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Optimized EGFR blockade strategies in *EGFR* addicted gastroesophageal adenocarcinomas

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Abbreviations:

COG: Cancer Oesophagus Gefitinib (Gefitinib for oesophageal cancer progressing after chemotherapy (COG): a phase 3, multicentre, double-blind, placebo-controlled randomised trial)

FMI: Foundation Medicine, Inc. (Cambridge, Massachusetts, USA)

GEA: Gastric and gastroesophageal adenocarcinoma

INT: Istituto Nazionale Tumori (Milan, Italy)

IRCC: Istituto per la Ricerca e la Cura del Cancro (Candiolo, Torino, Italy)

PDX: Patient-derived xenograft

TRANSLATIONAL RELEVANCE

Prior clinical trials performed in unselected gastroesophageal adenocarcinoma patients failed to show survival improvement upon treatment with anti-EGFR therapies. We report the clinical activity of EGFR monoclonal antibodies in patients bearing high level (>8 copies) of *EGFR* gene amplification and show that in Patient-derived xenografts the combination of an EGFR mAb and a tyrosine kinase inhibitor is significantly more effective and long lasting than mAb monotherapy. We also identify mTOR pathway activation as a novel mechanism of resistance to EGFR targeted therapy and show that it can be overcome by the combination of EGFR/mTOR inhibitors. These findings recognize EGFR as an actionable target in a small but significant subgroup of patients bearing *EGFR* amplification and suggest the combination of a EGFR mAb and a TKI as the most effective treatment.

ABSTRACT

Purpose: Gastric and gastroesophageal adenocarcinomas (GEA) represent the third leading cause of cancer mortality worldwide. Despite significant therapeutic improvement, the outcome of patients with advanced GEA is poor. Randomized clinical trials failed to show a significant survival benefit in molecularly unselected patients with advanced GEA treated with anti-EGFR agents.

Experimental Design: We performed analyses on 4 cohorts: IRCC (570 patients), FMI (9397 patients), COG (214 patients) and INT (206 patients). Preclinical trials were conducted in patient-derived xenografts (PDXs).

Results: The analysis of different GEA patient cohorts suggests that *EGFR* amplification drives aggressive behaviour and poor prognosis. We also observed that EGFR inhibitors are active in patients with *EGFR* copy number gain and that co-amplification of other receptor tyrosine kinases or *KRAS* is associated with worse response. Pre-clinical trials performed on *EGFR*-amplified GEA PDX models revealed that the combination of an EGFR monoclonal antibody and an EGFR tyrosine kinase inhibitor was more effective than each monotherapy and resulted in a deeper and durable response. In a highly *EGFR* amplified non-responding PDX, where resistance to EGFR drugs was due to inactivation of the TSC2 tumor suppressor, co-treatment with the mTOR inhibitor everolimus restored sensitivity to EGFR inhibition.

Conclusions: This study underscores EGFR as a potential therapeutic target in gastric cancer and identifies the combination of an EGFR TKI and a monoclonal antibody as an effective therapeutic approach. Finally, it recognizes mTOR pathway activation as a novel mechanism of primary resistance that can be overcome by the combination of EGFR and mTOR inhibitors.

Keywords: EGFR; targeted therapy; gastric and gastroesophageal cancer; drug resistance; mTOR

INTRODUCTION

Gastric and gastroesophageal adenocarcinomas (GEA) represent the third leading cause of cancer-related deaths worldwide. Despite the introduction of novel systemic treatment options, the outcome of patients with metastatic gastroesophageal adenocarcinoma (mGEA) is still extremely unsatisfactory, with median overall survival (OS) of less than 12 months in most clinical trials (1).

While the identification of specific molecular subtypes has had profound implications for targeted strategies in other malignancies, the same progress has only been partially realized for patients with mGEA. Trastuzumab and ramucirumab (targeting HER2 and VEGFR2, respectively) are the only approved targeted agents in mGEA (2,3), whereas the promising role of immune checkpoint inhibitors (ICIs) such as pembrolizumab and nivolumab still needs to be confirmed by randomized clinical trials (RCTs) performed in properly selected patient subgroups.

The molecular landscape of GEA has been extensively described and the two main molecular classifications (4,5) identified a disease subtype characterized by chromosomal instability and amplification of receptor tyrosine kinases (RTKs). Epidermal growth factor receptor (*EGFR*) amplification has been reported in 3-5% of GEAs (4,6), while other genetic alterations (such as point mutations or translocations) are extremely uncommon. Several EGFR targeting drugs, comprising monoclonal antibodies (MoAbs) and tyrosine kinase inhibitors (TKIs), have been approved for the treatment of multiple tumor types, including *RAS* wild-type metastatic colorectal cancer, head and neck squamous cell carcinoma and *EGFR* mutated advanced non-small cell lung cancer (7). Conversely, three phase III Randomized Clinical trials evaluating the addition of cetuximab, panitumumab or gefitinib to the standard of care in molecularly unselected patients with advanced gastric or esophageal adenocarcinomas reported negative results (8-10). On the other hand, intriguingly, experimental data obtained in GEA preclinical models showed a positive correlation between cetuximab response and high EGFR expression/amplification (11). Consistent with these preclinical findings, the association between *EGFR* copy number gain (CNG) and better

OS has been shown by a phase II trial of cetuximab plus FOLFOX chemotherapy in patients with mGEA (12). In addition, a pre-specified subgroup analysis of the COG trial showed that patients with esophageal and gastroesophageal junction carcinomas bearing *EGFR* CNG derived a significant progression-free survival (PFS), OS and Health-Related Quality of Life (HRQoL) benefit from gefitinib compared to placebo, thereby providing the proof-of-concept for *EGFR* CNG as a predictive biomarker of efficacy of EGFR targeted agents (13).

Here we aimed to investigate the efficacy of several EGFR inhibition strategies in preclinical models of *EGFR* amplified GEAs, to describe the clinical and molecular features of patients with *EGFR* amplified tumors and their responsiveness to EGFR inhibition, and to extensively investigate common and potentially novel genomic mechanisms of resistance, with the ultimate goal to optimize EGFR targeted combinations for the development of future clinical trials.

MATERIALS AND METHODS

Patients

IRCC: Tumor samples (from gastric and gastroesophageal junction adenocarcinomas) and matched normal samples were obtained from patients undergoing surgery in 15 Italian Hospitals: Candiolo Cancer institute- FPO, IRCCS (Torino); Ordine Mauriziano Hospital; San Giovanni Battista Hospital (Torino); San Luigi Gonzaga Hospital, Orbassano (Torino); Humanitas-IRCCS, Rozzano, Milano; San Raffaele Hospital (Milano); Treviglio-Caravaggio Hospital (Bergamo); Brescia Hospital; Borgo-Trento Hospital (Verona); Santa Maria delle Scotte Hospital (Siena); Forli' Hospital; Fondazione Macchi Hospital (Varese); Pisa Hospital; Fondazione IRCCS Istituto Nazionale dei Tumori (Milano); Ospedale Niguarda Ca' Granda (Milano). All patients provided written informed consent; samples were collected and the study was conducted under the approval of the Review Boards of all the Institutions. The study was done in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonization and Good Clinical Practice guidelines and GDPR (General Data Protection Regulation). Clinical and pathologic data were entered and maintained in our prospective database. All the samples have been anonymized before being shipped to Candiolo. No reference to the patients can be inferred from the histological and molecular characterization presented in the work.

FMI: Tumor samples from patients with GEA were submitted during routine clinical care for CGP. Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act waiver of authorization, was obtained from the Western Institutional Review Board (protocol no. 20152817).

Cell lines and drugs

293T cells were obtained from ATCC, OE21 from Sigma Aldrich (Saint Luis, MI, USA). The genetic identity of the cell lines was confirmed by short tandem repeat profiling (Cell ID, Promega,

Madison, WI, USA). Erlotinib and everolimus were purchased from Carbosynth (UK). Cetuximab and lapatinib were provided by the Hospital Pharmacy.

Primary cell cultures and organoids

GEA primary cells were derived from PDXs as described in(14), while GEA primary organoids were obtained as described in (15). The genetic identity of the *in vitro*-derived material with the original tumor has been verified by short tandem repeat profiling (Cell ID, Promega). GTR0078 cells were used for the *in vitro* experiments soon after tumor dissociation, as they do not permanently grow in culture.

Western blot analysis and immunoprecipitation

Cells/organoids were treated with the indicated drugs: lapatinib or erlotinib 100 nM for 2 hours; cetuximab 0.5µg/ml for 16 hours. Whole-protein extracts were prepared using Laemmli buffer and quantified using the BCA Protein Assay kit (Pierce, Rockford, lL, USA). EGFR immunoprecipitation was performed with cetuximab on organoids (stimulated with EGF 100ng/ml for 15', treated or not with erlotinib 100nM for 2h) previously washed out from matrigel with Cell Recovery Solution (#354253, Corning, Glendale, AZ, USA) and lysed with EB (Triton 1%, Tris-HCl pH7.4 20 mM, EDTA pH8 5mM, Glycerol 10%, NaCl 150mM). Primary antibodies: anti-EGFR (1005:sc-03) and anti-Actin were from Santa Cruz Biotechnology (Inc., Dallas, Texas, USA), antibodies against phosphorylated EGFR (Tyr 845), ERK (Thr202/Tyr204), phosphorylated AKT (Ser473) (Clone D9E), total AKT, and ERK were from Cell Signaling (MA, USA). Antibody against phosphorylated EGFR (Tyr1068) (ab5644) was from Abcam (Cambridge, UK). Antibody directed against aa 1172–1186 of human EGFR was described in (16). Antibody anti-EGFR extracellular epitope (111.6 antibody) was from Thermo Fisher Scientific (MA, USA). Secondary antibodies were from Amersham. Detection was performed with ECL system (Amersham, UK).

Transfection and transduction procedures

OE21 cells were transfected with siRNAs using Lipofectamine2000 (Thermo Fisher Scientific). Transfection reagents plus siRNAs at final concentration of 20nM were used following standard

protocols. Seventy two hours after transfection, cells were lysed and WB performed. *TSC2* silencing was achieved using SMARTpool ON-TARGETplus siRNA (Dharmacon, Lafayette, CO, USA).

Lentiviruses were produced as described in (17). OE21 cells were transduced with a pool of lentiviral particles containing of four *TSC2* silencing shRNAs (Sigma, #40179, #40178, #40454, #40455). Cells were selected with puromicin, checked for TSC2 silencing and subcutaneously injected in NOD/SCID mice ($5*10^{\wedge 6}$ cells/mouse) in SF medium: Matrigel (Corning) 1:1.

Analyte extraction

Genomic DNA was isolated using the Blood & Cell Culture DNA Midi Kit (Qiagen, Germany). DNA concentrations were quantified using the Qubit Fluorometer (Thermo Fisher Scientific).

CNV Evaluation by qReal Time PCR

Quantitative PCR experiments for estimation of *EGFR, MET, FGFR2 and KRAS* copy number variations were performed in triplicates using 2ng total gDNA as a template, with the following Human TaqMan Copy Number Assays: for *HER2* assay ID Hs02876245_cn, for *EGFR* assay ID Hs04942325_cn, for *MET* assay ID: Hs04993403_cn, for *FGFR2* assay ID Hs01472955_cn, for *KRAS* assay ID Hs06936191 and the TaqMan Copy Number Reference Assay RNase P 4316831 and GREB1 Hs01738470_cn (Applied Biosystems, Foster City, CA, USA). PCR runs were performed with ABI Prism 7900HT (Applied Biosystems).

AMNESIA panel

In a case-control study setting, we identified a panel of gene alterations (including *EGFR/MET/KRAS/PI3K/PTEN* mutations and *EGFR/MET/KRAS* amplifications) able to predict primary resistance to trastuzumab therapy in HER2-positive metastatic gastric cancer patients (18). We applied the same panel of gene alterations (substituting *EGFR* mutation/amplification with *HER2* mutation/amplification) in the context of EGFR driven tumors.

Phospho- Kinase Array

Cells were treated with the indicated drugs: lapatinib or erlotinib 100 nM for 2 hours; cetuximab 0.5µg/ml for 16 hours. The analysis of the phosphorylation profiles of kinases was performed using

the Human Phospho-Kinase Antibody Array (R&D Systems, Minneapolis, MN, USA), according to manufacturer instructions. Signal quantification was performed using Image Lab 5.2.1 Software (BioRad, Hercules, CA, USA).

PDX generation

Gastric PDX generation was performed as described in (19). All animal procedures adhered to the 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) standards and were approved by the Ethical Commission of the Candiolo Cancer Institute (Candiolo, Torino, Italy), and by the Italian Ministry of Health.

PDX xenotrials

PDXs were passaged and expanded for >2 generations until production of a cohort of mice. Established and randomized tumors (average volume 250 mm^3) were treated for the indicated days with the following regimens (either single agent or combination): vehicle (saline) per os; cetuximab 20 mg/Kg, twice weekly ip; lapatinib 100 mg/kg, daily, per os; erlotinib 50 mg/kg, daily, per os; everolimus 6 mg/kg, daily, per os. 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 is the minor tumor axis and D is the major tumor axis. 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 (20). Statistical testing for pharmacological experiment was performed with GraphPAD PRISM Software 8.0, using Two-way ANOVA followed by Bonferroni multiple comparisons experiments. Statistical significance: ns= not significant; *p <0,05; **p <0,01; ***p <0,001.

Genomic sequencing

IRCC samples: DNA extracted from PDX models along with a sample of normal germline DNA from each patient were utilized for next generation sequencing. Using standard methods, Illumina sequencing libraries were generated and subjected to hybrid capture with a focused targeted bait set

of 243 genes selected based upon their alteration in prior studies of gastroesophageal cancer (21,22).

FMI samples: Comprehensive genomic profiling (CGP) was performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified, New York State and CAP (College of American Pathologists)-accredited laboratory (Foundation Medicine, Inc., Cambridge, MA). In brief, ≥50ng DNA was extracted from 40 microns of formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 4,337 cases of gastric carcinoma. The samples were assayed by CGP using adaptor-ligation and hybrid capture was performed for all coding exons of from 180-395 cancer related genes plus select introns from 14-34 genes frequently rearranged in cancer. Sequencing of captured libraries was performed to a mean exon coverage depth of >500X, and resultant sequences were analyzed for genomic alterations including mutations (base substitutions, insertions and deletions), copy number alterations (focal amplifications and homozygous deletions), and select gene fusions or rearrangements, as previously described(23). *EGFR* amplification was defined as *EGFR* copy ≥8.

COG samples: RTK copy numbers were determined using Affymetrix OncoScan® CNV FFPE Assay following the manufacturer recommended protocol. DNA was extracted from histologically confirmed oesophageal and GEJ adenocarcinomas as described previously(13) and quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) following the manufacturer's recommended protocol, using 80ng for each case, normalised to a concentration 12 ng/μL. Array fluorescence intensity data (CEL files), generated by Affymetrix® GeneChip® Command Console® (AGCC) Software version 4.0 were processed using OncoScan® Console software version 1.1.034 to produce OSCHP files and a set of QC metrics. Features were quantile normalised and genome wide allele specific copy number assessed using the Affymetrix TuScan algorithm to allow adjustment for both tumor ploidy and nonaberrant cell admixture(24). Genome wide copy number variation was assessed across all cases using AffymetrixNexus Express for OncoScan (version 3.1.). Significant copy number variation events across the genome were identified using a 'Significance Testing for Aberrant Copy number' (STAC) approach(25).

INT samples: Formalin-fixed paraffin-embedded archival tumor tissue blocks obtained prior to any treatment were used for the purpose of this study. Next-generation sequencing was performed as in (26), to detect gene mutations, whereas *EGFR*, *HER2* and *MET* status were determined by SISH analysis and *KRAS* GCN gain was assessed by PCR, as previously described in (18).

Survival analysis

OS was calculated from the date of enrollment (for the COG trial) or from the date of diagnosis of metastatic disease (for the INT dataset) until the date of death or last follow-up for alive patients. The OS curves for *EGFR* amplified vs non-amplified subgroups were calculated with the Kaplan-Meier method and compared with the log-rank test. Survival analysis for COG was undertaken using IBM SPPS statistics 22, further details see(10,13).

In situ Hybridization and Immunohistochemistry

EGFR gene status was assessed by bright-field dual-color SISH (Ventana Medical Systems). The Colorado scoring system was adopted to classify samples into ISH strata according to the frequency of cells with each *EGFR* gene copy number and referred to the chromosome 7 centromere. *EGFR* SISH-negative cases had no or low genomic gain for *EGFR* gene copy number (disomy, low trisomy, high trisomy, and low polysomy), whereas the distinction between high polysomy and gene amplification was defined by the presence of gene clusters only in *EGFR*-amplified cases. *EGFR* FISH in the COG cohort was performed and scored as described in (13).

IHC for EGFR was performed using the CONFIRM® anti-EGFR (5B7) rabbit monoclonal primary antibody (Ventana Medical Systems, Tucson, AZ) that recognizes the internal domain of EGFR and the monoclonal mouse anti human anti-EGFR (E30) antibody (Dako, Glostrup, Denmark) that recognizes an external domain of EGFR. IHC was carried out on an automated immunostainer (BenchMark Ultra; Ventana Medical Systems) using the Optiview DAB Detection Kit (Ventana Medical Systems). IHC for P-EGFR was performed using anti-P-EGFR Y1173 53AS from Cell Signaling (MA, USA).

Transcriptome profiling

RNA-seq libraries were prepared using the Illumina TruSeq Stranded Total RNA Library Prep Gold kit and sequenced generating 75 bp paired-end reads. PDX RNA-seq data were first deconvoluted for mouse contamination with Xenome (27) software (version 1.0.1). Non-host reads (those classified as “graft”, “ambiguous” or “both”) were then mapped to UCSC hg38 reference genome with HISAT2 (28) aligner with default parameters. Gene expression estimate was performed with HTSeq (29) in “intersection-nonempty” mode against GENCODE v33 annotation.

RESULTS

Prevalence of *EGFR* amplification in GEA patients

We evaluated *EGFR* copy number in four different cohorts: 1) a proprietary cohort (IRCC cohort) of 570 primary GEAs (real time PCR analysis); 2) the Foundation Medicine Inc. (FMI) dataset of 4337 gastric and 5060 esophageal/gastroesophageal junction [GEJ] adenocarcinomas (comprehensive genomic profiling); 3) the subgroup of 214 patients with esophageal or GEJ adenocarcinoma enrolled in the COG trial (NCT01243398) of second-line gefinitib versus placebo(10) (fluorescence in-situ hybridization, FISH); 4) the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) of Milan dataset of 206 mGEA patients (in-situ hybridization, SISH). In the IRCC cohort we identified 44 primary tumors (7.8%) with *EGFR* CNG (≥4 gene copies), with 10 of them (1.8% of all samples) bearing > 8 gene copies (the suggested threshold of biologically meaningful amplification in the *HER2* and *MET* context (30)) and 8 of them (1.4% of all samples) bearing a heterogeneous *EGFR* amplification (one tumor area >8 copies and one tumor area ≤ 8 copies) (Figure 1 and Supplementary Table 1). In the FMI dataset 3.4% of gastric and 7.6% of esophageal carcinomas showed *EGFR* amplification equal or higher than 8 copies, while in the COG and INT datasets the frequencies of *EGFR* amplification were 7.0% and 4.9 %, respectively (Figure 1). In both COG and INT cohorts, no significant association between *EGFR* amplification and baseline clinic-pathological characteristics was observed (Supplementary Table 2 and 3).

EGFR amplification drives aggressiveness and poor prognosis in gastroesophageal adenocarcinomas

To investigate if *EGFR* amplification is associated with poor prognosis of gastroesophageal adenocarcinomas, we took advantage of a cohort of pre-treated patients with esophageal and GEJ adenocarcinomas enrolled in the COG trial and randomized to placebo (10). Among 102 cases with available *EGFR* FISH status, patients with *EGFR* amplification had a significantly inferior median

OS compared to those without EGFR amplification (3.1 versus 3.5 months; HR=1.23, 95% CI: 1.03-1.48; p=0.026; Figure 2A, left panel). All patients with *EGFR* amplified tumors died within 4 months.

Similarly, when focusing on the INT dataset, patients with *EGFR* amplification had inferior median OS as compared to those with *EGFR* SISH negative tumors (17.0 versus 18.9 months; HR=1.95, 95% CI: 0.90-4.21; p=0.083; Figure 2A, right panel). These results have been also confirmed in primary gastric tumors analyzing the TCGA data, in which tumor *EGFR* amplification correlated with significantly inferior OS and DFS (Figure 2B).

Activity of EGFR inhibitors in patients with *EGFR* amplified metastatic gastric cancer and landscape of primary treatment resistance

To determine whether patients with *EGFR* amplified mGEA may respond to EGFR inhibitors and to eliminate the potentially confounding effect of the combination with chemotherapy, we focused on patients with *EGFR* amplified mGEA treated at INT with the anti-EGFR monoclonal antibody panitumumab as single-agent after failure of standard treatment options. Three patients with *EGFR* amplification confirmed by SISH were identified (Supplementary Figure 1A); their molecular profile is summarized in Supplementary Figure 1B and their clinical history is reported in Figure 3. Briefly, INT#1 patient had *KRAS* co-amplified mGEA and showed progressive disease (PD) at the first radiological re-assessment; INT#2 had no co-occurring alterations in *HER2*, *MET*, *KRAS* or *PIK3CA* and showed a partial response (PR) lasting 6 months; INT#3 had co-occurring heterogeneous *KRAS* amplification and showed a PR lasting only 10 weeks and followed by rapid clinical progression and death.

To verify if RTK pathway activation is associated with EGFR inhibitor resistance in GEA, we investigated the relationship between RTK copy number gain and survival following treatment with gefitinib in 12 *EGFR* FISH positive gastroesophageal adenocarcinomas (7 with amplification and 5 with high polysomy) of the COG trial. All 12 tumors analyzed had copy number gain (defined as ≥4

gene copies) of at least one RTK (*HER2, HER3, HER4, MET, FGFR1, FGFR2, FGFR3, FGFR4, IGF1R, PDGFR2, VEGFR1, VEGR2* and *VEGFR3*). We found a significant inverse correlation between the extent of co-amplification of the RTKs and OS (Figure 4A). This observation of shorter survival following gefitinib treatment with activation of RTKs other than EGFR suggests optimizing inhibition of downstream signal transduction pathways could produce durable clinical responses.

To investigate the prevalence of potential genetic predictors of primary resistance to anti-EGFR treatment, we interrogated the TCGA dataset for the presence of resistance alterations included in our previously published AMNESIA panel (18) among cases with *EGFR* amplification and showed the co-occurrence of other genomic events in 53% of samples (Suppl. Figure 2). Finally, since the available in-silico datasets mainly represent a collection of primary GEAs, we investigated the prevalence of AMNESIA panel alterations in the 534 samples from *EGFR* amplified mGEA patients included in the FMI dataset. This analysis showed the co-occurrence of other genomic events of interest in 186 (35%) samples (Figure 4B).

Dual EGFR blockade is the most effective treatment of *EGFR*-amplified PDXs

Future trials might be prompted to re-assess the role of anti-EGFR MoAbs and TKI, either as monotherapy or in combination, in GEA molecularly selected patients. As already shown for dual HER2 blockade (trastuzumab plus pertuzumab or lapatinib) in HER2-positive breast and colorectal cancer (31-33), and despite the partially negative phase III data recently reported with this strategy in HER2-positive gastric cancer (34), dual EGFR blockade strategies with an anti-EGFR MoAb plus a TKI may be more effective than each drug as monotherapy.

A large series of human cancer specimens transplanted into mice (Patient-Derived Xenogratfs, PDXs) produce a study population that can be randomized for prospective treatment with targeted agents and so provide a strong strategy to perform precision medicine preclinical studies. This approach brings together the plasticity of preclinical analysis with the informative value of

population-based studies. From 570 gastric carcinoma samples (IRCC cohort), we generated a multi-level platform of GEA models, comprising 151 PDXs, primary cell lines and organoids (22). Despite conflicting evidence on the CNG threshold clearly defining gene amplification, preclinical and clinical data obtained from GEA displaying *HER2* or *MET* amplification suggested that the clinically relevant threshold is higher than 8 gene copies (30,35). Eleven PDXs harbored at least 4-8 *EGFR* copies and 4 PDXs > 8 *EGFR* copies (Suppl. Figure 3A: GTR0060: ~240 *EGFR* copies; GTR0078: ~700 copies; GTR0110: 12 copies; GTR0511: ~80 copies). These 4 models did not bear any other RTKs/*KRAS* CNV >8 copies (data not shown). SISH analysis and immunohistochemistry confirmed uniform *EGFR* amplification and expression (Suppl. Figure 3B). These PDX models were expanded to generate cohorts of mice, in order to evaluate the efficacy of the EGFR moAb cetuximab and the TKI erlotinib (EGFR-selective) and lapatinib (dual EGFR/HER2 inhibitor), as well as the combination of the moAb with a TKI. The original tumors were serially passaged *in vivo* until six tumor-bearing animals were produced per experimental group. When xenografts reached an average volume of ~250 mm^3, mice were randomized into six independent treatment cohorts: (i) vehicle (placebo); (ii) cetuximab; (iii) erlotinib; (iv) lapatinib; (v) cetuximab + erlotinib; (vi) cetuximab + lapatinib. Tumor response was evaluated according to RECIST-like Criteria (see Methods and Figure Legends).

As shown in Figure 5A, the GTR0060 PDX (240 *EGFR* copies) did not exhibit response to either of the TKIs used as monotherapy, but showed partial response (PR) upon cetuximab treatment. Notably, both the combo (cetuximab + TKI) treatments resulted in a complete response (CR). Interestingly, in 4 out of 6 mice in the combo arms, including 3/3 mice treated with erlotinib + cetuximab, the tumor mass did not reappear even after more than two months of drug removal (Figure 5B). Improved efficacy of the combo treatment was observed at long term also in a second model, GTR0110, characterized by a lower *EGFR* CNG (12 copies), uniformly distributed among tumor cells (Suppl. Figure 3B). While neither erlotinib nor lapatinib resulted in a clinical response and cetuximab conferred disease stabilization, cetuximab plus TKI combination treatment resulted

in a PR (Figure 5C). Moreover, at the end of the experiment, the tumor volume was significantly reduced in mice treated with the combination compared with those treated with the moAb alone. The xenotrial performed in the GTR0511 PDX (80 *EGFR* copies) cohort also showed response to anti-EGFR treatment. Even though neither cetuximab nor lapatinib monotherapies were effective, their combination resulted in a relevant response. Interestingly, in this PDX erlotinib was the only effective monotherapy (Figure 5D). To investigate the reason of the differential sensitivity of GTR0511 to erlotinib, we analyzed Whole Exome Sequence data but we did not detect *EGFR* alterations (data not shown). On the contrary, RNAseq analysis revealed a ten-fold decrease of the number of reads covering the last portion of the receptor (from exon 26 until the end of the mRNA, Suppl. Figure 4A). This resulted in the presence of an EGFR protein isoform lacking the C-terminal domain, together with an EGFR full length protein. As Kovacs et al. (36) showed that the loss of this portion of the tail, containing Y1068, determines a strong decrease in receptor activation, we immunoprecipitated (with an antibody directed against the EGFR extracellular portion) EGFR from organoids derived from the three PDXs. As shown in Suppl. Fig. 4B, in GTR0511 EGFR displayed only a modest activation, in spite of the high amount of the expressed protein, meaning that the ratio between phosphorylated/unphosphorylated receptor is much lower in GTR0511 compared to the other amplified models. As predicted by in silico data, two phosphorylated bands were detected only in GTR0511, and they were both effectively inhibited by erlotinib. Finally, stronger downstream signal blockade in GTR0511 vs GTR0110 and GTR0060 was seen in total cell lysates derived from the same organoids. In agreement with previously published data (36) we thus hypothesize that the lack of the EGFR C-terminal tail in GTR0511 can be responsible of its decreased activation and increased sensitivity to erlotinib treatment..

To investigate which pathways were inactivated by the different drugs/drug combinations in cases in which the combo resulted in a strongly enhanced response, we took advantage of PDX-derived primary cells in which *EGFR* amplification was maintained (Suppl. Figure 5A). Primary cells were treated with cetuximab, erlotinib and lapatinib, alone or in combination. Western blot analysis

showed that while lapatinib and erlotinib only slightly affected activation of downstream transducers such as AKT, MAPK and S6 (evaluated as read out of the PI3K, RAS/MAPK and mTOR pathways, respectively), a partial inhibition was induced by cetuximab. Interestingly, both the dual combinations resulted in a strong inhibition of signal transduction (Figure 5E). Phospho-array analysis of cellular kinases and RTKs confirmed these results but did not identify any other kinase specifically inhibited by the combo treatments (Suppl. Figure 5B). These *in vitro* data strongly support the results we obtained in the *in vivo* experiments where cetuximab induced SD while the two combos resulted in a complete and durable response. It is thus likely that when EGFR activation is exceptionally intense, the dual blockade with TKI+cetuximab is needed to improve the response.

TSC2 inactivation is a mechanism of resistance to EGFR targeted therapies.

We performed a preclinical trial, similar to those previously described, using the GTR0078 PDX harboring ~700 *EGFR* copies (Suppl. Figure 3). Despite the very high level of *EGFR* amplification we did not observe response to the TKIs, nor to cetuximab or cetuximab + TKI combos (Figure 6A). To understand the molecular basis for the observed resistance, we sequenced the tumor DNA and detected several genomic alterations; among these, we observed a fraction of *EGFR* gene copies displaying a deletion at the 5' gene portion, thus coding for a protein lacking the extracellular portion (Suppl. Figure 6A). Moreover, we also observed two missense *TSC2* mutations (p.M1300V and p.R1438Q), with an allelic frequency of 0.463 and 0.539, respectively (Figure 6B). The TSC2 protein forms a complex with TSC1, a critical negative regulator of mTORC1 (mammalian target of rapamycin complex 1) which controls anabolic processes to promote cell growth (37-39). TSC2 inactivation (due to homozygous mutations or gene loss), results in mTOR increased activation (40). Interestingly, when we interrogated cBioPortal for the possible co-occurrence of *EGFR* and *TSC2* functional genomic alterations in six gastric cancer datasets (4,41-45), we found a significant correlation (Suppl. Figure 6B). Moreover, alterations in the mTOR

pathway co-occurrent with *EGFR* CNG have been identified in the FMI dataset as well, although co-occurrence with *EGFR* amplification was uncommon (Suppl. Figure 6C).

To support the causative role of TSC2 in EGFR target therapy resistance, we silenced TSC2 in OE21 cells, harboring *EGFR* gene amplification (46). In *in vitro* experiments, upon TSC2 silencing, we observed the constitutive activation of the mTOR pathway, revealed by the activation of the downstream transducer S6, which was maintained even in the presence of anti-EGFR treatment (Suppl. Figure 7A). To validate these data *in vivo*, we transduced OE21 cells with either Ctrl shRNA (shC) or a pool of TSC2 shRNAs and we injected them in immunocompromised mice. As shown in Suppl. Figure Fig. 7B, shC mice underwent tumor regression in response to EGFR blockade, while partially TSC2 silenced tumors experienced only disease stabilization, reinforcing the idea that TSC2 silencing impairs the response to anti-EGFR therapy.

We thus wondered if treatment of GTR0078 tumors with a mTOR inhibitor (such as everolimus) could restore sensitivity to EGFR inhibitors. While treatment with everolimus alone did not show any clinical efficacy (Suppl. Figure 6D), the combination of everolimus with erlotinib resulted in a significant clinical response (Figure 6C). Experiments performed in PDX-derived cells showed that while treatment of GTR0078 cells with either EGFR inhibitors or everolimus was unable to block mTOR activation, the association of the two drugs resulted in a sustained inhibition of the pathway. Indeed, only the concomitant inhibition of the EGFR and mTOR pathway inactivated the downstream transducer S6 kinase (Figure 6D).

DISCUSSION

In unselected patients with advanced gastric/esophageal adenocarcinoma, the addition of an anti EGFR antibody to first-line standard chemotherapy failed to show a significant survival benefit in two randomized clinical trials (8,9). Similar negative results were also observed when the small molecule TK inhibitor gefitinib was compared to placebo from the second-line setting and beyond (10). Sporadic responses to EGFR inhibitors observed in these trials, however, lead several researchers to postulate the existence of a subset of metastatic patient with *EGFR*-addicted tumors, potentially vulnerable to EGFR blockade (13). The amplification of the *EGFR* gene is found in 3-5% of primary GEA tumors (4,6) and highly correlates with poor prognosis (47). By exploiting four different datasets, we have shown here that *EGFR* amplification has similar prevalence and is associated with poorer survival in the metastatic setting. This was also confirmed in the non-metastatic setting, analyzing the TCGA data. In a pre-specified exploratory analysis of one of those data sets, the COG trial randomizing 209 chemo-resistant metastatic patients to gefitinib or placebo (10), *EGFR* amplification is a positive predictive marker for EGFR targeting, whereas a smaller advantage is observed in patients with chromosome 7 polysomy (13). Response to the anti-EGFR moAb cetuximab, used alone or in combination with chemotherapy, was reported in a small set of seven *EGFR* amplified patients; albeit the role of the cytotoxic backbone contribution cannot be ruled out in three responders, one response was induced by EGFR blockade alone (48). Such results clearly mirror those achieved in patients receiving panitumumab monotherapy by our study. All together these observations suggest that *EGFR* is an oncogenic driver, with potentially exquisite sensitivity to EGFR targeting drugs, in a small but clinically consistent subgroup of GEAs. On the other hand, in these *EGFR*-amplified tumors, we observed the presence of selected co-occurring driver alterations. Specifically *MET/HER2/KRAS* co-amplifications and *KRAS/PK3CA/PTEN* co-mutations were identified in 53% and 35% of patients in the *EGFR* amplified subgroups included in the TCGA and FMI datasets, respectively; this result highlights that only a subset of patients with *EGFR* amplified gastroesophageal cancer may significantly benefit from single-agent anti-EGFR

therapy. Here, we have for the first time functionally identified *TSC2* mutations as a potential new mechanism conferring resistance to EGFR inhibition in GEAs. TSC2 is a GTPase-activating protein, whose loss or inactivating mutation result in the constitutive load of Rheb with GTP and activation of mTORC signaling (39). Interestingly, according to cBioPortal, *TSC1*/*TSC2* mutations are significantly associated with *EGFR* amplification (but not with other RTKs) in GEAs, possibly indicating that mTORC constitutive activation can sustain the oncogenic role of *EGFR*. Our preclinical trial in an *EGFR* amplified/*TSC2* mutated GEA PDX confirms this hypothesis. The pharmacological inhibition of TSC2-sustained mTORC activation by everolimus, a clinical grade small molecule mTOR inhibitor, overcame primary resistance and restored sensitivity to EGFR inhibition. Our data are reinforced by a recently published paper from Arteaga and coll. (49), in which they showed that hyperactivation of the mTORC pathway drives resistance to therapies targeting another member of the HER family, namely HER2, in HER2-mutant breast cancer. In their work, similarly to what we have observed, the combination of the TORC1 inhibitor everolimus and neratinib overcame resistance.

Resistance is a common occurrence of RTK inhibition across diseases, targets and drugs. Several cell autonomous mechanisms sustaining resistance to driver RTKs have been identified so far, including mutations of the target itself, activation of downstream transducers, activation of parallel pathways and transdifferentiation. Moreover, in many cases the amplified RTK is not located in the natural genomic site, but it is rather extrachromosomal. This results in a mechanism favoring rapid adaptation of cancer cells to environmental changes. Indeed, as extrachromosomal DNA lacks centromeres, it is unequally segregated during cell division, leading to increased tumor heterogeneity and different cellular fitness in diverse contexts. Cancer cells in which oncogenes are extrachromosomal can thus become resistant to RTK inhibitors either by increasing the number of gene copies (thus titrating the amount of the available inhibitor) or by progressively decreasing the number of gene copies. Both the mechanisms are sustained by experimental data. For example, Nathanson et al. (50) showed that glioblastoma cells can become resistant to erlotinib, eliminating

extrachromosomal copies of the mutant *EGFR* gene. This "adaptation" to the treatment can be acquired and expanded along tumor evolution, enabling tumors to maintain their intratumoral heterogeneity. In previous works (51,52) we have shown that in *MET* hyper amplified gastric cancer cells (where the amplified gene was extrachromosomal) resistance was due to further acquisition of gene copies; this resulted in an amount of activated receptor overcoming the inhibitory ability of the drugs at tolerable doses.

To bypass primary and prevent secondary resistance to EGFR-targeted drugs in *EGFR* amplified GEAs we leveraged our large platform of 151 primary GEAs patient-derived mouse avatars (22), enriched for 15 cases with *EGFR* gene copy gain, including 4 avatars with more than 8 *EGFR* copies (confirmed as amplified -i.e. non polysomic- by silver in situ hybridization). EGFR inhibition, in absence of chemotherapy, resulted in a clinical response in three out of four cases. Notably, one of these cases featured 12 *EGFR* copies, a range of amplification that is just above the threshold (8 copies) considered biologically relevant and that has not been investigated previously (48). Interestingly, a complete response was achieved only in the PDX with the highest *EGFR* CNG, suggesting that a higher level of gene amplification may be associated with a greater magnitude of treatment benefit, as it is known for *HER2*-amplified GEA and breast cancer (30,53).

The pharmacological space of EGFR targeted drugs is well populated by antibodies and small molecule TKIs, both experimental and approved for use in clinically diverse settings (54,55).

In our preclinical trials in *EGFR*-amplified GEA avatars we compared the efficacy of randomly allocated TKIs and cetuximab, delivered as single agent or in combinations. Erlotinib and cetuximab showed single agent excellent activity in one and two models respectively, while in a third model cetuximab treatment resulted in disease stabilization. Importantly however, the dual EGFR blockade resulted in a sustained significant response in all three models suggesting that a strong inhibition of the downstream transducers is needed to eradicate the disease.

In conclusion, our study further corroborates *EGFR* amplification as an actionable therapeutic target in GEA, demonstrates that a dual EGFR blockade may be needed to maximize the therapeutic

efficacy, and identifies potential mechanisms of primary resistance, specifically the mTORC pathway, paving the way for experimentally driven clinical trials. In fact, the next generation clinical trial landscape in *EGFR* amplified GEAs may not be at a dead end. The combination of lapatinib and cetuximab has already been proven safe in a phase 1 trial (56), potent second-generation antibodies mixtures against different, non-overlapping epitopes of EGFR – such as Sym004 and MM-151 (57,58) – are into clinical development and the TORC pathway is targetable with commercially available drugs. Given the diversity of clinically relevant genomic alterations and lack of benefit from EGFR targeted therapies in unselected GEA populations, broad based genomic profiling is thus necessary to reliably detect *EGFR* gene amplification in addition to other potential drivers and mechanisms of resistance.

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AUTHOR CONTRIBUTIONS

SG, SC, FP and MA conceived and supervised the study, contributed to design the experiments, wrote the manuscript; MD, RR, UF, SDP, GS, ER, GLB, SM, GDM, MB; SS, AS-B, FM, SC, MP, MDB provided patient material and data; C.Migliore, AP, DC, LDE, SD, DMR, SEB, SU performed experiments; C.Marchio', A.Sapino, AG performed the pathologic analysis; JL, SMA, JSR, BA, VAM and ABS provided data from FMI; A.Sottile provided technical support; ADS, ZM, BA, JSR, RP provided data from the COG study; S.Marsoni managed the GEA Study. All the authors revised the manuscript.

FIGURE LEGENDS

Figure 1. *EGFR* copy number gain. The graphs illustrate the percentage of tumors displaying *EGFR* copy number gain (CNG) in four different cohorts. Real time PCR analysis of IRCC gastric/gastroesophageal junction (GEJ) adenocarcinomas displaying *EGFR* gain (4-8 copies or more than 8 copies) or heterogeneity (significantly different *EGFR* CNG in diverse analyzed samples from the same tumor, with one tumor sample >8 copies and one tumor sample ≤ 8 copies). Comprehensive genomic profiling of FMI gastric and esophageal/GEJ cases, FISH analysis of COG esophageal/GEJ cases and SISH analysis of INT gastric/GEJ adenocarcinomas.

Figure 2. Survival analysis of patients with *EGFR* CNG. **(A)** The graphs show the cumulative survival (Cum Survival) of patients of the COG (left) and INT (right) cohorts related to *EGFR* CNG. **(B)** The graphs show the overall survival (left) and the disease-free survival (right) of patients of the gastroesophageal TCGA dataset, related to *EGFR* CNG.

Figure 3. Clinical history of patients treated with EGFR-targeted drugs. Summarized clinical course of INT patients with *EGFR* CNG. Red-lined boxes indicate periods of administration of the indicated therapeutic agents. Blue vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsies, as well as dates of tumor assessment by CT scan. PD, progressive disease; SD, stable disease, according to RECIST 1.1. EOX: epirubicin, oxaliplatin and capecitabine; 5FU: 5 fluorouracil; TCF: docetaxel, carboplatin and 5-fluorouracil; FOLFIRI: folinic acid, 5-fluorouracil, irinotecan; XELOX: capecitabine and oxaliplatin; CCDP: cisplatin, vinorelbine, ifosfamide and epirubicin; OGD: oesophago-gastro-duodenoscopy.

Figure 4. RTK/KRAS pathway activation in *EGFR* amplified cases. (A) The scatter plot shows a significant inverse correlation between the extent of RTKs co-amplification (*HER2, HER3, HER4, MET, FGFR1, FGFR2, FGFR3, FGFR4, IGF1R, PDGFR2, VEGFR1, VEGR2* and *VEGFR3* ≥4 gene copies) and OS in 12 *EGFR* FISH positive GEAs treated with gefitinib in the COG trial. Red

dots indicate cases with high polisomy; blue dots represent cases with *EGFR* amplification. **(B)** The graph shows the co-occurrence of *EGFR* amplification and genomic events affecting the RTK/*KRAS* pathway in *EGFR* amplified GEA tumors in the FMI dataset.

Figure 5. Dual EGFR blockade is the most effective treatment in *EGFR*-amplified PDXs
Tumor growth curves in mice cohorts derived from GTR0060 **(A)**, GTR0110 **(C)** and GTR0511 **(D)** patients treated with the EGFR inhibitors cetuximab (CETUX), erlotinib (ERL), lapatinib (LAP), alone or in combination, as indicated. The red lines indicate the day when treatment was started. 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. **(B)** Spaghetti plot illustrating drug response in the xenotrial performed on the cohort of mice derived from PDX GTR0060. Individual lines represent, for each mouse, the percentage variation in tumor burden, from treatment start (day 0). Blue lines: CETUX + LAP treated mice; red lines: CETUX + ERL treated mice. Dashed line indicates treatment stop. **(E)** Western blot analysis of the activation state of EGFR and its downstream targets (AKT, MAPK and S6) in GTR0060 tumor-derived cells treated with the indicated drugs/drug combinations. Actin was used as loading control. Statistical significance is indicated **<0.01; ***<0.001

Figure 6. TSC2 inactivation is a mechanism of resistance to EGFR targeted therapies.
(A) Tumor growth curves in the mice cohorts derived from GTR0078 treated with the EGFR inhibitors cetuximab (CETUX), erlotinib (ERL), lapatinib (LAP), alone or in combination, as indicated. The arrow indicates the day when treatment was started. **(B)** The table shows the two *TSC2* mutations identified in GTR0078 PDX. **(C)** Tumor growth curves in the mice cohorts derived from GTR0078 treated with erlotinib (ERL) or the combo erlotinib + everolimus (ERL+ EVEROL). The red line indicates the day when treatment was started. **(D)** Western blot analysis of the activation state of EGFR and its downstream targets (AKT, MAPK and S6) in GTR0078 tumor-

derived cells treated with the indicated drugs/drug combinations. Actin was used as loading control. Statistical significance is indicated ***<0.001

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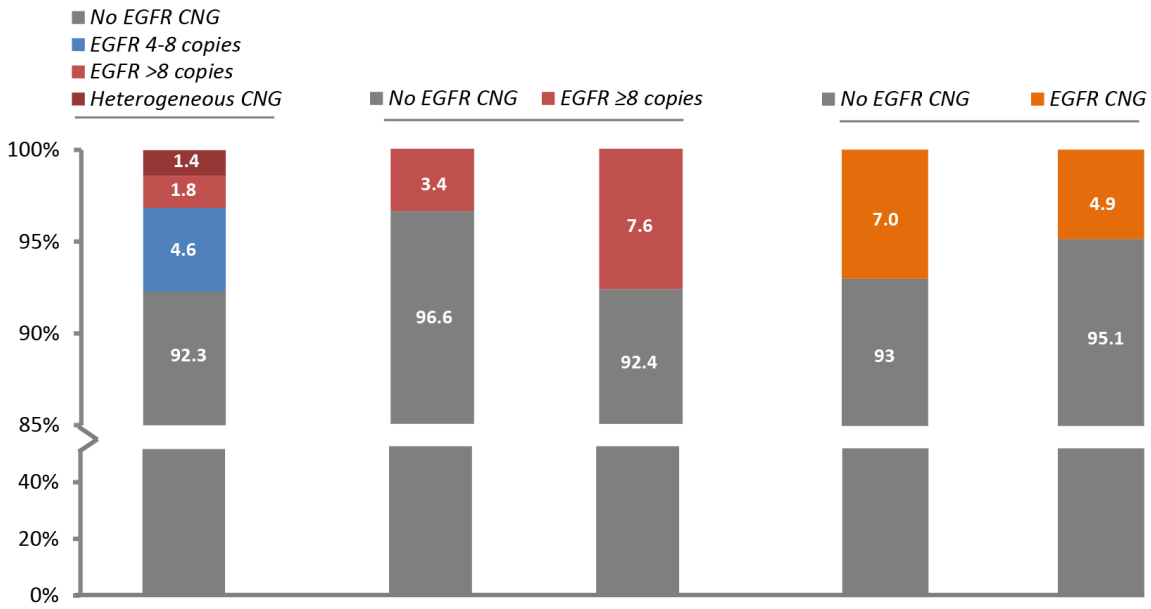
Figure 1

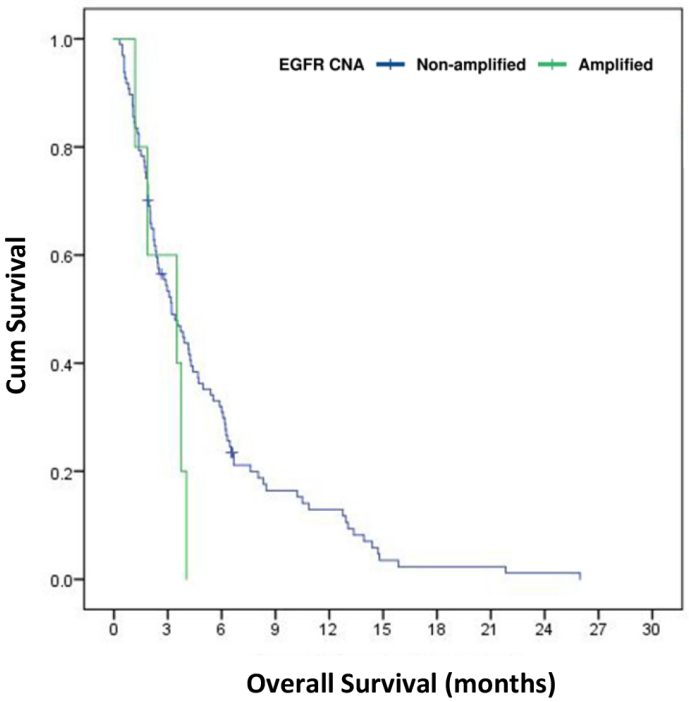


COHORT NAME	IRCC	FMI		COG	INT
SAMPLE TYPE	**Gastric/GEJ**	**Gastric**	**Esophageal/GEJ**	**Esophageal/GEJ**	**Gastric/GEJ**
SAMPLE SIZE	**N=570**	**N=4337**	**N=5060**	**N=214**	**N=206**
EGFR CNG DETECTION	**qReal Time PCR**	**Genomic Profiling**	**Genomic Profiling**	**FISH**	**SISH**

Figure 2

A

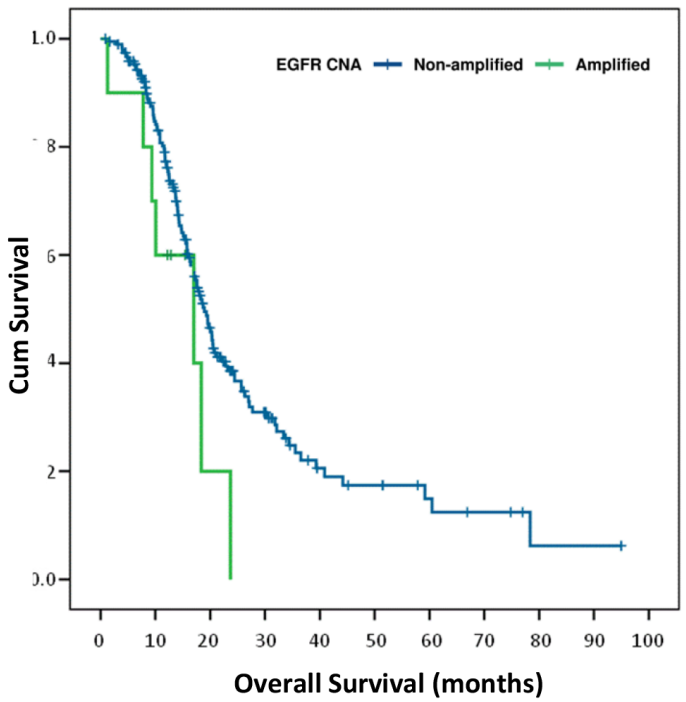
COG



Number at risk

94	50	29	19	12	3	2	1	1	0	0
8	3	0	0	0	0	0	0	0	0	0

INT

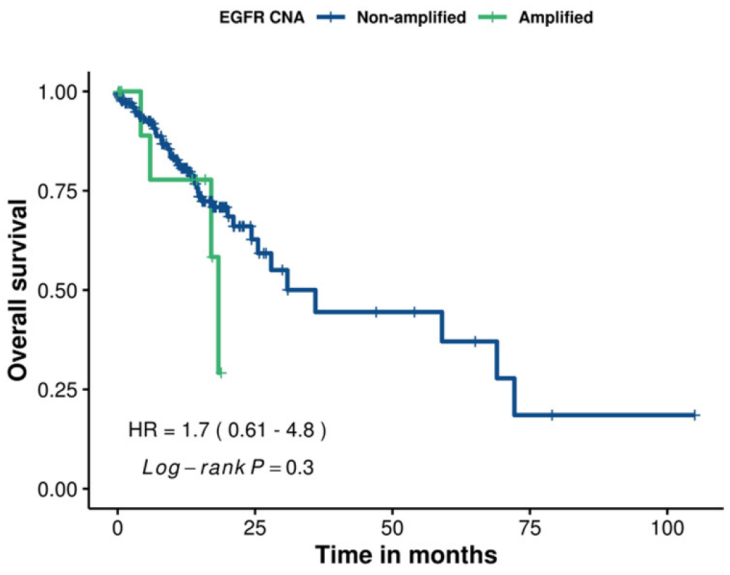


Number at risk

196	149	61	29	13	10	6	4	1	1	0
10	7	1	0	0	0	0	0	0	0	0

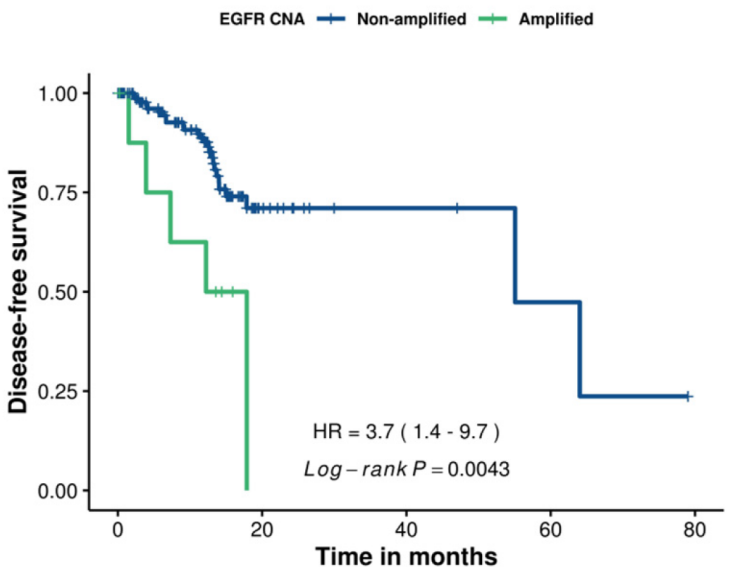
B

TCGA



Number at risk

217	18	7	2	1
12	0	0	0	0



Number at risk

152	13	4	2	0
9	0	0	0	0

Figure 3

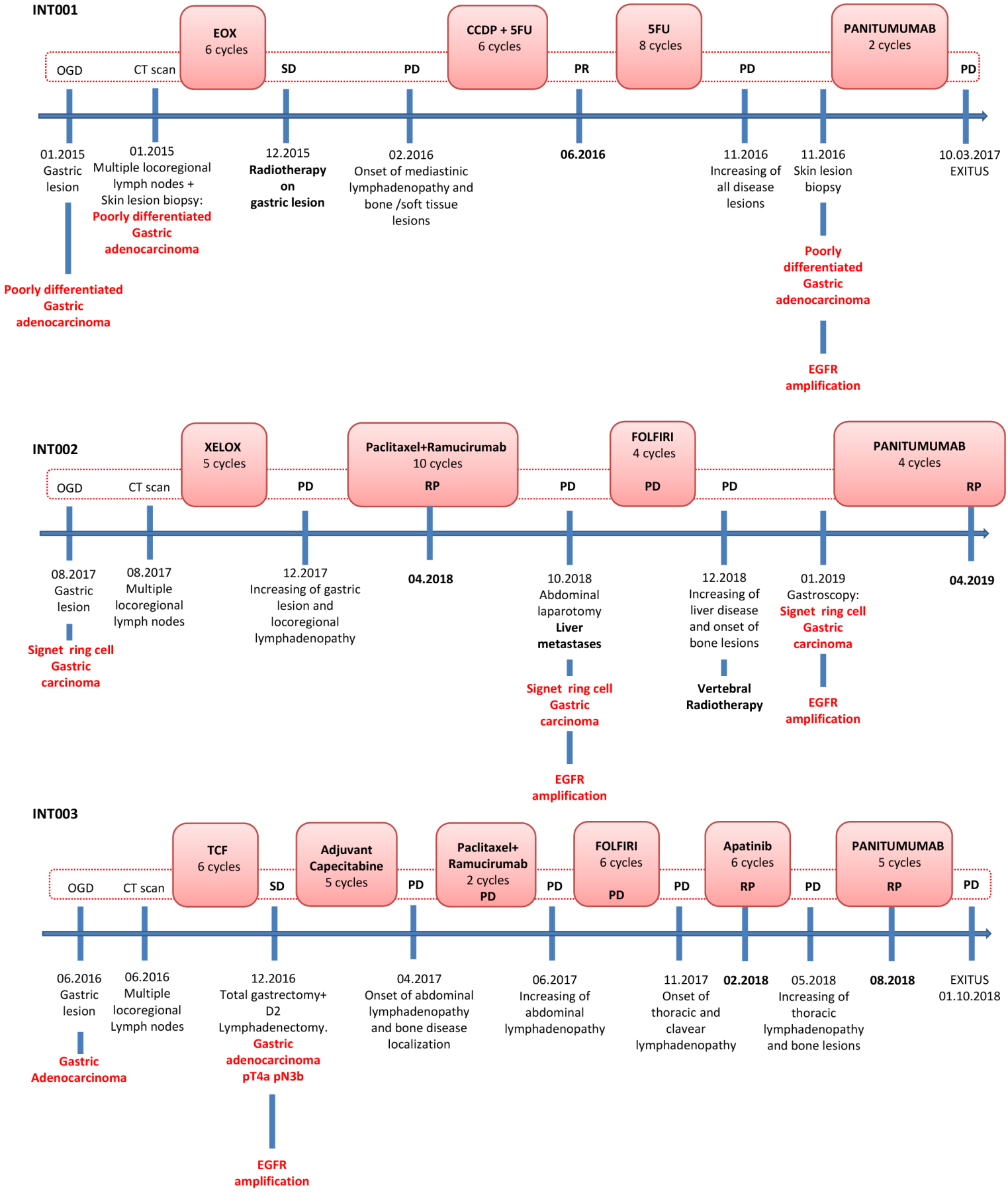
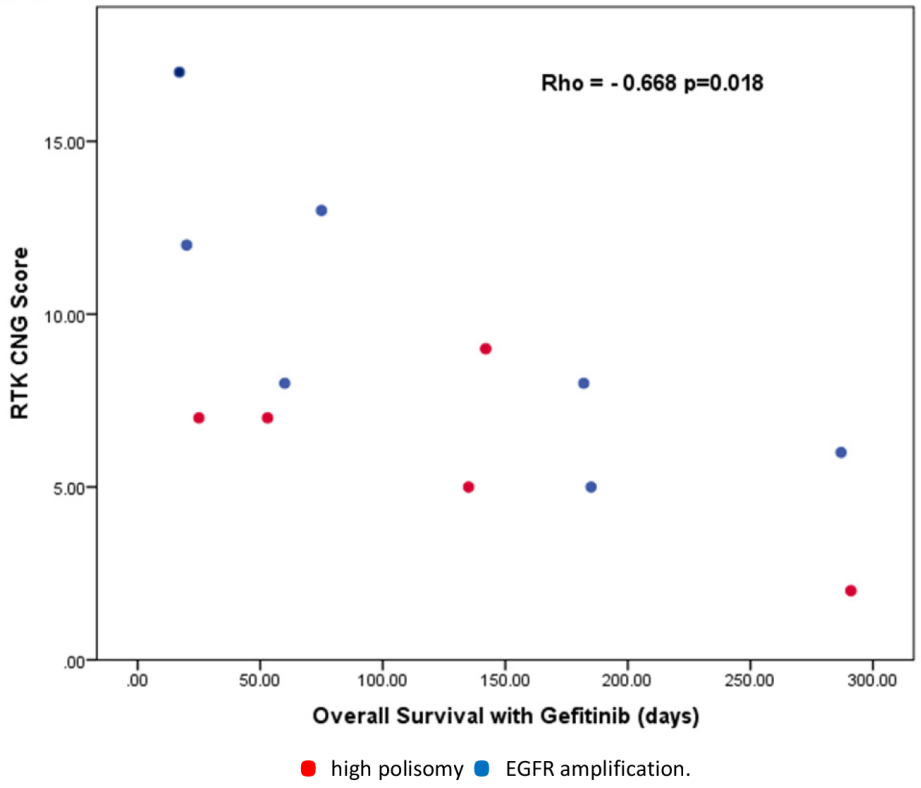

INT001
EOX
6 cycles
CCDP + 5FU
6 cycles
5FU
8 cycles
PANITUMUMAB
2 cycles
OGD
CT scan
SD
PD
PR
PD
PD
01.2015
Gastric lesion
01.2015
Multiple locoregional lymph nodes +
Skin lesion biopsy:
Poorly differentiated Gastric adenocarcinoma
12.2015
Radiotherapy on gastric lesion
02.2016
Onset of mediastinic lymphadenopathy and bone /soft tissue lesions
06.2016
11.2016
Increasing of all disease lesions
11.2016
Skin lesion biopsy
10.03.2017
EXITUS
Poorly differentiated Gastric adenocarcinoma
Poorly differentiated Gastric adenocarcinoma
EGFR amplification
INT002
XELOX
5 cycles
Paclitaxel+Ramucirumab
10 cycles
RP
FOLFIRI
4 cycles
PD
PANITUMUMAB
4 cycles
RP
OGD
CT scan
PD
PD
PD
PD
08.2017
Gastric lesion
08.2017
Multiple locoregional lymph nodes
12.2017
Increasing of gastric lesion and locoregional lymphadenopathy
04.2018
10.2018
Abdominal laparotomy
Liver metastases
12.2018
Increasing of liver disease and onset of bone lesions
01.2019
Gastroscopy:
Signet ring cell Gastric carcinoma
04.2019
Signet ring cell Gastric carcinoma
Signet ring cell Gastric carcinoma
Vertebral Radiotherapy
EGFR amplification
EGFR amplification
INT003
TCF
6 cycles
Adjuvant Capecitabine
5 cycles
Paclitaxel+ Ramucirumab
2 cycles
PD
FOLFIRI
6 cycles
PD
Apatinib
6 cycles
RP
PANITUMUMAB
5 cycles
RP
OGD
CT scan
SD
PD
PD
PD
PD
PD
PD
06.2016
Gastric lesion
06.2016
Multiple locoregional Lymph nodes
12.2016
Total gastrectomy+ D2 Lymphadenectomy.
Gastric adenocarcinoma pT4a pN3b
04.2017
Onset of abdominal lymphadenopathy and bone disease localization
06.2017
Increasing of abdominal lymphadenopathy
11.2017
Onset of thoracic and clavear lymphadenopathy
02.2018
05.2018
Increasing of thoracic lymphadenopathy and bone lesions
08.2018
EXITUS
01.10.2018
Gastric Adenocarcinoma
EGFR amplification

Figure 4

A


Rho = - 0.668 p=0.018
RTK CNG Score
15.00
10.00
5.00
.00
.00
50.00
100.00
150.00
200.00
250.00
300.00
Overall Survival with Gefitinib (days)
high polisomy
EGFR amplification.

B

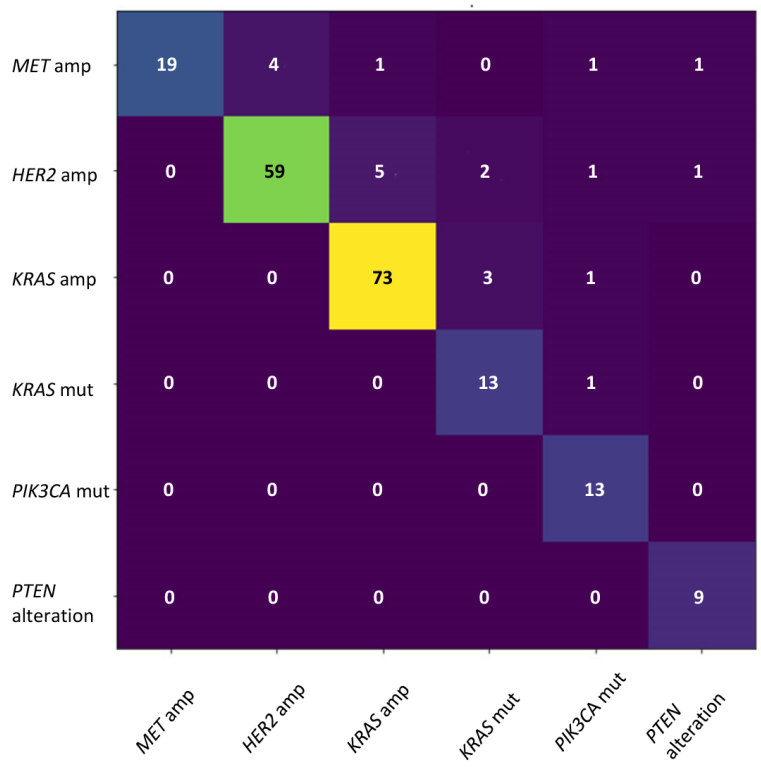
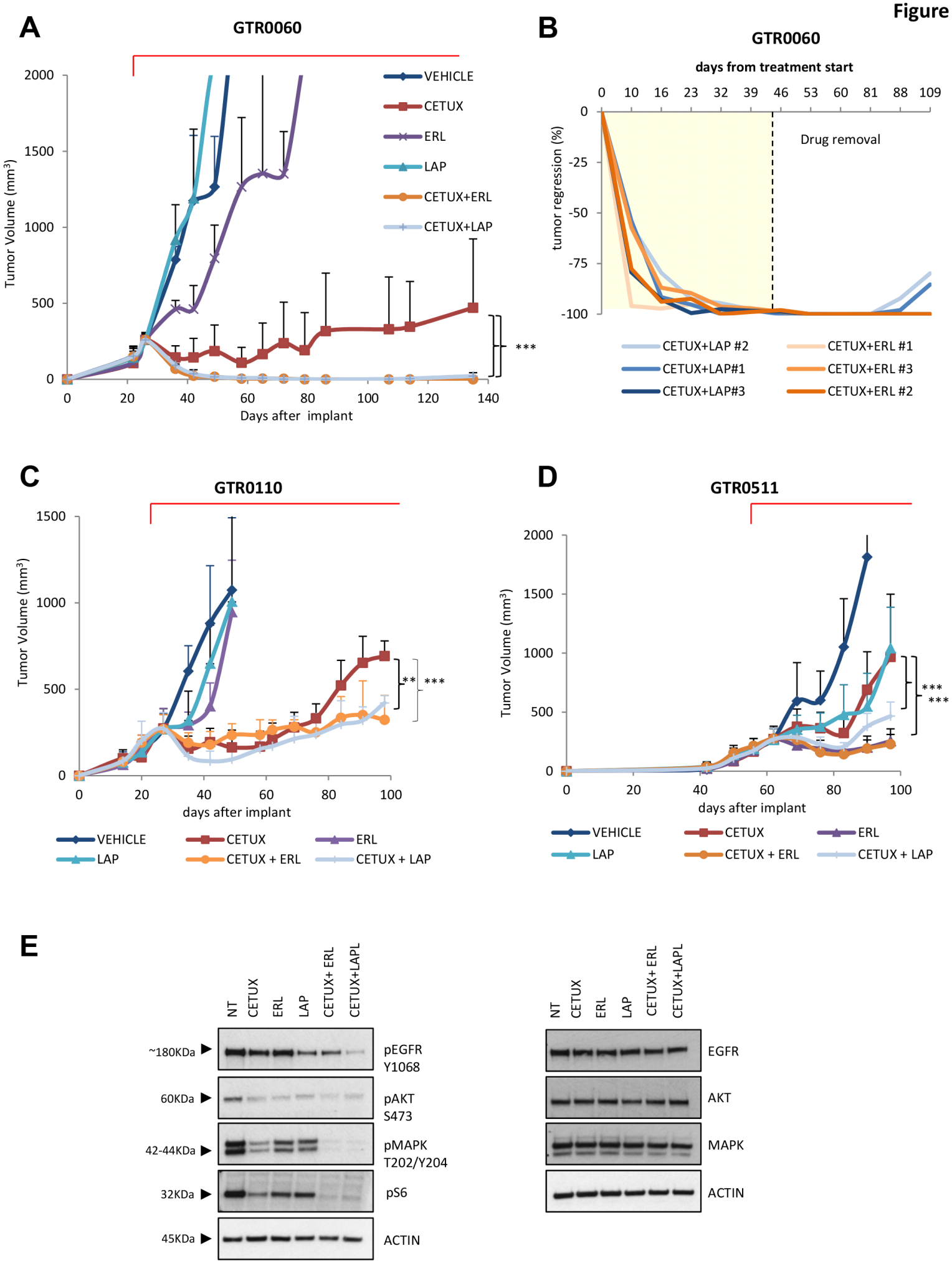
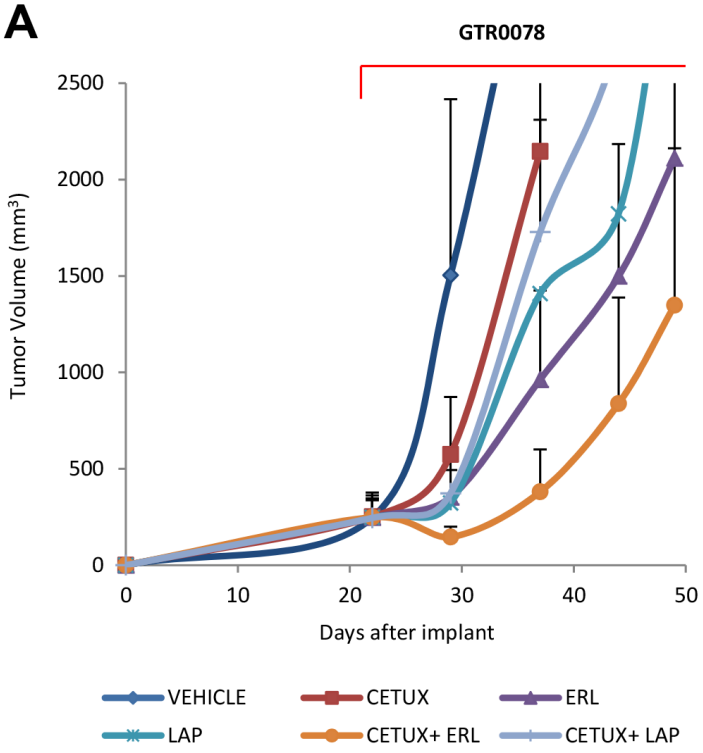

MET amp
HER2 amp
KRAS amp
KRAS mut
PIK3CA mut
PTEN alteration
19 4 1 0 1 1
0 59 5 2 1 1
0 0 73 3 1 0
0 0 0 13 1 0
0 0 0 0 13 0
0 0 0 0 0 9
MET amp
HER2 amp
KRAS amp
KRAS mut
PIK3CA mut
PTEN alteration

Figure 5


A
GTR0060
Tumor Volume (mm³)
Days after implant
VEHICLE
CETUX
ERL
LAP
CETUX+ERL
CETUX+LAP
***
B
GTR0060
days from treatment start
tumor regression (%)
Drug removal
CETUX+LAP #2
CETUX+LAP#1
CETUX+LAP#3
CETUX+ERL #1
CETUX+ERL #3
CETUX+ERL #2
C
GTR0110
Tumor Volume (mm³)
days after implant
**
***
VEHICLE
CETUX
ERL
LAP
CETUX + ERL
CETUX + LAP
D
GTR0511
Tumor Volume (mm³)
days after implant
***
***
VEHICLE
CETUX
ERL
LAP
CETUX + ERL
CETUX + LAP
E
NT
CETUX
ERL
LAP
CETUX+ ERL
CETUX+LAPL
~180KDa
pEGFR Y1068
60KDa
pAKT S473
42-44KDa
pMAPK T202/Y204
32KDa
pS6
45KDa
ACTIN
EGFR
AKT
MAPK
ACTIN

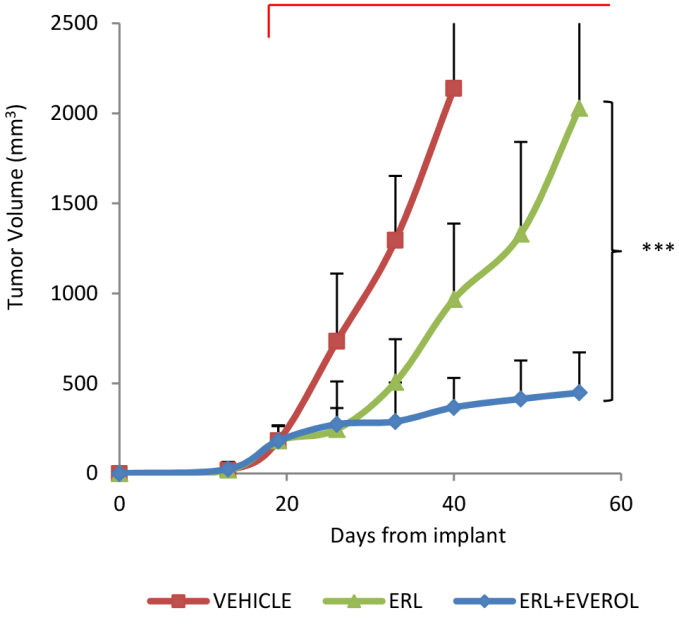
A



B

Hugo Symbol	Protein Change	cDNA Change	Variant Classification	dbsnp Site	Allele Fraction
TSC2	p.M1300V	c.3898A>G	Missense	NOVEL	0,463918
TSC2	p.R1438Q	c.4313G>A	Missense	COSMIC	0,539823

C



D