Genetic Variants in EGFR/PLCE1 Pathway Are Associated with Prognosis of Esophageal Squamous Cell Carcinoma after Radical Resection*

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Summary: Esophageal cancer (EC) is one of the most deadly malignant diseases. Several studies revealed that variations of the phospholipase C epsilon 1 (PLCE1) gene were associated with EC susceptibility. PLCE1 is located downstream of the epidermal growth factor receptor (EGFR) pathway. Presently, the single nucleotide polymorphisms (SNPs) of EGFR/PLCE1 genes and their associations with EC survival remain unclear. In this study, the associations between genetic variants in the EGFR/PLCE1 pathway and prognosis in 124 esophageal squamous cell carcinoma (ESCC) patients with radical resection were explored. The results showed that CC genotype of both PLCE1 rs17109671 and EGFR rs2072454 was associated with ESCC prognosis. Multivariate analysis revealed that patients with the two unfavorable genotypes had the worst overall survival (OS) or disease-free survival (DFS) (HR=6.099, 95%CI=1.903–19.552; HR=3.994, 95%CI=1.49–10.702, respectively). Additionally, combination of SNPs and tumor stage could better predict OS (for AUC, 0.774 *vs*. 0.709) and PFS (for AUC, 0.773 *vs*. 0.704) than tumor stage alone. In conclusion, genetic variants of the EGFR/PLCE1 may be predictors of the prognosis of ESCC after surgery. The individuals with the CC genotype of PLCE1 rs17109671 and EGFR rs2072454 should receive more aggressive treatments.
Key words: esophageal squamous cell carcinoma; prognosis; single nucleotide polymorphism; survival; epidermal growth factor receptor

Esophageal cancer (EC) is the eighth most common cancer worldwide, as well as the sixth most common cause of death from cancer. About 80% of the cases occur in developing countries, and China contributed to almost half of the global new EC cases in 2012[1, 2]. There were approximately 223 000 new cases and 197 200 deaths in China, accounting for 52.8% of incidence and 49.3% of mortality around the world in 2012[3]. EC has two main histological subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). In China, 90% of patients with EC are ESCC[4]. Despite major advances in the multimodal approach to the locally advanced esophageal and junctional cancer, the prognosis of EC patients treated with curative intent remains poor, with a five-year survival rate of 20.9% in China[5], and between 20% to 47% in the other countries[3, 6]. The majority of patients eventually succumb to this disease[7], thus early diagnosis and treatment are vital. However, the present clinical diagnosis, depending on radiology and endoscopic biopsy, is invasive, and most patients are at an advanced stage when accurate diagnosis is attained[8]. Therefore, it is urgently important to identify the effective biomarkers for the early detection of ESCC.

Genome-wide association studies (GWAS) of ESCC in Chinese populations have indicated that germline genetic variants in phospholipase C epsilon 1 (PLCE1, 10q23) are related to the risk of ESCC and the abnormal expression of PLCE1 seems to be associated with cancer prognosis[9, 10]. Phospholipase

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Cε, encoded by PLCE1, is one of the effectors of Ras-GTP, which is located downstream of the epidermal growth factor receptor (EGFR) pathway and links Ras to calcium release and protein kinase C activation[11]. Lin *et al*[12] reported that EGFR amplification may be useful in predicting overall survival (OS) in patients with ESCC. Recent studies indicated that coexpression of EGFR and periostin or c-Met is more likely to be of predictive value for ESCC development and poor progression[13, 14]. Liao *et al*[15] recently suggested that single nucleotide polymorphisms (SNPs) in PLCE1 and EGFR genes were associated with TP53 expression but not with the prognosis in hepatitis B virus-related hepatocellular carcinoma (HCC) in Chinese patients in Guangxi. TP53 protein is a key tumor suppressor and can prevent tumorigenesis and cancer progression. Although it is currently known that there exists PLCE1 germplasm variation in ESCC, very little is understood about the somatic changes of PLCE1 or EGFR and their associations with survival.

Therefore, this study aimed to explore the association between genetic variants in the EGFR/PLCE1 pathway and prognosis in ESCC patients with radical resection. Our findings may provide a novel target for the treatment of ESCC.

1 MATERIALS AND METHODS

1.1 Patients

This is a retrospective study on patients with ESCC who underwent esophagectomy at Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, China, between 2010 and 2012. Patients eligible for the analysis were those with confirmed R0 resection and ESCC by pathologists. The patients with distant metastasis, or who were given neoadjuvant therapy, or died in perioperative period were excluded. Tumor stage was determined by the American Joint Committee in Cancer Staging Manual (7th edition, 2010). The research protocol was approved by The Ethics Committee of Huazhong University of Science and Technology, China. Each patient signed a written informed consent before recruitment. Postoperative follow-up was conducted every 4 months in the first 2 years and every 6 months afterwards. At each follow-up, clinical information was collected and physical examination was performed. The last follow-up was done in May 2015.

1.2 Genotyping Method

Paraffin-embedded (FFPE) tissue samples were obtained from the Pathology Department of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. DNA was extracted from FFPE tissue using QIAamp DNA FFPE tissue kits (56404; Qiagen, Germany). By searching in the 1000 Genomes Project and Ensembl website, all SNPs that met the criteria of minor allele frequency (MAF) >0.1 in Southern Han Chinese, locating at 3′-UTR or 5′-UTR or synonymous variant or missense variant, and r^2 <0.8 according to linkage disequilibrium were screened out. Finally nine SNPs were selected. Genotypes of SNPs were analyzed by Sequenom MassARRAY®SNP combined with multiple PCR technique, MassARRAY iPLEX technique and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).

1.3 Statistical Analysis

Study endpoints included overall survival (OS) (from the operation day to the last follow-up or death) and disease-free survival (DFS) (from the operation day to tumor recurrence). Data analysis was conducted by SPSS Statistics 24.0 software. Patients were grouped according to their genotypes. Proportional hazards model was applied to evaluate hazard ratio (HR) and 95% confidence intervals (CIs). Multivariate analysis was conducted to adjust covariance. Receiver operating characteristic (ROC) curve was generated to compare the models with and without positive gene variants. The area under curve (AUC) in each group was compared using MedCalc statistical software. A P<0.05 was considered statistically significant.

2 RESULTS

2.1 Patient Characteristics and Prognosis

A total of 124 patients were enrolled, including 98 males and 26 females. Patient characteristics are listed in table 1. The median age was 59 years (range 40–76 years); 77 (62.1%) patients smoked, and 69 (55.6%) drank; 47.6% tumors located in the middle segment and 43.5% located in the lower segment; 50 (40.3%) patients were in stage Ⅱ and 25 (20.2%) were in stage III; 34.7% patients received adjuvant therapy.

The median follow-up time was 38 months (range 1–61 months). During the follow-up period, 38 patients (30.6%) died, and 46 patients (37.1%) relapsed. The median OS was 38 months, while the median DFS was 36 months. Multivariate analysis between patient-, tumor-, therapy-related factors and prognosis showed that patients in stage Ⅲ had decreased OS (HR=6.840, 95%CI=2.688–17.403, P<0.001) and DFS (HR=5.518, 95% CI=2.503–12.164, P<0.001) (fig. 1). Patients at more aggressive stage received adjuvant therapy, thus the decreased OS and DFS may be associated with adjuvant therapy (HR 2.480, 95% CI=1.307–4.706, P=0.005; HR 2.381, 95% CI=1.332–4.257, P=0.003). Sex, age, tobacco smoking, alcohol drinking and tumor location were not significantly associated with the prognosis of ESCC.

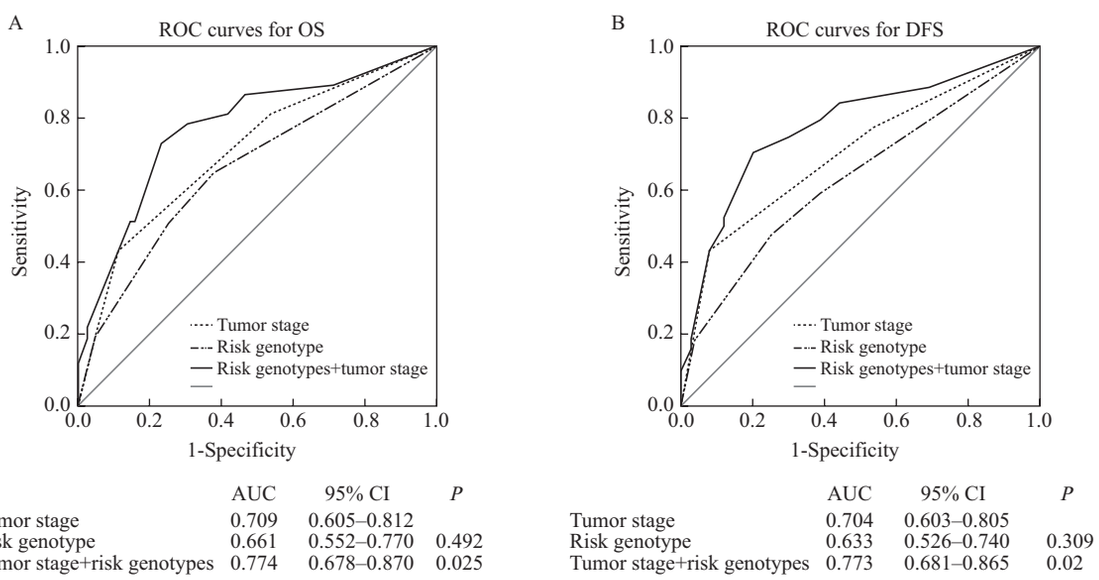
2.2 Effects of PLCE1 and EGFR Genotypes on Prognosis

The SNPs and their functional consequence minor

Table 1 Associations of demographics and clinicopathological characteristics with OS and DFS in Chinese ESCC patients

Parameters	No.	OS		DFS	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Gender					
Male	98	1		1	
Female	26	0.510 (0.137–1.897)	0.315	0.557 (0.195–1.590)	0.274
Age (years)					
<59	61	1		1	
≥59	63	1.551 (0.772–3.118)	0.218	0.968 (0.514–1.823)	0.921
Tobacco smoking					
Never	47	1		1	
Ever	77	0.908 (0.429–1.922)	0.801	0.931 (0.455–1.903)	0.844
Alcohol drinking					
Never	55	1		1	
Ever	69	1.186 (0.545–2.854)	0.667	0.660 (0.324–1.345)	0.253
Tumor location					
Upper	11	1		1	
Middle	59	1.267 (0.271–5.919)	0.764	1.910 (0.429–8.511)	0.396
Lower	54	2.369 (0.507–11.077)	0.273	2.330 (0.503–10.786)	0.279
Tumor stage					
Ⅰ	49	1		1	
Ⅱ	50	3.189 (1.230–8.269)	0.017	1.734 (0.766–3.930)	0.187
Ⅲ	25	6.840 (2.688–17.403)	<0.001	5.518 (2.503–12.164)	<0.001

OS: overall survival; DFS: disease-free survival; ESCC: esophageal squamous cell carcinoma; HR:, hazard ratio; CI: confidence interval



	AUC	95% CI	*P*
Tumor stage	0.709	0.605–0.812	
Risk genotype	0.661	0.552–0.770	0.492
Tumor stage+risk genotypes	0.774	0.678–0.870	0.025

	AUC	95% CI	*P*
Tumor stage	0.704	0.603–0.805	
Risk genotype	0.633	0.526–0.740	0.309
Tumor stage+risk genotypes	0.773	0.681–0.865	0.02

Fig. 1 Receiver operating characteristic (ROC) curves in esophageal squamous cell carcinoma (ESCC) patients
A: ROC curve for the prediction of overall survival (OS) by the tumor stage model, the risk genotypes model, and the combined tumor stage and risk genotypes model; B: ROC curve for the prediction of disease-free survival (DFS) by the tumor stage model, the risk genotypes model, and the combined tumor stage and risk genotypes model. *P* values were identified according to the area under curve (AUC) of the three different models.

allele frequency (MAF) are listed in table 2. Proportional hazards model showed that EGFR rs2072454 was associated with prognosis. Patients with CC genotypes had decreased OS (*P*=0.005) and DFS (*P*=0.016) (table 3). Multivariate analysis adjusted for patient sex, age, tobacco smoking, alcohol drinking, tumor location and tumor stage showed that PLCE1 rs17109671 and EGFR rs2072454 were associated with the prognosis of ESCC. Patients with both rs17109671 CC genotypes and rs2072454 CC genotypes presented decreased OS (rs17109671: HR=0.334, 95%CI=0.150–0.742, *P*=0.007; rs2072454: HR=0.394, 95%CI=0.192–0.807, *P*=0.011) and DFS (rs17109671: HR=0.458, 95%CI=0.219–0.958, *P*=0.038; rs2072454: HR=0.465,

Table 2 Genes and SNPs selected for analysis

Gene	SNP	Alleles	Functional consequence	MAF
PLCE1	rs17109671	T/C	Synonymous variant	0.481 (C)
	rs2274223	A/G	Missense	0.219 (G)
	rs11187870	G/C	3 prime UTR variant	0.157 (C)
	rs17417407	G/T	Missense	0.105 (T)
EGFR	rs884419	G/A	3 prime UTR variant	0.452 (G)
	rs2072454	C/T	Synonymous variant	0.386 (T)
	rs7334	C/A	3 prime UTR variant	0.224 (C)
	rs2293347	T/C	Synonymous variant	0.224 (T)
	rs1050171	G/A	Synonymous variant	0.124 (A)

SNP: single nucleotide polymorphism; MAF: minor allele frequency

95%CI=0.243–0.888, *P*=0.02). The other SNPs were not significantly associated with prognosis (table 3).

2.3 Combined Effect of SNPs on Prognosis

The CC genotypes of PLCE1 rs17109671 or EGFR rs2072454 were defined as "unfavorable" genotypes because they were associated with decreased OS and DFS. Patients were divided into three groups according to their number of unfavorable genotypes. Multivariate Cox regression analysis showed that patients with two unfavorable genotypes had the worst OS (95%CI=1.903–19.552) and DFS (95%CI=1.49–10.702); patients with one unfavorable genotype had significantly decreased OS (95%CI=1.264–6.748), but the DFS (95%CI=0.9–3.914) was not decreased significantly (table 4).

2.4 Prognosis Prediction Model Based on SNPs and Tumor Stage

A prognosis prediction model was established to identify whether combination of SNPs and tumor stage could better discriminate the prognosis compared to tumor stage or SNPs alone. According to the ROC curve analysis, the model with combination of SNPs and tumor stage showed better capability of predicting OS (95%CI=0.678–0.870) and DFS (95%CI=0.681–0.865), compared to the model with tumor stage alone (OS: 95%CI=0.605–0.812; DFS: 95%CI=0.603–0.805).

3 DISCUSSION

The TNM staging system, which is based on tumor depth (T), regional nodes with metastatic disease (N) and presence or absence of distant metastasis (M), is used to predict the prognosis of patients with cancer. However, the OS of patients with similar TNM stage varies, which may be attributed to overlooking the biological or molecular characteristics of each individual tumor. Our study evaluated the genetic variants of EGFR/PLCE1 and their potential

Table 3 Associations between genotypes and prognosis of patients with ESCC

Characteristic	*n*	OS	DFS
		HR (95% CI)	HR (95% CI)
PLCE1 rs17109671			
CC	26	1	1
TT+CT	93	0.334 (0.150–0.742)	0.458 (0.219–0.958)
PLCE1 rs2274223			
AA	67	1	1
GG+AG	57	0.986 (0.481–2.019)	0.994 (0.536–1.843)
PLCE1 rs2274223			
GG	73	1	1
CC+CG	41	1.032 (0.471–2.258)	1.046 (0.528–2.074)
PLCE1 rs17417407			
GG	100	1	1
TT+GT	20	1.616 (0.748–3.492)	1.599 (0.779–3.283)
EGFR rs884419			
AA	26	1	1
GG+AG	98	1.524 (0.624–3.721)	1.404 (0.647–3.044)
EGFR rs2072454			
CC	43	1	1
TT+CT	81	0.394 (0.192–0.807)	0.465 (0.243–0.888)
EGFR rs7334			
AA	77	1	1
CC+AC	44	0.672 (0.311–1.452)	0.621 (0.311–1.238)
EGFR rs2293347			
CC	55	1	1
TT+CT	66	1.199 (0.600–2.397)	1.241 (0.669–2.304)
EGFR rs1050171			
GG	88	1	1
AA+AG	35	1.506 (0.740–3.065)	1.291 (0.672–2.483)

Table 4 Associations between unfavorable genotypes and prognosis of patients with ESCC

No. of unfavorable genotypes	No. of patients	OS		DFS	
		HR (95% CI)	*P*	HR (95% CI)	*P*
0	66				
1	47	2.921 (1.264–6.748)	0.012	1.877 (0.9–3.914)	0.093
2	11	6.099 (1.903–19.552)	0.002	3.994 (1.49–10.702)	0.006

Multivariate analyses in this table were adjusted for patient sex, age, tobacco smoking, alcohol drinking, tumor location and tumor stage.

associations with the prognosis of ESCC patients who underwent radical resection. The results revealed that two SNPs, PLCE1 rs17109671 and EGFR rs2072454, were significantly associated with the OS and DFS of ESCC patients. The CC genotypes of PLCE1 rs17109671 and EGFR rs2072454 were significantly related to a decreased OS and DFS compared with the TC/TT genotype. Furthermore, these SNPs combined with tumor stage showed a cumulative effect on the prognosis of ESCC patients, and the patients with two unfavorable genotypes and advanced stage had the shortest OS and DFS. To the best of our knowledge, this is the first study to systematically investigate the association between gene variants of EGFR/PLCE1 pathway and survival in ESCC patients.

PLCE1 encodes a phospholipase C enzyme that mediates the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate the Ca^{2+}-mobilizing second messenger inositol 1,4,5-triphosphate and the protein kinase C-activating second messenger diacylglycerol[16, 17]. PLCE1 is activated by the Ras and Rho family GTPases and heterotrimeric G proteins, and is unique in the phospholipase C family[18]. The structure of PLCE1 renders it with functions associated with genome regulation, such as cell growth, differentiation, apoptosis, and angiogenesis[10, 19–22]. It has been demonstrated that germline genetic variants in the susceptibility locus (10q23) of PLCE1 gene were associated with the risk of ESCC[21]. Loss of PLCE1 dramatically decreased the invasion and proliferation capacity of EC cells *in vitro*. Moreover, such PLCE1 inactivated tumor grafts exhibit significantly decreased tumor size in mice[23]. Recently, Han *et al*[24] reported that miR-328 suppressed the survival of EC cells by directly targeting PLCE1. Cui *et al*[25] demonstrated that PLCE1 functioned as a tumor promoter in ESCC and was suppressed by miR-145 through inhibition of PLCE1 translation. Nevertheless, the predictive value of the PLCE1 gene variants on the prognosis of ESCC is still unknown. SNP rs2274223 of PLCE1, a missense mutation in the coding region, showed a genome-wide significance in ESCC but cannot be used as a predictive marker for the survival of ESCC patients in our study, which was in accordance with the results observed in the study of Zhou *et al*[26]. Shi *et al*[27] reported that rs17109671 in PLCE1 was significantly associated with remission, and our study revealed that patients with CC genotype of rs17109671 had worse survival than those with CT/TT genotype.

EGFR participates in several essential tumorigenic mechanisms, such as tumor survival, invasion, angiogenesis, and metastatic spread[28]. It is currently a major drug target in cancer treatment. EGFR overexpression and increased EGFR copy number are common events in ESCC[13]. EGFR amplification was significantly associated with advanced pathological stage and tumor lymph node metastasis in ESCC[12]. Study has reported that rs2072454 in EGFR is significantly associated with an increased risk of GC[29]. In our study, CC genotype of EGFR rs2072454 was associated with worse survival of ESCC.

However, there are several limitations in this study. As a retrospective study with a relatively small population, our findings need to be confirmed by large, multicenter, prospective studies. Due to the substantial ethnic variation in SNP frequencies, our results, which were demonstrated in Chinese Han population, should be validated in different ethnic backgrounds. Moreover, molecular mechanisms of rs17109671 and rs2072454 are needed to be further studied. Therefore, further research will be done to discover the impact of SNPs in the EGFR-PLCE1 pathway on cancer progression in our cohort.

In conclusion, genetic variants of EGFR-PLCE1 may be predictors of the prognosis of EC after surgery. The individuals with the CC genotype of PLCE1 rs17109671 and EGFR rs2072454 should receive more aggressive treatments and these patients may benefit from early prediction of an unfavorable prognosis by genotyping before the initiation of therapy.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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