

A method for establishing a patient-derived xenograft model to explore new therapeutic strategies for esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal carcinoma in China. The overall 5-year survival rate of ESCC patients is in the low range of 15-25%. One important reason for the poor prognosis is that the underlying molecular mechanisms are unclear. Furthermore, the development of effective therapeutic strategies to improve patient outcome is needed. Animal models can be beneficial to analyze the molecular mechanisms as well as specific clinical therapeutic strategies for esophageal cancer. In recent years, patient-derived xenografts (PDXs) have been widely used in numerous types of cancers to investigate the basic mechanisms and to conduct preclinical research. Accumulating evidence indicates that the PDX model is an important tool for basic and clinical research. Herein, we successfully established 14 ESCC PDXs. These PDX models preserved the patient pathological characteristics and effectively reflected the patient biological heterogeneity. Cancers exhibit diverse growth rates and tumor texture, even more, they have different signaling pathways. The PDX model is a superior strategy for understanding the underlying molecular mechanisms of ESCC and for screening new therapeutic strategies for ESCC patients.

Introduction

Esophageal cancer (EC) is one of the most common malignant diseases worldwide, particularly in China, where a high incidence of EC is noted. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal carcinoma in China (1). Several treatment approaches, including surgery, radiotherapy and chemotherapy are used in the clinic. However, the overall 5-year survival rate remains dismal (2). Therapeutic resistance at a late stage of ESCC is one of the significant reasons for the low survival rate (3).

Recent research indicates that tumorigenic signaling pathways are involved in the processes of ESCC, including tumor cell growth, cell cycling, apoptosis, angiogenesis and invasion (2,4). Hence, the key factors of signaling pathways can be effective therapeutic targets for the treatment of ESCC. Unlike breast cancer and non-small cell lung cancer (5-8), there is no reported data concerning the targeted therapy of ESCC. Therefore, there is needed to develop an effective strategy to investigate the mechanisms of esophageal carcinogenesis and explore therapeutic strategies for specific individuals to improve the prognosis of esophageal patients.

Animal models are appropriate tools to resolve both basic and clinical EC research problems. In recent years, patient-derived xenografts (PDXs) have been used to evaluate the effectiveness of targeted treatments for different types of tumors, such as breast and non-small cell lung cancer. The response of PDXs to chemotherapy resembles the patient response in different clinical treatment trials (9,10). Hence, PDX models provide a platform with which to study genetic and biological alterations as well as specific large-scale anti-cancer therapies for cancer patients (11).

In the present study, we established and characterized ESCC PDXs by transplanting 26 ESCC patient tumor specimens into severe combined immunodeficiency (SCID) mice. The 'success-rate' of the PDX models was 53.8%. Tumor growth in the first, second and third passage was observed. The pathology and the CK5/6, p63 and p40 expression levels

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Table I. Clinical characteristics of the studied patients.

No.	Age (years)	Gender	Tumor differentiation	TNM staging	Lymph node metastasis	Engrafted mode
EG1	63	Male	Poor	T4N0M0 III	No	No
EG2	64	Male	Well-Moderate	T2N0M0 IIa	No	Yes
EG3	74	Female	Well-Moderate	T2N0M0 IIa	No	Yes
EG4	60	Female	Moderate-poor	T3N0M0 IIa	No	No
EG5	61	Male	Moderate	T2N0M0 IIa	No	Yes
EG6	65	Female	Moderate-poor	T1N0M0 I	No	No
EG7	60	Female	Moderate	T3N2M0 III	Yes	No
EG8	63	Male	Moderate	T2N0M0 IIa	Yes	Yes
EG9	66	Male	Well-Moderate	T2N1M0 IIb	Yes	Yes
EG10	62	Female	Well-Moderate	T2N0M0 IIa	No	No
EG11	65	Male	Poor	T3N0M0 IIa	No	Yes
EG12	65	Female	Well-Moderate	T2N0M0 IIa	No	No
EG13	63	Male	Moderate	T3N0M0 IIa	No	Yes
EG14	59	Male	Moderate	T2N0M0 IIa	No	Yes
EG15	60	Male	Moderate	T2N1M0 IIb	Yes	No
EG16	58	Female	Moderate-poor	T3N1M0 III	Yes	No
EG17	82	Male	Moderate	T3N0M0 IIa	No	No
EG18	52	Male	Well-Moderate	T3N1M0 I-II	Yes	Yes
EG19	70	Male	Moderate	T1N1M0 II	Yes	Yes
EG20	46	Female	Moderate	T2N0M0 II	No	Yes
EG21	68	Female	Moderate-poor	T3N1M0 II-III	Yes	Yes
EG22	60	Male	Well	T2N0M0 II	No	No
EG23	61	Male	Moderate-poor	T3N1M0 II-III	Yes	No
EG24	47	Male	Well	T3N1M0 I	Yes	Yes
EG25	69	Male	Well	T2N0M0 I	No	Yes
EG26	64	Male	Moderate	T3N0M0 II	No	Yes

in the patient samples of the first and third passages were detected and compared with each other by hematoxylin and eosin (H&E), and immunochemical staining. In addition, tumor genetic signaling pathway kinases, including mTOR and p-mTOR (Ser2481), Stat1 and p-Stat1 (Tyr701), Stat3 and p-Stat3 (Tyr705), Akt1 and p-Akt (Ser473), Erk and p-Erk (Thr202/Tyr204) were detected in five PDX models by western blotting. These PDX models provided a platform to further understand the molecular mechanisms of ESCC and screen new specialized therapeutic regimens for ESCC patients in preclinical research.

Materials and methods

Patient tissue procurement. All 26 ESCC patients underwent surgical operations at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China), and ESCC tissues were obtained intraoperatively from October 2013 to October 2014. All patients had not received chemotherapy and radiotherapy prior to surgery. Written informed consent was provided by all patients for using the tissue samples. All research protocols were approved by the Research Ethics Committee of Zhengzhou University. Tissue histology was confirmed by two pathologists. Fresh harvested ESCC speci-

mens were obtained from the edge of whole tumor tissues to maintain high active and low necrotic part of the tissues. All tissues were transported to the laboratory in transport media [fetal bovine serum (FBS)-free, RPMI-1640 with penicillin and streptomycin].

Animals. Female CB17/SCID mice (6-8 weeks of age) with an average body weight of 18-20 g (Vital River, Beijing, China) were used in the study. Mice (4-5) were kept in a pathogen-free environment in light controlled rooms (12-h cycles) and were provided with food and water *ad libitum*.

Patient tumor xenografts. Tissue samples were placed in a Petri plate containing PBS with penicillin and streptomycin. The tissues were divided into three parts. One portion was implanted into SCID mice, the second portion was fixed in 10% formalin and the third portion was used for protein extraction. The mice were anesthetized using 0.3 ml of 0.4% (w/v) pentobarbital sodium for every 20 g of body weight. Then, they were subcutaneously implanted with tissues of different weight and size (0.10-0.12 g and ~3 mm). Animals were monitored periodically for their weight and tumor growth. Second passage was performed at a tumor size of ~1,500 mm³. The tumor specimen was cut into three parts and followed the same

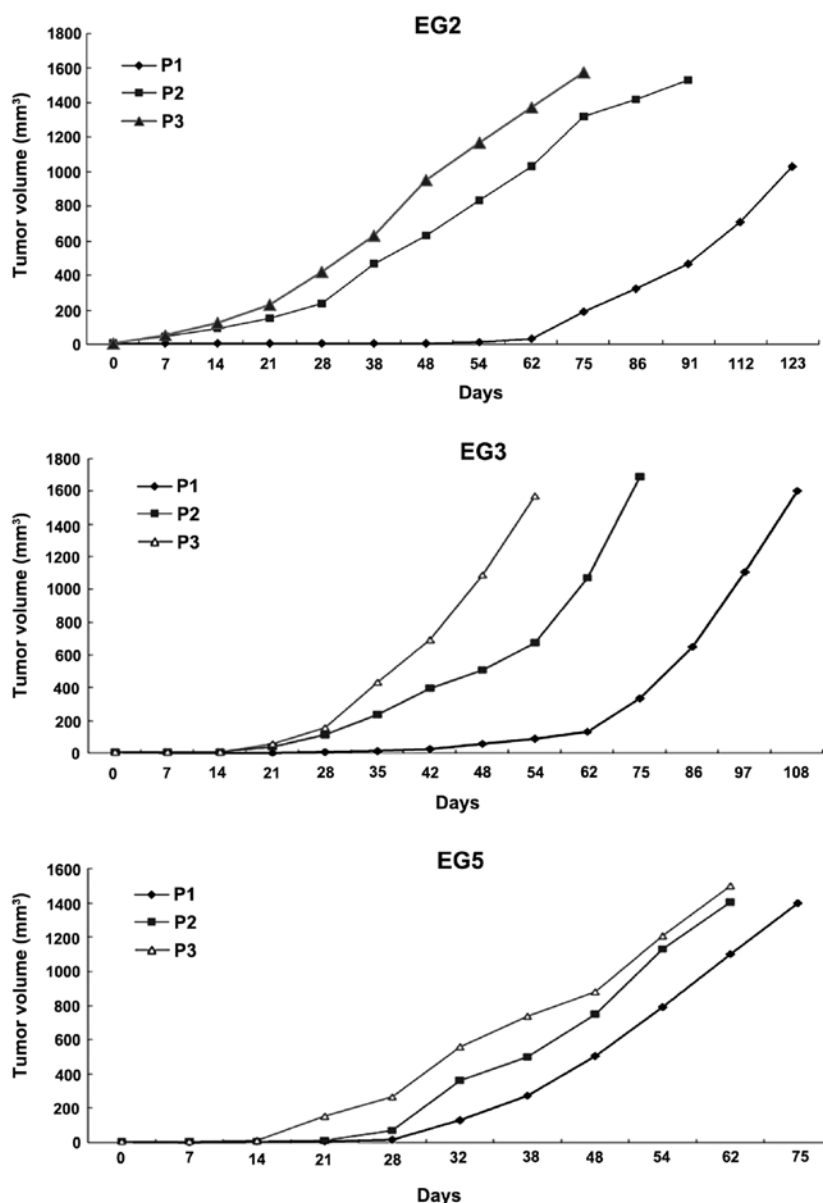


Figure 1. Growth curves of the ESCC xenografts in SCID mice. Growth curves of three passage xenografts (first, second and third passage) plotted as tumor volume over time. Xenografts were established from three ESCC patients (EG2, EG3 and EG5).

protocol as previously described. Successful tumor grafts were recognized as those with rapid tumor growth *in vivo* with more than three passages, and these were used for experimental studies.

Tumor growth measurements. The tumor xenograft size was measured with a Vernier caliper two times every week. The tumor volume was calculated using the formula: $V = LD \times (SD)^2 / 2$, where V is the tumor volume, LD is the longest tumor diameter and SD is the shortest tumor diameter. Tumor growth curves were plotted as tumor volume.

Histological and immunohistochemical analysis. The second part of the tumor was embedded in paraffin for histopathologic examination and immunohistochemical analysis. All of the slides were stained with Harris hematoxylin after dewaxing the 5- μ m sections with dimethylbenzene and were evaluated

by two pathologists. Tissue sections were stained with antibodies against CK5/6, p63, p40 (Abcam, UK) and were incubated overnight at 4°C. HRP-IgG secondary antibody was used at 37°C for 15 min. Then diaminobenzidine (DAB) was used for detection. All slides were observed and measured by an Olympus microscope (Japan).

Western blotting. The PDX tissues were ground in liquid nitrogen and were lysed by tissue lysate. Next, tissue protein was extracted by centrifugation at 12,000 rpm for 15 min at 4°C. The protein (50 μ g) was separated on 10% SDS-polyacrylamide gel, and then transferred to a PVDF membrane at 90 V for 2 h. The membrane was blocked with 5% non-fat milk for 60 min. Then the membrane was incubated with mTOR, p-mTOR (Ser2481), Stat1, p-Stat1 (Tyr701), Stat3, p-Stat3 (Tyr705), Akt1, p-Akt (Ser473), Erk1/2 and p-Erk1/2 (Thr202/Tyr204) antibodies (Cell Signaling Technology, USA) overnight at 4°C

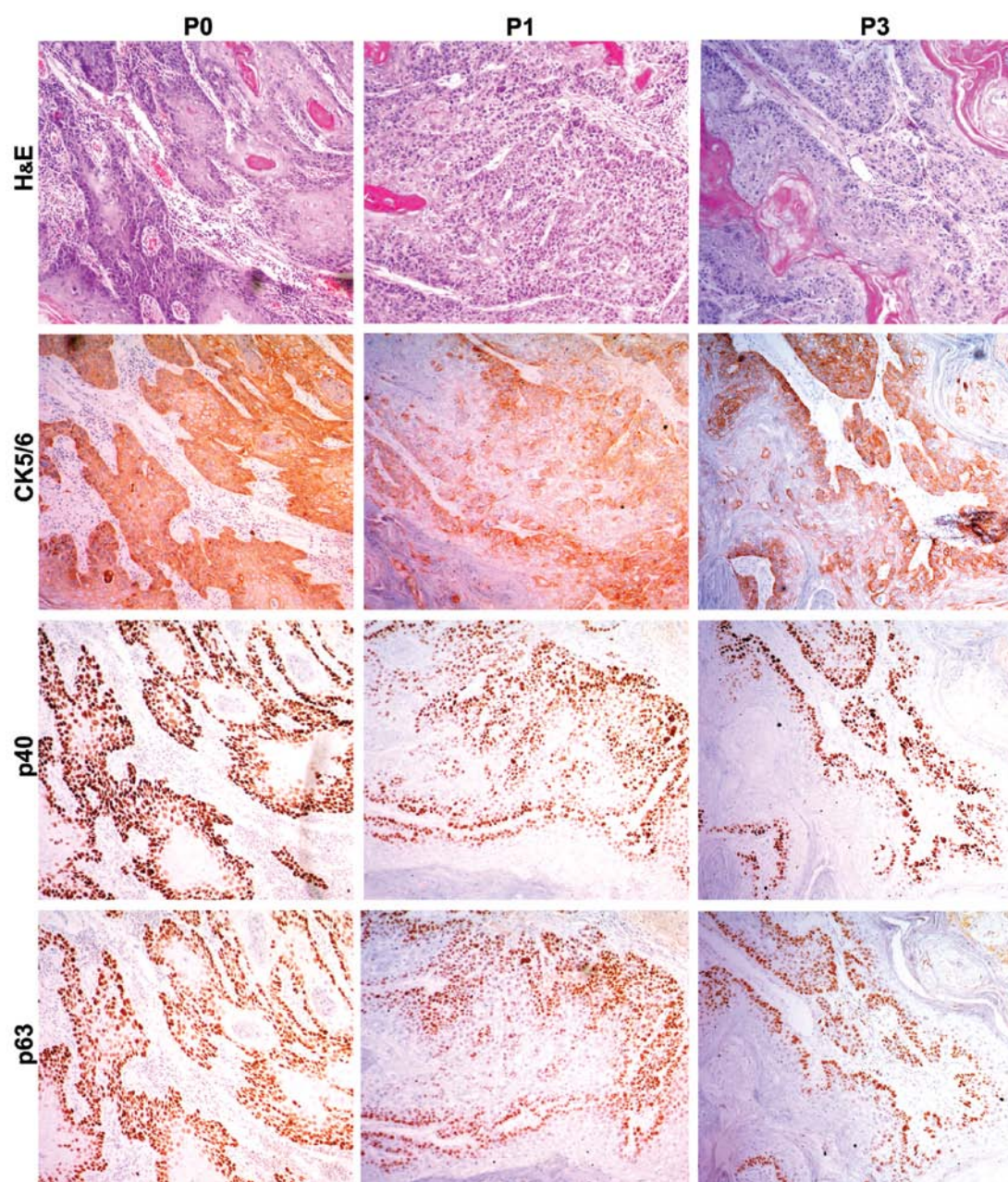


Figure 2. Comparative histology of the original patient tumors with first passage and third passage xenografts for sample EG2. The top row images show histological sections (H&E) of the esophageal patient tissue (P0), the first (P1) and the third passage (P3) xenograft tissues in the SCID mice. The second, third and last row of images show immunohistochemistry for CK5/6, p40, p63 in the esophageal patient tissue (P0), and the first (P1) and third passage (P3) xenograft tissues, respectively (magnification, x100).

(all antibody were used at 1:1,000, Stat3 antibody was from mouse and other antibodies were all from rabbit). HRP-IgG secondary antibody was incubated at room temperature for 2 h. Protein bands were visualized by an enhanced chemiluminescence detection kit (ECL; Pierce). The PVDF membranes were scanned by ImageQuant LAS 4000 and analyzed by ImageJ software v2.1.4.7.

Statistical analysis. All statistical analyses were performed using SPSS statistical software 17.0. Experimental values were reported as mean \pm standard error of the mean. One-way analysis of variance was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Clinical characteristics of the patients. In the present study, 26 ESCC subjects underwent surgical resection. The subjects consisted of 17 men and 9 women ranging from 46-82 years of age (62.6 ± 7.4 years). The subjects did not have any apparent distant metastases, and none had been previously treated. In regards to differentiation, 2 samples were established from 3 well differentiated samples, 4 were from 6 well-moderate differentiated samples, 7 were from 10 moderately differentiated samples, only 1 sample was established from 5 moderate-poorly differentiated samples while no sample was established from 2 poorly differentiated samples. Among these

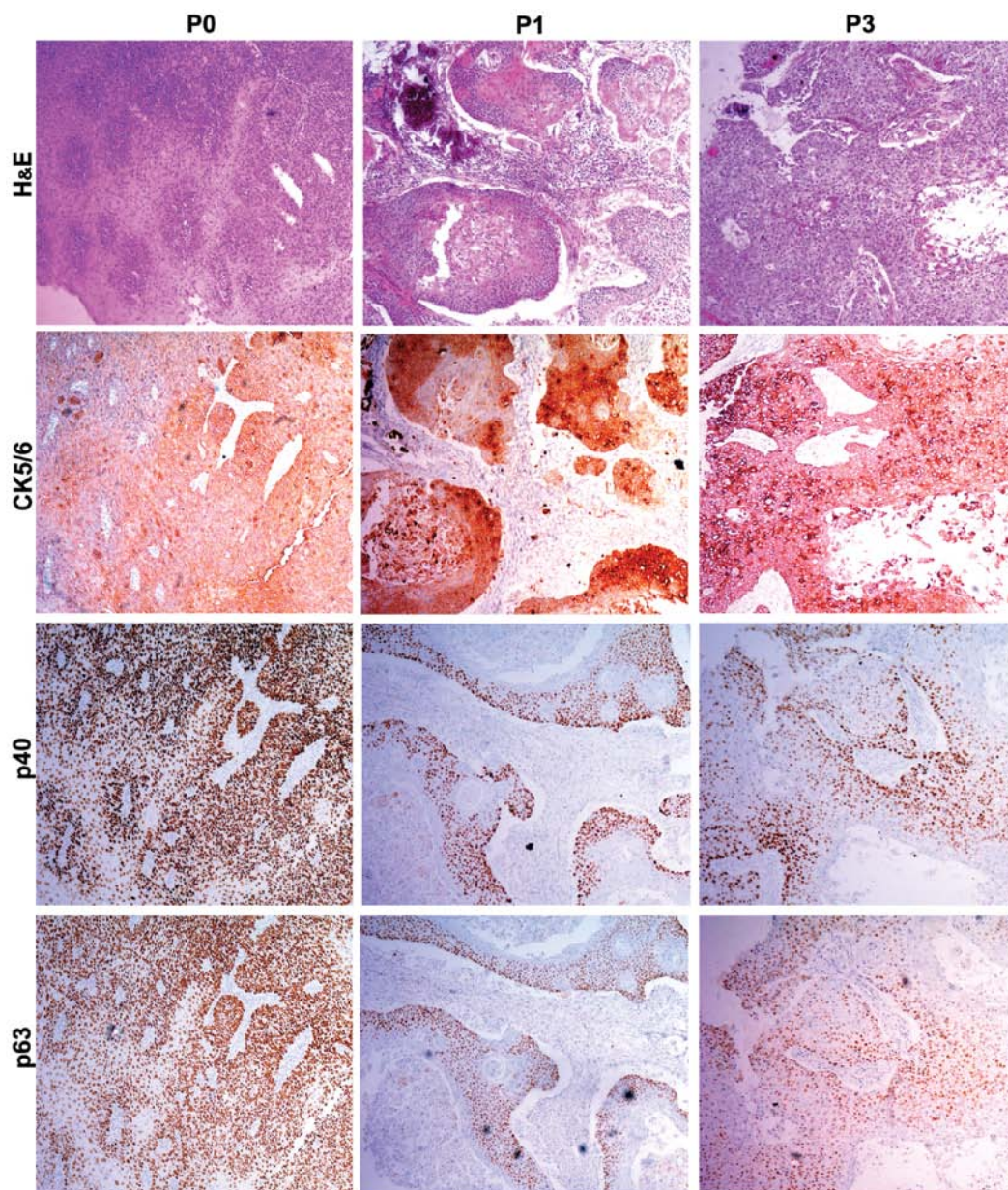


Figure 3. Comparative histology of the original patient tumors with first passage and third passage xenografts for sample EG3. The top row images show histological sections (H&E) of the esophageal patient tissue (P0), the first (P1) and the third passage (P3) xenograft tissues in the SCID mice. The second, third and last row of images show immunohistochemistry for CK5/6, p40 and p63 in the esophageal patient tissue (P0) and the first (P1) and third passage (P3) xenograft tissues, respectively (magnification, x100).

patients, 17 patients were stage II, 3 patients were stage III, 2 patients were stage II-III, 3 patients were stage I. One patient was stage I-II. According to lymph node metastasis, 10 samples had node metastasis and 6 samples were established, 16 samples had no node metastasis and 8 samples were established. Clinical data of all the patients are shown in Table I.

Growth of ESCC xenografts in SCID mice. Fourteen transplantable xenografts were established, and the success rate was 53.8%. EG2, EG3 and EG5 were chosen to assess the pathological characteristics. The growth curves of passage three xenografts (first, second and third passage) were plotted as tumor volume over time (Fig. 1). The growth time of the first passage was 123, 108 and 83 days respectively, the second

passage was 90, 74 and 81 days, and third passage was 62, 52 and 72 days. Every group period was shorter in the third passage than the first and second passage. In addition, the growth was stable after the third passage.

Comparison of the histology and immunohistochemistry between the xenografts and the patient tumors. In order to further evaluate the PDX xenografts, we compared the histology of the original patient tumors with the first and third passage xenografts for samples EG2, EG3 and EG5. We found that the pathological characteristics of the third passage xenografts were in accordance with the original patient samples. The expression of CK5/6, p40 and p63 was positive in the three passages by immunohistochemical staining (Figs. 2-4).

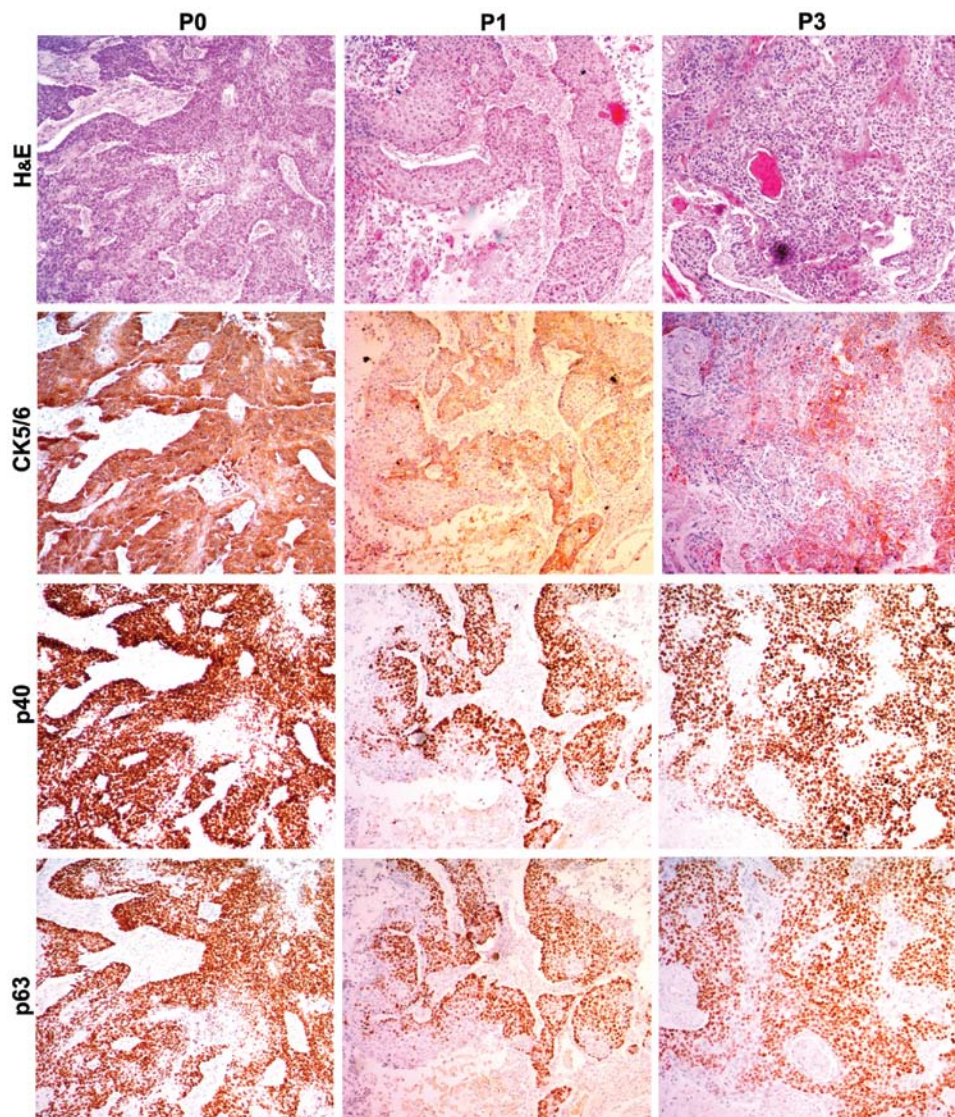


Figure 4. Comparative histology of the original patient tumors with first passage and third passage xenografts for sample EG3. The top row images show histological sections (H&E) of the esophageal patient tissue (P0), the first (P1) and the third passage (P3) xenograft tissues in the SCID mice. The second, third and last row of images show immunohistochemistry for CK5/6, p40 and p63 in the esophageal patient tissue (P0) and the first (P1) and third passage (P3) xenograft tissues, respectively (magnification, x100).

Activated signal transduction pathways in the established esophageal tumor xenografts. The levels of phosphorylated and total proteins of AKT-mTOR, Stat3 and Erk 1/2 were detected by western blotting. The level of p-mTOR (Ser2481) was higher in EG8 and EG9 than in EG2, EG3 and EG5, however, p-Akt (Ser473) was higher in EG9 than in EG2, EG3, EG5 and EG8 (Fig. 5A). The level of p-Stat3 (Tyr705) was highest in EG3, however, the level of p-Stat1 (Tyr701) was higher in EG3 and EG8 (Fig. 5B). Moreover, p-ERK1/2 (Thr202/Tyr204) was highest in EG3 (Fig. 5C). The difference in activated signal transduction pathways in these PDXs had statistical significance ($P=0.000$).

Discussion

Esophageal cancer (EC) is a serious malignancy with a high rate of mortality and poor prognosis. While many other types of cancer are expected to decrease in incidence over the next 10 years by 2025, the prevalence of EC is expected to increase

by 140% (12). ESCC is the most common histological type of EC, with a high incidence in China (13). Current clinical treatment strategies for ESCC include surgery, chemotherapy and radiotherapy. Recently, neoadjuvant chemoradiation followed by surgery has been used in the treatment of EC in the clinic (14). Unfortunately, the 5-year survival rate has not improved. Therefore, it is necessary to develop effective therapeutic strategies for ESCC patients.

Animal models are the most useful tool for preclinical evaluation of novel therapeutic strategies in cancer. PDX models can reproduce tumor development, proliferation and metastasis (11) and can predict phase II clinical trial performance (15). Therefore, PDX models play an important role in developing personalized treatment for EC patients (14). In the present study, we established 26 ESCC PDXs subcutaneously. In addition, the success 'take-rate' of the xenografts was 53.8% and it was higher compared to other EC PDX models for which the success rate was 38.5% (16). We further evaluated the established PDX models represented as EG2, EG3 and EG5. The

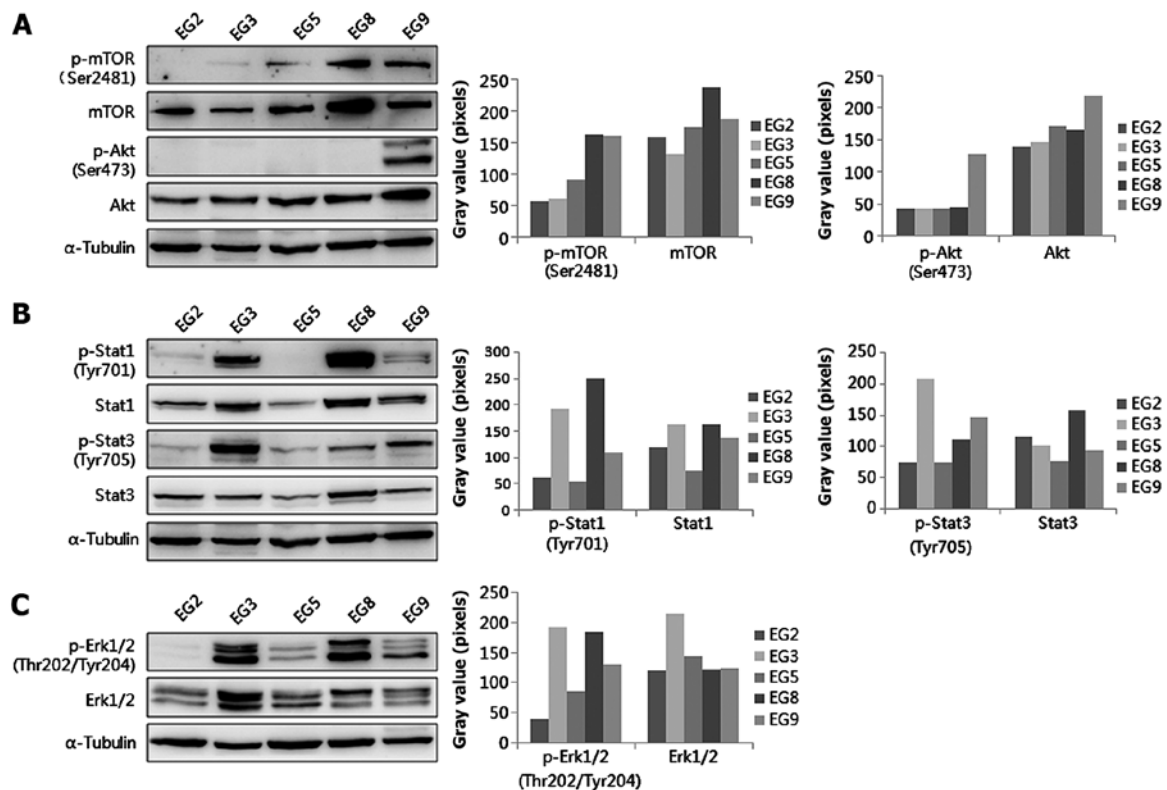


Figure 5. Activated signal transduction pathways [(A) Akt-mTOR axis, (B) Stat1 and Stat3], and (C) Erk1/2 in the established esophageal tumor xenografts. Right, the levels of phosphorylated and total proteins of the Akt-mTOR axis, Stat1 and Stat3, Erk are shown by western blotting. Each experiment was repeated three times. Left histograms, the results of western blotting. ImageJ was used to determine the gray value of the blots, and construct histograms using the gray value. SPSS 17.0 was used to analyze the data; $P=0.000$, statistical significant difference.

three established ESCC xenografts showed decreased degree of tumor growth curves from first passage to third passage (Fig. 1). In the three PDX models, the first passage reached 1,500 mm³ on day 133, 106 and 75 for EG2, EG3 and EG8, respectively. Next, we performed histology and growth curve analyses of the established xenografts. Expression of CK5/6, p63 and p40 was assessed by immunohistochemistry. Cytokeratin 5/6 is useful for differentiating epithelioid mesotheliomas from pulmonary carcinomas (17). P63 protein plays important roles in the carcinogenesis of ESCC through the Akt signaling pathway (18). An investigation into the combined expression of p63 may be helpful in early diagnosis and in evaluating the prognosis of ESCC (19). In our study, histological analysis and immunohistochemical analysis were used for assessing the characteristics of the PDX tumors. Expression of CK5/6, p63, p40 in patient samples was positive in the patient tissue and in the first and third passages. Our results indicated that the ESCC PDX models maintained the histological characteristics and the differentiation status of the original patient tumor. In a word, we successfully established ESCC PDX models from patient minimal surgical tissue. The established ESCC PDX models provide a superior *in vivo* preclinical platform to explore novel cancer therapeutics and analysis of drug activity, including the discovery of predictive biomarkers, the study of ESCC cancer stem-cell biology and stromal-tumor interactions in the clinic.

The drugs used for ESCC patient treatment are toxic to cells and have many side-effects. More effective and innovative treatment strategies are urgently needed (3). However, the molecular tumorigenesis mechanisms of ESCC have not

been fully elucidated. Many signaling pathways, for example Akt/mTOR, Erk1/2, Stat1 and Stat3 are involved in the processes of cell growth, cell cycling, apoptosis and invasion in ESCC (20-24). In the present study, we examined the expression of Akt and p-Akt (Ser473), mTOR and p-mTOR in five PDX models. Our results found that the level of p-Akt (Ser473) was positive only in EG9. Our results showed that all groups expressed total mTOR, however, the level of p-mTOR (Ser2481) was higher in EG8 and EG9 than in EG2 and EG5. Thus, we chose the activated Akt/mTOR pathway from our established PDX models to further research the role of the Akt/mTOR pathway in ESCC. The extracellular signal-regulated kinase (Erk1/2) signaling pathway is important for the regulation of cell growth by controlling transcription (25,26). In addition, p-Erk1/2 (Thr202/Tyr204) is higher in poorly differentiated tissues than in well and moderately differentiated tissues in ESCC (25,27). Our results indicated that Erk1/2 was activated in EG2 compared to the other patients. In addition, its levels were different in these five patient models.

Signal transducer and activator of transcription-3 (Stat3) plays a role in esophageal carcinogenesis by promoting cell proliferation, motility and suppression of apoptosis (28). In addition, high p-Stat3 (Tyr705) is correlated with poor prognosis in ESCC (29). However, Stat1 has opposing biological effects compared with Stat3. The loss of Stat1 expression significantly correlates with a worse clinical outcome in ESCC (30). Recent research found that Stat1 downregulates Stat3 and p-Stat3 (Tyr705), and the relative proportions of Stat1:Stat3 heterodimers decide the cell fate in ESCC (31).

In our study, we found that p-Stat1 and p-Stat3 were overexpressed in EG3, in contrast, there was absence of both in EG2 and EG5. In contrast, p-Stat1 (Tyr701) was lost and p-Stat3 (Tyr705) was activated in EG9. This indicated that the relationship between Stat3 and Stat1 is complicated. Investigation of the role of Stat1 and Stat3 and the relationship between both in ESCC are warranted using these xenografts.

In conclusion, we successfully established ESCC patient tumor-derived xenografts. These xenografts maintained the patient's pathological characteristics. They had different growth cycles and tumor texture. Notably, different signaling pathways were activated in ESCC, which reflected the biological heterogeneity observed in the patient population. Furthermore, these PDX models provide an important platform for understanding the molecular mechanisms of ESCC, for elucidating critical signaling pathways involved in esophageal tumorigenesis and progression. These models can also be used to identify potential individual therapeutic targets, investigate the biological activity and toxicity profiles of preclinical drugs and provide a more relevant system to test clinically directed hypotheses in ESCC.

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