Optimized EGFR Blockade Strategies in EGFR Addicted Gastroesophageal Adenocarcinomas

Simona Corso1,2, Filippo Pietrantonio3,4, Maria Apicella2, Cristina Migliore1,2, Daniela Conticelli1,2, Annalisa Petrilli2, Laura D’Errico1,2, Stefania Durando6, Daniel Moya-Rull2, Sara E. Bellomo2, Stefano Ughetto1,2, Maurizio Degiuli5, Rossella Reddavid5, Uberto Fumagalli6, Stefano De Pascale6, Giovanni Sgori7, Emanuele Rausa7, Gian Luca Baiocchi8, Sarah Molfino8, Giovanni De Manzoni9, Maria Bencivenga9, Salvatore Siena4,10, Andrea Sartore-Bianchi4,10, Federica Morano5, Salvatore Corallo8, Michele Prisciandaro3, Maria Di Bartolomeo3, Annunziata Gloghini11, Silvia Marsoni2, Antonino Sottile7, Anna Sapino2,12, Caterina Marchiò2,12, Asa Dahle-Smith13, Zosia Miedzybrodzka14, Jessica Lee15, Siraj M. Ali15, Jeffrey S. Ross15,16, Brian M. Alexander15, Vincent A. Miller15, Russell Petty17, Alexa B. Schrock18, and Silvia Giordano1,2

ABSTRACT

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

Experimental Design: We performed analyses on four cohorts: IRCC (570 patients), Foundation Medicine, Inc. (9,397 patients), COG (214 patients), and the Fondazione IRCCS Istituto Nazionale dei Tumori (206 patients). Preclinical trials were conducted in patient-derived xenografts (PDX).

Results: The analysis of different gastroesophageal adenocarcinoma patient cohorts suggests that EGFR amplification drives aggressive behavior and poor prognosis. We also observed that EGFR inhibitors are active in patients with EGFR copy-number gain and that coamplification of other receptor tyrosine kinases or KRAS is associated with worse response. Preclinical trials performed on EGFR-amplified gastroesophageal adenocarcinoma PDX models revealed that the combination of an EGFR mAb and an EGFR tyrosine kinase inhibitor (TKI) was more effective than each monotherapy and resulted in a deeper and durable response. In a highly EGFR-amplified nonresponding PDX, where resistance to EGFR drugs was due to inactivation of the TSC2 tumor suppressor, cotreatment 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 mAb 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.

Introduction

Gastric and gastroesophageal adenocarcinomas 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).
In addition, a prespecified subgroup analysis of the COG trial showed a positive correlation between cetuximab clinical activity of EGFR mAbs in patients bearing a 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 (TKI) 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 an EGFR mAb and a TKI as the most effective treatment.

Translational Relevance
Prior clinical trials performed in unselected patients with gastroesophageal adenocarcinoma failed to show survival improvement upon treatment with anti-EGFR therapies. We report the clinical activity of EGFR mAbs in patients bearing a 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 (TKI) 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 an EGFR mAb and a TKI as the most effective treatment.

The molecular landscape of gastroesophageal adenocarcinoma 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 (RTK). EGFR amplification has been reported in 3%–5% of gastroesophageal adenocarcinomas (4, 6), while other genetic alterations (such as point mutations or translocations) are extremely uncommon. Several EGFR-targeting drugs, comprising mAbs and tyrosine kinase inhibitors (TKI), 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 RCTs 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 gastroesophageal adenocarcinoma 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 FOLFIRI chemotherapy in patients with mGEA (12). In addition, a prespecified subgroup analysis of the COG trial showed that patients with esophageal and gastroesophageal junction carcinomas bearing EGFR CNG derived a significant progression-free survival, OS, and health-related quality of life benefit from gefitinib compared with 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 gastroesophageal adenocarcinomas, 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: Candido Cancer Institute-FPO, IRCCS (Torino, Italy), Ordine Mauriziano Hospital (Torino, Italy), San Giovanni Battista Hospital (Torino, Italy), San Luigi Gonzaga Hospital (Torino, Italy), Humanitas-IRCCS (Milano, Italy), San Raffaele Hospital (Milano, Italy), Treviglio-Caravaggio Hospital (Bergamo, Italy), Brescia Hospital (Brescia, Italy), Borgo-Trento Hospital (Verona, Italy), Santa Maria delle Scotte Hospital (Siena, Italy), Forlì Hospital (Forlì, Italy), Fondazione Macchi Hospital (Varese, Italy), Pisa Hospital (Pisa, Italy), Fondazione IRCCS Istituto Nazionale dei Tumori (Milano, Italy), and Ospedale Niguarda Ca’ Granda (Milano, Italy). 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 performed in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonisation and Good Clinical Practice guidelines, and General Data Protection Regulation. Clinical and pathologic data were entered and maintained in our prospective database. All the samples were anonymized before being shipped to Candido. No reference to the patients can be inferred from the histologic and molecular characterization presented in the work.

Foundation Medicine, Inc.

Tumor samples from patients with gastroesophageal adenocarcinoma were submitted during routine clinical care for comprehensive genomic profiling (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 the ATCC and OE21 from Sigma-Aldrich. The genetic identity of the cell lines was confirmed by short tandem repeat profiling (Cell ID, Promega). Erlotinib and everolimus were purchased from Carbosynth. Cetuximab and lapatinib were provided by the hospital pharmacy.

Primary cell cultures and organoids

Gastroesophageal adenocarcinoma primary cells were derived from patient-derived xenografts (PDX) as described in (14), while gastroesophageal adenocarcinoma primary organoids were obtained as described in (15). The genetic identity of the in vitro–derived material with the original tumor was verified by short tandem repeat profiling (Cell ID, Promega). GTR0078 cell cultures 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: 100 nmol/L lapatinib or erlotinib for 2 hours and 0.5 μg/mL cetuximab for 16 hours. Whole-protein extracts were prepared using Laemml buffer and quantified using the BCA Protein Assay Kit (Pierce). EGFR immunoprecipitation was performed with cetuximab on organoids (stimulated with 100 ng/mL EGFR for 15 minutes, treated or not with erlotinib 100 nmol/L for 2 hours) previously washed out from Matrigel with Cell Recovery Solution (#354253, Corning) and lysed with EB (1% Triton, 20 mmol/L Tris-HCl pH 7.4, 5 mmol/L EDTA pH 8, 10% glycerol, and 150 mmol/L NaCl). Primary antibodies, anti-EGFR (1005: sc-03) and anti-Actin, were from Santa Cruz Biotechnology, and antibodies against phosphorylated EGFR (Tyr 845), ERK (Thr202/Tyr204), phosphorylated AKT (Ser473) (Clone D9E), total AKT, and ERK were from Cell Signaling Technology. Antibody against phosphorylated
EGFR (Tyr1068) (ab5644) was from Abcam. Antibody directed against amino acid 1,172–1,186 of human EGFR was described in (16). Antibody anti-EGFR extracellular epitope (11.6 antibody) was from Thermo Fisher Scientific. Secondary antibodies were from Amersham. Detection was performed with ECL System (Amersham).

Transfection and transduction procedures
OE21 cells were transduced with siRNAs using Lipofectamine 2000 (Thermo Fisher Scientific). Transfection reagents plus siRNAs at final concentration of 20 nmol/L were used following standard protocols. Seventy-two hours after transfection, cells were lysed and Western blotting was performed. TSC2 silencing was achieved by using SMARTpool ON-TARGETplus siRNA (Dharmacon).

Lentiviruses were produced as described in (17). OE21 cells were transduced with a pool of lentiviral particles containing four TSC2 silencing short hairpin RNAs (shRNA; Sigma, #40179, #40178, #40454, and #40555). Cells were selected with puromycin, checked for TSC2 silencing, and subcutaneously injected in NOD/SCID mice (5 × 10^6 cells/mouse) in 1:1 SF medium:Matrigel (Corning).

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

Copy-number variation evaluation by qRT-PCR
Quantitative PCR experiments for estimation of EGFR, MET, FGFR2, and KRAS copy-number variations (CNV) were performed in triplicates using 2 ng total gDNA as a template, with the following primer sets:

- EGFR assay, ID Hs02876245_c1; for HER2 assay, ID Hs00492325_c1; for MET assay, ID Hs04993403_c1; for FGFR2 assay, ID Hs01472955_c1; for KRAS assay, ID Hs01472955_c1; and the TaqMan Copy-number Reference Assay RNase P 4316831 and GREB1 Hs01738470_cn (Applied Biosystems). 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/PIK3CA/PTEN mutations and EGFR/MET/KRAS amplifications) able to predict primary resistance to trastuzumab therapy in patients with HER2-positive metastatic gastric cancer (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: 100 nmol/L lapatinib or erlotinib for 2 hours and 0.5 μg/mL cetuximab for 16 hours. The analysis of the phosphorylation profiles of kinases was performed using the Human Phospho-Kinase Antibody Array (R&D Systems), according to the manufacturer’s instructions. Signal quantification was performed using Image Lab 5.2.1 Software (Bio-Rad).

PDX generation
Gastric PDX generation was performed as described in (19). All animal procedures adhered to the “Animal Research: Reporting of In Vivo Experiments” standards and were approved by the Ethical Commission of the Candido Cancer Institute (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) orally; cetuximab 20 mg/kg, i.p., twice weekly; lapatinib 100 mg/kg, daily, orally; erlotinib 50 mg/kg, daily, orally; and everolimus 6 mg/kg, daily, orally. Tumor size was evaluated once weekly by caliper measurements and approximate volume of the mass was calculated by using the formula 4/3π(D1/2)(D2/2)^2, where D is the minor tumor axis and D is the major tumor axis. The response in mice was evaluated using RECIST 1.1–like criteria, that is, progressive disease (PD): ≥35% increase from baseline, partial response (PR): ≥50% reduction from baseline; and stable disease (SD): intermediate variations from baseline (20). Statistical testing for pharmacologic 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).

Foundation Medicine, Inc. samples
CGP was performed in a Clinical Laboratory Improvement Amendments–accredited, New York State and College of American Pathologists–accredited laboratory [Foundation Medicine, Inc. (FM)]. In brief, ≥50 ng DNA was extracted from 40 μm 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 cancer-related genes from 180 to 395 plus select introns from 14 to 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 described previously (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’s recommended protocol. DNA was extracted from histologically confirmed esophageal and gastroesophageal junction adenocarcinomas as described previously (13) and quantified using the Quant-IT PicoGreen dsDNA Assay Kit (Life Technologies) following the manufacturer’s recommended protocol, using 80 ng for each case, normalized to a concentration 12 ng/μL. Array fluorescence intensity data (CEL files), generated by Affymetrix GeneChip Command Console 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 normalized and genome-wide allele-specific copy number was assessed using the Affymetrix TusScan algorithm to allow adjustment for both tumor ploidy and nonaberrant cell admixture (24). Genome-wide
CNV was assessed across all cases using Affymetrix Nexus express for OncoScan (version 3.1). Significant CNV events across the genome were identified using a “significance testing for aberrant copy number” (STAC) approach (25).

The Fondazione IRCCS Istituto Nazionale dei Tumori samples

FFPE 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 was determined by silver in situ hybridization (SISH) analysis and KRAS/GCN gain was assessed by PCR, as described previously 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 Fondazione IRCCS Istituto Nazionale dei Tumori (INT) dataset] until the date of death or last follow-up for alive patients. The OS curves for *EGFR*-amplified versus nonamplified subgroups were calculated with the Kaplan–Meier method and compared with the log-rank test. Survival analysis for COG was undertaken using IBM SPSS statistics 22, for further details see (10, 13).

ISH and IHC

*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) that recognizes the internal domain of EGFR and the monoclonal mouse anti-human anti-EGFR (E30) antibody (Dako) 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 phosphorylated EGFR was performed using anti-phosphorylated EGFR Y1173 53AS from Cell Signaling Technology.

Transcriptome profiling

RNA sequencing (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). Nonhost 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 patients with gastroesophageal adenocarcinoma

We evaluated *EGFR* copy number in four different cohorts: (i) a proprietary cohort (IRCC cohort) of 570 primary gastroesophageal adenocarcinomas (real-time PCR analysis), (ii) the FMI dataset of 4,337 gastric and 5,060 esophageal/gastroesophageal junction adenocarcinomas (CGP), (iii) the subgroup of 214 patients with esophageal or gastroesophageal junction adenocarcinoma enrolled in the COG trial (NCT01243398) of second-line gefitinib versus placebo (ref. 10; FISH), and (iv) the Fondazione IRCCS INT of Milan dataset of 206 patients with mGEA (ISH and 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; ref. 30) and eight of them (1.4% of all samples) bearing a heterogeneous *EGFR* amplification (one tumor area ≥8 copies and one tumor area ≤8 copies; Fig. 1, Supplementary Table S1). In the FMI dataset, 3.4% of gastric and 7.6% of esophageal carcinomas showed *EGFR* amplification equal or higher than eight copies, while in the COG and INT datasets, the frequencies of *EGFR* amplification were 7% and 4.9%, respectively (Fig. 1). In both COG and INT cohorts, no significant association between *EGFR* amplification and baseline clinicopathologic characteristics was observed (Supplementary Tables S2 and S3).

*EGFR* amplification drives aggressiveness and poor prognosis in gastroesophageal adenocarcinomas

To investigate whether *EGFR* amplification is associated with poor prognosis of gastroesophageal adenocarcinomas, we took advantage of a cohort of pretreated patients with esophageal and gastroesophageal junction 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 with those without *EGFR* amplification [3.1 vs. 3.5 months; HR, 1.23; 95% confidence interval (CI), 1.03–1.48; *P* = 0.026, Fig. 2A, left]. 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 with those with *EGFR* SISH–negative tumors (17 vs. 18.9 months; HR, 1.95; 95% CI, 0.90–4.21; *P* = 0.083; Fig. 2A, right). These results have also been confirmed in primary gastric tumors by analyzing The Cancer Genome Atlas (TCGA) data, in which tumor *EGFR* amplification correlated with significantly inferior OS and disease-free survival (Fig. 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* mAb, panitumumab, as single agent after failure of standard treatment options. Three patients with *EGFR* amplification, confirmed by SISH, were identified (Supplementary Fig. S1A); their molecular profile is summarized in Supplementary Fig. S1B and their clinical history is reported in Fig. 3. Briefly, INT#001 patient had KRAS-coamplified mGEA and showed PD at the first radiological reassessment, INT#002 patient had no cooccurring alterations in *HER2*, *MET*, *KRAS*, or *PIK3CA* and showed a PR lasting 6 months, and INT#003 patient had cooccurring heterogeneous *KRAS* amplification and showed a PR lasting only 10 weeks, followed by rapid clinical progression and death.

To verify whether RTK pathway activation is associated with *EGFR* inhibitor resistance in gastroesophageal adenocarcinoma, we
investigated the relationship between RTK CNG and survival following treatment with gefitinib in 12 EGFR FISH-positive gastroesophageal adenocarcinomas (seven with amplification and five with high polysomy) of the COG trial. All 12 tumors analyzed had CNG (defined as ≥4 gene copies) of at least one RTK (HER2, HER3, HER4, MET, FGFR1, FGFR2, FGFR3, IGF1R, PDGFR2, VEGFR1, VEGFR2, and VEGFR3). We found a significant inverse correlation between the extent of coamplification of the RTKs and OS (Fig. 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 cooccurrence of other genomic events in 53% of samples (Supplementary Fig. S2). Finally, because the available in silico datasets mainly represent a collection of primary gastroesophageal adenocarcinomas, we investigated the prevalence of AMNESIA panel alterations in the 534 samples from patients with EGFR-amplified mGEA included in the FMI dataset. This analysis showed the cooccurrence of other genomic events of interest in 186 (35%) samples (Fig. 4B).

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

Future trials might be prompted to reassess the role of anti-EGFR mAbs and TKIs, either as monotherapy or in combination, in molecularly selected patients with gastroesophageal adenocarcinoma. 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 mAb plus a TKI may be more effective than each drug as monotherapy.

A large series of human cancer specimens transplanted into mice (PDX) produce a study population that can be randomized for prospective treatment with targeted agents and thus, provides 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

![Figure 1.](image_url)
carcinoma samples (IRCC cohort), we generated a multi-level platform of gastroesophageal adenocarcinoma 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 gastroesophageal adenocarcinoma displaying HER2 or MET amplification suggested that the clinically relevant threshold is higher than eight gene copies (30, 35). Eleven PDXs harbored at least 4–8 EGFR copies and four PDXs had >8 EGFR copies (Supplementary Fig. S3A, GTR0060: ∼240 EGFR copies; GTR0078: ∼700 copies; GTR0110: 12 copies; and GTR0511: ∼80

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 OS (left) and the disease-free survival (right) of patients of the gastroesophageal TCGA dataset, related to EGFR CNG.

Corso et al. Clin Cancer Res; 2021 CLINICAL CANCER RESEARCH
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 and SD according to RECIST 1.1. 5FU, 5-fluorouracil; CCDP, cisplatin, vinorelbine, ifosfamide, and epirubicin; EOX, epirubicin, oxaliplatin, and capecitabine; FOLFIRI, folinic acid, 5-fluorouracil, and irinotecan; OGD, esophago-gastro-duodenoscopy; TCF, docetaxel, carboplatin, and 5-fluorouracil; XELOX, capecitabine and oxaliplatin.
These four models did not bear any other RTKs/KRAS CNV >8 copies (data not shown). SISH analysis and IHC confirmed uniform EGFR amplification and expression (Supplementary Fig. S3B). These PDX models were expanded to generate cohorts of mice to evaluate the efficacy of the EGFR mAb, cetuximab, and the TKIs, erlotinib (EGFR selective) and lapatinib (dual EGFR/HER2 inhibitor), as well as the combination of the mAb with a TKI. The original tumors were serially passaged in vivo until 6 tumor-bearing animals were produced per experimental group. When xenografts reached an average tumor volume of approximately 250 mm³, mice were randomized into six independent treatment cohorts: (i) vehicle (placebo), (ii) cetuximab, (iii) erlotinib, (iv) lapatinib, (v) cetuximab + erlotinib, and (vi) cetuximab + lapatinib. Tumor response was evaluated according to RECIST-like criteria (see Materials and Methods and figure legends).
As shown in Fig. 5A, the GTR0060 PDX (240 EGFR copies) did not exhibit response to either of the TKIs used as monotherapy, but showed PR upon cetuximab treatment. Notably, both the combination (cetuximab + TKIs) treatments resulted in a complete response (CR). Interestingly, in 4 of 6 mice in the combination arms, including 3 of 3 mice treated with erlotinib + cetuximab, the tumor mass did not reappear even after more than 2 months of drug removal (Fig. 5B).

Improved efficacy of the combination 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 ( Supplementary Fig. S3B). While neither erlotinib nor lapatinib resulted in a clinical response and cetuximab conferred disease stabilization, cetuximab plus TKI combination treatment resulted in a PR (Fig. 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 mAb 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 therapy. 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 therapy.

To investigate which pathways were inactivated by the different drugs/drug combinations in cases in which the combination resulted in 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 Supplementary Fig. S4B, 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 was much lower in GTR0511 compared with 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 versus 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 combination resulted in a strongly enhanced response, we took advantage of PDX-derived primary cells in which EGFR amplification was maintained (Supplementary Fig. S5A). 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 (Fig. 5E). Phospho-array analysis of cellular kinases and RTKs confirmed these results, but did not identify any other kinase specifically inhibited by the combination treatments (Supplementary Fig. S5B). These in vitro data strongly support the results we obtained in the in vivo experiments where cetuximab induced SD, while the two combinations 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 described previously, using the GTR0078 PDX harboring approximately 700 EGFR copies (Supplementary Fig. S3). Despite the very high level of EGFR amplification, we did not observe response to the TKIs, nor to cetuximab or cetuximab + TKI combination treatments (Fig. 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 (Supplementary Fig. S6A). Moreover, we also observed two missense TSC2 mutations (p.M1300V and p.R1438Q), with an allelic frequency of 0.463 and 0.539, respectively (Fig. 6B). The TSC2 protein forms a complex with TSC1, a critical negative regulator of mTOR complex (mTORC) 1, which controls anabolic processes to promote cell growth (37–39). TSC2 inactivation (due to homozygous mutations or gene loss) results in increased mTOR activation (40).

Interestingly, when we interrogated the BioPortal for the possible cooccurrence of EGFR and TSC2 functional genomic alterations in six gastric cancer datasets (4, 41–45), we found a significant correlation (Supplementary Fig. S6B). Moreover, alterations in the mTOR pathway cooccurred with EGFR CNG have been identified in the FMI dataset as well, although cooccurrence with EGFR amplification was uncommon (Supplementary Fig. S6C).

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 (Supplementary Fig. S7A). To validate these data in vivo, we transduced OE21 cells with either control shRNA (shC) or a pool of TSC2 shRNAs and we injected them in immunocompromised mice. As shown in Supplementary Fig. S7B, 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 whether treatment of GTR0078 tumors with an mTOR inhibitor (such as everolimus) could restore sensitivity to EGFR inhibitors. While treatment with everolimus alone did not show any clinical efficacy (Supplementary Fig. S6D), the combination of everolimus with cetuximab resulted in a significant clinical response (Fig. 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 (Fig. 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 RCTs (8, 9). Similar negative results were also observed when the
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), and 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, that is, PD: ≥35% increase from baseline; PR: ≥50% reduction from baseline; and SD: intermediate variations from baseline. B, Spaghetti plot illustrating drug response in the xenotrial performed on the cohort of mice derived from GTR0060 PDX. Individual lines represent, for each mouse, the percentage variation in tumor burden, from start of treatment (day 0). Blue lines, cetuximab + lapatinib–treated mice and red lines, cetuximab + erlotinib–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 (∗∗, P < 0.01; ∗∗∗, P < 0.001).
small-molecule TKI, gefitinib, was compared with placebo from the second-line setting and beyond (10). Sporadic responses to EGFR inhibitors observed in these trials, however, led several researchers to postulate the existence of a subset of metastatic patients with EGFR-addicted tumors, potentially vulnerable to EGFR blockade (13). The amplification of the EGFR gene is found in 3%–5% of primary gastroesophageal adenocarcinoma 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 nonmetastatic setting, by analyzing TCGA data. In a prespecified exploratory analysis of one of those datasets, the COG trial, randomizing 209 chemoresistant metastatic patients to gefitinib or placebo (10), found that EGFR amplification was a positive

Figure 6.

TSC2 inactivation is a mechanism of resistance to EGFR-targeted therapies. A, Tumor growth curves in the mice cohorts derived from GTR0078 and treated with the EGFR inhibitors, cetuximab (CETUX), erlotinib (ERL), and lapatinib (LAP), alone or in combination, as indicated. The red line 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 and treated with erlotinib or the combination 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 (***, $P < 0.001$).
predictive marker for EGFR targeting, whereas a smaller advantage was observed in patients with chromosome 7 polysomy (13). Response to the anti-EGFR mAb, cetuximab, used alone or in combination with chemotherapy, was reported in a small set of 7 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 gastroesophageal adenocarcinomas. On the other hand, in these EGFR-amplified tumors, we observed the presence of selected cooccurring driver alterations. Specifically, MET/HER2/KRAS coamplifications and KRAS/PIK3CA/PTEN comutations were identified in 53% and 35% of patients in the EGFR-amplified subgroups included in TCGA and the 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 gastroesophageal adenocarcinomas. TSC2 is a GTPase-activating protein, whose loss or inactivating mutation results 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 gastroesophageal adenocarcinomas, possibly indicating that mTORC constitutive activation can sustain the oncogenic role of EGFR. Our preclinical trial in an EGFR-amplified/TSC2-mutated gastroesophageal adenocarcinoma PDX confirms this hypothesis. The pharmacologic 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 article from Arteaga and colleagues (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 and colleagues (50) showed that glioblastoma cells can become resistant to erlotinib by 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-hyperamplified 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 gastroesophageal adenocarcinomas, we leveraged our large platform of 151 primary gastroesophageal adenocarcinomas patient-derived mouse avatars (22), enriched for 15 cases with EGFR gene copy gain, including four avatars with more than eight EGFR copies (confirmed as amplified, i.e., nonpolysomic, by silver ISH). EGFR inhibition, in absence of chemotherapy, resulted in a clinical response in three of four cases. Notably, one of these cases featured 12 EGFR copies, a range of amplification that is just above the threshold (eight copies) considered biologically relevant and that has not been investigated previously (48). Interestingly, a CR was achieved only in the PDX with the highest EGFR CNVs, 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 gastroesophageal adenocarcinoma and breast cancer (30, 53).

The pharmacologic 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 gastroesophageal adenocarcinoma 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 gastroesophageal adenocarcinoma, 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 gastroesophageal adenocarcinomas may not be at a dead end. The combination of lapatinib and cetuximab has already been proven safe in a phase I trial (56), potent second-generation antibodies mixtures against different, nonoverlapping 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 gastroesophageal adenocarcinoma populations, broad-based genomic profiling is thus necessary to reliably detect EGFR gene amplification in addition to other potential drivers and mechanisms of resistance.

Authors’ Disclosures

S. Corso reports grants from Italian Association for Cancer Research (AIRC) during the conduct of the study. F. Pietrantonio reports personal fees from Amgen, Roche, Sanofi, Bayer, Servier, and Merck-Serono and grants from BMS outside the submitted work. S. Siena reports other from AstraZeneca, Daiichi-Sankyo, Seattle Genetics, Merck, and CheckMab outside the submitted work. A. Sartore-Bianchi reports personal fees from Amgen, Bayer, Sanofi, and Servier outside the submitted work. F. Morano reports other from Servier outside the submitted work.
work. Z. Miedzybrodzka reports grants from University of Aberdeen development trust and nonfinancial support from Chief Scientist Office Scotland during the conduct of the study, as well as grants from Sanofi, Agen, Chief Scientist Office, and Akcea outside the submitted work; AstraZeneca funded costs of gefitinib in the original TRANSOC study (more than 3 years ago); and Stratified Medicine Scotland funded development work that led to this project, more than 3 years ago, CRUK funded the COG trial more than 3 years ago, and Chief Scientist Office Scotland funded TRANS-COG (translation part of COG) more than 3 years ago. J. Lee reports personal fees from Foundation Medicine and Roche during the conduct of the study and outside the submitted work. S.M. Ali reports personal fees from Foundation Medicine; and personal fees from Eli Lilly, P. Scotland funded development work that led to this project, more than 3 years ago, and personal fees from EQRx, and Revolution Medicines outside the submitted work. S.M. Ali reports personal fees from Foundation Medicine during the conduct of the study and outside the submitted work. J.S. Ross reports personal fees from Foundation Medicine during the conduct of the study. B.M. Alexander reports personal fees from Foundation Medicine, Roche, and Takeda during the conduct of the study. V.A. Miller reports other from Foundation Medicine, EQRx, and Revolution Medicines outside the submitted work, and had a patent 8501413 issued and with royalties paid from Memorial Sloan Kettering Cancer Center. R. Petty reports grants and personal fees from BMS and AstraZeneca; grants from Roche, MSD, Merck Serono, Clovis, Jansen, Five prime Therapeutics, and Jansen; and personal fees from Eli Lilly, Pfizer, Sanofi, and Servier outside the submitted work. A.B. Schrock reports personal fees from Foundation Medicine and other from Roche during the conduct of the study. No disclosures were reported by the other authors.

Authors’ Contributions

S. Corso: Conceptualization, formal analysis, supervision, funding acquisition, writing—original draft, writing—review and editing. F. Pietranieto: Conceptualization, resources, supervision, funding acquisition, writing—original draft, writing—review and editing. M. Apicella: Conceptualization, resources, supervision, investigation, writing—original draft, writing—review and editing. C. Migliore: Resources, data curation, formal analysis, investigation, writing—review and editing. D. Conticelli: Resources, formal analysis, investigation, writing—review and editing. A. Petrelli: Resources, formal analysis, validation, investigation, writing—review and editing. D. D’Errico: Resources, validation, investigation, writing—review and editing. L. D’Errico: Resources, validation, investigation, writing—review and editing. S. Durando: Resources, validation, investigation, writing—review and editing. D. Moya-Rull: Resources, data curation, formal analysis, validation, investigation, writing—review and editing. S.E. Bellomo: Resources, data curation, formal analysis, investigation, writing—review and editing. S. Ughetto: Resources, data curation, validation, investigation, writing—review and editing. M. Degiuli: Resources, data curation, writing—review and editing. R. Reddavid: Resources, data curation, writing—review and editing. U. Fumagalli: Resources, data curation, writing—review and editing. S. De Pascale: Resources, data curation, writing—review and editing. G. Sgroi: Resources, data curation, writing—review and editing. E. Bausa: Resources, data curation, writing—review and editing. V.A. Miller: Conceptualization, data curation, supervision, funding acquisition, writing—original draft, project administration, writing—review and editing. S. Giordano: Conceptualization, the submitted, formal analysis, supervision, funding acquisition, investigation, writing—original draft, project administration, writing—review and editing. S. De Pascale: Resources, data curation, writing—review and editing. S. Molfino: Resources, data curation, writing—review and editing. G. De Manzonieri: Resources, data curation, writing—review and editing. M. Benveni: Resources, data curation, writing—review and editing. Z. Miedzybrodzka: Data curation, writing—review and editing. S. Ross: Data curation, writing—review and editing. V.A. Miller: Data curation, writing—review and editing. R. Petty: Data curation, writing—review and editing. A.B. Schrock: Conceptualization, data curation, supervision, funding acquisition, writing—original draft, project administration, writing—review and editing. S. Giordano: Conceptualization, formal analysis, investigation, writing—review and editing. A. Sapino: Resources, formal analysis, supervision, funding acquisition, writing—original draft, project administration, writing—review and editing.

Acknowledgments

This work was funded by the Italian Association for Cancer Research, I.G.2010 (to S. Giordano), 21770 (to S. Corso), and I.G.2634 (to F. Pietranieto); Fondazione Piemontese per la Ricerca sul Cancro (FPRC, ONLUS) 5 × 1000 Min. Salute 2013 (to A. Sottile); FPRC 5 × 1000 2014 Min. Salute (to S. Giordano and A. Sapino); FPRC 5 × 1000 2015 Min. Salute (to S. Giordano and A. Sapino); FPRC 5 × 1000 2015 Min. Salute “Strategy” (to S. Giordano); Ricerca Corrente 2019, Min. Salute (to A. Sapino). We thank Dr. Adam Bass for performing sequencing analysis of IRCC samples; our colleagues of GIRC (“Gruppo Italiano Ricerca Canceroma Gastroenterico”) for their support; G. Manessi for experimental help; B. Martinoglio, M. Buscarino, and M. Montone for technical support with real-time PCR and Cell-ID; I. Sarotto, D. Balmaiotto, E. Maldi, M. Volante, and A. Rigutto for pathologic analysis; animal facility employees; and L. Trusolino and A. Bertotti for helpful scientific discussion. S. Giordano and S. Corso are EuroOPDX Consortium members.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 13, 2020; revised December 4, 2020; accepted February 1, 2021; published first February 4, 2021.

References


