Establishment and characterization of esophageal squamous cell carcinoma patient-derived xenograft mouse models for preclinical drug discovery

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The purpose of this study was to establish and characterize patient-derived esophageal squamous cell carcinoma xenograft (PDECX) mice for utilization in antitumor drug discovery. A total of 96 esophageal squamous cell carcinoma (ESCC) tissues from Chinese patients were transplanted subcutaneously into immunodeficient mice. Histology, *EGFR*, *K-ras*, *B-raf*, and *PIK3CA* mutations, and *HER2* gene amplifications were analyzed in both patient tumors and mouse xenograft tissues using immunohistochemistry, mutant-enriched liquid chip sequencing and fluorescence *in situ* hybridization assays, respectively. Furthermore, *in vivo* efficacy studies using five PDECX mice harboring a variety of genetic aberrations were performed using the chemotherapy agents 5-fluorouracil (5-FU) and cisplatin. Thirty-seven PDECX mouse models were successfully established in immunodeficient mice. Pathological analysis revealed similar histological architecture and degrees of differentiation between patient ESCC and xenografted tumors. No mutations were identified in *EGFR*, *K-ras*, and *B-raf* genes in either xenograft models or patient ESCC tissues. In contrast, *PIK3CA* gene mutations were detected in 12.5% (12/96) ESCC patients and 18.9% (7/37) PDECX models. Interestingly, patient ESCC tissues exhibiting *HER2* overexpression or gene amplification were unable to survive in immunodeficient mice. Further analysis showed that PDECX models carrying *HER2* 2+ expression had no response to 5-FU/cisplatin, compared with *HER2*-negative models. In conclusion, a panel of PDECX mouse models, which include *PIK3CA* mutant and *HER2*-positive models, was established and characterized thus mimicking the current clinical genetic setting of esophageal carcinoma. The sensitivity of *HER2*-negative ESCC models to chemotherapy supports stratification approaches in the treatment of esophageal carcinoma patients and warrants further investigation of the impact of *PIK3CA* on treatment response.

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Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent cancers worldwide and occurs at a relatively high frequency (> 50 per 100 000 population) in some areas of China.^{1,2} Furthermore, it has been suggested that ethnic factors can contribute to esophageal cancer susceptibility.¹ ESCC is a complex disease, the mortality rate associated with ESCC is similar to the incidence rate due to diagnosis at a generally advanced stage, its aggressive characteristics, and a paucity of effective treatment strategies.³ In spite of its poor prognosis, however, ESCC has not been well studied to date.

Surgery remains one of the fundamental treatment strategies, however, the 5-year survival for nonmetastatic disease

is 20–40%.⁴ Patients with metastatic disease who are treated with palliative chemotherapy using a combination of 5-fluorouracil (5-FU) and cisplatin have a median survival of less than 1 year with response rates of 25–45% in phase II and III trials.^{3,5} Salvage options for patients with refractory ESCC are rare.⁶ Recently, neoadjuvant treatment approaches using a combination of chemotherapy and radiotherapy have been performed on locally advanced EC, with some improvements observed in clinical trials.^{7–9} Despite these limited improvements, the prognosis for these patients remains poor.¹⁰ In addition, the trimodal therapeutic model (use of cetuximab in combination with definitive

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chemoradiotherapy) appeared to offer hope in a phase IB/II clinical trial,¹¹ but failed in a later phase IIB/III clinical trial.¹² Furthermore, the efficacy of new therapeutic drugs remains to be tested in ESCC as most of the previous trials have included predominantly esophageal adenocarcinoma patients.^{13–19}

Despite some successes in the treatment of cancers,⁹ more novel rational therapies targeting specific cancer-related molecules are urgently needed. The increasing body of knowledge around molecular and genetic events involved in oncogenesis and tumor progression has led to the identification of new therapeutic targets and the synthesis of novel therapeutic agents. Animal models have been used in front-line preclinical studies for predicting the efficacy and possible toxicities of anticancer drugs in cancer patients.²⁰ Although the advent of cancer cell-line culture techniques fuelled an acceleration and expansion of cancer biology discovery that continues to this day, the harsh reality is that there is a high failure rate among new oncology agents when attempting to translate preclinical efficacy into clinical benefit. One of the most frequently cited reasons is the lack of preclinical models that recapitulate the heterogeneity of tumors in patients.^{21,22}

Human tumor fragments obtained from patients and directly transplanted into immunodeficient mice, known as primary xenografts or tumor grafts, are one category of recognized models used as tools for preclinical assays.^{23–28} Although the methods have been established for decades, these preclinical models are only now being consistently characterized and applied to drug development in oncology. There is a strong evidence that a greater genetic divergence exists between a primary tumor and the corresponding cell line derived from that tumor, *versus* a direct xenograft.^{22,29}

Future preclinical evaluation of these and other agents for ESCC requires the development of animal models of human ESCC. To obtain preclinical results with high predictive value for clinical trials, the choice of the tumor models on which new compounds and novel drug combinations are evaluated is critical.

In the present study, we used fresh patient ESCC tissues to establish patient-derived esophageal squamous cell carcinoma xenograft (PDECX) models in immunodeficient mice to support drug discovery and development.

MATERIALS AND METHODS

Patients and Tissue Samples

From March to August 2010 with the assistance of the Department of Thorax Surgery, Zhongshan Hospital, 96 patient primary ESCC tissues without any preoperative treatment were obtained intraoperatively during esophagectomy resection at Zhongshan Hospital. None of the patients received any therapies before surgery. Prior written informed consent was provided from all patients and the study protocol received Ethics Board approval at Zhongshan

Hospital, Fudan University. Where sufficient residual tumor tissue was available, histopathological examination and related analyses were performed. The tumor specimens were collected at surgery and divided into three parts for the following purposes: implantation into immunodeficient mice for xenograft model establishment, snap freezing in liquid nitrogen for DNA/RNA extraction, and fixed in 10% formalin buffer for 24 h and embedded into paraffin for immunohistochemical (IHC) analysis.

Clinical Data Collection

The diagnosis of ESCC was confirmed by histological analysis in all cases. Then, the following patient characteristics were collected for research study only, including: gender, age, tumor site (upper, middle, and lower of esophagus), histological grade, coagulative necrosis, nerve and vascular infiltration, mitotic index (number recorded as <50/10 HPF, or ≥50/10 HPF), lymph node metastasis, and stage as previously reported.⁴

Establishment of PDECX Mouse Models

Eight- to ten-week-old female nude (*nu/nu*) and the severe combined immune-deficient (SCID) mice (Vital River, Beijing, China) were used for xenograft model generation in this study. All experiments with immunodeficient mice were performed in accordance with the guidelines approved by IACUC. The PDECX models were established with fresh patient ESCC tissues, surgically removed from ESCC patients, as described in a previous study.³⁰ In brief, surgically removed patient's ESCC tissues (F0 tissue) were cut into fragments of approximately 15 mm³ in volume and implanted subcutaneously via Trocar needle into female SCID mice within 2 h after surgery. Patient tumor-implanted mice were observed daily until 90 days. Tumors were measured once a week by caliper when tumors started to grow up in mice. The ~500 mm³ xenografted ESCC tumors subsequently harvested from the mice were further implanted and expanded in nude mice. After three consecutive mouse-to-mouse passages, the xenograft was considered to be stable and was characterized. The xenografted tumor specimens in each passage of tumor-bearing mice were harvested and divided into three parts for the following purposes. The first was implanted into immunodeficient mice for the next generation of the xenograft model. The second part was used for DNA/RNA extraction by snap freezing in liquid nitrogen. The third part was fixed in 10% formalin buffer for 24 h and embedded into paraffin for IHC analysis. Fresh tumor fragments at passages 3–5 were frozen with 20% FCS in liquid nitrogen for further model recovery and utilization for drug discovery.

H&E and IHC Staining

Tissues from all PDECX models and the corresponding ESCC patient tumors were harvested and fixed in 10% buffered formalin within 30 min after resection according to our

previous study. Sections were stained with hematoxylin and eosin and reviewed by two pathologists to confirm the ESCC diagnosis. Dual cores from each tissue were made on tissue microarray (TMA) for IHC and fluorescence *in situ* hybridization analysis. For the IHC, all incubations were at room temperature and washes were performed with TBST. Antigen retrieval was performed in a pressure cooker at 110 °C for 5 min in retrieval buffer (S2367, DAKO). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (S2023, DAKO). Sections were then incubated with anti-HER2 antibody (Herceptest™, DAKO) for 30 min. Immunocomplexes were detected by incubation with DAKO K5204 for 30 min and were visualized with diaminobenzidine (K3468, DAKO) for 10 min. For HER2-negative cases, the experiment was repeated on the whole section in order to exclude heterogeneity. The IHC scoring criteria on ESCC followed Hoffman's criteria on human gastric cancer:³¹ no staining or <10% tumor cell-positive staining as 0/negative; faintly or barely perceptible staining on >10% tumor cell membrane as 1+; weak to moderate positive staining on >10% tumor cells as 2+; as tumor tissues on TMA mimic the biopsy samples, cohesive moderate to strong staining on the membranes were scored as 3+.

HER2 Fluorescence *In Situ* Hybridization Assay

TMA sections were dewaxed and dehydrated. Tissue pretreatment was performed according to the SpotLight Tissue pretreatment kit manual (Invitrogen, Cat #00-8401). After pretreatment, 10 µl HER2/CEP17 probes were applied onto each TMA slide, covered with coverslip and circled around the edge of coverslip with rubber cement (Mpbio, Cat #11FIX00125). Sections together with probes were codenatured at 75 °C for 4 min on OmniSlide *In-Situ* hybridization System, then incubated at 37 °C overnight. After codenaturation and incubation, probes were washed off sequentially with 0.3% NP40/1 × SSC at 75.5 °C for 3 min, and 0.3% NP40/2 × SSC at 75.5 °C for 2 min, 2 × SSC at room temperature 2 min, two times, then dehydrated sequentially in 70, 85 and 100% ethanol, and air dried. Sections were mounted with 0.3 µg/ml DAPI mounting medium (diluted from Vector, Cat #H-1200), and stored at 4 °C to avoid light for at least 30 min before scoring.

In each case, 50 tumor nuclei were evaluated. Cases showing average gene copy number of HER2 >6 or a gene/CEN17 fluorescence ratio ≥2 were considered gene amplified. Amplification was also defined in cases where HER2 signals formed a tight gene cluster. The above criteria are based on Hoffmann's criteria³¹ and related investigations.^{32,33}

Gene Mutation Screening

Formalin-fixed paraffin-embedded tumor blocks were reviewed for quality and tumor content. A single representative block, from either the patient primary tumor or

the xenograft model, containing at least 70% of neoplastic cells, was selected for gene analysis. Genomic DNA was extracted using the QIAamp Mini kit (Qiagen) according to the manufacturer's instructions. Based on the prevalence of gene mutations and molecular segmentation in other tumor types (as well as our previously published data; Hou *et al*),³⁴ 'hot spot' EGFR, K-ras, B-raf, and PIK3CA gene mutations were screened for using the mutant-enriched PCR method.³⁵ The method was performed by SurExam Bio-Tech Co. Ltd., Guangzhou Technology Innovation Base, Science City, Guangzhou, PRC. Positive results were further confirmed by PCR-direct sequencing. The primers for exon 9 of PIK3CA gene were 5'-GTCTTAGATTGGTTCTTTCCTG-3' for forward and 5'-GCATTTAATGTGCCAACTACC-3' for reverse, for exon 20 of PIK3CA gene were 5'-TTTGTCTACGA AAGCCTCTCTA-3' for forward and 5'-CCATCACTTTT CCTTCTCCAT-3' for reverse.

Antitumor Efficacy of Cytotoxic Agents

For *in vivo* efficacy studies, xenograft tumor-bearing nude mice (tumor volumes of 150–250 mm³) were sorted randomly and assigned to treatment groups (eight animals per group) or the vehicle control. The treatment groups received the chemotherapy drugs 5-FU/cisplatin (5-FU at 10 mg/kg/qd for 5 days a week intraperitoneally, and cisplatin at 5 mg/kg/qd/once a week on the first day of the week, intravenously). Subcutaneous xenografted tumors in nude mice and mice body weight were measured twice a week. Tumor volumes were calculated by measuring two perpendicular diameters with calipers. Tumor volumes (V) were calculated by using the formula: $V = (\text{length} \times [\text{width}]^2) / 2$. Percentage tumor growth inhibition (%TGI) was calculated using the formula: $\%TGI = [1 - (\text{change of tumor volume in treatment group} / \text{change of tumor volume in control group})] \times 100$ and was used for the evaluation of antitumor efficacy. Four stratification groups were defined based on criteria in previous publications:²⁴ (a) high responders, in which the treatment induced complete regressions (the established tumors being no longer palpable after treatment); (b) moderate responders, in which TGI was superior to 50%; (c) low responders, with TGI between 40 and 50%; and (d) the non-responders, in which the growth parameters were not significantly altered by the treatment.

Statistical Analysis

A χ^2 test was used for univariate analyses, cross-tabulations with qualitative variables were analyzed with the Fisher's exact, with a *P* value less than 0.05 recorded as significant in clinical data analysis. To evaluate statistical significance in our antitumor efficacy studies, the Student's *t*-test was used to compare TGI in treatment groups to the control group. Statistical tests were two sided, with *P* < 0.05 considered significant.

RESULTS

Establishment of Patient-Derived ESCC Xenograft Mouse Models

A total of 96 ESCC samples obtained from esophageal cancer patients were implanted subcutaneously into SCID mice, and growing xenograft tissues then implanted into nude mice starting from the second generation of xenograft model. Thirty-seven PDECX mouse models were established in consecutive passages in nude mice (Figure 1). The PDECX model success rate was 53.1% (51/96) in the first generation in SCID mice, and 92.2% (47/51), 89.4% (42/47), 88.1% (37/42) from second to fourth generations in nude mice, respectively. After the fifth generation in nude mice, PDECX mouse models became stable without further change in model survival. The overall survival rate of the PDECX models during model establishment was 38.5% (37/96; defined as the generation of a stable PDECX model in an immunodeficient mouse from initial implantation of primary patient ESCC tissue). All 37 established PDECX mouse models showed kinetic growth (data not shown), similar to

the growth patterns obtained in our previous studies.³⁰ Examination of autopsies in ESCC-bearing mice from these xenograft mouse models at 2–3 months post-implantation revealed no evidence of metastases in the brain, lung, liver, or kidney. All established PDECX models could be recovered in mice after freeze/thawing of xenograft tumor fragments using liquid nitrogen (data not shown). Pathology assessment by H&E staining and analysis by pathologist revealed similar morphologies in PDECX tissues compared with that of the corresponding patient primary ESCC tissues in most cases (Figure 2a).

Analysis of Potential Factors Affecting PDECX Model Establishment Success

Results of our correlation analyses between the success rates of PDECX model establishment and patient clinical parameters are listed in Table 1. Of all analyzed parameters, nerve invasion, necrosis, and HER2 amplification or over-expression in patient ESCC tissues showed correlative trends, but unfortunately did not reach statistical significance. ($P = 0.088, 0.118, 0.106$, respectively; Table 1). In 96 patient esophageal carcinoma tissues, 3 cases showed HER2 3+ expression by IHC analysis and 1 case showed *HER2* gene amplification. A total of 4.2% (4/96) cases were evaluated as *HER2* gene amplified or overexpressing. Surprisingly, none of the ESCC tissues showing *HER2* amplification or IHC 3+ expression were able to grow up in immunodeficient mice. Compared with *HER2* as a potential negative factor, *PIK3CA* mutation represents an unlikely factor in the successful establishment of PDECX mouse models (Table 1). A total of 41.6% (5/12) of patient ESCC tissues harboring *PIK3CA* mutation grew up in immunodeficient mice (Table 1).

Characterization of PDECX Mouse Models

In order to characterize our established PDECX mouse models at both gene and protein levels, we screened for the major known genetic aberrations in esophageal carcinoma, both in model tissues and patient tumors, and performed IHC analysis. The list included mutations in *PIK3CA*, *EGFR*, *K-ras*, *B-raf*, and expression of HER2 protein in our 37 established PDECX models and the corresponding patient ESCC tissues.

HER2 positivity (IHC score 1+, 2+ or 3+) was identified in 24.3% (9/37) of patient ESCC samples and 32.4% (12/37) of PDECX models. A total of 76% (28/37) of PDECX models showed consistent HER2 expression (positive or negative) compared with the corresponding patient ESCC tissues (Table 2 and Figure 2b).

Gene mutations within *EGFR* (exons 18, 19, 20, 21), *K-ras* (exons 2 and 3), *B-raf* (exons 11 and 15), and *PIK3CA* (exons 9 and 20) were screened for within all 37 established PDECX model and patient ESCC tissues. Interestingly, no mutations were detected in *EGFR*, *K-ras*, or *B-raf* in any of the samples. *PIK3CA* mutation, however, was identified in 13.5% (5/37) of patient ESCC tissues (Figure 3) and 18.9% (7/37) of PDECX

Figure 1 Success rates of PDECX model development at different passages.

Figure 2 Comparison of pathology and HER2 expression between patient ESCC tissues and corresponding PDECX models. In most cases, tumors show similar architecture, differentiation, and HER2 expression. Some variations in HER2 expression were observed in EC047, EC001, and EC104.

models. A total of 71% (5/7) of PDECX models showed consistent *PIK3CA* mutation when compared with the corresponding patient ESCC tissues. Interestingly, *PIK3CA* mutation in two additional PDECX models was not detected in the corresponding patient ESCC tissues.

PDECX Chemotherapy Anti-Tumor Efficacy Studies

To evaluate whether our PDECX mouse models responded to treatment with standard ESCC antitumor drugs, five available PDECX models were treated with the clinically used

cytotoxic agents, 5-FU and cisplatin (Figure 4). Our data demonstrated that two PDECX models with genetic aberrations (HER2 IHC 2+ in EC044; HER2 IHC 2+ and *PIK3CA* mutation in EC039) had no significant response to cytotoxic agents ($P > 0.05$). Interestingly, however, three PDECX mouse models with HER2 negativity and wild-type *PIK3CA* were sensitive to 5-FU/cisplatin (49% TGI in EC004, $P < 0.05$; 47% TGI in EC016, $P < 0.05$; and 84% TGI in EC054, $P < 0.001$). EC004 and EC016 were categorized as low responders, whereas EC054 was a moderate responder based on predefined response criteria.²⁴

Table 1 Clinical features of ESCC patients and *in vivo* tumor take rate (%) by univariate analysis based on 96 cases

Clinicopathological features	Patient number (n)	Successful xenograft (tumor take rate)	P value
Sex			0.639
Male	80	30 (38%)	
Female	16	7 (44%)	
Age at diagnosis (years)			0.824
<60	35	14 (40%)	
≥60	61	23 (38%)	
Tumor site			0.628
Upper + middle	34	12 (35%)	
Lower	62	25 (40%)	
Differentiation			0.831
G2	61	24 (39%)	
G3	35	13 (37%)	
Nerve invasion			0.088
Yes	34	17 (50%)	
No	62	20 (32%)	
Vascular invasion			0.722
Yes	19	8 (42%)	
No	77	29 (38%)	
Necrosis			0.118
Yes	58	26 (45%)	
No	38	11 (29%)	
Lymph node metastases			0.661
Yes	44	18 (41%)	
No	52	19 (37%)	
Mitoses (10 HPF)			0.843
<50	66	25 (38%)	
≥50	30	12 (40%)	
Stage			0.163
I	4	0 (0%)	
II	53	19 (36%)	
III	39	18 (46%)	
PIK3CA mutation			0.812
Yes	12	5 (42%)	
No	84	32 (38%)	
HER2 amplification or 3 +			0.106
Yes	4	0 (0%)	
No	92	37 (40%)	

Table 2 Comparison between patient ESCC tissue and corresponding patient-derived ESCC xenograft tissue

ID	Patient ESCC tissue		Patient-derived ESCC xenograft tissue	
	HER2 IHC	Exon 9 of PIK3CA	HER2 IHC	Exon 9 of PIK3CA
EC001	2 +	Wt	—	Wt
EC002	—	Wt	—	Wt
EC004	—	Wt	—	Wt
EC006	—	Wt	—	Wt
EC008	2 +	Wt	—	Wt
EC014	2 +	Wt	1 +	Wt
EC016	—	Wt	—	Wt
EC018	—	Wt	—	Wt
EC019	—	Wt	—	Wt
EC021	—	Wt	—	Wt
EC022	—	Wt	1 +	Wt
EC023	1 +	Wt	1 +	Wt
EC030	—	GAG > AAG	1 +	GAG > AAG
EC039	2 +	GAA > AAA	2 +	GAA > AAA
EC040	—	Wt	—	Wt
EC043	2 +	Wt	1 +	Wt
EC044	2 +	Wt	2 +	Wt
EC047	—	Wt	2 +	Wt
EC054	—	Wt	—	Wt
EC056	—	Wt	—	Wt
EC057	—	Wt	2 +	Wt
EC058	—	GAG > AAG	—	GAG > AAG
EC060	—	GAG > AAG	—	GAG > AAG
EC063	—	Wt	—	GAG > AAG / GAA > AAA
EC066	—	Wt	—	Wt
EC069	—	Wt	—	Wt
EC074	—	GAG > AAG	—	GAG > AAG
EC077	—	Wt	—	Wt
EC079	—	Wt	—	Wt
EC082	—	Wt	—	Wt
EC083	—	Wt	1 +	Wt
EC087	—	Wt	—	Wt
EC089	—	Wt	2 +	Wt
EC092	—	Wt	—	Wt
EC097	—	Wt	—	Wt
EC104	2 +	Wt	3 +	GAA > AAA
EC105	1 +	Wt	—	Wt

Wt, wild type.

in vitro culture conditions, resulting in xenograft tumors, which are mostly undifferentiated and bearing weak resemblance to the real human disease histology and tumor

DISCUSSION

For several decades, historic cancer drug screening efforts have relied heavily on the use of mouse tumor xenograft models. A key problem, however, is that the source of these xenografts are permanent cell lines kept under continuous

Figure 3 *PIK3CA* mutation in patient ESCC and the corresponding PDECX models. EC001 harbors a wild-type *PIK3CA* gene in primary tissue and xenografted model, EC039 contains a codon 542 GAA>AAA mutation, and EC058 has a codon 545 GAG>AAG mutation in exon 9 of the *PIK3CA* gene in both primary and xenografted tissues, EC074 has acquired a codon 542 GAA>AAA mutation as well as a codon 545 GAG>AAG mutation in xenografted tissue, EC104 acquired a codon 542 GAA>AAA mutation in xenografted tissue.

architecture.^{25,36} Development of esophageal animal xenograft models has been especially difficult but some success has been achieved,³⁷ either by the inoculation of EC cell lines into immunodeficient rodents (mice and rats) or rabbits.^{15,38–41} Unfortunately, again, however, these cell lines also suffer from the same issues of long *in vitro* culture times and loss of original tumor characteristics. Another problem faced by the field of esophageal oncology research is that most of the commercially available EC cell lines were originally derived from esophageal adenocarcinoma,^{15,39,40,42} which does not represent the predominant ESCC disease in Asian esophageal cancer patients. Furthermore, our recent experimental results

show that some of the commercially available EC cell lines have lost their original genetic aberrations, such as *PIK3CA* mutation, in the early generations. Therefore, the establishment of clinically relevant animal xenograft tumor models is necessary and urgently required to progress preclinical ESCC-targeted drug discovery. In recent years, patient-derived xenograft mouse models have been established through direct transplantation of fresh patient tumor tissue into immunodeficient mice.^{23,43} As early as 1981, Kitamura *et al*⁴⁴ established a panel of 17 PDECX models. Although similar models have been established in Western countries,⁴⁵ the majority of PDECX models were of adenocarcinoma origin. Therefore, our present study aimed to establish a panel of Chinese patient-derived ESCC xenograft mouse models, harboring a variety of genetic aberrations and thereby constituting a pharmacologic platform for targeted drug efficacy evaluation.

In our study, a total of 37 viable and recoverable PDECX mouse models were established by implanting 96 fresh Chinese ESCC tissues into immunodeficient mice. Our successful engraftment rate of 38.5% was in the range of engraftment rates observed in similar patient-derived xenograft mouse model studies.^{28,46–49} Correlative analysis between patient clinical characteristics and PDECX model establishment success rates demonstrated no statistically significant factors, but did, however, show potential negative trends associated with nerve invasion status and *HER2* amplification or overexpression.

Based on published molecular analyses of the esophageal genetic landscape, together with our own published data (Hou *et al*, Boland and Burtneis),^{34,50} we chose to focus on *EGFR*, *KRas*, *BRAF*, *HER2*, and *PIK3CA*. *HER2* amplification and protein overexpression have been observed in ESCC and many other tumor types^{33,51} and often associates with poor prognosis.^{31,52–54} We identified 4.2% (5/96) of patient ESCC tissues showing overexpression or amplification of the *HER2* gene, which was lower than previous reports.³³ Unexpectedly, patient ESCC tissues with *HER2* gene amplification or overexpression (*HER2* IHC 3+) were unable to form stable PDECX models in immunodeficient mice. The majority of the failures (3/5) occurred in generation 1 (F1), whereas two models survived in the first generation and then failed in the second generation. These data are consistent with published observations of breast cancer xenograft development, where the establishment of patient-derived xenograft mouse models harboring *HER2* amplification is rare.²⁴ Unfortunately, the underlying mechanism of how *HER2* amplification or overexpression could contribute to model failure is currently unclear. We also acknowledge that the relatively low incidence of *HER2* gene amplification or overexpression in this data set (4.2%) could likely introduce some bias in our statistical analyses.

In contrast to the negative impact of *HER2* gene amplification on successful PDECX model establishment, *PIK3CA* gene mutation had no apparent impact. In all, 41.6% (5/12) of patient ESCC tissues harboring *PIK3CA* mutation grew up

Figure 4 *In vivo* efficacy of chemotherapy in five PDECX mouse models. Tumor-bearing mice were treated with 5-FU and cisplatin starting from tumor volumes of 100–200 mm³. Subcutaneous tumor volumes were measured using calipers and calculated and expressed as mean ± s.e.m.

in immunodeficient mice, similar to the overall success rate (38.5%) of model establishment in this study. Interestingly, two additional PDECX models were identified as *PIK3CA* mutant, whereas the corresponding patient ESCC tissues were identified as *PIK3CA* wild type. We also observed that a further two PDECX models lost HER2 expression (from IHC 2+) in the corresponding patient ESCC tissues to negative (IHC 0) in the PDECX models. Contrasting with this, three PDECX models showed HER2 2+ expression but the HER2 positivity was not detected in the corresponding patient

ESCC tissues. Multiple repeat experiments were performed to exclude technical error as a possible explanation for this result and the authors suggest tumor heterogeneity as a likely cause. Indeed, the location of the tissues used to establish the PDECX mouse models was different to that used to prepare formalin-fixed paraffin-embedded sections and perform genetic analysis. Another possibility may be clone enrichment or disappearance of human genetic aberrations within the mouse environment, however, this is speculation and is the subject of ongoing investigations. Taken together, these

findings highlight the necessity for thorough characterization of patient-derived xenograft models before drug testing in preclinical drug discovery and development.

In our previously published study,³⁰ Trastuzumab induced complete tumor regressions in PDECX mouse models with HER2 positivity (IHC 2+), but was inefficacious in models harboring either *PIK3CA* mutation or combined HER2 IHC 2+ positivity and *PIK3CA* mutation. In the present study, we extended these findings to demonstrate that PDECX models with HER2 negativity and wild-type *PIK3CA* were more sensitive to chemotherapy using 5-FU and cisplatin than PDECX models harboring genetic aberrations in HER2 or *PIK3CA* mutation.

In summary, a panel of PDECX mouse models, which included *PIK3CA* mutant and HER2-positive models, was established and characterized, thus mimicking the current clinical genetic setting of esophageal carcinoma. The sensitivity of HER2-negative ESCC models to chemotherapy supports stratification approaches in the treatment of esophageal carcinoma patients and warrants further investigation of the impact of *PIK3CA* on treatment response.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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