaw the organoids quickly as the DMSO is toxic to the cells at room temperature. To thaw the frozen vials, put them into the 37°C water bath for 1-2 minutes. Prepare 10ml of prewarmed media in a 15ml falcon and slowly add the thawed organoid suspension to the media in a dropwise manner. Centrifuge the samples down at 900xg, 4°C for 5 minutes. Plate the organoids as described above in method section 1.

3.3. Isolation of human PBMCs from blood

All blood samples were collected under the Memorial Sloan Kettering Review Board biospecimen protocol 14-244. Patients consented pre-procedure to the blood drawing.

PBMCs were collected in 4 CPT tubes per patient blood drawing and processed within 2-4 hours after drawing. The separation of PBMCs from whole blood was done by centrifugation for 2400rpm, 20min at RT (acc.3, brakes: 1). The plasma was collected and centrifuged at 1000xg,

10 min at RT to get rid of any remaining cells. 6 1.5ml plasma cryo vials were stored for future experiments.

PBMC were washed twice in cell wash buffer (PBS, 10% FBS) at 350xg, 10min, 4°C. Cells were then counted with a hematocytometer. PBMCs were frozen down at 4-6 Mio/ml in PBMC freezing media (90% FBS, 10% DMSO) and stored at –80°C.

3.4. Immune cell activation and expansion

For T cell activation and expansion, frozen or fresh PBMCS were used, frozen PBMCs were thawed as followed.

The cryo vial containing PBMCs in freezing media gets transferred to dry ice. Fast thawing by putting it in the water bath at 37°C for 1-2min is advised.

Transfer into 15ml Falcon and add 10ml of prewarmed TexMACS media to the PBMCs, while flicking the tube. Centrifuge at 350xg, 5 minutes at RT. Resuspend cell pellet in 1ml of TexMACS media containing 200mg/ml IL-2, Primocin and Pen/Strep and 40ug/ml anti-PD-1 antibody Nivolumab (optional). Count the cells with a hematocytometer.

Organoids were incubated the day before with 200ng/ml IFNgamma to maximize antigen presentation. On the day of the T cell organoids from the same patient in cell recovery solution to disrupt the BME matrix. Incubate for 30min to 2 hours at 4°C. Collect the organoids and centrifuge at 900xg, 5min at RT. Wash once with ADF wash buffer. To dissociate the organoids to single cells, add 2ml TrypLE dissociation reagent and incubate in the water bath at 37 degree for 5-12 minutes. Check under the microscope frequently if more than 80% of organoids

have been disrupted to single cells, vortex in between. Then add ADF wash buffer + 10% FBS to stop the reaction and centrifuge at 900xg, 5min, 4°C. Count the cells with a hematocytometer.

For culture calculate the number of organoids and T cells in a ratio of 1:20 - organoids: T cells. Mix them accordingly in TexMACS media containing IL-2, Primocin, Pen/Strep and 40ug/ml anti-PD-1 antibody Nivolumab. Prepare a 96well U bottom plate and add TransACT T cell activation reagent 2µL/well. Add 1.2-1.5 x 10^6 cell suspension / well dropwise to each well. Incubate at 37degree for 2-3 days to select for T cells.

After the selection period carefully collect the T cells, centrifuge them at 350xg, 4°C, 5min to get rid of the transactivation reagent. Count the cells and plate them with fresh TexMACs media containing IL-2, Primocin and Pen/Strep at 1 x 10^6 cells/well.

Monitor the T cells closely and split them 1:2 every second day, if they are over 90% confluent. Otherwise take half of the medium out and add fresh media containing IL-2 to the wells. T cells can be expanded up to 14 days. Experiments were always performed after 10 days of expansion.

3.5. Co culture set-up

The organoids should be ideally grown for 7 days after dissociation to reach maturity. Aspirate media and add cell recovery solution to the plate. Incubate while shaking at 4°C for at least 1 hour. Collect cells in a tube and spin down 600xg, 5min (5 brakes/acc) at 4°C to harvest organoids. Wash them once with ADF wash buffer. Spin down at 600xg,

5min (5 brakes/acc) to get rid of any dead cells and without compromising the integrity of the organoid structure. Resuspend in 10ml ADF wash buffer and take 200µL aliquot out. Add 2ml TrypLE to the solution and incubate for 5-10 min at 37°C, wash once with ADF+ 10% FBS and count the number of cells. Calculate the volume needed for 20.000 cells/well.

In the meantime, collect T cells, expanded for 10 days and count them using the hematocytometer.

Resuspend the organoids at a concentration of 20.000 cells/well (40µl) in the required amount of media, mix the pellet well. Then add Matrigel (50%). Leave on ice!

- Plating:

It is very important to perform the whole experiment on ice, suspension tube, reservoirs and plates. Follow the schema to plate 2,000 organoids/well in 1:1 Matrigel: Gastric media and only 1:1 Matrigel: Gastric media for T cells only controls in white 96 well plates, avoid the outer wells of the plate. Spin down the plates at 100g, 1 min, 4°C, 5 brakes/acc. Take out the plates out of centrifuge and incubate them at 37°C for 30 min. Add 4,000 T cells/well as 50 µl/well and add rest of the 50 µl media per well containing drug as per the sample condition or 100 µL media in the conditions without T cells. Lastly, add PBS to the outer wells to avoid evaporation.

- CellTitreGlo Viability assay

The CellTitreGlo assay measures ATP content by reading the luminescence of the CellTitreGlo reagent.

First you need to add 11 µL lysis buffer (1:100) for Day0 CellTitre Glo plate and incubate for 1 hr. The CellTitreGlo reagent needs to be mixed with the CellTitreGlo reagent in a 1:1 ratio. The mixed reagent is then added to the wells of the 96well plate in a dropwise manner. Followed by an incubation of 10 min in the dark on a shaker at room temperature. Subsequently, the plate will be put in the Biotek microscope for measuring luminescence of ATP. Statistical analysis is done by xxxx

- Flow cytometry

Before performing flow cytometry, please see method section 4.1 for plating of the organoid T cell co culture. After the 48 hours of incubation in the incubator at 37°C. Spin down the plate briefly so that all the cells go down. (500 g, 5 min, 4°C). Remove part of the media (~50µl) from each well and put it in another plate for cytokine analysis, seal, snap freeze on dry ice for 10 min, store at -80°C. (Important: try not to collect cells.)
Next, pool all the replicates together for each condition. Add 500 µL cell recovery solution (Cornig) for each tube. And add additional 200 µL cell recovery solution in each well using multichannel pipette. Try to disrupt the Matrigel by pipetting up and down but be careful to avoid the filter blockage. Also collect organoids for single stain and T cells only in tubes. Keep them at 4°C at least for an hour. Prepare the plate for flow

cytometric analysis with antibody cocktails during this time. For single staining controls, add 1 drop of beads in 100 µL flow buffer and recommended amount of antibody. Next, prepare antibody mixes in 50 µL buffer/sample.

Sr No.	Target	Clone	Flurochrome	Expected expression
1	CD4	SK3	PerCP (dim)	Moderate -high
2	CD8	RPA-T8	AlexaFluor700 (dim)	Moderate -high
3	CD3	SK7	PE-Cy7 (bright)	High
4	PD-1	MIH4	BV421 (very bright)	Moderate - low
5	TIM3	7D3	PE-CF594 (very bright)	Moderate -low
6	EpCAM	Ber-EP4	FITC (moderate)	high
7	Viability		Near IR Viability: Zombie	high

Table 1 Flow cytometry antibodies

Collect some organoids for staining controls. Put them in cell recovery and perform all the subsequent steps and dissociate them in TrypLE too. Pool the cell recovery solution from plate into the respective tubes and pipette well and wash with ADF wash buffer (500 g, 5 min, 4°C) Repeat the washing and spin down again. Remove the supernatant and add 2 mL TrypLE in samples, untreated control and zombie only. Mix well and incubate it for 7 min at 37°C. Mix well and shake every minute. After 7 min of TrypLE, add 5mL ADF wash buffer to each tube. Plate back in U-bottom 96 well plate and spin plates (330g, for 5min at 4°C) to pellet

cells. It is important to work on ice from this step on and keep fluorochromes protected from light. Wash one time with 200 µL staining buffer. Incubate in 50µl 1:400 Zombie NIR in PBS for 15-30min at RT. Spin down 330g, 5min. Wash once with 200 µL staining buffer. Resuspend at 50 µl/sample, using flow buffer containing human Fc block (20 µl/ml). Incubate at RT for 10 min. Add 50 µL of the antibody mix to each well. Mix well without causing air bubbles. Incubate for 30 min at 4 °C, protected from light. Wash twice with 200 µL of FACS buffer per well (after each wash centrifuge the cells at 330g, for 5 min, for 4 °C and then remove the supernatant). Resuspend cells in 200-300 µL of FACS buffer and record on a suitable flow cytometer. It is possible to store the samples in 50–100 µL of FACS buffer overnight at 4 °C in the dark and continue with the recording the morning after. Add 40 µl counting beads per experimental samples just before acquisition. (Vortex well!).

3.6. HER2 characterization

For the characterization of HER2 receptor expression, organoids were grown for 7 days in 1:1 matrigel: gastric media. To perform the staining for HER2 in live imaging first aspirate media and add 3ml/6-well cell recovery solution. Incubate shaking at 4°C for 30-60min. Collect cells in a tube. Wash with ADF wash buffer (add up to 10ml) – pipette up and down at least 10x. Spin down 900xg, 5min. Repeat washing step. Count the number of cells by using a hematocytometer. Calculate for 20k-40k cells/well in 8-well chamber slide. Centrifuge at 900xg, 5min. Resuspend in calculated amount of gastric media +/- Wnt and aliquot, spin down again in Eppendorf tube. Add 10% Matrigel + 90% Media to each well (180µl Media, 20µl Matrigel). Incubate for 20 min at 37°C. Matrigel will

have polymerized at the bottom, aspirate medium on top. Add Matrigel (20%) - for 8-well chamber slide: 40µL total volume/well. Keep buried in ice. Centrifuge for 1 min, 100g (so no organoids are settled at the edges) – put it on 6-well plate lid and wrap it in parafilm. Incubate at 37°C for 10-20 min. Add 500µL of Media - Wnt containing 1:1000 Hoechst nuclei dye reagent and 1µg/ml AlexaFluor647 anti-HER2 antibody. Incubate for 1 hour at 4°C. Set up the ZEISS microscope and the 37°C heating chamber. Let the plate come to RT for 1-2 hours to allow the antibody to bind. Perform the live imaging at 20x or 40x magnitude.

3.7. Statistical analysis

For all statistical analysis a non-randomized Mann-Whitney U –test was performed in GraphPad Prism.

4. Results

Patient-derived EGD organoid culture

Tissue samples were obtained from the MSKCC hospital in collaboration with Dr. Yelena Jangjian. All patients consented to the MSK biospecimen protocol 14-244 pre-procedure.

In total 62 tissue and blood samples were collected from 62 patients with a range of esophagogastric cancer subtypes. Samples have been processed within an hour of procedure. As seen in Fig. 1A, the tissues were processed and dissociated into single cells. Optimization of the gastric growth media as well as processing of tissue samples to increase successful initiation of organoid cultures and minimize the advent of carrying along normal gastric glands was done. For complete formation of the organoid structure, cells were kept in cell culture for 6-8 weeks. The success rate of organoid development from patient tissue samples was 80%. Overall, 62 gastric cancer organoid cultures could be initiated. We selected four organoid lines for further analyses based on their high growth rate with a splitting ratio of 1:2/1:3 once a week. Most samples were obtained through endoscopic procedures or biopsies, leading to an elevated risk of contamination. Contaminated samples. were treated with the appropriate antibiotical, antifungal or anti-yeast reagent. Mycoplasma testing was performed once a month or after successful cultivation of the cells. Morphology of organoids was investigated by standard light microscopy over the course of 6 – 8 weeks. These organoid lines showed divergent morphological phenotypes, indicating different molecular genotypes (figure 1B).

The classification of organoids was done according to the Lauren classification system into the 3 described types., the intestinal, diffuse and intermediate subtype [38]. The intestinal subtype shows a glandular morphology of tumor cells with a distinct formation of lumen and a layer of endothelial cells with frequent metastasis to the lung and liver. In contrast, the diffuse subtype is characterized by a non-coherent diffuse growth of cancer cells with characteristic singlet-ring cells and a frequent metastatic spread to the peritoneum. The intermediate subtype consists of a mixture of the two other described subtypes (Fig. 1C). Further classification was done according to genomic patient data obtained from the Memorial Sloan Kettering Hospital Genomic Core Facility. The 4 subtypes, chromosomal instability (CIN), microsatellite instability (MSI-HIGH), Epstein-Barr virus (EBV) positive and genomic stability (GS) was done according to the TMN cancer classification.
All organoid lines were continuously in culture for over 1 year without a change in growth behavior or morphological phenotype. Also, freeze and thaw cycles did not affect or change these growth characteristics (data not shown). Gastric cancer organoids proliferated at different rates, but all with a higher percentage than normal organoids (figure 1C). Overall, response differed markedly between organoid lines. Normal organoids did grow long term only in complete medium. To specifically select for tumor organoids, the gastric growth media was depleted of the supplement Wnt after successful organoid formation. In Figure 1C tumor organoids classified by the Lauren classification system are depicted. Due to the mutational tumor burden these organoids lost their dependence on the Wnt pathway. Omission of Wnt was without phenotypical impact on the cancer lines.

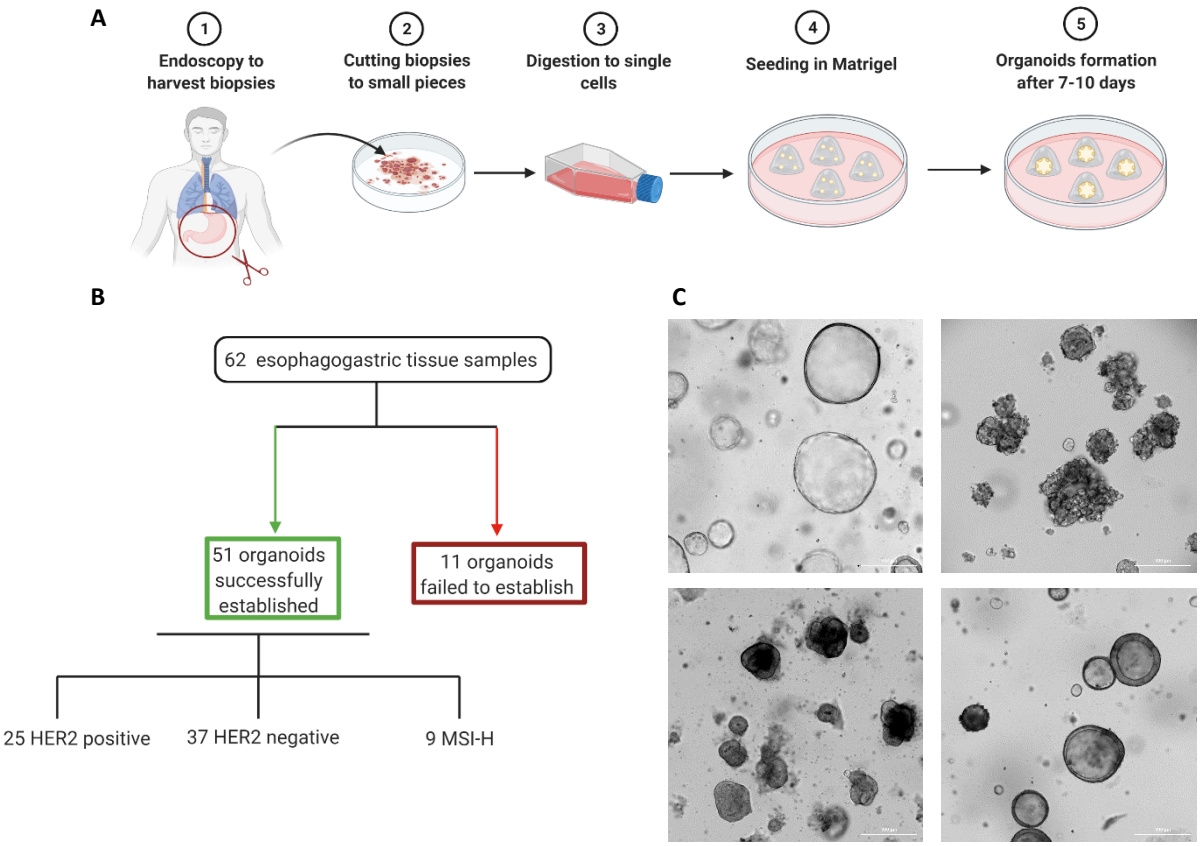

Fig. 1 Establishment of esophagogastric organoids
(A) Scheme of tissue processing. Patient tissue samples were harvested by endoscopic procedures, cut into small pieces with razor blades, dissociated into single cells by incubation in digestion buffer and seeded in 1:1 gastric media : Matrigel (B) Overview of esophagogastric biobank cases (C) Upper left: KG61N normal esophagogastric organoids, Upper right: KG65G Diffuse subtype EGC organoids, Lower left: KG55G intermediate subtype EGC organoids, Lower right: KG86G intestinal subtype EGC organoids

Characterization and validation of organoids

As ERBB2 amplification has long been known as an abundant mutation in Stage IV esophagogastric adenocarcinoma, the selected well-expanding organoid lines were investigated for that mutation. The

staining was performed by a ALEXAFlour647 labeled Trastuzumab which served as an anti-HER2 antibody, binding to the outer domain of the HER2 receptor. All organoid lines were split, and transferred into a 8-well chamber microscopy slide, then incubated with the cell nucleus stain and the labeled anti-HER2 antibody for 3-4 hours before live imaging under the ZEISS microscope was started. As clearly seen in Fig 2A, most organoid lines express HER2 abundantly but not heterogeneously. KG55G shows high expression of HER2 receptor in the so-called bulk of the organoid, whereas the core of the organoid displays no expression at all. Interestingly, the KG63G organoid line has higher expression in the core of the organoid and is reduced in the bulks. KG14G, KG86G and KG80G all indicate a homogenous amplification of HER2 in their membranes. KG59G serves as an example for an organoid line with baseline HER2 expression, which can be seen in figure 2A. The flow cytometry data of described organoid lines show the expression level of HER2 in comparison to the baseline with negative expression. As can bee seen in Figure 2B all organoid lines classified as HER2 positive show t=an higher expression of the receptor in flow analysis. All values have been normalized to the base protein B2M with constant expression in all lines. Also PD-L1 expression was investigated which showed no significant different expression than the unstained control. Hereby, no correlation with the provided clinical data in table X could be observed. The staining results on the contrary correlates with the clinical data obtained from IHC analysis of the primary tumor of the corresponding patients. IHC and FISH analysis measured the abundance of the HER2 receptor and MSK impact was run as proof of chromosomal amplification (as seen in Figure 2C). Though differences in amplification levels measured by IHC, FISH and MSKimpact analysis versus the live imaging

and flow cytometric analysis performed in the lab, can be observed. As the organoid lines are derived from a very small selected amount of tissue, the accurate replication of the clinical data is hard to grasp. Furthermore, HER2 amplification has been described as a dynamic mutation that can be lost and newly developed by every mutational clone, which can be seen in our heterogenous organoid cultures. However, all patients clinically described as HER2 positive could be verified in organoid culture.

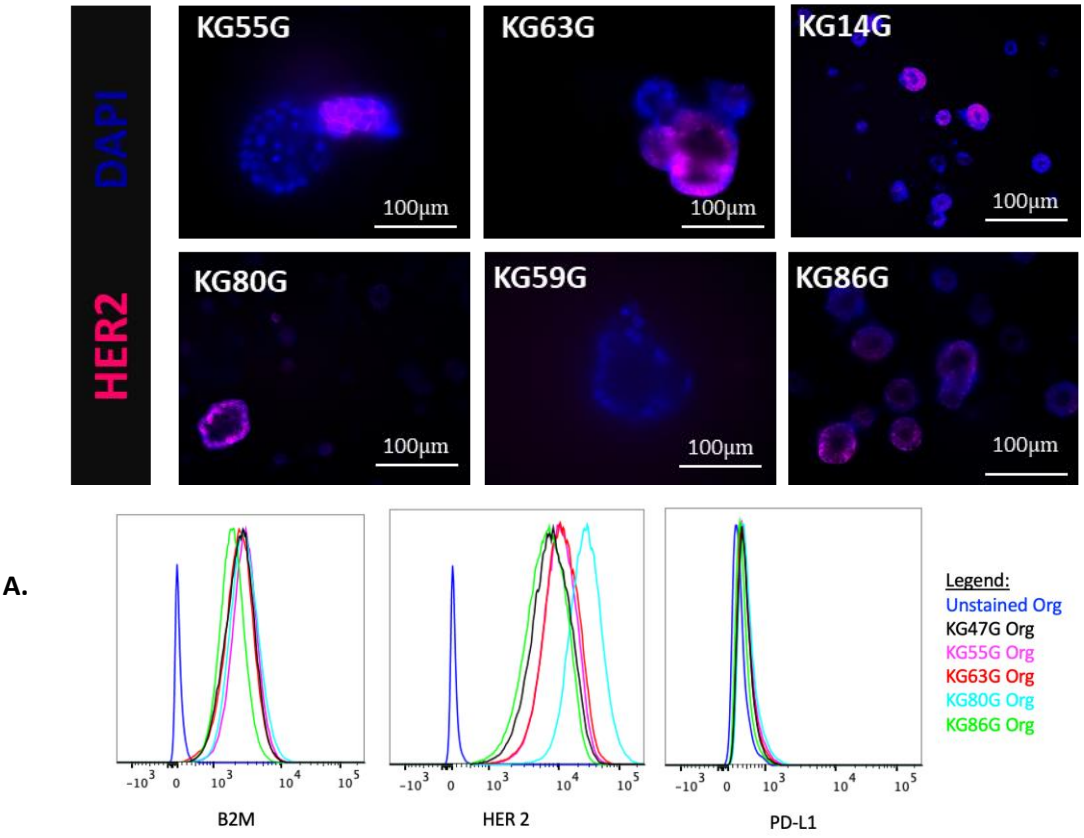

Figure 2. HER2 characterization of EGC organoids
(A) Live imaging of EGC organoids embedded in matrigel and gastric media with DAPI nucleus stain and anti-HER2 antibody conjugated with AlexaFluor647 (B) Flow cytometric analysis, stained for B2M as control, HER2 and PD-L1

Drug response of EGD organoids

All characterized organoid lines were further used to investigate the drug response of clinically proven but also investigational substances. As Trastuzumab is the FDA approved first-line treatment in Her2 positive advanced EGD cancer patients, we used this antibody to optimize the experimental set-up of our organoid drug response assay. Furthermore, the antibody-drug conjugate Trastuzumab-Derutexan, subsequently described as TdxD, was used as it has been described to be more potent than the antibody alone. It has also be reported that this ADC TdxD exhibit a bystander effect on non-HER2-amplified cells. Therefore, one HER2 positive and one HER2 negative organoid line were grown to confluency, dissociated into single cells and transferred into a 96well plate enclosed in the extracellular matrix Matrigel and incubated for 2-3 days until organoids were formed. They were subsequently treated with 0, 10nM, 100Nm and 1uM of the anti-Her2 antibody Trastuzumab and the antibody drug conjugate TdxD for 2, 4 and 6 days, respectively. The viability assay measuring the ATP content clearly shows the effect of Trastuzumab on the HER2 amplified organoid line KG47G. After 48 hours of incubation with Trastuzumab no significant decrease in viability was observed but after 4 and 6 days respectively a significant reduction in cancer organoid viability was observed. This is in line with the clinical assessment as the tissue was obtained after the patient has become resistant to the administration of this antibody. The antibody drug conjugate TdxD on the contrary shows a significant drug response after 48 hours to around 75% at 10nM, decreasing to 50% with a dose of 1uM. After 4 days of incubation both anti-Her2 drugs show significant reduction in viability and 6 days incubation didn’t show any interpretable results.

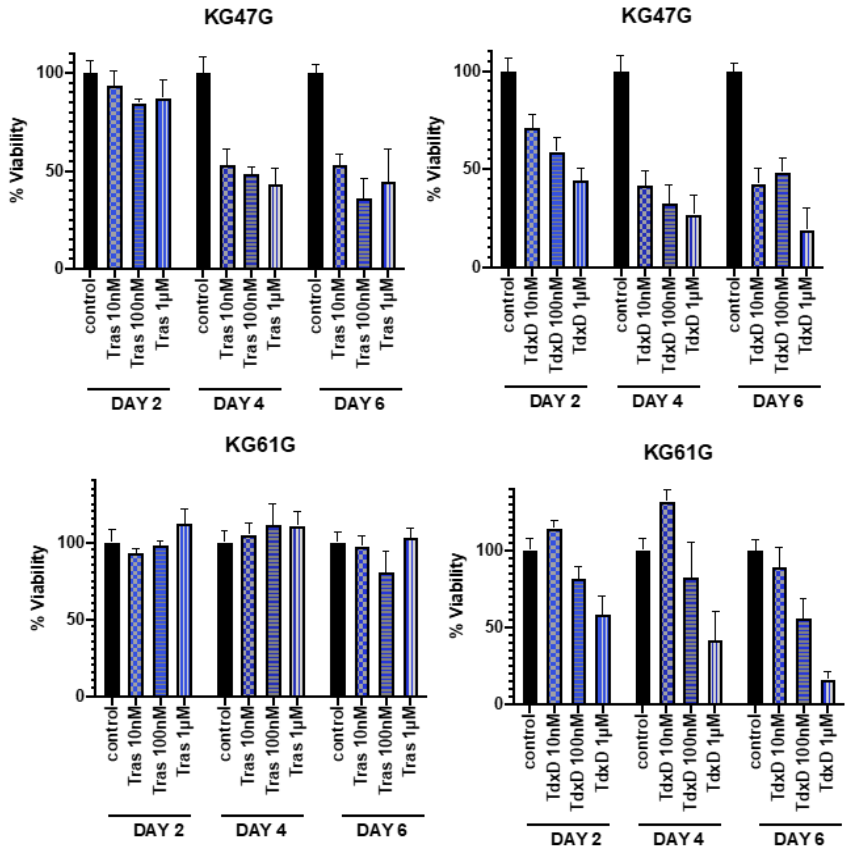

Fig 3. Optimization of anti-HER2 antibody dosage in organoids
CellTitreGlo viability assay was performed on 20,000 organoids in 6 replicates (n=3). Viability was calculated as seen in Method section. KG47G organoid line was used as HER2 positive control, KG61G organoid line was used as HER2 negative control

To estimate the ideal drug dosage, the Her2 negative organoid line KG61G was treated the exact same way within the same experiment. As expected Trastuzumab addition shows no significant changes in the viability (Fig 3C) at any timepoint or concentration, whereas high doses of TdxD decreased the viability at all timepoints. This is most likely due to the before mentioned bystander effect, in which the conjugate derutexan functions as internalized topoisomerase I inhibitor.

Interestingly, addition of only 10nM results in a slight increase in viability, which could indicate a form of activation of the proliferation. This phenomenon has been described in [8], where low doses of anti-HER2 drugs can first trigger the cancer cells to proliferate. These results indicated that 48 hours is the ideal time to see the effect of the drug on cancer organoids, mimicking the process in vivo the most accurate.

To further estimate the best drug dosage, all above selected EGD cancer organoid lines were investigated. Therefore, IC50 curves were obtained. IC50 is the value at which 50% of all cancer cells or organoids have been killed. Dosages of 0uM, 1nm, 10nM, 100nM and 1uM of aq variety of different anti-HER2 drugs were administered. As seen in Figure 4, the effect of the drugs vary for every organoid line. Experiments were performed in triplicates, with 6 biological replicas respectively. KG61G, the MSI-H HER2- organoid line shows no response to Trastuzumab but some effect could be seen with TdxD and TDM1, and the TKIs Neratinib and Afatinib, though even at the highest dose, the viability is still above 50%, resulting in no IC50 value. Across all investigated HER2+ organoid lines, KG14G, KG55G, KG63G, KG80G and KG86G, Trastuzumab leads to only a slight decrease in viability. The TKI Neratinib also show almost no significant effect on these lines with responses at very high concentrations above 100nM. Both antibody-drug conjugates proved to have the best response in cancer organoid lines with varying degrees of efficiency.

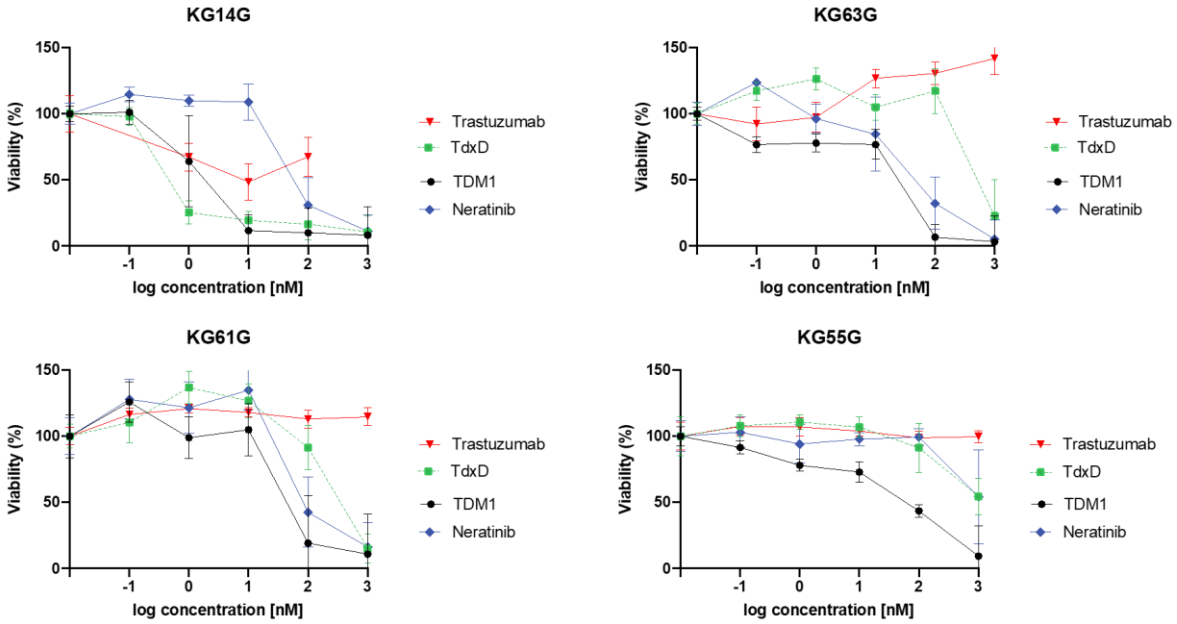

Figure 4. Evaluation of IC50 value in EGC organoids
EGC organoids were plated as 2,000 intact organoids/well. Viability was observed via CellTitreGlo reagent after 6 days of incubation with anti-HER2 antibody Trastuzumab, antibody-drug conjugate TdxD and TDM-1 or TKI Neratinib respectively in 6 biological replicas (n=3)

The IC50 values of the organoid lines can be correlated with the clinical responses in all investigated cases. The values indicate the optimal treatment window of 3D in vitro EGD cancer organoid lines, is around 100nM after 48 hours.

T cell organoid co-culture

The aim of this study was to investigate the effect of the combination therapy of anti-HER2 drugs with immunotherapy in patient derived EGD cancer organoids. For this purpose, the organoids and T cells were cultivated as described before. As seen in Figure X, the organoids have been incubated with IFNgamma overnight to increase antigen presentation on the cancer organoids. The T cells were treated in two different cultures, on with the addition of anit-PD-1 antibody Nivolumab and one without. As described in previous studies, treatment with anti-PD-1 antibody often leads to a permanent inhibition of the PD-1/PD-L1 pathway. Therefore, the latter addition of this antibody in the setting of the co-culture platform should not have any additional effect on the cancer targeting. To investigate the described phenomena in depth, the cancer organoid line KG61G was used. Figure 6B shows the viability of cancer organoids after 48 hours of culture with the respectively isolated T cells activated in the presence of anti-PD-1 antibody Nivolumab. The addition of Pembrolizumab for 48 hours does not lead to any significant difference in cancer targeting. On the contrary, the addition of Pembrolizumab on T cells that were not activated in the presence of Nivolumab, show a significant effect on the viability of the KG61G cancer organoids, reducing the viability another 10%. In both cases however, the T cells effectively target and kill the cancer organoids. We further investigated the activation of T cells and the effect of anti-PD-1 antibodies on them. The exhaustion marker TIM-3 was not significantly changed (data not shown). The expression of activation marker PD-1 is increased upon culture with organoids and decreased when Pembrolizumab is added.

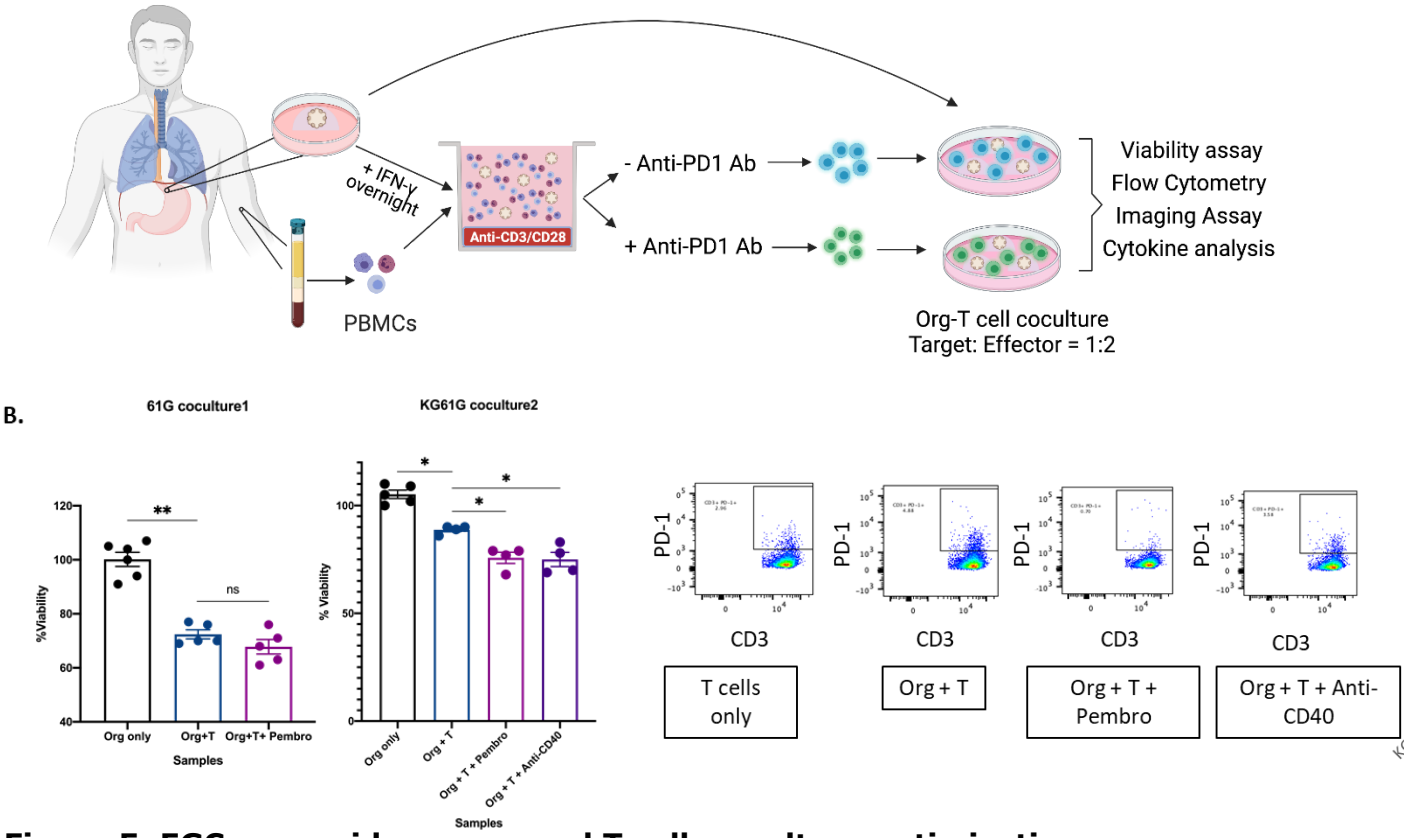

Figure 5. EGC organoid and T cell co culture optimization
(A) Experimental scheme of co culture model. PBMCs were collected from patients blood by Ficoll centrifugation and cultivated (B) CellTitreGlo viability assay after 48 hours of co culture. Organoid line used HER2 negative, MSI-H KG61G, incubated with respective isolated T cells (T) and Pembrolizumab or anti-CD40 antibody (C) Flow cytometric analysis of CD3 positive organoid cells

Patient-derived organoid and T cell co-culture platform

As described previously, the aim of this study was to compare the treatment effect of combination therapies to the clinical response of the respective patient. To study these effects in depth a variety of assays were performed. The coculture system of cancer organoids and T cells were tested for viability with the CellTitreGlo assay, Flow Cytometry to

investigate activation and exhaustion markers and imaging to keep track of viability and morphology. In Figure 6, two patient-derived organoid lines were selected, in which one patient KG63G was proven to be clinically resistant to Trastuzumab administration, whereas the other patient KG55G showed promising response to this therapy.

The clinical data of the patient KG55G suggest a reliable response to the combination treatment of Trastuzumab and Pembrolizumab with a significant decline in tumor size. The same effect can be investigated in our co-culture system. Upon addition of Trastuzumab the cancer cell viability is significantly lower than the control with only cancer organoids and T cells. Additional administration of Pembrolizumab is not affecting the viability significantly. This was expected as the T cells were activated with anit-PD-1 antibodies beforehand. (see Fig 5). Due to the bystander effect discussed earlier, it was expected to see lower viability in TdxD administered organoids. As seen in Figure 7 this reduction can be observed.

The KG63G organoid line was derived from a patient after progression on Trastuzumab treatment. Upon administration of this anti-HER2 drug, the change in viability is not observable, thereby enforcing the hypothesis on Trastuzumab resistance within that tumor. Better response can be observed upon TdxD administration leading to a significant decrease in viability. The overall response of all organoid lines tested under the mentioned treatment regimens, Trastuzumab, only, Trastuzumab and Pembrolizumab, TdxD only and TdxD plus Pembrolizumab, TdxD only and TdxD and Pembrolizumab is depicted in Figure 6.

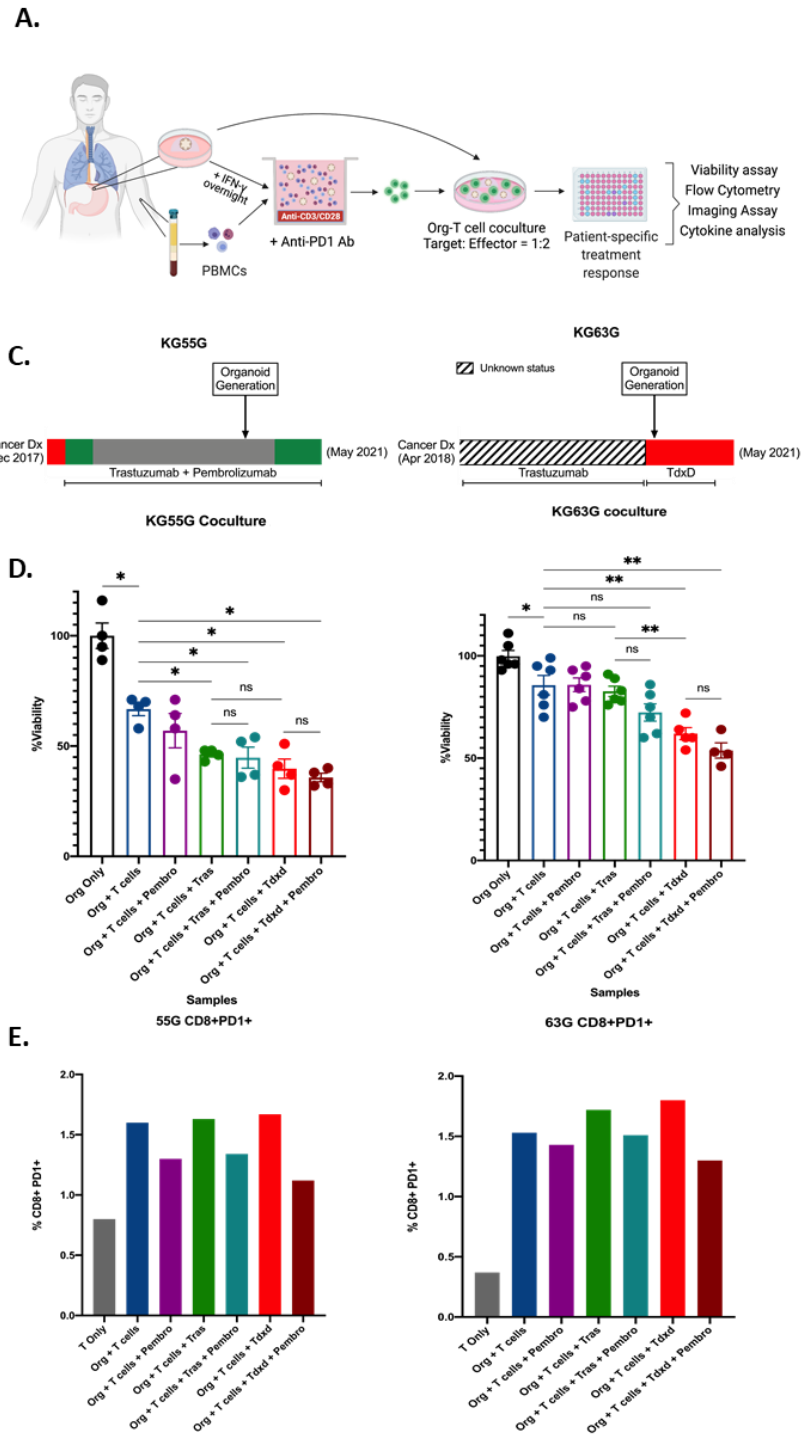
A.
+ IFN-γ overnight
Anti-CD3/CD28
+ Anti-PD1 Ab
PBMCs
Org-T cell coculture
Target: Effector = 1:2
Patient-specific treatment response
Viability assay
Flow Cytometry
Imaging Assay
Cytokine analysis
C.
KG55G
Organoid Generation
Cancer Dx (Dec 2017)
(May 2021)
Trastuzumab + Pembrolizumab
KG55G Coculture
KG63G
Unknown status
Organoid Generation
Cancer Dx (Apr 2018)
(May 2021)
Trastuzumab
TdxD
KG63G coculture
D.
%Viability
Org Only
Org + T cells
Org + T cells + Pembro
Org + T cells + Tras
Org + T cells + Tras + Pembro
Org + T cells + Tdxd
Org + T cells + Tdxd + Pembro
Samples
55G CD8+PD1+
63G CD8+PD1+
E.
% CD8+ PD1+
T Only

Figure 6.Co culture model of EGC organoids and T cells recapitulate patient response (A) Overview of co culture system platform (C) Graphical chart of patient's treatment history. Length of the graph correlates directly to time span of the respective therapy (D) CellTitreGlo viability assay of coculture system in 6 biological replicas, statistical analysis was done by Mann-Whitney U-test (E) Flow cytometrc analysis of the exhaustion marker PD1 in CD8+ cells after cultivation with the respective organoids

To analyze activation and exhaustion states of the patient-derived T cells, the levels of PD-1 were investigated in sorted CD8+ T cells. In both lines expression of PD-1 was significantly elevated in the presence of the stimuli presented by the organoids. As expected, the addition of Pembrolizumab to single agent anti-Her2 antibody reduced the PD-1 expression by further 20%.

All the additional organid lines are depicted in Figure 7. Various treatment responses. Overall, there is a significant better response to the combination treatment in comparison to the single agent treatment.

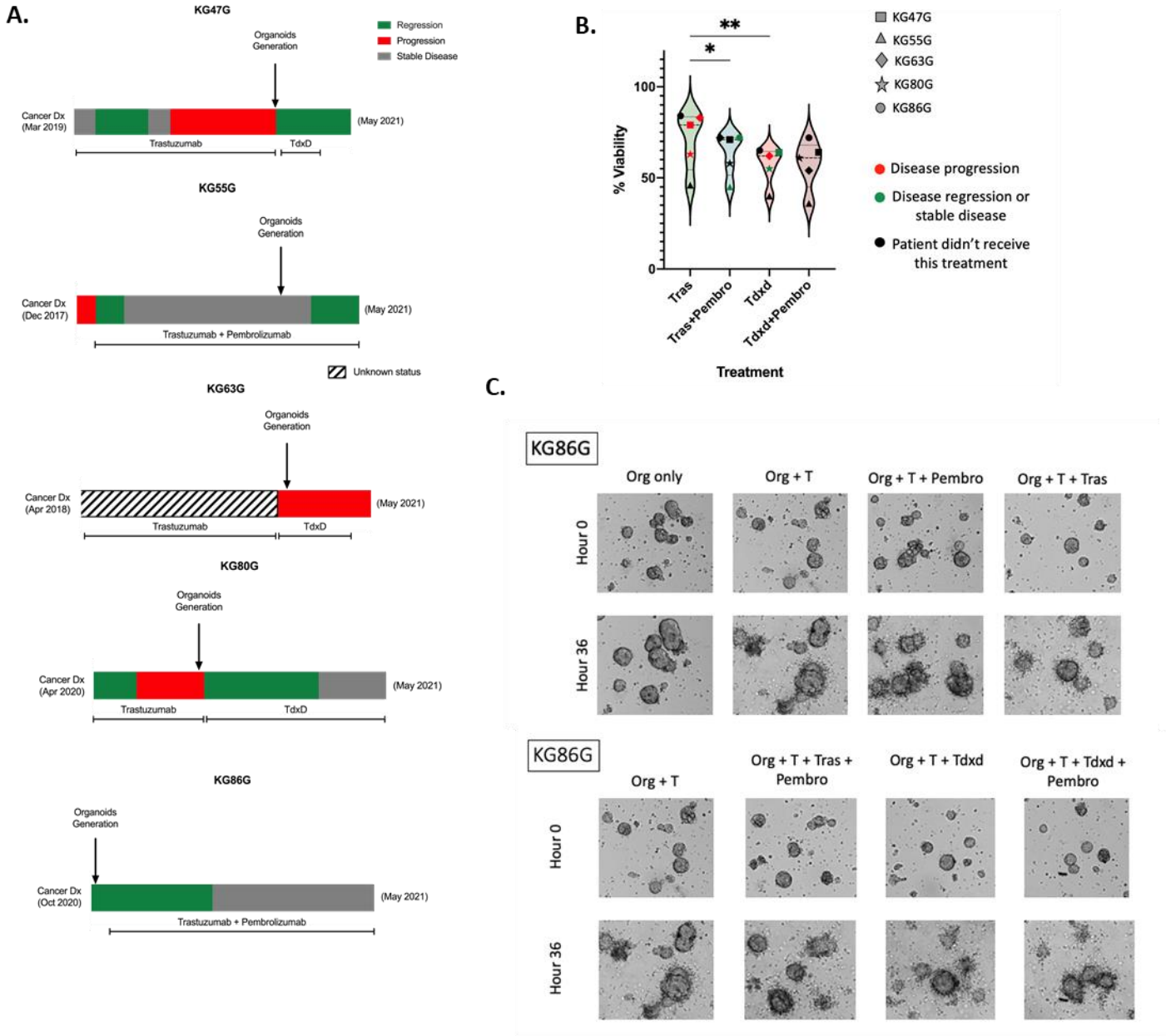

Figure 7. EGC organoid lines in coculture platform correlate with patient response over a variety of treatments

Discussion and Conclusion

The establishment of the esophagogastric cancer biobank, characterized by HER2 analysis and phenotypical observation proved the concept of the feasibility of patient derived EGC organoid formation. Co culturing with the respective isolated T cells provided an advanced insight into the tumor microenvironment. Clinical trials with immunotherapy could be investigated in that approach. To our knowledge was this the first study with in Matrigel embedded intact organoids and T cells to examine the efficacy of administered drugs and correlation to the patient response. Limitation of the data include the low number of replicas. For now, this preliminary data can be interpreted as observative. More replicas are needed to deeply analyze the correlations.

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