Original Article

A subset of esophageal squamous cell carcinoma patient-derived xenografts respond to cetuximab, which is predicted by high EGFR expression and amplification

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Background: Epidermal growth factor receptor (EGFR) is reportedly overexpressed in most esophageal tumors, but most targeted therapies showed no efficacy in non-selected patients. This study aims at investigating the adaptive cetuximab subset in a cohort of esophageal squamous cell carcinoma (ESCC) patient-derived xenografts (PDXs).

Methods: A large panel of ESCC PDXs has been established. The copy number, mRNA expression and immunohistochemistry (IHC) of key EGFR pathways have been examined along with cetuximab response. A preclinical trial on a randomly selected cohort of 16 ESCC PDXs was conducted, and the genomic annotations of these models were compared against the efficacy readout of the mouse trial.

Results: The trial identified that 7 of 16 (43.8%) responded to cetuximab ($\Delta T/\Delta C < 0$ as responders). The gene amplification and expression analysis indicated that EGFR copy number ≥5 (P=0.035), high EGFR mRNA expression (P=0.001) and IHC score of 2–3 (P=0.034) are associated with tumor growth inhibition by cetuximab, suggesting EGFR may function as a single predictive biomarker for cetuximab response in ESCC.

Conclusions: Overall, our results suggest that an ESCC subtype with EGFR amplification and overexpression benefits from cetuximab treatment, which warrants further clinical confirmation.

Keywords: Biomarker; patient-derived xenograft (PDX); epidermal growth factor receptor (EGFR); cetuximab; esophageal squamous cell carcinoma (ESCC)

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Introduction

Esophageal cancer (EC) is the sixth leading cause of cancer death in the world (1). The prognosis of EC remains poor even with significant advancements in new surgical techniques and other treatment approaches (2). Due to the high recurrence post esophagectomy or definitive chemoradiotherapy (3), additional strategies are thus needed to increase systemic treatment options. Over the past decades, drugs targeting specific oncogenic alterations have been developed to treat cancers, with much success particularly for lung, breast and colorectal cancers. However, adaptive targeted treatment has not yet been approved for treating EC, which is an unmet and urgent need.

Epidermal growth factor receptor (EGFR) (ERBB1) is a member of the ERBB receptor tyrosine kinase family, also including ERBB2, ERBB3 and ERBB4 (4). EGFR is reported to be overexpressed in many types of tumors including head and neck (90%), colorectal (72%), lung (50%), bladder (65%) and esophageal (92%) (5-7), which correlates with poor prognosis. Cetuximab, a mouse-human chimeric antibody, binds to EGFR blocking phosphorylation and activation of EGFR (8). Over the past years, cetuximab has been approved in treating head and neck squamous carcinoma and KRAS-mutation metastatic colorectal cancer (9,10). For esophageal squamous cell carcinoma (ESCC), the EGFR inhibitor down regulates the level of EGFR and correlated downstream genes to inhibit the growth of EGFR overexpressed cells and increase cell sensitivity to chemo-/radiotherapy in some *in vitro* studies (11,12). For EGFR tyrosine kinase inhibitors (TKIs), five small clinical phase 2 trials reported the objective response rate in unselected patients with advanced EC was 2.8% to 16.7% (13-17). In the COG trial, the only randomized phase 3 study of second line therapy in EC, the progression free survival and patient reported outcome were improved in the gefitinib group (18). Moreover, in biomarker analysis, EGFR copy number and overexpression might potentially be used in predicting the efficacy in patients treated with EGFR TKIs (19,20). However, the randomized phase 2/3 and 3 clinical trials of SCOPE1 and RTOG 0436 showed that the addition of cetuximab to concurrent chemoradiotherapy did not improve overall survival for non-selected ESCC (21,22). As for the other EGFR monoclonal antibody, the EORTC power trial also revealed that the addition of panitumumab to chemotherapy provided no additional benefit and the biomarker analysis is on going (23). So far, most studies investigated the efficacy of cetuximab in combination with chemo-/radiotherapy, no suitable ESCC subtype was confirmed to cetuximab treatment and no established biomarkers were reported to predict tumor response to cetuximab.

Patient-derived tumor xenografts (PDXs) are used in predicting clinical activity of drugs and exploring biomarkers (24-27). We previously reported the discovery of a predictive biomarker for cetuximab response in CRC (28,29) and gastric (30) carcinoma via mouse clinical trial (MCT) using cohorts of PDXs. In this present study, we set out to investigate the activity of cetuximab in ESCC PDXs. We established 16 ESCC-PDXs by transplanting untreated tumor tissues from patients into immunocompromised BALB/c nude mice via subcutaneous inoculation, followed by extensive characterization and tests for response to cetuximab in an MCT. After therapeutic responders and non-responders were identified, candidate biomarkers were then assessed by genomic/phenotypical properties.

Methods

PDX establishment

The engraftment of transplant patient tumor fragments to mice was previously reported (31). In brief, freshly surgically resected ESCC tumor samples that are in excess of surgical pathologic diagnosis were obtained from the patients in Shanghai Cancer Hospital with the Institutional Review Boards of the hospital and the informed consents from patients. Tumor tissues were cut into 3×3×3 mm^3 fragments mixed with 10% Matrigel at 4 °C and subcutaneously implanted into immune deficient mice (BALB/c nude, 6 to 8 weeks old female mice,), followed by expansion, banking, histopathology and molecular characterizations, and pharmacology characterization as previously described (31). Most procedures for genomic and histopathology analysis have been thoroughly described before (30,31). All animal procedures were conducted at Crown Bioscience SPF facility and in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee of the Ethics of Animal Experiments of Crown Bioscience (Crown Bioscience IACUC Committee).

PDX therapeutic treatment

MCTs using cohorts of PDXs have also been described previously (28,30). When tumors reached on average 100–150 mm^3, mice were grouped equally by tumor volume into

Table 1 Summary of the patient information of the enrolled cohort of ESCC-PDXs in the present study

Characteristic	Numbers, n (%)
Age, median [range] years	59 [53–76]
Gender	
Male	8 [50]
Female	8 [50]
Stage*	
I–II	10 (62.5)
III–IV	6 (37.5)
Differentiation	
Low-middle	1 (6.3)
Middle	7 (43.8)
Middle-high	4 (25.0)
High	4 (25.0)
Tumor location	
Cervical + upper thoracic	1 (6.3)
Middle + lower thoracic	14 (87.5)
Double/multiple primary tumor	1 (6.3)
Tumor length, median (range) cm	5 (3–7.5)
Neoadjuvant treatment	
Yes	0 (0)
No	16 [100]

*, UICC 6th. ESCC, esophageal squamous cell carcinoma; PDXs, patient-derived xenografts.

treatment and control groups, each group comprising 5 mice. Vehicle controls received PBS intraperitoneal injection weekly, while the treatment group received weekly intraperitoneal injection with cetuximab (50 mg/kg, Merck KGaA). Both groups received the treatment for 3 weeks. Tumor volume was measured twice weekly, the mice were sacrificed when the volume reached 2,000 mm^3 or after 30 days-post treatment. $\Delta T/\Delta C$ value was calculated for assessing tumor response to the treatment (ΔT = tumor volume change in the treatment group and ΔC = tumor volume change in the control group). At the end point, tumors were fixed in formalin for pathological examination, and snap-frozen banking in liquid nitrogen for gene analysis occurred.

Gene copy number and mutation analysis

For copy number assay using Affymetrix SNP 6.0 chips, genomic DNA was isolated and purified using the Genomic DNA Tissue and Blood Isolation Kit (Qiagen) following manufacturer's instruction. DNA processing was performed following a standard Affymetrix protocol (http://media.affymetrix.com/support/down-loads/manuals/genomewidesnp6_manual.pdf). Gene copy number analysis was performed by PICNIC and/or PennCNV methods and a copy number ≥5 is considered positive. The confirmation of hotspot mutations was conducted for some mutation alleles as previously described (31).

IHC analysis

For all of the samples, the relative EGFR protein expression level was determined by immunohistochemistry (IHC), anti-human antibodies including EGFR (CST, Beverly, MA, USA), P-EGFR (Abcam, Cambridge, MA, USA), HER3 (CST, Beverly, MA, USA), P-HER3 (CST, Beverly, MA, USA), MET (CST, Beverly, MA, USA), P-MET (CST, Beverly, MA, USA), Akt (CST, Beverly, MA, USA), P-Akt (CST, Beverly, MA, USA), ERK (CST, Beverly, MA, USA), P-ERK (CST, Beverly, MA, USA) were applied to stain the positive sections. The test specimens were then scored independently by three investigators in a blinded fashion per the following criteria: score 0 representing no specific section staining within the tumor while 1+, 2+, 3+ represent different staining intensity of the nucleus or membrane.

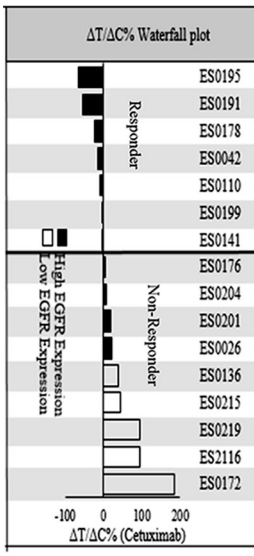
Statistical analysis

Pearson correlation tests and linear regression were applied for comparing the data of the two groups. All data analyzes were completed using SPSS (version 19.0, SPSS Inc., Chicago, IL, USA), $P<0.05$ was considered statistically significant.

Results

Characterization of patients and PDXs

One hundred and ten surgically removed ESCC patient tumor tissues were implanted into immunocompromised mice, and 61 of 110 were found to grow: take-rate of 55.5%. None of the patients received neoadjuvant treatment before surgery. The summary of patient information for the randomly selected 16 patients with PDX established/enrolled in this study is shown in *Table 1*. Among them, 10 (62.5%) were stages I–II, 6 (37.5%) were stages III–IV,



	ΔT/ΔC%	Copy number						mRNA intensity (Log2)					
		AKT	EGFR	HRAS	KRAS	NRAS	PIK3CA	AKT	EGFR	HRAS	KRAS	NRAS	PIK3CA
ES0195	-64.11	3	5	2	3	2	4	6.90	4.33	6.17	3.61	4.02	3.84
ES0191	-54.05	4	14	2	3	3	5	6.21	6.31	5.70	3.15	2.81	2.11
ES0178	-22.4	3	11	2	3	3	4	6.47	5.06	5.90	2.51	3.99	2.39
ES0042	-14.68	4	5	2	2	3	7	6.83	4.37	6.28	2.55	5.12	4.42
ES0110	-9.66	2	14	2	3	2	2	6.51	7.02	7.10	3.51	4.47	3.52
ES0199	-4.01	3	3	1	3	3	3	6.05	4.88	6.18	3.63	5.07	3.33
ES0141	-1.63	3	3	2	4	2	5	6.86	3.85	6.00	4.21	4.45	3.43
ES0176	4.8	3	3	2	4	3	4	5.88	4.32	5.89	4.06	4.45	3.79
ES0204	7.77	3	4	4	4	3	5	5.97	3.77	6.76	2.32	3.62	2.82
ES0201	19.78	2	5	1	5	2	5	6.00	3.95	5.75	5.24	4.49	3.99
ES0026	23.25	5	4	3	3	3	6	6.42	4.59	6.54	2.86	3.81	3.36
ES0136	38.32	1	3	1	6	2	4	5.84	4.23	5.92	4.60	4.21	3.31
ES0215	44.19	2	2	2	2	3	4	6.33	3.36	4.75	1.70	4.33	3.07
ES0219	94.6	1	2	1	6	3	4	5.95	2.13	5.59	5.26	5.49	5.29
ES2116	95.23	3	4	5	3	3	3	5.94	1.18	6.61	3.21	3.76	1.94
ES0172	183.8	3	4	3	3	2	6	6.68	2.35	7.04	3.35	3.40	4.09

Figure 1 The copy number and mRNA levels of EGFR pathway genes (*AKT*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA*) were not significant related with cetuximab response. EGFR, epidermal growth factor receptor.

there were no material bias in patients' stage. As 1 (6.3%) was low-middle differentiation, 7 (43.8%) were middle differentiation, 4 (25%) were middle-high differentiation and 4 (25%) were high differentiation, the distributions of differentiation were basically equal.

Many of these PDX tumors were then extensively characterized, including the 16 PDXs enrolled in the study, e.g., histopathology confirmed, transcriptome sequenced, SNP 6.0 analyzed (gene copy number), IHC analyzed (protein expression), etc. In particular, the activated oncogenic pathways commonly seen in cancers were carefully examined using these profiling data. Specifically, only a few oncogenic mutation alleles were identified by transcriptome sequencing (RNAseq) (also confirmed by hotspot mutation analyses), with a few oncogene amplifications also revealed by SNP 6.0 GeneChip analysis (*Figure 1*), among the commonly activated pathways in cancers (e.g., EGFR, HRAS, AKT, KRAS, NRAS, PI3KC). The expression or overexpression of the relevant genes have also been examined per RNAseq and IHC, as summarized in *Table 2*.

A subset of ESCC-PDXs responded to cetuximab

An MCT on the cohort of 16 ESCC-PDXs, randomly enrolled from 61 PDXs, was conducted ("n=10 format": 10 mice per arm, two arms: vehicle and cetuximab treatment; 1 mg per mouse, once weekly for 3 weeks, initiated when tumor volume reached 150–200 mm^3) to assess cetuximab efficacy. The tumor response to cetuximab was quantified by ΔT/ΔC, as summarized in *Figure 2*. The tested ESCC-PDXs fell into two distinct categories according to the drug activities: 7 of 16 (43.8%) responded to cetuximab treatment (ΔT/ΔC <0); 9 of 16 (56.3%) did not, with ΔT/ΔC >0. Among the responders, 2 of 7 (28.6%) reached nearly complete response (ΔT/ΔC =–64.11%/–54.05%) while the other 5 showed partial response (ΔT/ΔC ranged from –22 to 0). Representative tumor response curves are shown in *Figure 3*. ES0191 and ES0195 are examples of cetuximab sensitive models, while ES0172 and ES0219 are resistant models. Our data clearly suggests that a subset of patients might potentially benefit from cetuximab treatment in EC, or in other words, EGFR is an oncogenic driver for these patients, at least for maintaining their disease state.

EGFR expression seems to positively predict cetuximab response in ESCC-PDXs

Cetuximab targets surface expressed EGFR, therefore it is reasonable to first suspect that the status of EGFR could be related to drug response. We previously reported similar studies of cetuximab treatment on cohorts of CRC (28) and gastric (30) cancers, where the status of EGFR

Table 2 Correlation between protein level and tumor response

Protein	Expression level	Tumor response (−ΔT/ΔC%)		P value
		Low	High	
EGFR	Low	5	0	0.034*
	High	4	7	
p-EGFR	Low	7	1	0.041*
	High	2	6	
HER3	Low	7	3	0.302
	High	2	4	
p-HER3	Low	6	6	0.585
	High	3	1	
MET	Low	7	2	0.126
	High	2	5	
p-MET	Low	4	4	1
	High	4	4	
Akt	Low	4	6	0.145
	High	5	1	
p-Akt	Low	5	6	0.308
	High	4	1	
Erk	Low	6	4	1
	High	3	3	
p-Erk	Low	3	4	0.615
	High	6	3	

*, P<0.05. IHC results of EGFR were divided into 2 categories: high expression (score 2–3) and low expression (score 0–1), the EGFR protein level associated with tumor response. IHC, immunohistochemistry; EGFR, epidermal growth factor receptor.

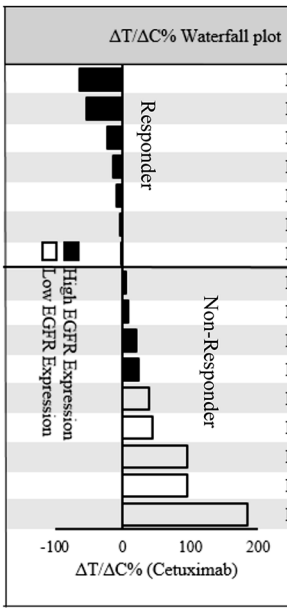
played completely different roles. While little role was seen in CRC, EGFR amplification and/or overexpression clearly demonstrates positive correlation to drug response in gastric cancer (30). It would be interesting to determine whether the status of EGFR in ESCC plays a role in response to cetuximab.

We first tested the copy number of EGFR by Affymetrix SNP 6.0, demonstrating amplification rate of 37.5% (or 6/16), similar to those previously reported (19,20,32). More importantly, the EGFR amplification was significantly correlated with the ESCC-PDXs response to cetuximab (P value of 0.035, *Figure 2*).

We next examined whether there is a correlation between EGFR mRNA levels and ΔT/ΔC. As shown in *Figure 2*, the majority of ESCC (13/16) expressed some degree of EGFR mRNA (Log2 >3), as is known in ESCC patients. Interestingly, the higher expressors are all responders (P value of 0.00). Furthermore, EGFR mRNA levels had a negative correlation with ΔT/ΔC (correlation coefficient: –0.748; P=0.001) (*Figure 4*). The linear regression analysis showed the same results as R^2=0.559 (P=0.001), indicating that a higher EGFR mRNA expression showed a better tumor response to cetuximab (*Figure 4*).

We next examined protein levels of EGFR by IHC and their correlation with drug response. The tumor tissue microarray of these models was stained by either anti-EGFR or anti-pEGFR antibodies. The signals were semi-quantitatively determined using a score system. The results showed positive EGFR and pEGFR immunostaining in 14/16 (87.5%) models. Among EGFR positive models, 3/16 had staining intensity score of 1+, 5/16 of 2+, 6/16 of 3+, while 6/16 of 1+, 5/16 of 2+ and 3/16 of 3+ were observed for pEGFR staining. The typical EGFR staining of score 0–3+ is shown in *Figure 5*. To further explore the correlation between tumor protein expression and cetuximab response, the IHC results of EGFR and pEGFR were divided into 2 categories: high expression [2–3] and low expression [0–1]. By Chi-square test, the P value was 0.034 for EGFR and 0.041 for pEGFR, indicating that the IHC scores for EGFR and pEGFR associated with tumor response. For EGFR protein expression, its predictive accuracy was 75% (12/16), high expression of EGFR predictive accuracy of 63.6% (7/11), low expression of EGFR predictive accuracy was 100% (5/5). These IHC observations are consistent with the mRNA data, although less significant and consistent, likely due to IHC providing poorer quantitation than mRNA. Overall, EGFR may function as a single predictive biomarker for cetuximab response in ESCC, with similarity to that seen in gastric carcinoma but not in CRC. In this study, we also explored the relationship between expression of different proteins. EGFR was associated with the expression of p-EGFR (P=0.004) and HER3 (P=0.036), but not of other molecules. The expression of p-EGFR was not only related to the expression of EGFR, but also related to the expression of HER3 (6/16, P=0.002) and MET (7/16, P=0.002).

As EGFR copy number and protein expression were correlated with cetuximab response in ESCC, we further combined EGFR copy number and IHC data to see whether there was increased predictive value. We found it was significantly correlated with ESCC-PDXs response to cetuximab (P value =0.021, *Table 3*).



ΔT/ΔC% Waterfall plot		ΔT/ΔC%	EGFR Copy number	EGFR mRNA intensity (Log2)	t-EGFR IHC Score	p-EGFR IHC Score
	ES0195	-64.11	5	4.33	3	2
	ES0191	-54.05	14	6.31	3	3
	ES0178	-22.4	11	5.06	2	2
	ES0042	-14.68	5	4.37	2	0
	ES0110	-9.66	14	7.02	3	3
	ES0199	-4.01	3	4.88	3	3
	ES0141	-1.63	3	3.85	2	2
	ES0176	4.8	3	4.32	3	2
	ES0204	7.77	4	3.77	3	1
	ES0201	19.78	5	3.95	2	2
	ES0026	23.25	4	4.59	2	1
	ES0136	38.32	3	4.23	1	1
	ES0215	44.19	2	3.36	1	0
	ES0219	94.6	2	2.13	0	1
	ES2116	95.23	4	1.18	0	1
	ES0172	183.8	4	2.35	1	1
		P value (correlation with ΔT/ΔC%)	0.035	0.000	0.030	0.041

Figure 2 ΔT/ΔC% of cetuximab in 16 ESCC-PDX models significantly correlated with EGFR amplification (copy number: P=0.035) and expression (mRNA: P=0.000, t-EGFR: P=0.030). EGFR, epidermal growth factor receptor.

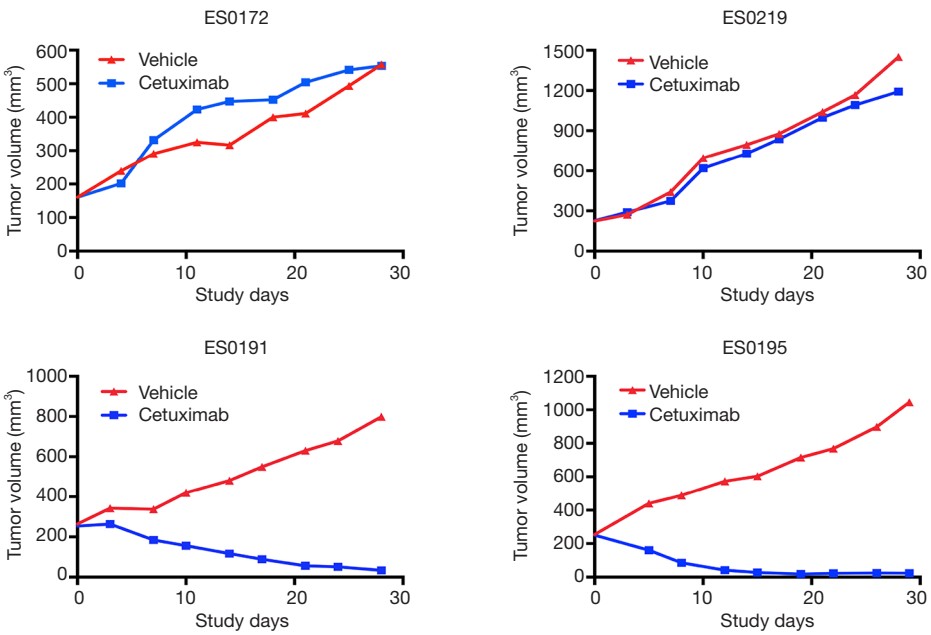


Figure 3 Growth curve of 4 representative mice. ES0172 and ES0219: 2 models did not respond to cetuximab; ES0191 and ES0195: 2 models nearly reached complete response.

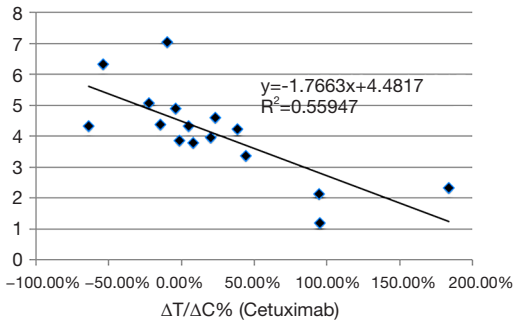


Figure 4 Linear regression analysis of EGFR mRNA level and cetuximab efficacy. The result of linear regression analysis: R^2=0.559 (P=0.001), indicating that a higher mRNA expression of EGFR had a better tumor response to cetuximab. EGFR, epidermal growth factor receptor.

Evaluation of other common oncogenes as potential predictive biomarkers

It is possible that other commonly seen oncogenes may also play roles in cetuximab response as in CRC (28), therefore the 5 most reported genes (*MET*, *HGF*, *ERBB2*, *ERBB3*, *IGF1R*) and 4 main downstream effectors (AKT1, AKT2, MAPK1, MAPK3) were also analyzed. The oncogene mutation alleles were found to be less frequent (*Figure 6*) than in CRC, similar to those in gastric cancers (30). These mutations thus seem to playing minor roles in cetuximab response in ESCC, or also as likely as predictive biomarker. There also seems no apparent role of gene copy numbers or expression levels of these oncogenes as well (*Figure 1*), although there is differential

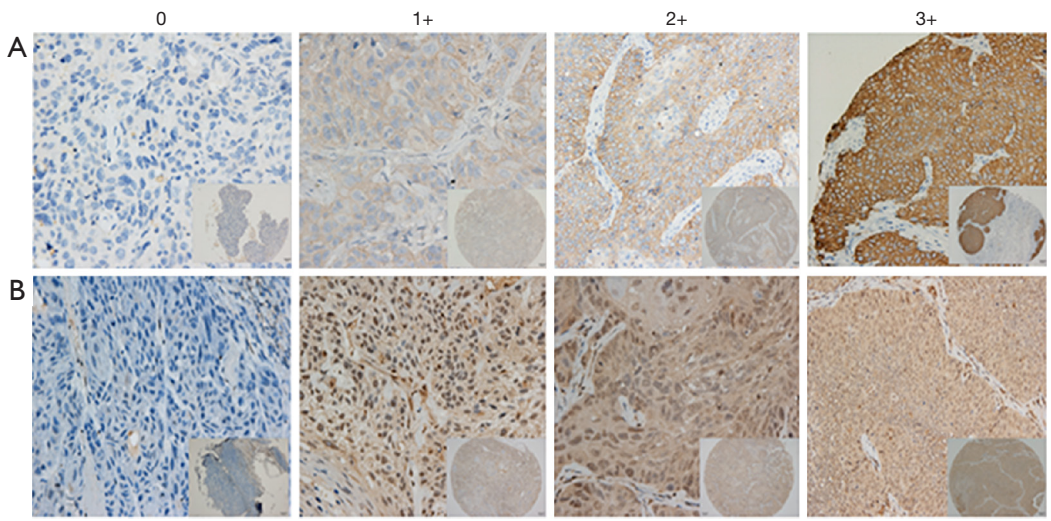
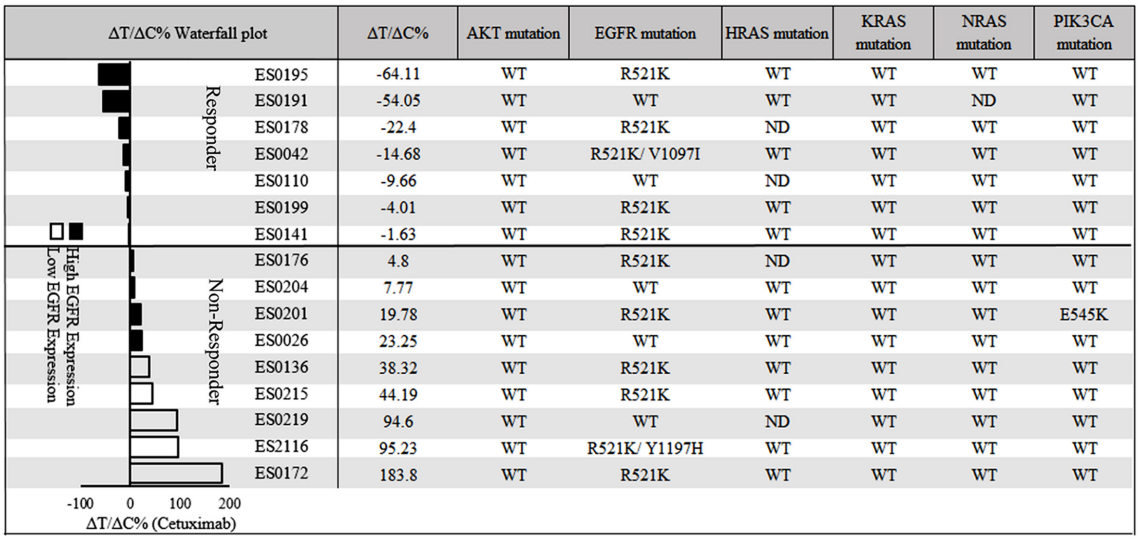


Figure 5 Typical EGFR and Akt staining of score 0–3+. EGFR and Akt staining as determined by immunohistochemistry. Panels show representative examples of esophageal tumor specimens stained for total EGFR (A) and Akt (B). EGFR, epidermal growth factor receptor.

Table 3 The cetuximab response was significantly correlated with both EGFR amplification and t-EGFR IHC

Status of EGFR copy number and t-EGFR IHC	Non-responder	Responder	P value
Both positive	1	5	
Only one positive	3	2	0.021
Both negative	5	0	

IHC, immunohistochemistry; EGFR, epidermal growth factor receptor.



ΔT/ΔC% Waterfall plot		ΔT/ΔC%	AKT mutation	EGFR mutation	HRAS mutation	KRAS mutation	NRAS mutation	PIK3CA mutation
Responder	ES0195	-64.11	WT	R521K	WT	WT	WT	WT
	ES0191	-54.05	WT	WT	WT	WT	ND	WT
	ES0178	-22.4	WT	R521K	ND	WT	WT	WT
	ES0042	-14.68	WT	R521K/ V1097I	WT	WT	WT	WT
	ES0110	-9.66	WT	WT	ND	WT	WT	WT
	ES0199	-4.01	WT	R521K	WT	WT	WT	WT
	ES0141	-1.63	WT	R521K	WT	WT	WT	WT
Non-Responder	ES0176	4.8	WT	R521K	ND	WT	WT	WT
	ES0204	7.77	WT	WT	WT	WT	WT	WT
	ES0201	19.78	WT	R521K	WT	WT	WT	E545K
	ES0026	23.25	WT	WT	WT	WT	WT	WT
	ES0136	38.32	WT	R521K	WT	WT	WT	WT
	ES0215	44.19	WT	R521K	WT	WT	WT	WT
	ES0219	94.6	WT	WT	ND	WT	WT	WT
	ES2116	95.23	WT	R521K/ Y1197H	WT	WT	WT	WT
	ES0172	183.8	WT	R521K	WT	WT	WT	WT

Figure 6 The oncogene mutation alleles of ESCC were not frequent. ESCC, esophageal squamous cell carcinoma.

expression of ERBB and MET mRNA among the 16 models with a standard deviation (SD) >1.

Whether mRNA level and gene amplification of the same gene had a relationship was detected by Pearson correlation test. Statistically, EGFR, ERBB2, ERBB3 and MET were considered correlated in mRNA and gene amplification, while the upstream genes (*HGF*, *MAPK*) and downstream (*AKT1*, *AKT2*, *MAPK1*, *MAPK2*) were not. The correlation between upstream and downstream gene expression was also tested. MET and MAPK1 were reported as positively correlated (correlation coefficient: 0.684; P=0.003) while IGF1R and MAPK3 had negative correlation (correlation coefficient: −0.628; P=0.009). No expression of other molecules was found to be correlated (ERBB2, ERBB3, HGF, IGFR, MET, MET, AKT1/2, MAPK1/3).

Discussion

To our knowledge, this is the first study to use PDX models to investigate the effectiveness of cetuximab in EC, and to establish a subgroup of patients who are sensitive to cetuximab therapy. In this study, 16 PDX models of EC were established and 7 of 16 (43.8%) responded to cetuximab monotherapy.

Our data found that EGFR amplification was closely correlated to cetuximab response and EGFR expression, which was similar to the observation in the gastric MCT described previously (30). Moreover, our data clearly confirmed a strong positive correlation between cetuximab response in ESCC and EGFR mRNA expression levels, which is also confirmed for protein levels per IHC staining, albeit to a lesser degree. The fact that no low-EGFR expression models responded to cetuximab also confirmed the conclusion above. The certain degree of discrepancy in the correlation between protein and mRNA is likely due to the semi-quantitative nature of IHC. Furthermore, the combination of EGFR copy number and IHC analysis could predict cetuximab response more precisely.

PDX closely mimic the original patient in both histology and molecular pathology (33), and also with demonstrated similar drug response (34,35). A cohort of ESCC-PDXs representing diversity of the disease could be potentially useful in assessing the activity of cetuximab on ESCC and in determining potential predictive biomarkers through an MCT in the preclinical setting. The PDX trial may also have actual advantages over a human trial for clearly demonstrating drug activity by defined experimental conditions and minimized individual differences, e.g., patients' general conditions and different PK among individuals, etc. The present MCT study seems to confirm cetuximab activity in ESCC and identify EGFR as a predictive biomarker, which warrants further clinical confirmation.

Although many ESCC express EGFR, EGFR monoclonal antibodies, such as cetuximab, have yet to be confirmed effective in non-selective ESCC in the clinic (13-17). In ESCC, an EGFR inhibitor could downregulate the level of EGFR and correlated downstream genes, to inhibit high

EGFR expressed cell growth, and to increase cell sensitivity to chemo-/radiotherapy in some *in vitro* studies (11,12). For clinical trials, a phase 2 study from Lorenzen *et al.* is the only randomized clinical trial to confirm the efficacy of cetuximab in high-EGFR expressed ESCC patients. 62 patients were enrolled in the study, 32 receiving cetuximab plus CF (cisplatin + 5-flurouracil) and 30 CF only. With a median follow up of 21.5 months, the median overall survival was 9.5 and 5.5 months for cetuximab + CF and CF respectively (P=0.32). However, considering the poor prognosis of high EGFR expressed ESCC patients (PFS: 3.6 months, OS: 5.5 months), the efficacy of group cetuximab + CF was encouraging (36). Several retrospective studies also suggested cetuximab-combined therapy increased overall survival of high EGFR ESCC patients, even better than EGFR negative patients who were supposed to have a better prognosis (37,38). However, none of these studies have definitely confirmed activity in the EGFR-high expressors with statistical significance, which could be due to small number of subjects involved in the trials, or the retrospective nature of study.

For EGFR TKIs, in the phase 3 COG trial, gefitinib improved the PFS (median PFS, 1.57 *vs.* 1.17 months, P=0.020) both in adenocarcinomas and squamous cell carcinomas over placebo. In the gefitinib subgroup, the EGFR FISH-positive patients had longer PFS and OS, which was similar to other TKIs therapies in gastric or gastroesophageal carcinoma (18). Consistent with COG, a phase 2 trial showed that icotinib had favorable activity in ESCC patients with EGFR overexpression or amplification (16). In our study, we confirmed the predictive value of EGFR amplification (P=0.035) and overexpression (mRNA: P=0.001; IHC: P=0.034) in cetuximab treatment in ESCC patients, and also found the combination of EGFR copy number and IHC results could increase the predictive value (P=0.021), which was similar to the findings in ESCC EGFR TKIs therapy.

Compared with a higher frequency of EGFR/KRAS mutations in CRC and non-small cell lung cancer, these commonly seen oncogene mutations are rarely detected in ESCC tumors (39,40). In the present study, no effective EGFR/KRAS/HRAS/NRAS mutations were detected in 16 PDX models and the overexpression of EGFR also seemed to have little correlation with increased EGFR copy number. One of 16 (6.3%) patients had a PIK3CA mutation, whose frequency was similar to that previously reported 7.4% (41).

MET and HER3 activation have been reported to effect tumor resistance to EGFR inhibitors. During anti-EGFR therapy of CRC, MET amplification was significantly correlated with resistance to that EGFR blockade, which could be reversed by a MET inhibitor (42). In Engelman's team, the same conclusion has been reached, and it has also been shown that MET amplification causes tumor resistance to EGFR inhibitors by activation of HER3 (43). In the present study, we investigated the relationship between EGFR, MET and HER3 by Spearman correlation test. The expression of p-EGFR correlated not only with EGFR (P=0.004), but also with HER3 (P=0.002) and MET (P=0.002). The results were similar to those previously reported. PI3K/Akt and MAPK/Erk are two main downstream pathways of EGFR and PI3K/Akt, which are closely related with cell apoptosis and tumor prognosis. In Li's study, a higher level of p-Akt was observed in 5-FU resistant ESCC and the inhibition of the PI3K/Akt pathway could repress cell proliferation (44). This study also investigated copy number, mRNA and IHC levels of these common EGFR downstream oncogenes, however, none seemed to be a promising biomarker of cetuximab efficacy.

Of course, there are still many deficiencies in our study, such as the small sample size. We are expanding the number of PDXs to verify our results and improving the SNP method to measure gene copy number more accurately. At the same time, we are starting to conduct clinical trials based on this result and explore the favorable subtype of cetuximab treatment which is still on going.

In conclusion, our results suggest that an ESCC subtype with overexpression of EGFR copy number, mRNA and protein levels benefits from cetuximab treatment, which warrants further clinical confirmation.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest

to declare.

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