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Original contribution

Three-gene immunohistochemical panel predicts progression and unfavorable prognosis in esophageal squamous cell carcinoma☆,☆☆

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Summary Esophageal squamous cell carcinoma (ESCC) is a highly invasive disease with a poor long-term survival rate. Although there has been progress in understanding the pathogenesis of ESCC, there are currently no molecular biomarkers that are used in routine clinical practices to determine prognosis. Therefore, the aim of this study was to determine a small immunohistochemical panel that could predict the prognosis of patients with ESCC. Phospholipase C epsilon-1 (PLCE1), IKKα, IKBα, p65, and p53 were highly expressed in ESCC tissues. The high expression level of PLCE1, IKBα, and p53 showed a significant positive correlation with a short overall survival ($P = .022$, .009, and .024, respectively). This 3-biomarker panel (ie, PLCE1, IKBα, and p53) was found to be a predictor of ESCC, with a worse overall survival as each positive marker was added (hazard ratio, 1.553; 95% confidence interval, 1.166-2.067; $P = .003$). In another cohort (including 1922 esophageal endoscopic biopsy tissues), the lesions of 28 patients were aggravated. Three proteins (PLCE1, 12/28 [42.86%]; IKBα, 16/28 [57.14%]; p53, 16/28 [57.14%]) were immunoreactive in patients with progressive disease. Our study identified and validated that this immunohistochemical biomarker panel of 3 proteins, consisting of PLCE1, IKBα, and p53, is not only independently associated with an unfavorable outcome for ESCC patients but also able to predict disease progression to precancerous lesions.

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1. Introduction

Esophageal squamous cell carcinoma (ESCC) is the predominant histologic type of esophageal carcinoma in the "Asian esophageal cancer belt" (such as Turkey, Iran, Kazakhstan and northern and central China) and accounts for 480 000 new cases and 400 000 deaths in developing countries worldwide each year [1]. The overall survival (OS) of patients with ESCC has increased owing to the development of endoscopic and pathological biopsy screening. However, most patients are still diagnosed at the late stage, and the 5-year OS rate of ESCC patients is only 10% to 20% [2]. In addition, more than 99% of ESCC cases are associated with esophageal precancerous lesions [3]. Thus, effective biomarkers are required to be identified to predict the prognosis of ESCC and the risk of precancerous lesion development.

Our group has previously described a novel polymorphism in phospholipase C epsilon-1 (PLCE1), which is related to the susceptibility of ESCC, by using large-scale, genome-wide association studies of the Chinese Han and Kazakh populations [4]. One independent study identified that PLCE1 overexpressed in ESCC tissues could be screened via immunohistochemistry (IHC) to facilitate prognostication in patients with ESCC. Inflammation is a fundamental protective response and is a major cofactor in the pathogenesis of ESCC [5,6]. Our previous study showed that NF-κB pathway proteins (ie, p65, IKKα, and IKBα) were overexpressed in the Xinjiang Kazakh ESCC tissues [7]. Many studies in the human ESCC tissue have shown that the overexpression of NF-κB results in a poor prognosis of patients [8]. p53 was the most common protein with abnormalities found in ESCC. The mutation rate of the DNA-binding region in p53 gene was 83%, and the mutated p53 protein was shown to functionally promote cell invasion and metastasis [9]. However, these proteins need further study in the prognosis of ESCC.

Nonetheless, not all identified targets have equal prognostic value, and simultaneous detection of various ESCC-associated proteins can improve the accuracy of prognostic evaluation in patients with ESCC. Therefore, to facilitate clinical utility, we aimed to create a small optimized panel of IHC markers that could be used to segregate patients into different prognostic groups. In this study, 5 candidate proteins, PLCE1, IKKα, IKBα, p65, and p53, were first screened in tissue microarrays, including 164 ESCC samples and 100 adjacent normal esophageal tissues, via IHC. The high expression levels of PLCE1, IKBα, and p53 were significantly associated with the unfavorable prognosis of ESCC patients. When combined with the TNM stage of ESCC patients, this panel was able to further stratify the risk of ESCC patients. Finally, when detected in another independent cohort of 1922 esophageal endoscopic biopsy patients, this panel was able to predict disease progression to precancerous lesions. Thus, this IHC biomarker panel has the ability to predict disease progression and prognosis in patients with ESCC.

2. Materials and methods

2.1. Patients and samples

Surgically resected primary ESCC tissues from 164 patients and esophageal endoscopic biopsies from 1922 patients from Xinjiang, China, were collected for this present study. The 164 surgically resected ESCC tissues were collected between 1997 and 2016 at the First Affiliated Hospital of Shihezi University in Xinjiang, China. Our research protocol was approved by the medical ethics and human clinical trial committee of the Shihezi University School of Medicine, and all recruited subjects were enrolled with written informed consent. All of the operative samples were used as residual specimens after diagnostic sampling. The deadline for follow-up for all ESCC patients was December 2017, which was 1 to 10 years after radical resection. None of the patients received chemotherapy or radiotherapy treatment before surgery, and patients who survived less than 1 month after surgery were excluded. The clinicopathological characteristics of the 164 ESCC patients are described in Supplementary Table S1.

The 1922 biopsies used in this study were collected by endoscopic screening between 2007 and 2016 at the endoscopy center of the First Affiliated Hospital of Shihezi University in Xinjiang, China. With endoscopic follow-up 1 to 5 years after the initial biopsy examination, 28 patients, including 9 normal epithelia and 19 precancerous lesions (8 low-grade intraepithelial neoplasia [LGIN] and 11 high-grade intraepithelial neoplasia [HGIN]), who had undergone a progression from normal esophageal squamous epithelium to precancerous/carcinoma since the initial biopsy were identified. All of the biopsy specimens in this study were used as surplus tissues after diagnostic sampling. The clinicopathological characteristics and disease progression of the 28 patients with endoscopic esophageal biopsy are summarized in Supplementary Tables S2 and S3, respectively.

2.2. Sample preparation and IHC procedure

Tissue microarray and endoscopic biopsy tissues were fixed in formalin and embedded in paraffin. The tissue sections were prepared for IHC staining using the EnVision method (DAKO, Glostrup, Denmark). Each 4-μm section was dewaxed by a dimethylbenzene gradient and dehydrated using an alcohol gradient. The antigen retrieval step involved using high-pressure heat recovery for 8 minutes in a boiling antigen retrieval solution (citric acid buffer [pH 6.0] for PLCE1, IKKα, P65, and p53; EDTA buffer [pH 9.0] for IKBα). The antigen retrieval solution was cooled to 37°C for 30 minutes, and then the endogenous peroxidase was eliminated. After incubation in 5% fetal bovine serum for 30 minutes at 37°C, the tissues were washed 3 times with phosphate-buffered saline for 5 minutes each time. The tissue sections were incubated with anti-PLCE1 rabbit polyclonal antibody (1:500 dilution, HPA015598; Sigma-Aldrich,

St Louis, MO), anti-IKKα rabbit monoclonal antibody (1:100 dilution, ab32041; Abcam, Cambridge, MA), anti-IKBα rabbit monoclonal antibody (1:200 dilution, ab32518; Abcam), anti-P65 rabbit monoclonal antibody (1:3200 dilution, ab32536; Abcam), and anti-p53 mouse monoclonal instant type antibody (Stoste, Do-7; Gene Tech, Shanghai, China) at 4°C for 8 hours. The tissue sections were then washed with 1 × Tris-buffered saline with Tween 20 and incubated in EnVision Two antibody for 30 minutes at 37°C. Finally, 3,3′-diaminobenzidine and hematoxylin were used for protein detection and analysis of the tissue architecture, respectively.

2.3. Assessment of IHC results

The expression levels of PLCE1, IKKα, IKBα, p65, and p53 were assessed semiquantitatively according to the percentage of positive cells and the staining intensity of the cytoplasm or nucleus. Scores were given as follows: 0 (0-5% positive cells), 1 (6%-25% positive cells), 2 (26%-50% positive cells), 3 (51%-75% positive cells), or 4 (≥76% positive cells). The IHC staining intensities of tumor cells were scored on a scale from 0 to 3 as follows: 0 (negative), 1 (buff), 2 (yellow), and 3 (brown). The percentage of positive epithelial cells and the staining intensities were then multiplied to generate an immunoreactivity score (IS) for each case. For example, if the staining intensity was brown (3) and the percentage of positive cells was greater than 75% (4), then the IS would be determined as 3 × 4 = 12. Five random fields were selected for scoring from each slide, and the mean score for each slide was used for final analysis. For example, the IS would range between 0 and 12. All cases were divided into 2 groups: the high group (score range> 4) and the low group (score range ≤ 4). Two independent observers performed the IHC scoring for each slide.

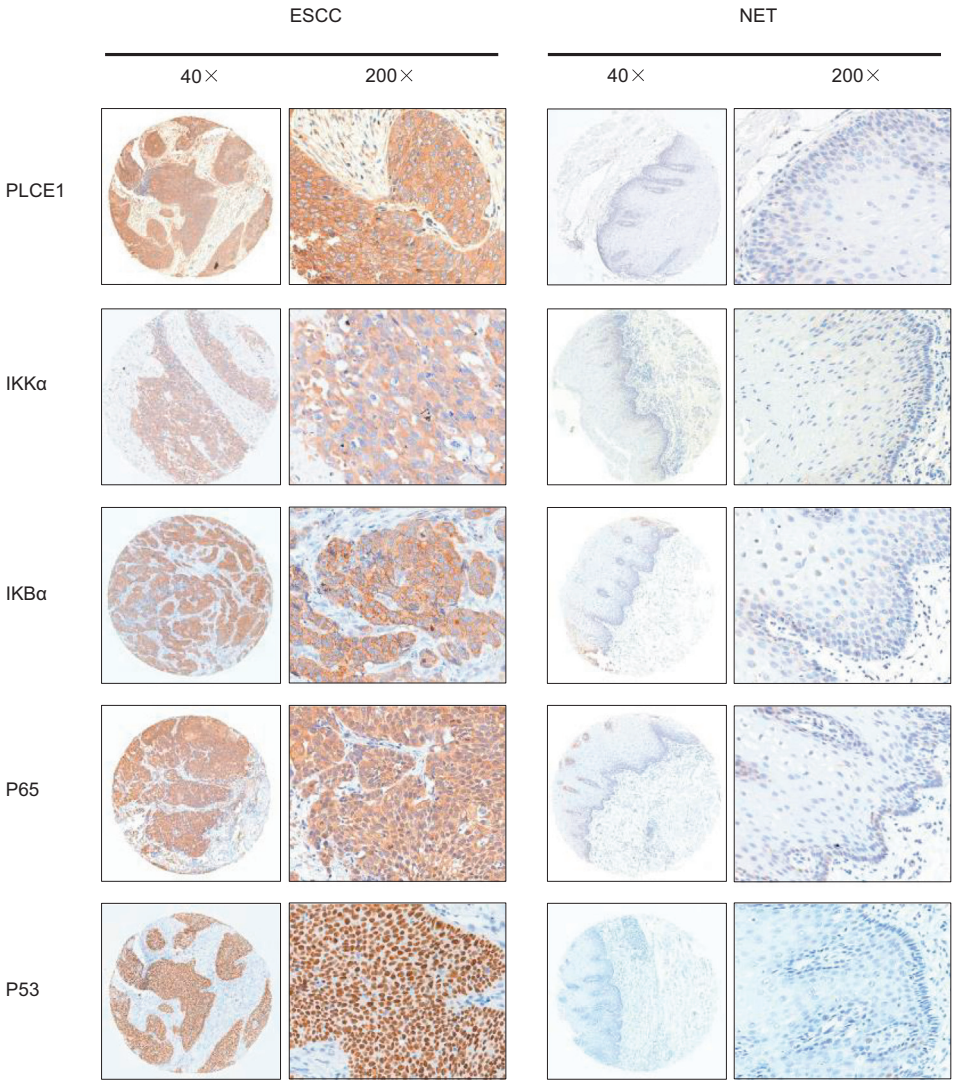

Fig. 1 Representative IHC images of PLCE1, IKKα, IKBα, p65, and p53. IHC results revealed that these proteins were highly expressed in ESCC tumors, but were minimally or not expressed in adjacent normal tissues. Original magnification ×40 and ×200.

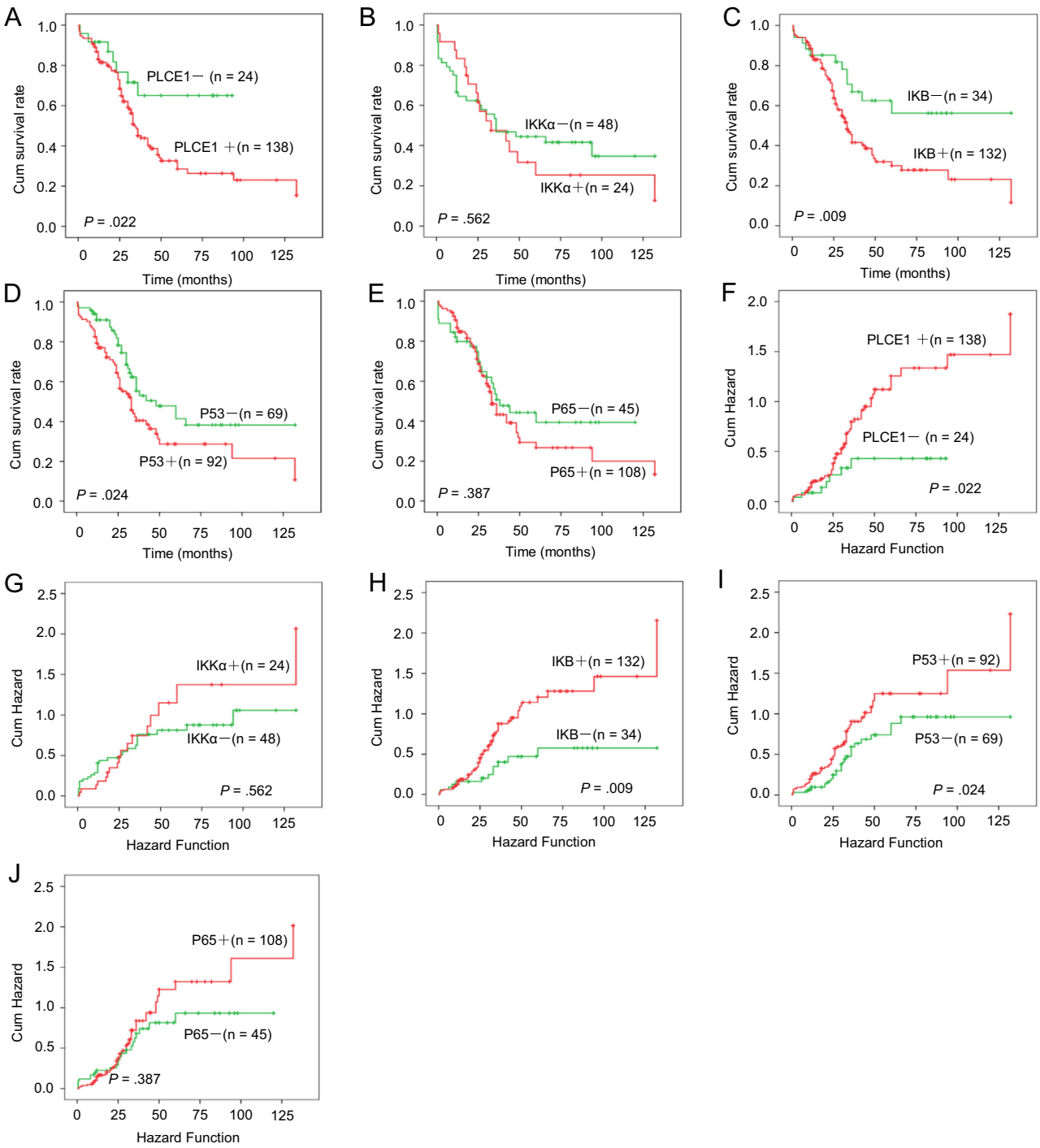

Fig. 2 Kaplan-Meier plot estimates and hazard functions plot estimates of ESCC-specific survival probabilities based on the expression levels of PLCE1, IKKα, IKBα, p65, and p53. A-E, The OS analysis result. F-J, The overall risk of death result. A and F, PLCE1 expression in 162 ESCC patients. B and G, IKKα expression in 72 ESCC patients. C and H, IKBα expression in 166 ESCC patients. D and I, p53 expression in 161 ESCC patients. E and J, p65 expression in 153 ESCC patients. Red graph indicates patients with "PLCE1 high," "IKKα high," "IKBα high," "p53 high," or "p65 high." Green graph indicates patients with "PLCE1 low," "IKKα low," "IKBα low," "p53 low," or "p65 low."

2.4. Statistical analysis

Comparison of the expression levels of proteins between ESCC and adjacent normal tissues was performed using paired *t* test analysis. Categorical data were compared by χ^2 test and Fisher exact test to assess the correlation of protein expression levels with clinicopathological parameters. For survival analyses, Kaplan-Meier curves were plotted using the log-rank test. The clinical end point in the study was OS, which was defined as the time from surgery to death from

ESCC or last contact. The data of the patients who were alive at the end of the study were censored. A Cox regression model was carried out to identify the independent factors that had a significant impact on patient survival. The sensitivity and specificity of ESCC prognosis predictions were assessed by receiver operating characteristic (ROC) curves. For all tests, a difference was considered significant if the *P* value was less than .05.

3. Results

3.1. Evaluation of the expression levels of PLCE1, IKKα, IKBα, p65, and p53 proteins in ESCC tissues

Immunoreactivity against PLCE1, IKKα, IKBα, p65, and p53 was detected in ESCC samples, with high expression found in 84.75% (139/164), 79.2% (57/72), 78.66% (129/

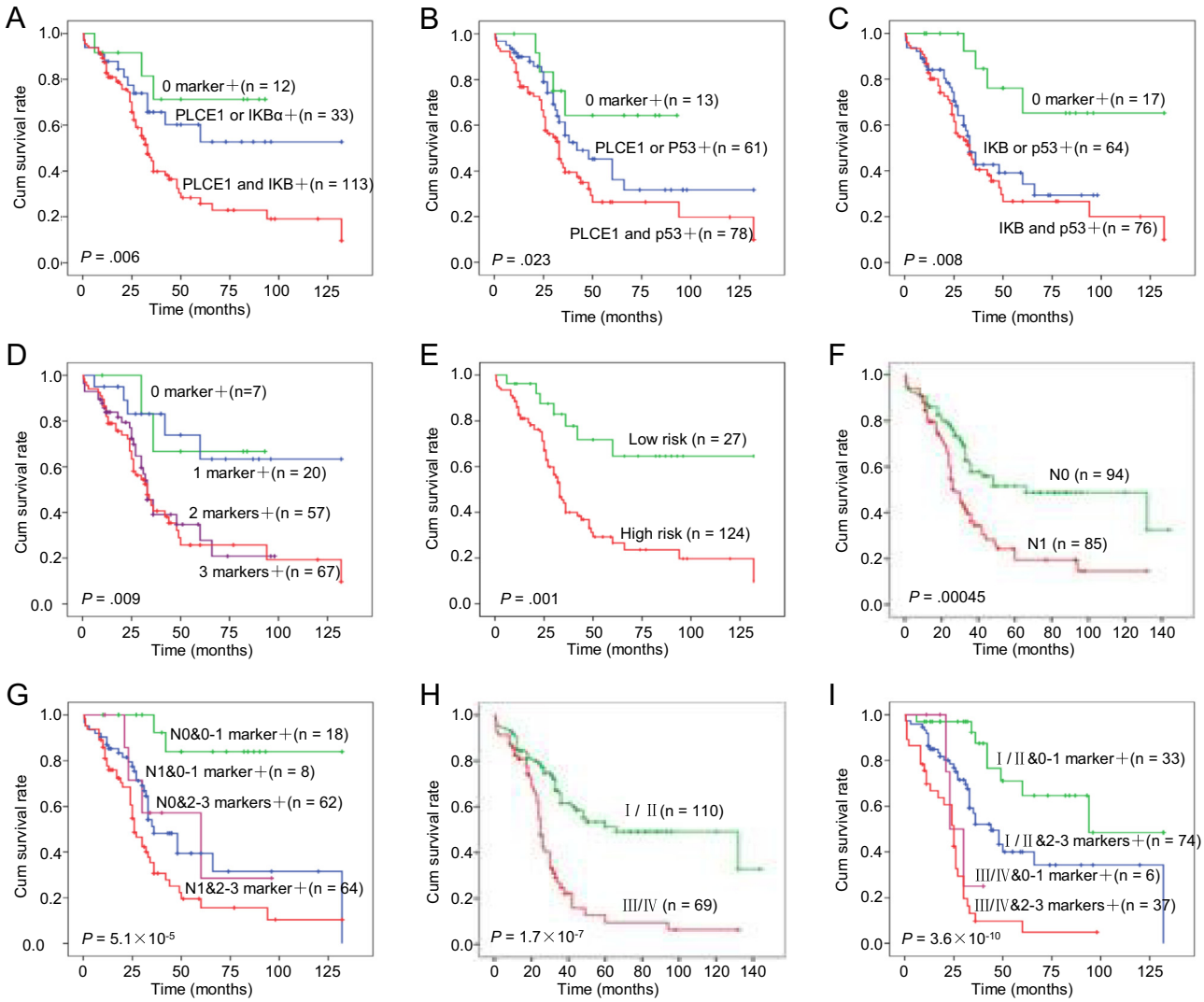

Fig. 3 OS analysis based on protein panel and clinicopathological parameters in ESCC tumors. A-C, ESCCs were divided into 3 groups: good prognosis (high expression level of 0 markers, green graph), average prognosis (high expression level of 1 marker, blue graph), and poor prognosis (high expression level of 2 markers, red graph). D, ESCCs were divided into 4 groups in the 2 cohorts or together: good prognosis (high expression level of 0 markers, green graph), good prognosis (high expression level of 1 marker, blue graph), average prognosis (high expression level of 2 markers, purple graph), and poor prognosis (high expression level of 3 markers, red graph). E, ESCCs were divided into 2 groups: the low-risk group ($Y \leq 2.3154$, green graph) and the high-risk group ($Y > 2.3154$, red graph). F, ESCCs were divided into 2 groups: good prognosis (with no lymph node metastasis, green graph) and poor prognosis (with lymph node metastasis, red graph). H, ESCCs were divided into 2 groups: good prognosis (ESCCs of early TNM staging, green graph) and poor prognosis (ESCCs of advanced TNM staging, red graph). G and I, Each group of ESCC patients in panels F and H were divided into subgroups.

Table 1 Relationship between the expression of PLCE1, IKBα, and p53 and the clinicopathological characteristics of ESCC patients

Parameters	n	PLCE1			IKBα			p53		
		Low (n = 25)	High (n = 139)	*P*	Low (n = 35)	High (n = 129)	*P*	Low (n = 70)	High (n = 94)	*P*
Age (y)				.850			.998			.524
<60	75	11	64		16	59		25	50	
≥60	89	14	75		19	70		45	44	
Sex				.201			.306			.491
Male	110	14	96		26	84		49	61	
Female	54	11	43		9	45		21	33	
Location				.891			.977			.606
Up	10	2	8		2	8		5	5	
Mi	110	16	94		24	86		44	66	
Lo	44	7	37		9	35		21	23	
Grade				.246			.044 [a]			.650
G1	48	7	41		16	32		23	25	
G2	77	15	62		14	63		32	45	
G3	39	3	36		5	34		15	24	
T				.780			.034 [a]			.203
T1-T2	68	11	57		20	48		33	35	
T3-T4	96	14	82		15	81		37	59	
N				.009 [a]			.313			.949
N0	85	19	66		21	64		36	49	
N1	79	6	73		14	65		34	45	
TNM stage				1.30×10^{-4} [a]			.682			.717
I-II	80	21	59		16	64		33	47	
III-IV	84	4	80		19	65		37	47	

[a] Statistically significant difference.

164), 65.85% (108/164), and 57.32% (94/164) of the samples, respectively. The high expression level of PLCE1, IKKα, IKBα, p65, and p53 in normal operative margins was found in 43% (43/100), 28% (28/100), 29% (29/100), 65.85% (108/164), and 11% (11/100) of samples, respectively. The expression level of the 5 proteins significantly differed between ESCC and adjacent normal epithelial tissues. PLCE1, IKKα, IKBα, and p65 were primarily detected in the membrane or cytoplasm of tumor cells, but not in stromal cells. The high expression level of p53 was mainly presented in

Table 2 Univariate and multivariate Cox regression analyses of OS of ESCC patients

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex (male, female)	1.028 (0.674-1.568)	.899		
Age (<60 y, ≥60 y)	0.745 (0.492-1.128)	.164		
Location (lo, up/mi)	0.941 (0.594-1.490)	.796		
Grade (G2/G3, G1)	1.279 (0.812-2.013)	.289		
T (T3-T4, T1-T2)	1.402 (0.921-2.134)	.115		
N (N1-N3, N0)	2.049 (1.354-3.099)	6.83×10^{-4} [a]	0.902 (0.370-2.196)	.820
M (M1, M0)	2.968 (1.419-6.208)	.004 [a]	2.432 (0.877-6.745)	.088
TNM (III/IV, I/II)	3.306 (2.168-5.039)	2.73×10^{-8} [a]	2.793 (1.095-7.121)	.0031 [a]
PLCE1 (>4, ≤4)	2.382 (1.094-5.185)	.029 [a]	1.043 (0.346-3.146)	.940
IKKα (>4, ≤4)	1.744 (0.735-4.138)	.207		
IKBα (>4, ≤4)	2.197 (1.189-4.062)	.012 [a]	0.833 (0.343-2.023)	.686
p65 (>4, ≤4)	1.237 (0.758-2.017)	.394		
p53 (>4, ≤4)	1.659 (1.059-2.598)	.027 [a]	1.201 (0.494-2.918)	.686
PLCE1 + IKB + P53	1.553 (1.166-2.067)	.003 [a]	1.173 (0.588-2.341)	.651

Abbreviation: HR, hazard ratio.

[a] Statistically significant difference.

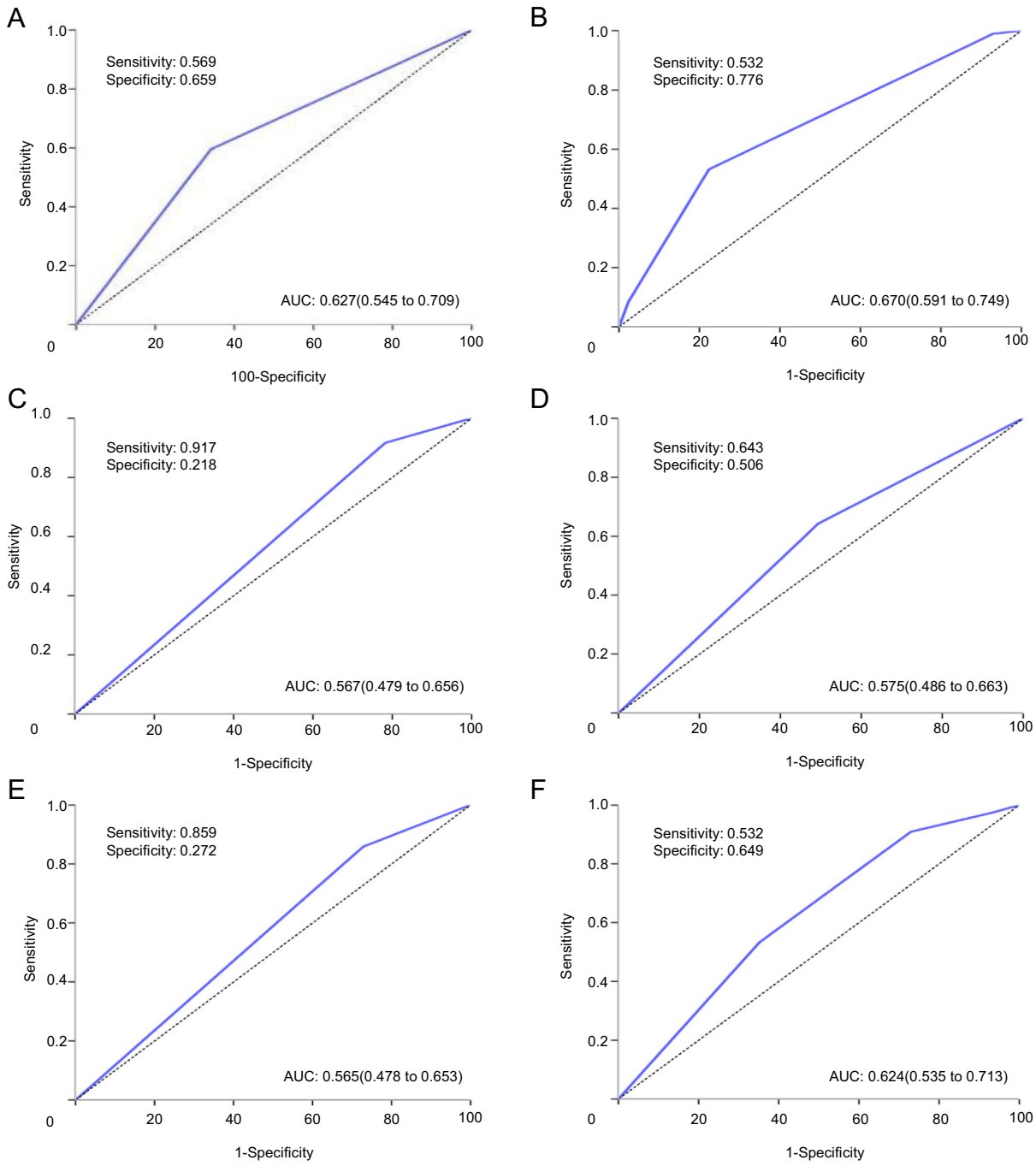

Fig. 4 ROC curve analysis for ESCC prognosis. Area under the curve estimation for the N stage (A), TNM staging (B), PLCE1 expression (C), p53 expression (D), IKBα expression (E), and protein panel expression (F).

the tumor cell nucleus. Cells positive for PLCE1, IKKα, IKBα, and p65 showed diffuse distribution of these proteins throughout the tumor cell cytoplasm. The high expression level of PLCE1 (5.593 ± 2.9395), IKKα (4.792 ± 3.5561), IKBα (6.679 ± 3.8743), p65 (6.864 ± 3.4358), and p53 (4.605 ± 4.4712) in tumor cells and low expression levels of PLCE1 (2.6267 ± 2.2586), IKKα (1.385 ± 0.1062), IKBα (2.353 ± 2.1336), p65 (2.825 ± 2.5975), and p53 (0.680 ± 1.1903) in stromal cells are shown in Fig. 1.

3.2. Association between the expression of PLCE1, IKBα, and p53 and the survival of ESCC patients

To determine the relevance of PLCE1, IKBα, IKKα, p65, and p53 protein expression in predicting disease prognosis, we reviewed the follow-up information of the patients. Kaplan-Meier OS curves for patients with ESCC based on the expression of PLCE1, IKBα, IKKα, p65, and p53 are illustrated in Fig. 2. The high expression level of PLCE1

($P = .022$), IKBα ($P = .009$), and p53 ($P = .024$) was associated with poor survival in ESCC (Fig. 2A, C, and D), whereas no correlation was found between the protein expression of IKKα or p65 and prognosis (Fig. 2B and E). Patients with a high expression level of PLCE1 ($P = .022$), IKBα ($P = .009$), and p53 ($P = .024$) had a greater risk of death when compared with patients with low expression (Fig. 2F, H, and J). Different combinations of these 3 proteins (ie, PLCE1, IKBα, and p53) could be used to divide the patients into subgroups with different prognoses (Fig. 3A-C). OS deteriorated gradually with the increase in the co-expression of PLCE1, IKBα, and p53, and the patient group with tumors positive for all 3 proteins had significantly worse survival than that of other groups ($P = .009$; Fig. 3D). The ISs of PLCE1, IKBα, and p53 were introduced into the esophageal cancer prognosis equation as follows: $Y = 0.541 \times \text{PLCE1} + 0.463 \times \text{IKB}\alpha + 0.379 \times \text{p53}$. According to the median value of $Y = 2.3154$, patients were divided into 2 different prognosis groups (high-risk group >2.3154; low-risk group ≤2.3154; Fig. 3E). The 1-, 2-, and 3-year survival rates of high-risk group were 77.4%, 57.4%, and 19.1%, respectively. Kaplan-Meier analysis revealed that scoring of PLCE1, IKBα, and p53 effectively discriminated the risk of postoperative survival in ESCC patients.

3.3. Relationship between the expression level of PLCE1, IKBα, and p53 and the clinicopathological characteristics of ESCC patients

To further evaluate the protein level of PLCE1, IKBα, and p53 in ESCC tissues, we analyzed the expression of PLCE1, IKBα, and p53 by IHC staining. We determined the correlations between the expression of PLCE1, IKBα, and p53 and the clinicopathological characteristics in 164 ESCC samples. No statistically significant association was found between p53 expression and any clinicopathological feature. By contrast, expression of PLCE1 was significantly associated with lymph node metastasis ($P = .009$) and clinical stages (TNM; $P = 1.3 \times 10^{-4}$; Table 1). The expression of IKBα was significantly associated with differentiation ($P = .009$) and infiltration depth ($P = .034$; Table 1).

3.4. Determination of the prognostic value of the 3-protein IHC panel

Given that the 3 proteins (ie, PLCE1, IKBα, and p53) showed significant prognostic value, we further determined if they were able to provide additional prognostic information independent of clinicopathological characteristics by use of multivariate Cox regression analysis. The univariate analysis showed that differentiation degree, lymph node metastasis, clinical stages, PLCE1, IKBα, p53, and PLCE1 + IKBα + p53 were associated with OS, whereas sex, age, location, infiltration depth, IKKα, and p65 showed no prognostic significance for OS. In the multivariate Cox hazard regression analysis, only clinical stages were identified as independent prognostic factors for OS ($P = .002$; Table 2). When compared with only lymph node metastasis or clinical stages, the combination of the panel and lymph node metastasis or clinical stages could stratify patients accurately ($P = 3.6 \times 10^{-10}$; Fig. 3F-I).

Table 3 Progression of esophageal lesions with positive PLCE1, IKBα, and p53 expression in endoscopy screening

PLCE1	IKBα	P53	Start time	Start diagnosis	End point time	End point diagnosis	Alteration
−	−	+	January 2012	HGIN	February 2012	ESCC	Progressed
+	+	+	January 2012	HGIN	February 2012	ESCC	Progressed
+	+	−	May 2010	HGIN	August 2010	ESCC	Progressed
−	−	+	August 2011	ES	April 2012	LGIN	Progressed
+	+	−	June 2011	HGIN	September 2011	ESCC	Progressed
−	+	+	October 2011	HGIN	December 2011	ESCC	Progressed
+	−	−	May 2012	ES	June 2013	LGIN	Progressed
+	+	+	June 2012	LGIN	August 2012	ESCC	Progressed
+	+	−	April 2013	LGIN	July 2013	HGIN	Progressed
+	+	+	January 2013	LGIN	April 2013	ESCC	Progressed
+	+	+	March 2013	HGIN	December 2013	ESCC	Progressed
+	+	+	October 2013	HGIN	March 2014	ESCC	Progressed
+	+	+	March 2014	LGIN	June 2014	HGIN	Progressed
−	+	+	December2014	ES	February 2015	HGIN	Progressed
−	+	+	August 2014	HGIN	April 2015	ESCC	Progressed
+	+	+	February 2014	LGIN	July 2015	ESCC	Progressed
+	+	+	May 2015	HGIN	July 2015	ESCC	Progressed
−	+	+	August 2015	LGIN	December 2015	ESCC	Progressed
−	−	+	January 2015	LGIN	July 2015	HGIN	Progressed
−	+	+	September 2016	LGIN	October 2016	ESCC	Progressed

Abbreviation: ES, esophagitis.

3.5. Three-protein IHC panel as a good predictive model for the OS of ESCC patients

To establish a sensitive model for predicting the outcomes of patients with ESCC, we combined expression of PLCE1, IKBα, and p53 with lymph node metastasis and TNM staging to create a prognostic score system. The ROC analysis showed that the predictive value of the 3-protein panel (PLCE1, IKBα, and p53; AUC [95% confidence interval {CI}], 0.624 [0.535-0.713]) approached that of TNM staging (AUC [95% CI], 0.670 [0.591-0.749]). The 3-protein panel revealed statistically superior prognostic value compared with PLCE1 (AUC [95% CI], 0.567 [0.479-0.656]), IKBα (AUC [95% CI], 0.575 [0.486-0.663]), and p53 (AUC [95% CI], 0.565 [0.478-0.653]) proteins alone and lymph node metastasis (Fig. 4). These test results from the ROC test analysis suggest that the 3-protein panel (PLCE1, IKBα, and p53) is a suitable predictive model for the OS of ESCC patients.

3.6. Validation of the biomarker panel in an independent cohort with the progression of esophageal precancerous lesions from endoscopy screening

We collected biopsy tissues from 1922 patients who underwent endoscopic examination of the esophagus to detect the

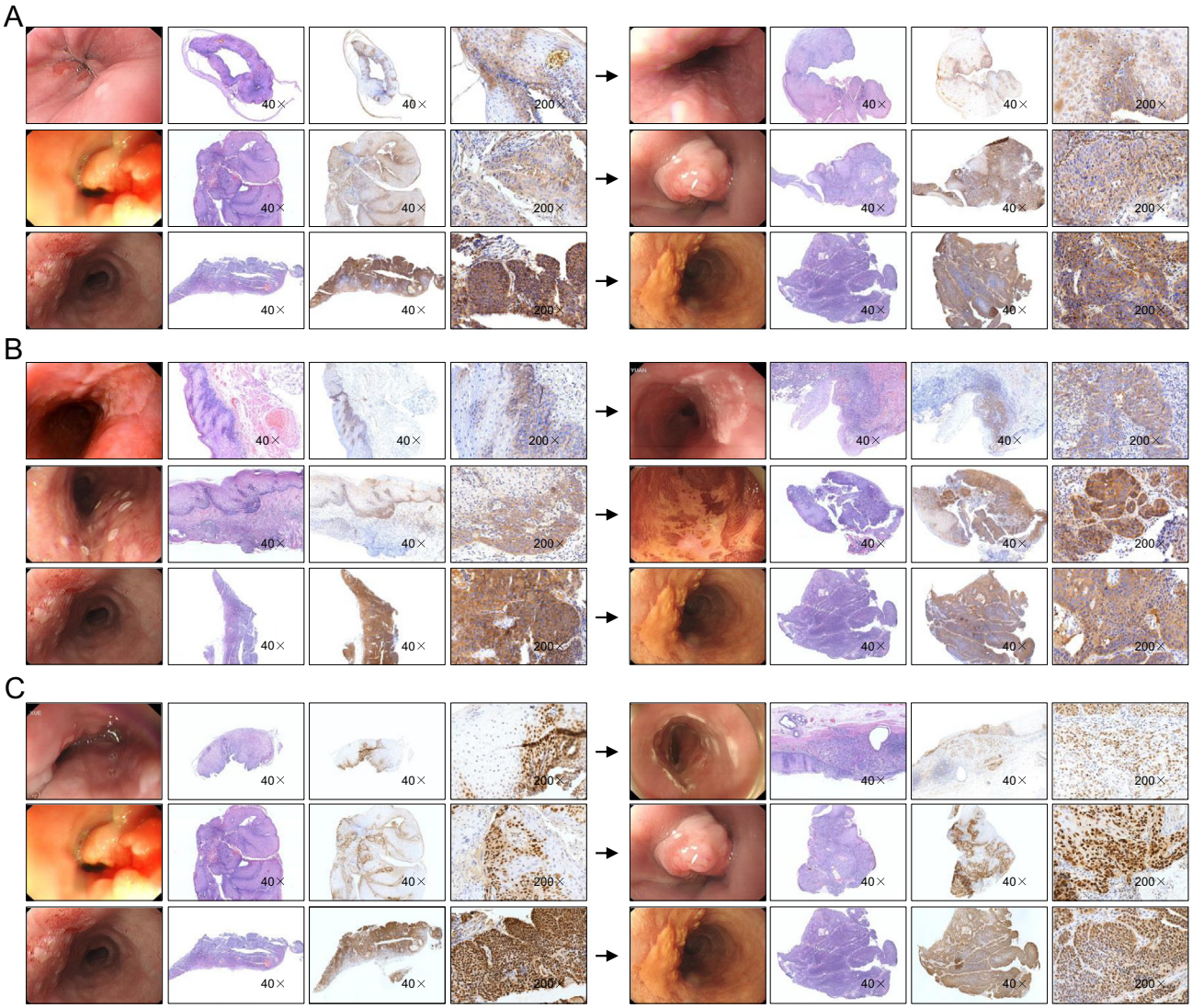

Fig. 5 Representative images show the positive expression of PLCE1, IKBα, and p53 proteins in esophageal biopsy tissue during disease progression. Typical immunostaining patterns of PLCE1 (A), IKBα (B), and p53 (C) were found in 3 patients of different progression levels. The first and fourth column images are the hematoxylin and eosin staining of each tissue (original magnification ×40). Patients who progressed to LGIN from normal (top image of panel A), ESCC from LGIN (middle image of panel A), and ESCC from HGIN (bottom image of panel A) are PLCE1 positive (all magnifications ×40 and ×200). Patients who progressed to HGIN from LGIN (top image of panel B), ESCC from LGIN (middle image of panel B), and ESCC from HGIN (bottom image of panel B) are IKBα positive (all magnifications ×40 and ×200). Patients who progressed to ESCC from normal (top image of panel C), ESCC from LGIN (middle image of panel C), and ESCC from HGIN (bottom image of panel C) are p53 positive (all magnifications ×40 and ×200).

expression of the biomarker panel. The availability of follow-up endoscopic data for 10 years after the initial biopsy examination allowed us to identify 28 patients who had undergone a progression from normal esophageal squamous epithelium to precancerous/carcinoma after the initial biopsy. Expression levels of PLCE1, IKBα, and p53 were analyzed in the 28 cases with precancerous lesions, and 8 cases (9/28; 28.57%) with positive expression of the 3 biomarkers progressed from precancerous to carcinoma after the initial biopsy. Twelve cases presented positive PLCE1 staining (12/28; 42.86%), including 1 case with esophagitis, 5 with LGIN, and 6 with HGIN. Expression of IKBα was observed in 16 cases (16/28; 57.14%), including 1 case with esophagitis, 7 cases with LGIN, and 8 cases with HGIN. Expression of p53 was observed in 16 cases (16/28; 57.14%), including 2 cases with esophagitis, 7 cases with LGIN, and 7 cases with HGIN. The detailed condition changes in patients positive for PLCE1, IKBα, and p53 are shown in Table 3. The positive expressions of PLCE1, IKBα, and p53 proteins in biopsy tissue during disease progression are shown in the representative images in Fig. 5.

4. Discussion

For the first time, this study demonstrates that an IHC panel, which is composed of 3 molecular markers (ie, PLCE1, IKBα, and p53) that were generated and validated among independent cohort patients who had esophageal lesions, could be used to aid prognosis prediction in patients with ESCC. Furthermore, this IHC panel was found to be associated with prognosis and could be used as an adjunct to the current staging systems.

Previous investigations by Cui et al [7] showed that positive expressions of IKKβ and p65 are detected in 53.3% and 57.78% of ESCC tumors, respectively. Ping et al [10] reported that the positive immunoreaction for p65 is detected in 54.5% of ESCC tissues. In the present study, the overexpression levels of IKKα and p65 were observed in 79.2% and 65.85% of ESCC tumors, respectively, but were insignificantly correlated with poor survival of ESCC patients. Therefore, they were excluded from the prognostic panel. The difference in the levels of IKKα and p65 between our IHC results and previously published reports might be due to the differences in sources and clones of the antibodies, antigen retrieval methods, incubation time, and the detection system.

PLCE1 is a prognostic factor in multiple tumors. New research has found that positive expression of PLCE1 is significantly related to the low 5-year survival rate in gastric cancer [11]. Previous research in our laboratory has found that the increase in the expression of PLCE1 is associated with lymph node metastasis and TNM staging, and the high expression level of PLCE1 is an independent prognostic factor of patients with ESCC [5]. However, PLCE1 has been shown to function as a tumor suppressor, decreasing the incidence of colorectal carcinoma and hepatocellular carcinoma [12-16]. Liao et al [16] found that the low expression of PLCE1 combined with any serum α-fetoprotein level is associated with a decreased risk of hepatitis B virus–related hepatocellular carcinoma recurrence. The prognostic value of PLCE1 in our study was in agreement with those observed for gastric cancer. Cui et al [7] previously described that overexpression of IKBα is associated with ESCC I/II TNM staging. In our current study, we not only found that the high expression level of IKBα was related to middle differentiation and late infiltration, but also that IKBα-positive patients had poor long-term prognosis. Huang et al [17] indicated that the high expression level of p53 protein in ESCC significantly correlated with tumor grade and N stage and was associated with the poor survival rate of ESCC patients. Our data indicate that p53 is a prognostic factor of ESCC patients; however, we did not find a relationship between p53 expression and clinicopathological characteristics. For these reasons, we incorporated PLCE1, IKBα, and p53 into the ESCC prognostic panel.

The combination of the protein panel and clinicopathological characteristics could be used to divide the patients into subgroups with different prognoses. A panel of PI3K-p85, EGFR, and p53 and another panel of EGFR, TRIM44, and SIRT2 have been used to determine the prognosis of ESCC and esophageal adenocarcinoma, respectively [18,19]. In the present study, we identified a 3-protein prognostic panel (PLCE1, IKBα, and p53), which was not dependent on any clinicopathological feature. The outcome of patients with ESCC varied greatly. Although the stage of cancer was the same at the time of surgery, there were some patients who were positive for all 3 proteins and died of the disease soon after surgery, whereas others were negative for all the 3 proteins and experienced long-term survival. Thus, our results suggest the stage of cancer and 3-protein staining index could be an accurate prognostic tool for patients with ESCC and might be beneficial as clinical adjunctive therapy for improving the outcome of patients.

The initial purpose of our study was to identify a biomarker portfolio for clinical implementation and accurate prediction of patient prognosis. The ultimate goal, however, was to apply these molecules to targeted therapies for ESCC. At present, many molecular factors have been used to evaluate the efficacy of neoadjuvant therapy for ESCC. Unlike that of other types of tumors, such as breast and non–small cell lung cancers, the targeted therapy of ESCC is still in the early stages. The proposed prognostic potential of the combination of 3 proteins not only provided prognostic information for patients but also identified the patients who might benefit from targeted therapies. For example, p53 is now considered a potential therapeutic target for many cancers, such as B-cell acute lymphoblastic leukemia [20]. p53 is a popular tumor suppressor and exhibits the function of proto-oncogenes in some cases. Previous studies in our laboratory have found that knockdown of the PLCE1 gene can increase the expression of the p53 protein and promote apoptosis of ESCC cells (unpublished results). Previous authors have noted that an inhibitor for the interaction of p53 with MDM2, spiro-oxindoles, is undergoing clinical testing and shows promising prospect [20]. Inhibitors that target the prognostic

proteins associated with ESCC are expected to be therapeutic targets for improving the prognosis of ESCC.

We further studied the expression levels of PLCE1, IKBα, and p53 in endoscopic biopsies of esophageal precancerous lesions combined with follow-up information. Among 28 patients with progression, 8 patients were positive for all 3 proteins, including 5 LGIN and 4 HGIN patients. Specifically, 2 LGIN patients progressed to HGIN, and the remaining 7 patients progressed to ESCC. The positive rates of PLCE1, IKBα, and p53 in progressed precancerous lesions were 42.86% (12/28), 57.14% (16/28), and 57.14% (16/28), respectively. These results suggest that PLCE1, IKBα, and p53 have the potential to serve as a predictor biomarker panel for the malignant progression of esophageal precancerous lesions. The 5-year OS rate of Chinese ESCC patients was less than 10%. However, the 5-year OS rate was up to 95% after endoscopic treatment and rigorous follow-up [21]. Therefore, the development of early diagnostic and prognostic biomarker expression might reduce the risk of ESCC formation. These results suggest that esophageal mucosal biopsy coupled with rigorous follow-up is necessary for patients with esophageal precancerous lesions that are 3-protein positive. In the future, we will extend the existing research to a prospective study to clarify the potential clinical value of a 3-protein biomarker panel, consisting of PLCE1, IKBα, and p53, in predicting the progression of esophageal precancerous lesions.

In summary, our study identified and validated a 3-protein IHC biomarker panel, consisting of PLCE1, IKBα, and p53, that not only is independently associated with an unfavorable prognosis in patients with ESCC but also predicts the disease progression of precancerous lesions. This study also identified novel molecular targets that should be further investigated to determine if they could offer tailored therapeutic options to patients with ESCC.

Author contributions

Xiaobin Cui designed the research study, review and editing the passage. Jie Yu participated in the writing the passage and analyzed the data. Yi Zheng helped with data curation and writing. Hao Peng and Hong Zhou contributed editing the paper and analyzed the data. Yunz-hao Chen, Lijuan Pang, and Feng Li contributed essential reagents, tools and materials. Xue ping Han helped with specimen preparation, IHC staining and scoring all IHC slides, and also revised the manuscript. All authors have read and approved the final version of the manuscript.

Supplementary data

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