AREG and EREG are Predictive Biomarkers of Response to EGFR Inhibition in Gastroesophageal Cancer

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Abstract

EGFR is a potential therapeutic target in gastroesophageal cancer (GEA). However, negative results from several phase II/III clinical trials have hindered the approval of EGFR inhibitors for treating GEA. Preclinical and clinical results have shown that EGFR targeting is effective in GEA patients harboring *EGFR* amplification. Retrospective analyses also suggest that a subset of GEA patients lacking *EGFR* amplification may benefit from the treatment, thus underscoring the need to identify reliable predictive biomarkers of response. Through the screening of 27 GEA primary cancer cell lines and 10 patient-derived xenograft (PDX) models, we identified a subset of GEA lacking EGFR quantitative alterations but sensitive to EGFR targeting. Molecular characterization of the sensitive models revealed overexpression of the EGFR ligands AREG or EREG. Post-hoc analysis of patients on the COG trial treated with the EGFR inhibitor gefitinib demonstrated a significant correlation between overall survival and AREG/EREG expression level. No predictive power of EGFR ligand expression was observed in the presence of *KRAS* mutations. In conclusion, this study proposes the existence of a subgroup of GEA patients with susceptibility to EGFR inhibition driven by overexpression of the EGFR ligands AREG and EREG.

SIGNIFICANCE: Elevated levels of AREG or EREG in gastroesophageal cancer confers sensitivity to EGFR inhibition, providing a low-toxicity treatment option for the subpopulation of patients overexpressing the EGFR ligands.

INTRODUCTION

Gastroesophageal adenocarcinoma (GEA) represents the third cancer-related cause of death worldwide. In the last years, targeted therapies have improved the clinical management of patients with GEA: at present, the anti-HER2 monoclonal antibody Trastuzumab (and the derived antibody-drug–conjugate Trastuzumab-deruxtecan), the anti-Claudin18.2 mAb zolbetuximab and Ramucirumab (anti-VEGFR-2 mAb) have been approved for advanced disease. In addition, PD1 inhibitors in combination with chemotherapy (and trastuzumab in HER2-positive disease) are recommended in selected patients as first line treatment in advanced disease, due to the positive results obtained in several phase 3 randomized clinical trials (1-3).

Epidermal Growth Factor Receptor (EGFR), a tyrosine kinase receptor activated by several ligands, represents an attractive target for GEA patients for several reasons: a) it is amplified in a subset of patients and is expressed in most tumors (4), b) in other tumors, such as colorectal cancer, EGFR targeting is effective in absence of *EGFR* genetic alterations (such as amplification and/or mutation) due to functional receptor activation (5); c) EGFR inhibitors are guideline-recommended options in several tumor types (6). Thus, EGFR targeting in GEA patients has been actively investigated, but negative results of several phase II/III clinical trials determined its resting far from the approval (7-9). The main reason for this failure can be ascribed to lack of patient selection, mainly due to the absence of reliable predictive markers of response/resistance to therapy. Nevertheless, several reports of response and benefit from EGFR blockade have been published, thus underscoring the urgent need of identifying patients who may take advantage from this treatment (10-13).

Using PDXs (patient derived xenografts) we have previously demonstrated that *EGFR* amplified GEAs can benefit from treatment with the anti-EGFR monoclonal antibody cetuximab, alone or in combination with a Tyrosine Kinase Inhibitor (Lapatinib or Erlotinib), opening the possibility to offer a new therapeutic option to this subgroup of patients (14). Our preclinical results have been recently confirmed by the success of EGFR targeting in GEA patients harboring *EGFR* gene amplification (15). In this retrospective analysis of the COG trial (9), while *EGFR* amplified tumors benefited from gefitinib and *EGFR* non-amplified did not overall, a small fraction of GEA without genetic alterations of this receptor, did appear to benefit from EGFR targeting and it would be valuable to identify predictive biomarkers for these patients. This evidence is in line with other hints in literature (10), highlighting the urgent need for the identification of reliable markers of selection of patients.

Taking advantage of a proprietary annotated platform of GEA PDXs and of their corresponding primary cell lines, we verified the existence of non-*EGFR* amplified GEAs sensitive to EGFR targeting. Responsive tumors were characterized by the overexpression of the EGFR ligands Amphiregulin (AREG) and Epiregulin (EREG), whose role as biomarkers of response to anti-EGFR treatment was clinically validated in the COG trial. Our results reveal that a

subpopulation of patients not displaying an increased *EGFR* copy number but overexpressing AREG and/or EREG ligands can benefit from EGFR targeting.

MATERIALS AND METHODS

Primary cell cultures and drugs. In this work we used 27 primary cell lines (16 obtained from male and 11 from female patients) derived from PDXs as described in (16), and grown in 2D monolayers in collagen I coated plates. Most of them were maintained in culture in Iscove's medium supplemented with 10% Fetal Bovine Serum and antibiotics while GTR0128, GTR0533, GTR0558 and GTR0615 cells were grown in 50% Iscove's medium and 50% conditioned media from L-WRN cells (RRID:CVCL_DA06) supplemented with 10% Fetal Bovine Serum and antibiotics. Genetic identity between primary cells and the original tumor was verified by short tandem repeat profiling (Cell ID, Promega). Mycoplasma testing was routinely performed using the PCR Mycoplasma Detection Kit (Applied Biological Materials Inc., Richmond, BC, Canada). Verified cells are generally thawed few weeks before the experiments and kept in culture for 3-6 months. Erlotinib and gefitinib were purchased from Carbosynth (UK). SN38 was purchased from Selleckchem (USA). Cetuximab, panitumumab, lapatinib, U3-1402, epirubicin, 5-FU, oxaliplatin were provided by the Hospital Pharmacy.

Preclinical trials in PDXs. Experiments were performed on 8 weeks old female immunocompromised NOD/SCID mice (Charles River). GTR0125 (n=6/arm), GTR0187 (n=6/arm), GTR0210 (n=7/arm), GTR0221 (n=6/arm), GTR0247 (n=5/arm), GTR0498 (n=6/arm), GTR0539 (n=6/arm), GTR0558 (n=6/arm), GTR0640 (n=6/arm), GTR0687 (n=6/arm) PDXs were expanded for 2-3 generations to obtain 6-7 mice per treatment arm. Genetic identity between patient tumor and the PDX was verified by short tandem repeat profiling (Cell ID, Promega) before cohort expansion. When tumors reached an average volume of 220-250 mm^3 mice were randomized in a computerized assisted manner and treated for the indicated days with either vehicle (saline) cetuximab 20 mg/Kg, twice weekly ip. Tumor size was evaluated once weekly by caliper measurements and approximate volume of the mass was calculated using the formula $4/3\pi(D/2)(d/2)^2$, where D and d are the major and minor tumor axes, respectively. As often done in PDX models, the response in mice has been evaluated using RECIST 1.1-like criteria, i.e. progressive disease (PD): ≥35 % increase from baseline; partial response (PR): ≥ 50% reduction from baseline; stable disease (SD): intermediate variations from baseline (16). Statistical analysis was performed with GraphPad PRISM 9.0 (RRID:SCR_002798), using the 2-way ANOVA Bonferroni corrected method. Statistical significance: ns = not significant; *P<0.05; **P<0.01; *** P<0.001; ****P<0.0001. No a priori criteria were used for including and excluding animals, experimental units or data points; no confounders were controlled.

Ethics approval: This study took advantage from samples collected in the COG clinical trial approved by the National Research Ethics Service Committee (REC reference: 08/H0505/127), as in (9). Collection of samples to generate PDX models has been authorized by the Ethical Committee of Candiolo IRCCS (CE IRCCS 153/2014). All patients provided written informed consent. Guidelines for Care and Use of Laboratory Animals were followed during investigation. All animal procedures were approved by Ethical Commission of the IRCC in Candiolo and the Italian Ministry of Health (n° 58/2021-PR).

Western blot analysis. The following primary antibodies were used: HER3 (CST#12708 RRID:AB_2721919), HER2 (CST#2165 RRID:AB_10692490), VINCULIN (Millipore 05-386 RRID:AB_11212640), human EGFR (described in (17)). Intensity of the signal of each receptor was quantified by ImageJ software (RRID:SCR_003070) and normalized to vinculin signal.

Viability, cell death, cytotoxicity, apoptotic assays and cell cycle analysis. Cell viability was assessed by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). 2500/well primary cells were seeded in collagen coated 96-well plates in the presence of increasing concentrations of EGFR targeting agents (0.156-10µg/ml for cetuximab and panitumumab; 7.8-500nM for erlotinib, lapatinib and gefitinib) for 6 days. Normalized values have been used to compute the Area Under the Curve (AUC) using *GRmetrics* R package (18) and plotted as box plot (statistical analyses were performed in *ggpubr* R package (RRID:SCR_021139) using two-tails unpaired Student's t-test assuming equal variances of group samples). Viability assays testing chemotherapeutic agents have been conducted similarly, with increased concentrations of chemotherapy alone (epirubicin 3.13-200nM, oxaliplatin 0.31-20µM, SN38 0.78-50nM, 5-FU 0.31-20µM) or in combination with cetuximab (0.156-10µg/ml). For cell death measurement, $5*10^4$ cells were seeded in a 6 well costar and treated or not with 5µg/ml cetuximab for 6 days. Cells stained with trypan blue and the percentage of not viable cells (blue) calculated. For apoptosis quantification, cells were treated with 5µg/ml cetuximab for 72 hours and apoptotic cells detected using FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen V13242), according to manufacturer's instructions, using BD LSRFortessa™ Cell Analyzer. The evaluation of caspase 3 activity and cytotoxicity were performed by Caspase-Glo® 3/7 Assay and CellTox™ Green Cytotoxicity Assay (Promega) respectively according to manufacturer's instructions. For cell cycle analysis, 10^5 cells/condition were seeded and treated with 5µg/ml cetuximab for 72 hours or left untreated, harvested, fixed using 70% ethanol and stained overnight with Propidium Iodide working solution (Triton X-100 0.1% v/v, RNAse A 50 µg/ml Sigma-Aldrich R6513, Propidium Iodide 25 µg/ml). Cell cycle phases (G1-G0, S and G2/M) were evaluated through flow cytometry (BD LSRFortessa™ Cell Analyzer).

Transduction procedures. Lentiviruses were produced as described in (19). GTR0539, GTR0640, GTR0498, GTR0687 cells were transduced with a pool of lentiviral particles containing of three silencing shRNAs against AREG

(Sigma Aldrich, #423298, #426883, #420315) and/or three silencing shRNAs against EREG (Sigma Aldrich, #09832, #40086, #40084). Cells were selected with puromicin, checked for silencing and plated for viability assay.

Analyte extraction and real time qPCR. Genomic DNA was isolated using the Blood & Cell Culture DNA Midi Kit (Qiagen, Germany). RNA was extracted with Maxwell® RSC miRNA tissue Kit (Promega). Retrotranscription was performed by High-Capacity cDNA Reverse Transcription Kit. The following primers were used for real time qPCR: Epiregulin (fw: 5'-GCTCTGCCTGGGTTTCCATC-3'; rev: 5'-CCACACGTGGATTGTCTTCTGTC-3'), Amphiregulin (fw: 5'-GTCCTCGGGAGCCGACTAT-3'; rev: 5'-GGGGGCTTAACTACCTGTTCAA-3'), TGFa1 (fw: 5'- CAGCATGTGTCTGCCATTCTG-3'; rev: 5'-GGTGATGGCCTGCTTCTTCT-3'), EGF (fw: 5'-AGCTAACCCATTATGGCAACA-3', rev: 5'-AGTTTTCACTGAGTCAGCTCCAT-3'), HB-EGF (fw: 5'-CCCTCCCACTGTATCCACG-3', rev: 5'-AGTGACTCTCAAAAGGTCCAGA-3'), β-cellulin (fw: 5'-ATCTGCCACCACACAGTGAA-3', rev: 5'-AGAGCGGGGTTGATGGAC-3'), Epigen (fw:5'-ACAGCCCAGCAAGGTAACTG-3', rev: 5'-GTGTGAGAACTTCAAGGCTATGG-3').

Whole exome sequencing. DNA extracted from PDX models along with normal germline DNA from each patient were utilized for paired-end whole exome sequencing, performed in outsourcing by Macrogen (Seoul, South Korea RRID:SCR_014454) and Macrogen Europe (Amsterdam, The Netherlands RRID:SCR_014454). Samples were prepared using Sureselect Human All Exon V7 probe set, and libraries were sequenced using Illumina HiSeq 2000 sequencer at 100X coverage (RRID:SCR_020130). The base calling files were converted into FASTQ by Illumina package bcl2fastq v2.20.0 (RRID:SCR_015058). Raw reads quality control was performed using FastQC v0.11.7 tool (RRID:SCR_014583). Reads were aligned using BWA-MEM v0.7.17 (RRID:SCR_010910) to both human (hg38) and mouse (mm10) reference genomes, to distinguish the reads originating from the mouse stromal tissue in the PDX sample. Data were sorted and duplicate-marked using Picard v2.18.9 (RRID:SCR_006525). Each normal-tumor pair aligned bam files were passed all together to the Mutect2 tool (GATK v4.0.8.1 RRID:SCR_001876), to identify somatic point mutations and short indels. Variants detected in PDXs which were also identified in murine DNA were filtered and removed. Filtered variants were annotated using BCFtools v1.9 (RRID:SCR_005227).

EBV and MSI analysis. EBV-positivity and microsatellite status were evaluated as in (16).

Definition of molecular subtypes according to TCGA. Molecular subtypes were assigned to PDXs following TCGA decisional tree in line with previous publication (16).

Immunohistochemistry and RNA in situ hybridization. Immunohistochemistry was performed according to standard protocols using HER3 (D22C5) XP, CST #12708 RRID: AB_2721919. RNA Chromogenic in situ hybridization was performed on Formalin-Fixed Paraffin-Embedded samples, using RNAScope 2.5 RED technology (Advanced Cell Diagnostics, Hayward, California, USA). Sections were pretreated according to the manufacturer's protocols and then

incubated in HyBEZ Oven for two hours at 40°C with Hs-AREG (#313111) or Hs-EREG (313081) probe. After amplification steps, the signal was detected using fast RED substrate and expression was evaluated by bright-field microscopy (Zeiss – Scope A1). H-score was assigned in blind of clinical information by two independent pathologists.

Statistical analysis. Seventy-five COG samples were analyzed for the expression of AREG, EREG and HER3. Five samples were excluded due to lack of *EGFR* amplification data. One, three and one samples were excluded from AREG, EREG and HER3 scoring, respectively, due to lack of material. Because this was a pilot study, a formal power calculation was not required. The correlation between AREG, EREG and HER3 H-scores in specimen from gefitinib-treated arm in the COG clinical trial was assessed using Spearman's rank correlation in *ggpubr* R package (RRID:SCR_021139). The Kaplan-Meier method was used for survival curves in both COG (9) and ACRG (20) datasets, and the statistical significance was calculated using the Log-rank (Mantel-Cox) test with GraphPad PRISM 9.0 (RRID:SCR_002798). Cases were divided into two groups based on the dichotomization of AREG and EREG expression levels, using quartiles (Q4 versus Q1-Q3) as cut-off. Group with high AREG or EREG expression was defined when at least the expression of one of the two ligands was in Q4. Multivariate analyses were performed by Cox proportional hazard model. Statistical significance was recognized at p-values less than 0.05.

Data availability. The used cell lines are available upon request for collaborative purposes. PDX models data and metadata are openly available in PDX Finder (21) and in the EurOPDX data portal (https://www.cancermodels.org/) that will be updated with the newly generated models. Genomic data has been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS50000000966. Further information about EGA can be found at https://ega-archive.org and "The European Genome-phenome Archive of human data consented for biomedical research". GTR0221 was subjected to focused targeted bait set of 243 genes, as described in (16).

RESULTS

Identification of a subset of primary gastro-esophageal cancer cell lines and PDXs sensitive to EGFR targeting.

In the past years, we have set up a unique proprietary biobank of GEA Patients-Derived Xenografts (PDXs) and of their derivatives, organoids and primary cell lines (16). This multi-level platform easily allows the simultaneous testing of many different compounds *in vitro* (in organoids/primary cell lines) and their validation *in vivo* (in mice cohorts, Fig. 1) (16,22,23). Taking advantage from this platform, we had previously shown that EGFR inhibitors are active in patients with *EGFR* copy-number gain and that co-amplification of either other receptor tyrosine kinases or *KRAS* is associated with resistance (14).

Since the post-hoc analysis of the COG trial highlighted the existence of a small subgroup of GEA patients responsive to EGFR targeting, in spite of being devoid of *EGFR* genomic alterations (9,24) we decided to experimentally validate this observation. We thus selected from our platform 27 GEA primary cancer cell lines (not displaying *EGFR* amplification or mutation) representative of the pathological and molecular subtypes identified by the TCGA (25) (Supplementary Fig. S1A). Since in colon cancer the anti-EGFR monoclonal antibody cetuximab is an effective EGFR-targeting drug in tumors devoid of *EGFR* alterations, we evaluated cell viability in PDX-derived cell lines treated with increasing concentrations of cetuximab (in the range of its plasmatic concentration in humans). The heatmap shown in Fig. 2A reveals that 8/27 lines (29.6%) displayed sensitivity to EGFR targeting (residual viability <50% after 6 days of treatment), while the remaining 19 cell lines were resistant to cetuximab. These two groups were also clearly identified by evaluation of the AUC (area under the curve, Fig. 2B). MSI status was not significantly enriched in any group (Supplementary Fig. S1A). As expected, similarly to what observed in colorectal cancer, *KRAS* mutations significantly correlate with resistance to cetuximab (Supplementary Fig. S1B).

Since evidences from literature show that the activity of the different EGFR targeting drugs is not always superimposable (26), we tested the sensitivity of the panel of primary cell lines to other anti-EGFR agents already in clinics: the monoclonal antibody panitumumab (approved in colorectal cancer), the EGFR tyrosine kinase receptor inhibitors (TKIs) erlotinib and gefinitib (approved in non-small cell lung cancer) and the dual EGFR/HER2 TKI lapatinib (approved in breast cancer). As shown in Fig. 2B and in Supplementary Fig. S2A-D, cetuximab sensitive cells responded to the other targeted drugs as well, with a high Pearson correlation coefficient (Supplementary Fig. S2E-L). Few cell lines, non-responsive to cetuximab exhibited sensitivity to other EGFR targeted drugs. In some cases, this could be explained by the presence of co-alterations in other RTKs (i.e. lapatinib responsive GTR0233 cells display *HER2* amplification (22)); in other cases, no mechanism was found (i.e. GTR0607 and GTR0221 not displaying any *EGFR/HER2* genetic alteration responded to EGFR TKIs but not to monoclonal antibodies).

Cetuximab responsive (GTR0539, GTR0640 and GTR0247), but not resistant cells (GTR0125, GTR0498 and GTR0687), underwent cell death upon cetuximab treatment, as shown in Fig. 2C and Supplementary Fig. S3 A-C. Increased apoptosis in cetuximab sensitive models was paralleled by an augmented percentage of cells arrested in G0/G1 (Supplementary Fig. S3D).

To validate *in vivo* the *in vitro* results, we performed xenotrials on the PDXs from which the primary cells were originally derived. Briefly, a piece of the patient tumor was implanted subcutaneously in mice and serially transplanted until cohorts of six mice bearing the same tumor were obtained and treated with cetuximab or saline solution as control. As shown in Fig. 3, we observed a perfect overlap between *in vitro* and *in vivo* results, as GTR0539, GTR0640, GTR0558, GTR0187 and GTR0247, were responsive to the treatment (according to modified RECIST criteria (16)),

while GTR0221, GTR0210, GTR0125, GTR0498 and GTR0687 did not show any benefit from treatment with cetuximab. Importantly, in two models we observed a complete response (CR), further validating the effectiveness of the treatment.

All together these results highlight the existence of a subgroup of GEAs responsive to EGFR targeting, in absence of *EGFR* genetic alterations.

Identification of biomarkers of response.

The identification of biomarkers predictive of response to EGFR targeting is crucial for the correct patients' selection and the design of successful clinical trials able to impact on clinical practice.

We reasoned that if EGFR targeting affects the viability of sensitive models, some molecules linked to the EGFR pathway could be deregulated. EGFR belongs to a family composed by four Receptor Tyrosine Kinases (RTKs; HER2, HER3, HER4 and EGFR itself) and can bind seven different ligands (EGF, EREG, AREG, TGFA, EPGN, BTC and HB-EGF). Thus, we investigated the expression of these molecules in cetuximab sensitive *vs* resistant tumors. *KRAS* mutated (intrinsically not responsive) and *HER2* amplified (for which other therapeutic options are available) models were excluded from further analysis. GTR0221 and GTR0607 were left apart as well, to avoid confounding results due to their sensitivity to TKIs but not to EGFR monoclonal antibodies.

When we investigated the expression of the EGFR ligands we noticed a significant increase in the levels of *AREG* and *EREG* mRNA in cetuximab sensitive *vs* resistant tumors (Fig. 4 A,B,C). As shown in Fig. 4D, overexpression of at least one of the ligands (high = at least one ligand in Q4 - quartile of higher expression) was a significant predictor of response to cetuximab. No significant difference in the expression of the other ligands was detected (Supplementary Fig. S4A-E).

To unravel the role of these ligands in EGFR targeting, we silenced *AREG* and *EREG* expression by means of lentiviral transduction of shRNAs in both cetuximab sensitive and resistant cells and evaluated their viability. As shown in Fig. 4 E-H, AREG and EREG silencing (either alone or in combination) strongly impaired the viability of cetuximab sensitive (GTR0539 and GTR0640) but not of the resistant cells (GTR0498 and GTR0687). Moreover, cetuximab treatment further decreased the viability of sensitive cells.

While HER2 and EGFR were not significantly differentially expressed between sensitive and resistant cells (Supplementary Fig. S5A-C), we noticed that HER3 protein was overexpressed in cetuximab sensitive cells (Fig. 5 A-B).

We wondered whether we could exploit the presence of high levels of HER3 in cetuximab sensitive cells as a pharmacological target. Despite the attempts done to tackle HER3 in clinics, no approved drugs are available so far.

Encouraging results from clinical trials (confirmed by FDA Breakthrough Therapy designation) shed light on a novel antibody drug conjugate (ADC), patritumab-deruxtecan (U3-1402), where the HER3-directed monoclonal antibody patritumab is linked to the DXd topoisomerase I inhibitor payload. As shown in Fig. 5 C-D, treatment of cetuximab sensitive/HER3 overexpressing cells (GTR0539 and GTR0640) with this compound induced a marked reduction in cell viability, while no effect was seen in cetuximab resistant/HER3 low expressing cells (GTR0125, GTR0498 and GTR0687). Moreover, the combo of cetuximab and patritumab deruxtecan further decreased cell viability of sensitive models (Fig. 5 E-F), thus opening the possibility of exploring combinatorial treatments to achieve a better response.

All together these data suggest that, in agreement with colon cancer (27-29), AREG and EREG increased expression can be considered a positive predictor of response to EGFR inhibition. Moreover, concomitant HER3 over-expression can be exploited as a pharmacological target for the design of combinatorial treatments.

Cetuximab treatment does not compromise chemotherapy efficacy.

While in other tumors, such as colon cancer, patients benefit from the combination of cetuximab plus chemo (30), reports for gastroesophageal cancer are controversial (7,31,32). We thus evaluated if cetuximab treatment in *EGFR* not amplified cases impinges on the response to the different drugs that have been tested in FISH positive tumors (8,9,33,34). Epirubicin (anthracyclines), oxaliplatin (platinum-based class), SN38 (active metabolite of irinotecan, topoisomerase I inhibitor) and 5FU (pyrimidine analog) were tested alone or in combination with cetuximab in GTR0539 and GTR0640 sensitive cells and in GTR0498 and GTR0687 resistant cells (Fig. 6). The experiments show that the addition of cetuximab to the tested chemotherapeutic drugs either enhanced or left unaffected the response to chemo in cetuximab sensitive cells. No detrimental or paradoxical effects were recorded in any condition tested. Notably, for cells very sensitive to chemo, cetuximab addition did not further increase the response.

AREG and EREG overexpression identifies patients responsive to anti EGFR treatment.

To validate our preclinical data, we chose to analyze the patients treated with gefitinib in the COG clinical trial (9,22) (Table 1), with the theoretical advantage that the EGFR TKI was given as single-agent without chemotherapy. In this post-hoc analysis, 19 out of 70 (27,1%) patients with *EGFR* FISH negative tumors showed disease control (Fig. 7A). We thus explored the expression of *AREG*/*EREG* (67 FFPE samples - by means of RNA in situ hybridization) and HER3 (69 FFPE samples - by means of IHC). Positivity was quantified and an H-score assigned in blind of clinical information by two independent pathologists (Supplementary Fig. S6 and S7A) whose evaluations were highly concordant (Supplementary Fig. S7B; for details see the material and Methods section). From this analysis, no correlation between HER3 expression and OS was revealed (Supplementary Fig. S8). Vice versa, a significant

association between *AREG*/*EREG* ligands overexpression and gefitinib response was observed (Fig. 7B). Indeed, the overexpression of one of the two ligands (either *AREG* or *EREG*) identified patients benefitting from anti-EGFR treatment and showing a significantly longer Overall Survival (HR=0.58, CI= 0.34-0.99, p=0.044), thus confirming our preclinical results. Moreover, in a COX regression model, dichotomized ligand expression was significantly associated with disease control (Fig. 7C). Interestingly, the expression of *AREG* and *EREG* was highly correlated (Supplementary Fig. S7D). Notably, *AREG*/*EREG* overexpression did not reveal per-se any prognostic value, as shown by the analysis of the Asian Cancer Research Group (ACRG) (20) cohort (Supplementary Fig. S9). As expected, the forest plot reported in Fig. 7D shows that both a worse performance status and the presence of *KRAS* mutations represent negative prognostic factors for OS in patients receiving gefitinib.

All together these data indicate that AREG/EREG overexpression may represent a positive biomarker of response to EGFR-targeted therapies in patients without *EGFR* amplification. This poses the basis for future clinical trial design.

DISCUSSION

In the past years, several trials explored EGFR as a target in gastroesophageal tumors. In particular, two phase III trials (REAL3 and EXPAND) showed that in unselected patients with advanced GEA the addition of an anti-EGFR monoclonal antibody to first line standard chemotherapy failed to show a significant survival benefit (7,8). Negative results were obtained also in the COG trial, in which the pure effect of EGFR targeting was investigated in unselected patients with gastroesophageal cancer treated with the TKI gefitinib versus placebo from the second line setting and beyond (9). Even if no difference in OS was observed between gefitinib and placebo groups, the increased disease control and PFS observed in gefitinib-treated patients suggested the existence of a gefitinib-responsive subgroup. A post-hoc analysis of the COG trial suggested that patients with *EGFR* amplified tumors could benefit from EGFR blocking (24). Indeed, recently, preclinical and clinical data confirmed that patients with *EGFR* amplified GEAs derive significant benefit from EGFR targeting (14,15). An additional analysis of the COG trial evidenced the existence of a subgroup of patients with *EGFR*-FISH negative GEAs and benefit from EGFR targeting. To validate this observation, we selected from our platform 27 GEA PDX-derived primary cancer cell lines not bearing *EGFR* gene amplification. Eight of them (29.6%) displayed sensitivity to cetuximab, a percentage very similar to that observed in the COG trial (27%). Interestingly, we observed that the eight models were generally sensitive to several EGFR targeting drugs beyond cetuximab and including panitumumab, gefitinib, lapatinib and erlotinib.

The existence of a subgroup of patients, in addition to those with *EGFR* amplified GEAs, that could benefit from EGFR targeting, despite carrying *EGFR* copies below the cut-off used to define a gene amplification or high polysomy, raises the challenge of how to identify these patients and makes the discovery of predictive biomarkers mandatory. We

reasoned that if EGFR targeting affects the viability of sensitive models, this could be due to de-regulation of molecules involved in the EGFR pathway. Possible candidates were the EGFR ligands. Analysis of their expression showed that only AREG and EREG were correlated with sensitivity to EGFR targeting. Interestingly, EREG stabilizes different dimeric conformations of the EGFR extracellular region, inducing less stable EGFR dimers but eliciting more sustained EGFR signaling than seen with EGF (35). The analysis of the Asian Cancer Research Group (ACRG) cohort (20) showed that *AREG*/*EREG* overexpression did not reveal any prognostic value. On the contrary, the increased expression of at least one of the ligands (either *AREG* or *EREG*) in the tumors of patients enrolled in the COG trial and treated with gefitinib identified patients benefiting from anti-EGFR treatment. Notably, AREG and EREG are considered positive predictors of response to cetuximab/panitumumab in colorectal cancer (27-29) where they are highly co-expressed, both at the transcriptional and protein levels. Similarly, we showed that AREG and EREG increased expression was highly correlated in GEA as well. From a molecular point of view, it is likely that the continuous ligand-mediated EGFR stimulation renders tumor cells "addicted" to EGFR functional activation, driving the permanent activation of the RAS/MAPK pathway. Indeed, EGFR targeting lead to decreased MAPK and pS6 activation (Supplementary Figure S10) and the concomitant presence of *KRAS* mutations was associated with resistance.

Since a possible clinical use of EGFR targeting could be a combinatorial treatment, together with chemotherapeutic drugs, we evaluated the possible detrimental effect of the addition of chemotherapy to EGFR blocking drugs. Few years ago, Smyth et al. tested the effect of panitumumab addition to EOX (epirubicin, oxaliplatin and capecitabine) in *EGFR* amplified gastric cancer patients recruited in the REAL3 trial (8) and found no benefit (31). Furthermore, they investigated in one *EGFR* amplified patient-derived organoid the effect of the addition of epirubicin to cetuximab and observed a small increase in viability. In our work, we have tested the effect of the treatment of both sensitive and resistant primary cells with the same drugs, alone or in combination with cetuximab. Differently with what reported, we have never observed an impairment of chemotherapy when associated with cetuximab, meaning that EGFR targeting does not interfere with the antitumor effect of chemotherapeutic drugs. It would thus be clinically relevant to test the effectiveness of anti-EGFR drugs as single agents as an additional line of therapy.

Based on our results, we propose the existence of a subgroup of patients with GEA with susceptibility to EGFR inhibition driven by either cell autonomous *EGFR* genomic alterations, such as the previously and well described existence of *EGFR* amplification, or alternatively by paracrine and/or autocrine mechanisms, thus potentially leading to EGFR ligands AREG and/or EREG over-expression and EGFR functional activation as described here. Based on these results, a novel wave of EGFR targeting drugs including bispecific monoclonal antibodies and antibody-drug conjugates could be clinically investigated in adequately selected and stratified molecular subgroups of patients with GEA.

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REFERENCES

1. Janjigian YY, Kawazoe A, Yañez P, Li N, Lonardi S, Kolesnik O, *et al.* The KEYNOTE-811 trial of dual PD-1 and HER2 blockade in HER2-positive gastric cancer. Nature **2021**;600:727-30
2. Janjigian YY, Shitara K, Moehler M, Garrido M, Salman P, Shen L, *et al.* First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. Lancet **2021**;398:27-40
3. Nevo Y, Ferri L. Current management of gastric adenocarcinoma: a narrative review. J Gastrointest Oncol **2023**;14:1933-48
4. Birkman EM, Ålgars A, Lintunen M, Ristamäki R, Sundström J, Carpén O. EGFR gene amplification is relatively common and associates with outcome in intestinal adenocarcinoma of the stomach, gastro-oesophageal junction and distal oesophagus. BMC Cancer **2016**;16:406
5. Xie YH, Chen YX, Fang JY. Comprehensive review of targeted therapy for colorectal cancer. Signal Transduct Target Ther **2020**;5:22
6. Santos EDS, Nogueira KAB, Fernandes LCC, Martins JRP, Reis AVF, Neto JBV, *et al.* EGFR targeting for cancer therapy: Pharmacology and immunoconjugates with drugs and nanoparticles. Int J Pharm **2021**;592:120082
7. Lordick F, Kang YK, Chung HC, Salman P, Oh SC, Bodoky G, *et al.* Capecitabine and cisplatin with or without cetuximab for patients with previously untreated advanced gastric cancer (EXPAND): a randomised, open-label phase 3 trial. Lancet Oncol **2013**;14:490-9
8. Waddell T, Chau I, Cunningham D, Gonzalez D, Okines AF, Okines C, *et al.* Epirubicin, oxaliplatin, and capecitabine with or without panitumumab for patients with previously untreated advanced oesophagogastric cancer (REAL3): a randomised, open-label phase 3 trial. Lancet Oncol **2013**;14:481-9
9. Dutton SJ, Ferry DR, Blazeby JM, Abbas H, Dahle-Smith A, Mansoor W, *et al.* Gefitinib for oesophageal cancer progressing after chemotherapy (COG): a phase 3, multicentre, double-blind, placebo-controlled randomised trial. Lancet Oncol **2014**;15:894-904
10. Adua D, Di Fabio F, Rojas Llimpe FL, Pini S, Pinto C. Long-term survival in an advanced gastric cancer patient treated with cetuximab in association with FOLFIRI: a case report. J Gastrointest Oncol **2014**;5:E13-7
11. Pinto C, Di Fabio F, Barone C, Siena S, Falcone A, Cascinu S, *et al.* Phase II study of cetuximab in combination with cisplatin and docetaxel in patients with untreated advanced gastric or gastro-oesophageal junction adenocarcinoma (DOCETUX study). Br J Cancer **2009**;101:1261-8

12. Lordick F, Luber B, Lorenzen S, Hegewisch-Becker S, Folprecht G, Wöll E, *et al.* Cetuximab plus oxaliplatin/leucovorin/5-fluorouracil in first-line metastatic gastric cancer: a phase II study of the Arbeitsgemeinschaft Internistische Onkologie (AIO). Br J Cancer **2010**;102:500-5

13. Catenacci DVT, Moya S, Lomnicki S, Chase LM, Peterson BF, Reizine N, *et al.* Personalized Antibodies for Gastroesophageal Adenocarcinoma (PANGEA): A Phase II Study Evaluating an Individualized Treatment Strategy for Metastatic Disease. Cancer Discov **2021**;11:308-25

14. Corso S, Pietrantonio F, Apicella M, Migliore C, Conticelli D, Petrelli A, *et al.* Optimized EGFR blockade strategies in EGFR addicted gastroesophageal adenocarcinomas. Clin Cancer Res **2021**;27:3126-40

15. Maron SB, Moya S, Morano F, Emmett MJ, Chou JF, Sabwa S, *et al.* Epidermal Growth Factor Receptor Inhibition in Epidermal Growth Factor Receptor-Amplified Gastroesophageal Cancer: Retrospective Global Experience. J Clin Oncol **2022**;40:2458-67

16. Corso S, Isella C, Bellomo SE, Apicella M, Durando S, Migliore C, *et al.* A Comprehensive PDX Gastric Cancer Collection Captures Cancer Cell-Intrinsic Transcriptional MSI Traits. Cancer Res **2019**;79:5884-96

17. Sigismund S, Algisi V, Nappo G, Conte A, Pascolutti R, Cuomo A, *et al.* Threshold-controlled ubiquitination of the EGFR directs receptor fate. EMBO J **2013**;32:2140-57

18. Clark NA, Hafner M, Kouril M, Williams EH, Muhlich JL, Pilarczyk M, *et al.* GRcalculator: an online tool for calculating and mining dose-response data. BMC Cancer **2017**;17:698

19. Vigna E, Naldini L. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J Gene Med **2000**;2:308-16

20. Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, *et al.* Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med **2015**;21:449-56

21. Conte N, Mason JC, Halmagyi C, Neuhauser S, Mosaku A, Yordanova G, *et al.* PDX Finder: A portal for patient-derived tumor xenograft model discovery. Nucleic Acids Res **2019**;47:D1073-D9

22. Ughetto S, Migliore C, Pietrantonio F, Apicella M, Petrelli A, D'Errico L, *et al.* Personalized therapeutic strategies in HER2-driven gastric cancer. Gastric Cancer **2021**;24:897-912

23. Petrelli A, Rizzolio S, Pietrantonio F, Bellomo SE, Benelli M, De Cecco L, *et al.* BRCA2 Germline Mutations Identify Gastric Cancers Responsive to PARP Inhibitors. Cancer Res **2023**;83:1699-710

24. Petty RD, Dahle-Smith A, Stevenson DAJ, Osborne A, Massie D, Clark C, *et al.* Gefitinib and EGFR Gene Copy Number Aberrations in Esophageal Cancer. J Clin Oncol **2017**;35:2279-87

25. Network CGAR. Comprehensive molecular characterization of gastric adenocarcinoma. Nature **2014**;513:202-9

26. Ayati A, Moghimi S, Salarinejad S, Safavi M, Pouramiri B, Foroumadi A. A review on progression of epidermal growth factor receptor (EGFR) inhibitors as an efficient approach in cancer targeted therapy. Bioorg Chem **2020**;99:103811

27. Williams CJM, Elliott F, Sapanara N, Aghaei F, Zhang L, Muranyi A, *et al.* Associations between AI-Assisted Tumor Amphiregulin and Epiregulin IHC and Outcomes from Anti-EGFR Therapy in the Routine Management of Metastatic Colorectal Cancer. Clin Cancer Res **2023**;29:4153-65

28. Jacobs B, De Roock W, Piessevaux H, Van Oirbeek R, Biesmans B, De Schutter J, *et al.* Amphiregulin and epiregulin mRNA expression in primary tumors predicts outcome in metastatic colorectal cancer treated with cetuximab. J Clin Oncol **2009**;27:5068-74

29. Seligmann JF, Elliott F, Richman SD, Jacobs B, Hemmings G, Brown S, *et al.* Combined Epiregulin and Amphiregulin Expression Levels as a Predictive Biomarker for Panitumumab Therapy Benefit or Lack of Benefit in Patients With RAS Wild-Type Advanced Colorectal Cancer. JAMA Oncol **2016**;2:633-42

30. Li R, Liang M, Liang X, Yang L, Su M, Lai KP. Chemotherapeutic Effectiveness of Combining Cetuximab for Metastatic Colorectal Cancer Treatment: A System Review and Meta-Analysis. Front Oncol **2020**;10:868

31. Smyth EC, Vlachogiannis G, Hedayat S, Harbery A, Hulkki-Wilson S, Salati M, *et al.* EGFR amplification and outcome in a randomised phase III trial of chemotherapy alone or chemotherapy plus panitumumab for advanced gastro-oesophageal cancers. Gut **2021**;70:1632-41

32. Pinto C, Di Fabio F, Siena S, Cascinu S, Rojas Llimpe FL, Ceccarelli C, *et al.* Phase II study of cetuximab in combination with FOLFIRI in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma (FOLCETUX study). Ann Oncol **2007**;18:510-7

33. Narahara H, Iishi H, Imamura H, Tsuburaya A, Chin K, Imamoto H, *et al.* Randomized phase III study comparing the efficacy and safety of irinotecan plus S-1 with S-1 alone as first-line treatment for advanced gastric cancer (study GC0301/TOP-002). Gastric Cancer **2011**;14:72-80

34. Meemanage M, Spender LC, Collinson D, Iannetta J, Challapalli P, Turbitt J, *et al.* Interactions between anti-EGFR therapies and cytotoxic chemotherapy in oesophageal squamous cell carcinoma: why clinical trials might have failed and how they could succeed. Cancer Chemother Pharmacol **2021**;87:361-77

35. Freed DM, Bessman NJ, Kiyatkin A, Salazar-Cavazos E, Byrne PO, Moore JO, *et al.* EGFR Ligands Differentially Stabilize Receptor Dimers to Specify Signaling Kinetics. Cell **2017**;171:683-95.e18

Table 1. Clinical Features of the Patients Evaluable for AREG and EREG Expression

Age at assignment, years, mean (SD)	64.3 (10.3)
Sex, No. (%)	
Male	60 (85.7)
Female	10 (14.3)
Original diagnosis, No. (%)	
Adenocarcinoma	70 (100)
Squamous	0 (0)
Undifferentiated	0 (0)
Disease site, No. (%)	
Esophageal	47 (67.1)
Type I junctional	10 (14.3)
Type II junctional	13 (18.6)
Performance Status, No. (%)	
0	19 (27.1)
1	39 (55.7)
2	12 (17.1)
Previous treatments, No. (%)	
0	0 (0)
1	46 (65.7)
2	23 (32.9)
3	1 (1.4)
BMI, (kg/m^2), mean, SD, No.	25.4, 4.22, 68
BMI grouped, No. (%)	
<18.0	2
18.1-24.9	34
25.0-29.9	22
>= 30	10
Missing	2

Abbreviations: BMI, body mass index; COG, Cancer Esophagus Gefitinib; AREG, Amphiregulin; EREG, Epiregulin; SD, Standard Deviation.

Figure Legends

Fig.1. Workflow of the experimental plan.

Collection of GEA samples, subcutaneous implant in not immunocompetent mice, generation of *in vitro* material, evaluation of *in vitro* response to EGFR targeted drugs, *in vivo* validation. (Created in BioRender. Corso, S. (2025) https://BioRender.com/ejv17qx).

Fig. 2. A subset of gastroesophageal tumors not displaying *EGFR* gene amplification are sensitive to anti-EGFR drugs.

(A) Heatmap showing the viability (in percentage *vs* untreated) of 27 primary cell lines treated for 6 days with the indicated doses of the monoclonal antibody cetuximab. The heatmap represents the average of three independent experiments. **(B)** Boxplot showing the sensitivity of the 27 primary cells (divided in resistant and sensitive on the basis of the response to cetuximab) to the indicated drugs. Each dot represents the mean AUC (Area Under the Curve) computed by the mean of three independent experiments. Statistical significance was calculated by Student's t-test. (* $p<0.05$, **** $p<0.0001$). The labelled dot indicates a HER2 amplified cell line (GTR0233). **(C)** Boxplots showing the percentage of not viable cells (positive for trypan blue staining), not treated (-) or treated with cetuximab (+, 5µg/ml for 6 days). The average of four independent experiments performed on three cetuximab sensitive (GTR0539, GTR0640 and GTR0247) and three resistant cell lines (GTR0125, GTR0498 and GTR0687) is shown. Statistical significance was calculated using Ratio Paired t-test. (* $p<0.05$, ** $p<0.01$).

Fig. 3. Preclinical trials confirm the sensitivity to cetuximab of a subset of GEA PDXs.

Tumor growth curves of the PDX cohorts derived from five responsive and five resistant models according to *in vitro* data. After reaching an average tumor volume of 220-250 mm^3, PDXs were randomized and treated with either placebo (vehicle, pale blue or pale red lines) or cetuximab (20 mg/Kg, twice weekly ip; dark blue or dark red lines). Lines represent average tumor volume + st. dev. N=5-7 animals/group. The response has been evaluated using RECIST 1.1-like criteria: progressive disease (PD): ≥35% increase from baseline; partial response (PR): ≥50% reduction from baseline; stable disease (SD): intermediate variations from baseline. The clinical response of each PDX cohort is indicated. Arrow = treatment start. Statistical significance was calculated using the Two-way ANOVA with Bonferroni correction. At the end of the trial (GTR0187, GTR0247) or at the time of mice sacrifice (GTR0539, GTR0558 and GTR0640) the cetuximab arm was compared with the vehicle arm. (**** $p<0.0001$).

Fig. 4. Cetuximab sensitive tumors overexpress the EGFR ligands *AREG* or *EREG* and rely on their expression for viability.

(A-B) Dot plots depicting *AREG* and *EREG* mRNA expression levels in cetuximab resistant *vs* sensitive PDX models. Statistical significance was calculated using unpaired t-test. * $p<0.05$, ** $p<0.01$**. (C)** Heatmap representing *AREG* and

EREG relative mRNA expression in cetuximab resistant and sensitive PDX models. **(D)** Stacked barplot showing dichotomized analysis of cetuximab response groups in PDX models according to *AREG*/*EREG* expression. Low: both ligands in first-third quartiles; High: at least one ligand in the fourth quartile. Statistical significance was calculated using the Chi-square test. *p<0.05. **(E-H)** *AREG* and *EREG* expression was silenced in cetuximab sensitive (GTR0539 -panel E- and GTR0640 -panel F) and resistant (GTR0498 -panel G- and GTR0687 -panel H) cells. Silenced and control cells were treated with the indicated doses of cetuximab for 6 days and viability was tested. The bar plots show the average of three independent experiments. Statistical significance was calculated using the Two-way ANOVA with Turkey correction. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Fig. 5. Cetuximab sensitive tumors overexpress HER3 protein and are responsive to HER3 targeting.

(A) Western blot analysis of HER3 expression in 18 primary GEA cell lines. Vinculin was used as loading control. Blue cell lines: sensitive cells; red cell lines: resistant cells. **(B)** Box plot showing the quantification of the intensity of HER3 signal normalized to vinculin in cetuximab sensitive *vs* resistant cells. Statistical significance was calculated using Student's t-test (**p<0.01). **(C-D)** Barplots showing the viability of cetuximab sensitive/HER3 over-expressing cells (GTR0539 and GTR0640, C) and cetuximab resistant/HER3 low-expressing cells (GTR0498, GTR0125, GTR0687, D) treated with 10µg/ml and 20µg/ml of the anti-HER3 patritumab deruxtecan U3-1402 for 6 days. The bar plots show the average of three independent experiments. Statistical significance was calculated using the Two-way ANOVA with Dunnet correction. **(E-F)** Barplots showing the viability of cetuximab sensitive cells treated with cetuximab (CTX), U3-1402 (U3) or their combination (CTX+U3) for 6 days. Drugs were used at concentrations near the IC50: 1.25µg/ml CTX and 10µg/ml for GTR0539 and 2.5µg/ml and 20µg/ml for GTR0640. The barplots show the average of three independent experiments. Statistical significance was calculated using the One-way ANOVA with Turkey correction. NT: not treated.

Fig. 6. Cetuximab treatment does not impair the efficacy of chemotherapeutic treatments in cetuximab sensitive cells.

Dotplots showing the AUC of GTR0539, GTR0640 (sensitive), GTR0498 and GTR0687 (resistant) cells treated with epirubicin (EPI) **(A)**, oxaliplatin (OXA) **(B)**, SN38 **(C)** and 5-FU **(D)**, alone or in combination (combo) with cetuximab (CTX). Three independent experiments were performed. The horizontal line represents the mean value of the three experiments. Statistical significance was calculated using the One-way ANOVA with Turkey correction. (ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Fig. 7. Overexpression of either AREG or EREG identifies patients responsive to EGFR targeting.

(A) Retrospective analysis of the clinical response to gefinitib of COG patients. Response was dichotomized for disease control or progressive disease. **(B)** *AREG* and *EREG* expression was quantified by RNA in situ hybridization in 67

tumor samples from gefitinib-treated patients enrolled in the COG clinical trial. The expression of the ligands was divided into quartiles. Low: first-third quartiles; High: at least one ligand in the fourth quartile. Kaplan Meier showing the Overall Survival probability in patients either low or high ligand expression. Statistical evaluation was performed with Log-rank (Mantel-Cox) test. **(C)** Stacked barplot showing dichotomized analysis of disease control in COG patients according to AREG/EREG expression. Low: both ligands in first-third quartiles; High: at least one ligand in the fourth quartile. Statistical significance was calculated using the Chi-square test. *$p<0.05$. **(D)** Forest plot of Multivariate analyses were performed by Cox proportional hazard model.

FIGURE 1

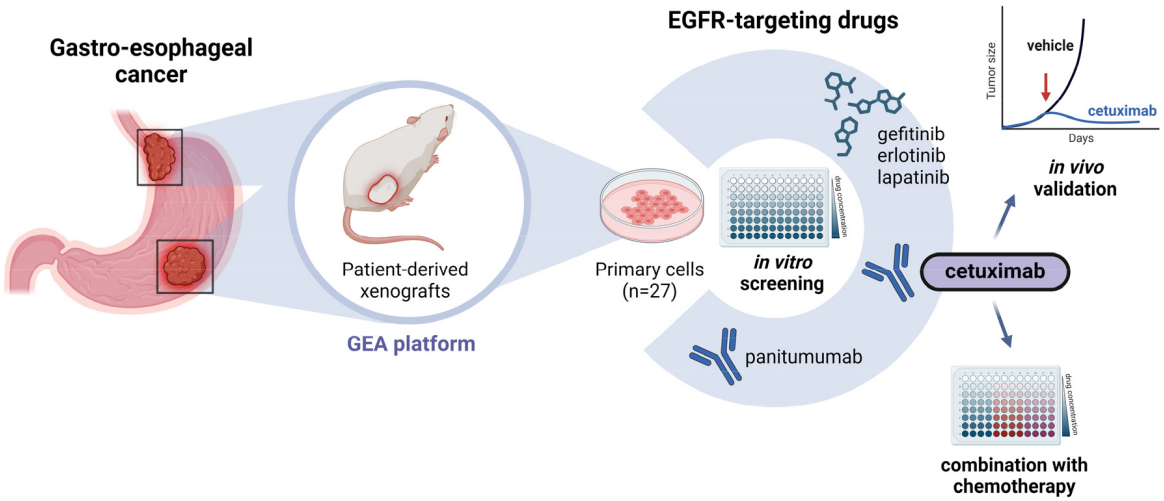
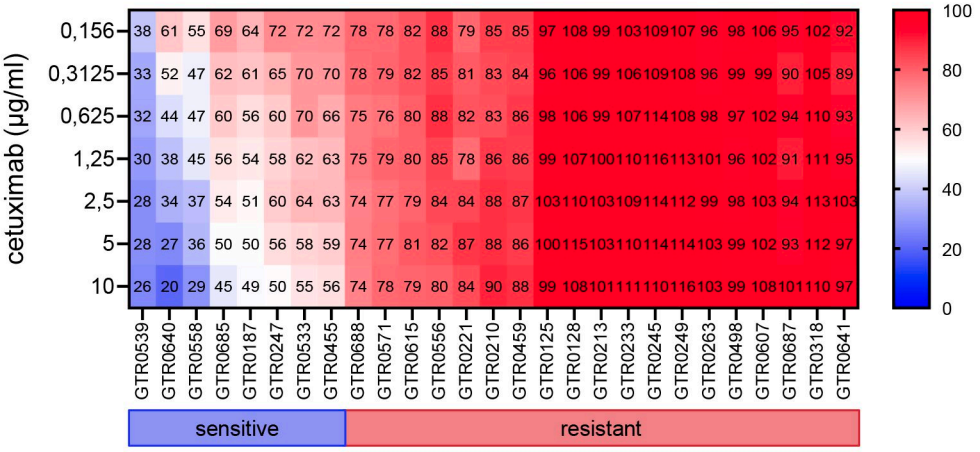
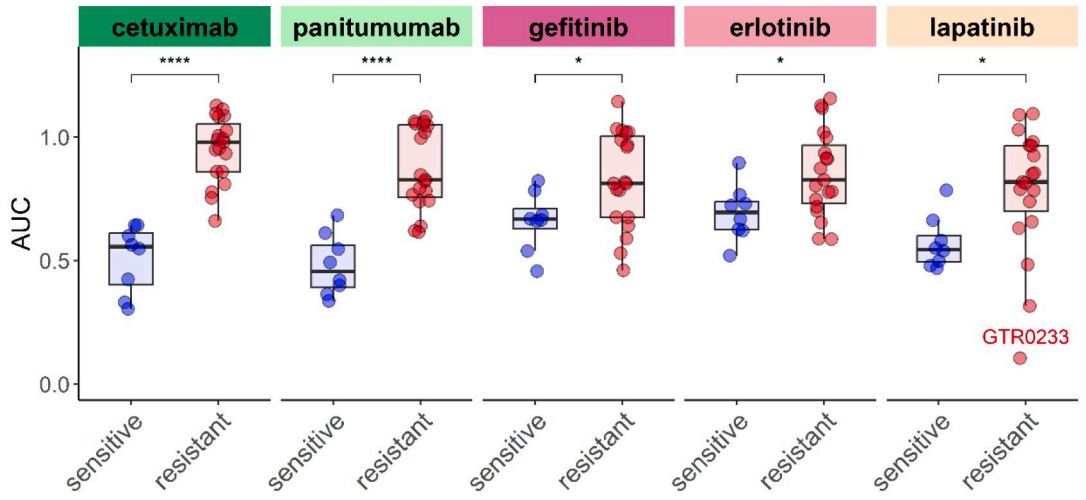

Gastro-esophageal cancer
Patient-derived xenografts
GEA platform
EGFR-targeting drugs
gefitinib
erlotinib
lapatinib
Primary cells (n=27)
in vitro screening
drug concentration
panitumumab
cetuximab
Tumor size
vehicle
cetuximab
Days
in vivo validation
drug concentration
combination with chemotherapy

FIGURE 2

A


cetuximab (µg/ml)
0,156
0,3125
0,625
1,25
2,5
5
10
38 61 55 69 64 72 72 72 78 78 82 88 79 85 85 97 108 99 103 109 107 96 98 106 95 102 92
33 52 47 62 61 65 70 70 78 79 82 85 81 83 84 96 106 99 106 109 108 96 99 99 90 105 89
32 44 47 60 56 60 70 66 75 76 80 88 82 83 86 98 106 99 107 114 108 98 97 102 94 110 93
30 38 45 56 54 58 62 63 75 79 80 85 78 86 86 99 107 100 110 116 113 101 96 102 91 111 95
28 34 37 54 51 60 64 63 74 77 79 84 84 88 87 103 110 103 109 114 112 99 98 103 94 113 103
28 27 36 50 50 56 58 59 74 77 81 82 87 88 86 100 115 103 110 114 114 103 99 102 93 112 97
26 20 29 45 49 50 55 56 74 78 79 80 84 90 88 99 108 101 111 110 116 103 99 108 101 110 97
100
80
60
40
20
0
GTR0539 GTR0640 GTR0558 GTR0685 GTR0187 GTR0247 GTR0533 GTR0455 GTR0688 GTR0571 GTR0615 GTR0556 GTR0221 GTR0210 GTR0459 GTR0125 GTR0128 GTR0213 GTR0233 GTR0245 GTR0249 GTR0263 GTR0498 GTR0607 GTR0687 GTR0318 GTR0641
sensitive
resistant

B


cetuximab
panitumumab
gefitinib
erlotinib
lapatinib
****
****
*
*
*
AUC
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0.5
0.0
GTR0233
sensitive
resistant
sensitive
resistant
sensitive
resistant
sensitive
resistant
sensitive
resistant

C

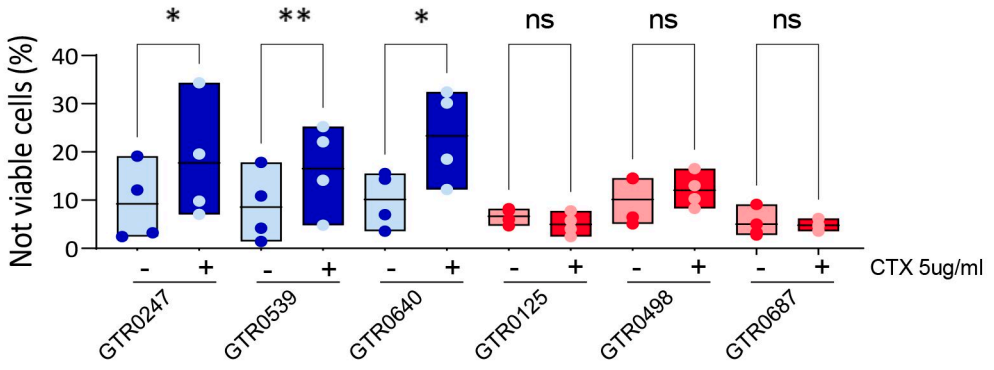

*
**
*
ns
ns
ns
Not viable cells (%)
40
30
20
10
0
- + - + - + - + - + - +
CTX 5ug/ml
GTR0247
GTR0539
GTR0640
GTR0125
GTR0498
GTR0687

FIGURE 3

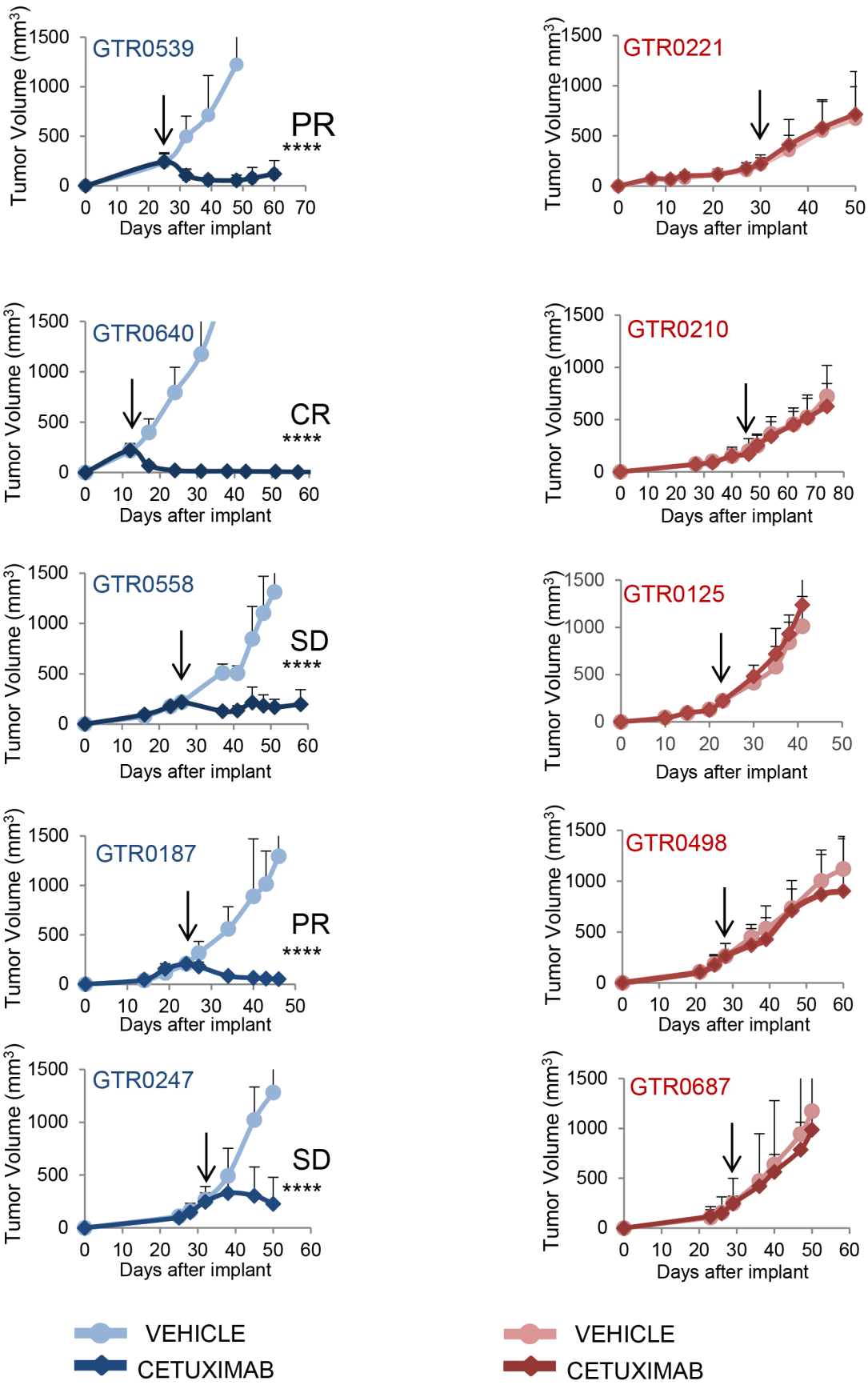
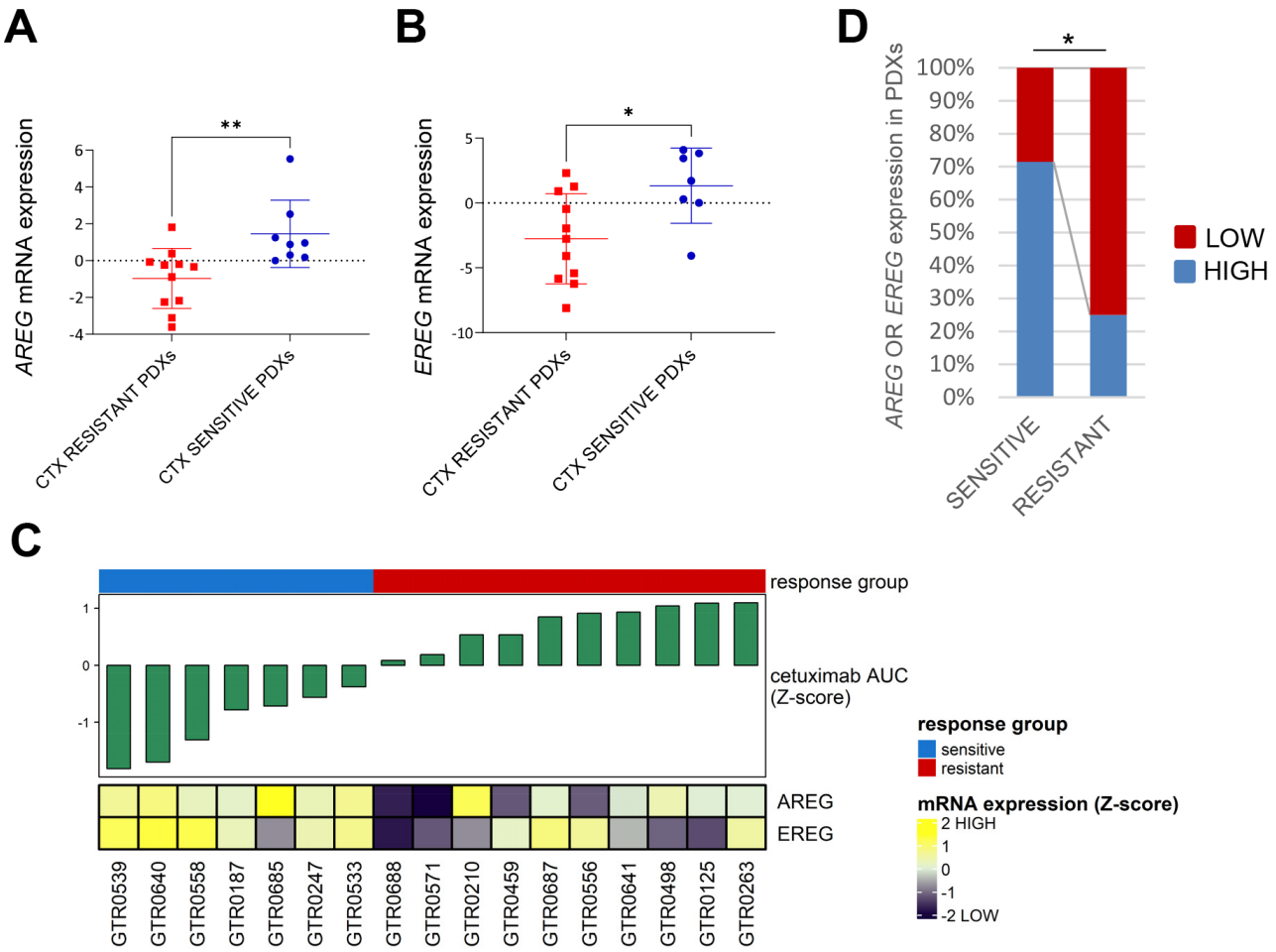
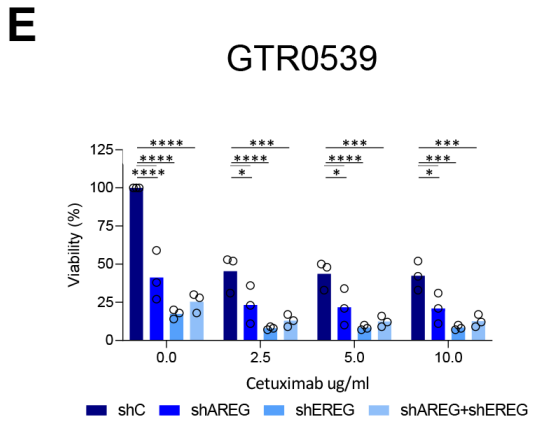
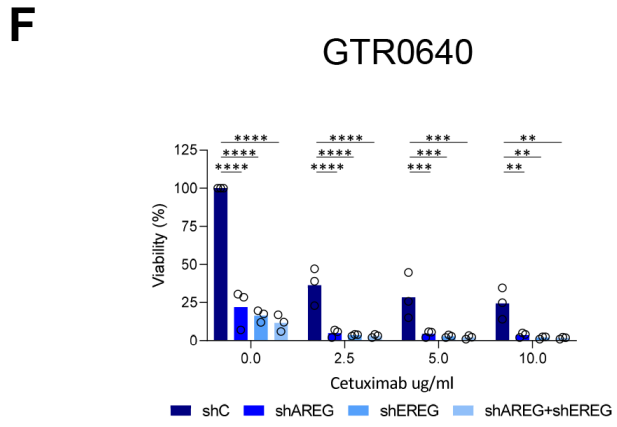
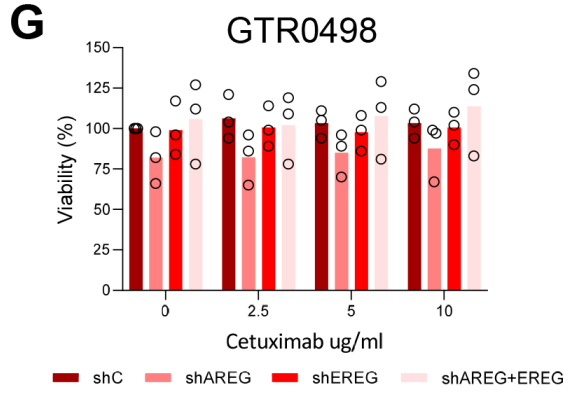

GTR0539
PR
****
GTR0221
GTR0640
CR
****
GTR0210
GTR0558
SD
****
GTR0125
GTR0187
PR
****
GTR0498
GTR0247
SD
****
GTR0687
Tumor Volume (mm3)
Days after implant
VEHICLE
CETUXIMAB
VEHICLE
CETUXIMAB

FIGURE 4


A
AREG mRNA expression
**
CTX RESISTANT PDXs
CTX SENSITIVE PDXs
B
EREG mRNA expression
*
CTX RESISTANT PDXs
CTX SENSITIVE PDXs
D
AREG OR EREG expression in PDXs
*
LOW
HIGH
SENSITIVE
RESISTANT
C
response group
cetuximab AUC (Z-score)
AREG
EREG
GTR0539
GTR0640
GTR0558
GTR0187
GTR0685
GTR0247
GTR0533
GTR0688
GTR0571
GTR0210
GTR0459
GTR0687
GTR0556
GTR0641
GTR0498
GTR0125
GTR0263
response group
sensitive
resistant
mRNA expression (Z-score)
2 HIGH
1
0
-1
-2 LOW


E
GTR0539
Viability (%)
Cetuximab ug/ml
shC
shAREG
shEREG
shAREG+shEREG


F
GTR0640
Viability (%)
Cetuximab ug/ml
shC
shAREG
shEREG
shAREG+shEREG


G
GTR0498
Viability (%)
Cetuximab ug/ml
shC
shAREG
shEREG
shAREG+EREG

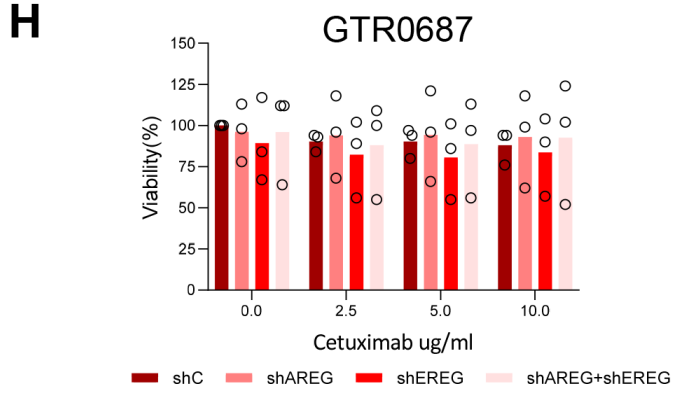

H
GTR0687
Viability(%)
Cetuximab ug/ml
shC
shAREG
shEREG
shAREG+shEREG

FIGURE 5

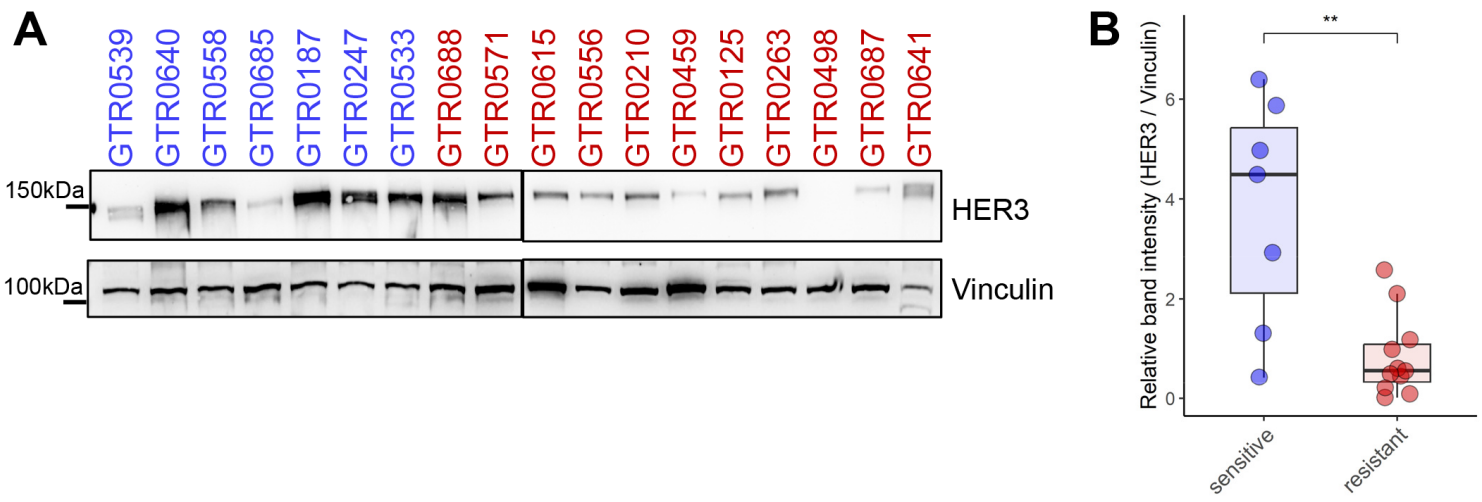

A
GTR0539
GTR0640
GTR0558
GTR0685
GTR0187
GTR0247
GTR0533
GTR0688
GTR0571
GTR0615
GTR0556
GTR0210
GTR0459
GTR0125
GTR0263
GTR0498
GTR0687
GTR0641
150kDa
HER3
100kDa
Vinculin
B
Relative band intensity (HER3 / Vinculin)
sensitive
resistant
C
Viability(%)
U3-1402 (µg/ml)
GTR0539
GTR0640
D
Viability (%)
GTR0125
GTR0498
GTR0687
E
GTR0539
Viability (%)
NT
U3
CTX
U3+CTX
F
GTR0640

FIGURE 6

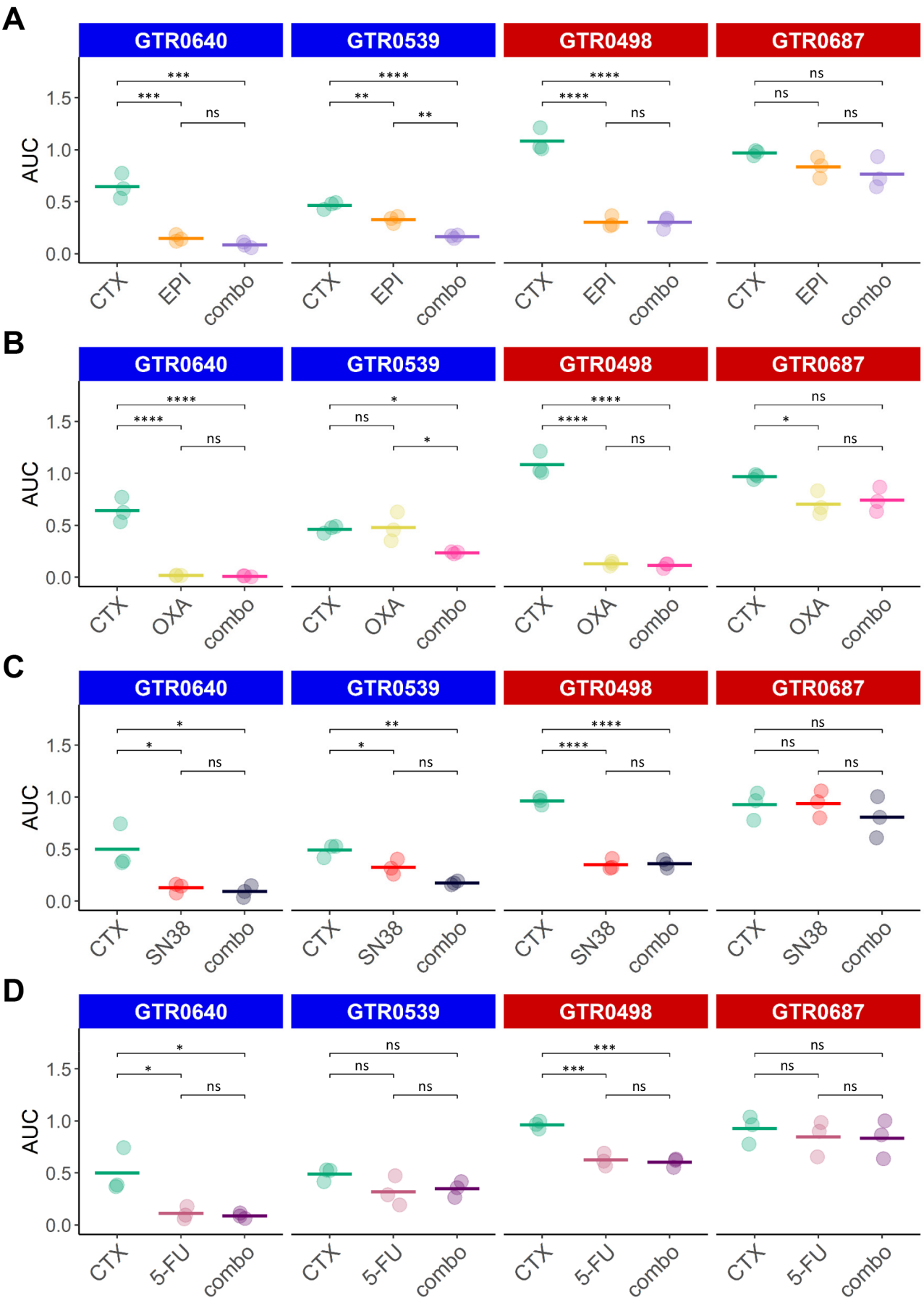
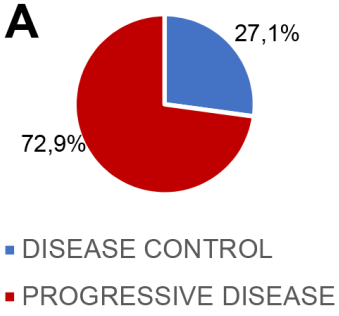
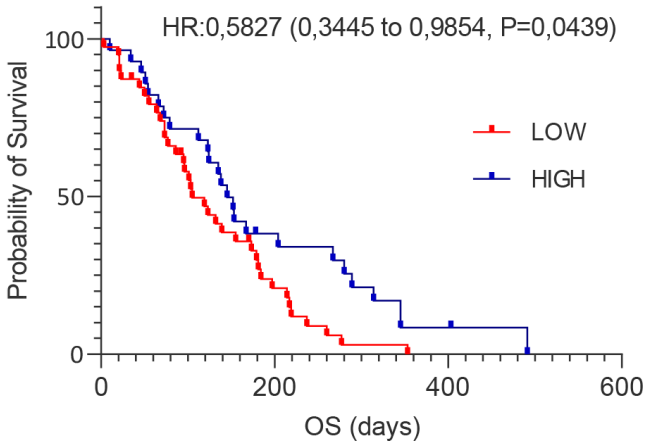

A
B
C
D
GTR0640
GTR0539
GTR0498
GTR0687
AUC
0.0
0.5
1.0
1.5
CTX
EPI
OXA
SN38
5-FU
combo
ns

FIGURE 7

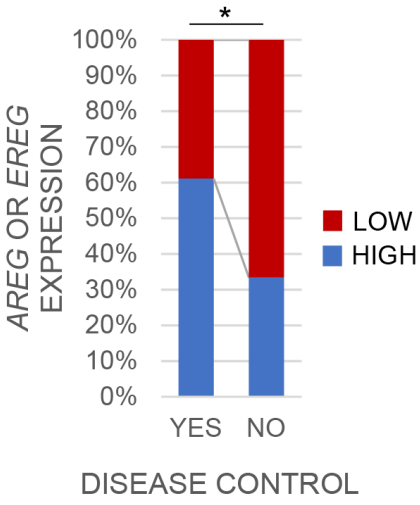
A



B



C



D

Variable		N	Hazard ratio	p
Gender	Male	57	Reference	
	Female	9	1.30 (0.55, 3.05)	0.55
Age	< 64.7	33	Reference	
	≥ 64.7	33	1.02 (0.54, 1.93)	0.95
BMI	< 24.9	33	Reference	
	≥ 24.9	33	1.50 (0.83, 2.71)	0.18
WHO	0	17	Reference	
	1	37	1.53 (0.74, 3.16)	0.25
	2	12	13.12 (4.65, 37.05)	<0.001
EGFR fish status	Disomy	28	Reference	
	Trisomy	20	0.84 (0.44, 1.60)	0.60
	Low polysomy	18	0.92 (0.45, 1.86)	0.81
KRAS	wt	62	Reference	
	mut	4	3.56 (1.15, 11.05)	0.03
PIK3CA	wt	64	Reference	
	mut	2	1.85 (0.39, 8.71)	0.44