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Overexpression of *PLCE1* in Kazakh esophageal squamous cell carcinoma: implications in cancer metastasis and aggressiveness

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Three recent large-scale genome-wide association studies (GWAS) in Chinese Han populations have identified an esophageal squamous cell carcinoma (ESCC) susceptibility locus within *phospholipase C epsilon 1* (*PLCE1*) gene, which encodes a phospholipase involved in intracellular signaling. The expressed *PLCE1* in ESCC, however, are inconsistent. This study examined PLCE1 expression by immunohistochemistry (IHC) from 110 ethnic Kazakh ESCC patients and 50 from adjacent normal esophageal tissues (NETs). The expressed PLCE1 was localized in cytoplasm, especially in the peripheral layers of cancer cell nests, which was significantly higher in tumors than in NETs ($p < 0.001$). Increased expression of PLCE1 was correlated with advanced tumor-node-metastasis (TNM) stages ($p = 0.015$) and lymph node metastasis ($p = 0.003$) in patients with ESCC. Of the 110 patients, we examined 50 paired ESCC tissues and corresponding NETs by quantitative RT-PCR (polymerase chain reaction) and the mean mRNA level of *PLCE1* in ESCC was 1.85-fold higher compared with those in corresponding NETs ($p = 0.0012$). Meanwhile, 4 of 5 ESCC cell lines also showed elevated expression of *PLCE1* mRNA. Furthermore, elevated expression of *PLCE1* mRNA in Kazakh ESCC was associated with its immunoreactivity ($\rho = 0.297$, $p = 0.040$), lymph node metastasis ($p < 0.001$), and advanced TNM stages of ESCC ($p = 0.013$). To our knowledge, this study demonstrates for the first time that *PLCE1* overexpression correlates with lymph node metastasis and advanced TNM stages of Kazakh ESCC, implicating a role of *PLCE1* in cancer metastasis and aggressiveness in ethnic Kazakh patients with ESCC. Furthermore, the current findings may warrant investigations into whether inhibiting *PLCE1* could be a strategy for targeted anticancer therapy particularly for Kazakh ESCC.

Key words: Kazakh esophageal squamous cell carcinoma; *phospholipase C epsilon 1* (*PLCE1*); cancer metastasis; aggressiveness.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies of the digestive system, especially in Chinese Kazakh ethnic population residing in Xinjiang, northwest of China. ESCC ranks the 6th in mortality and the 7th in incidence in China with great variations in geography, ethnicity, and sociocultures (1). Despite recent advances achieved in clinical treatment, patients with ESCC exhibit a poor prognosis with an average 5-year survival rate of less than 30% due to regional or distant metastases (2, 3). Compared with other ethnic populations residing within Xinjiang, China, ESCC of Kazakhs is characterized by a strong tendency toward familial aggregation and a higher incidence and mortality (age standardized rates of 90–150/100 000) (4), which places ESCC as a major cause of cancer-related deaths in the Kazak ethnic group in Xinjiang. For these reasons, defining novel biomarkers for early diagnosis and for suggestive of aggressiveness and/or metastasis of ESCC is thus imperative, particularly in the Xinjiang Kazakh ethnic population.

Recently, three large-scale GWAS of Chinese Han populations have identified a new ESCC susceptibility locus at chromosome 10q23, tagged by a non-synonymous SNP of rs2274223 with amino acid substitution of H1927R in *phospholipase C epsilon 1* (*PLCE1*) gene (5–7). The PLCE1 protein, a member of the phospholipase family, contains several Ras interactive domains including one CDC25 domain and two Ras-associating domains (8). *PLCE1* functions as an effector of Ras and serves as a phospholipase that catalyzes the hydrolysis of polyphosphoinositides to generate intracellular secondary messengers, such as diacylglycerol and inositol 1, 4, 5-trisphosphate (IP3), thereby contributing to intracellular signaling (9). This signaling process may play a role in regulating cellular growth, differentiation, apoptosis, and angiogenesis through its coactions with Ras family members (8, 10–12). Studies have shown that *PLCE1* plays crucial roles in carcinogenesis and progression of several cancers, including cancers of the skin (13, 14), intestine (15–17), bladder (18, 19), and head and neck (20, 21). The expressed *PLCE1* in ESCC, however, are inconsistent. PLCE1 protein expression has also been reported to be higher in ESCC tissues comparing to in normal tissues (6). In contrast, a study conducted by Hu et al. (22) showed that *PLCE1* mRNA level in esophageal cancerous tissues was significantly lower than that in adjacent normal tissues but no differences were detected at PLCE1 protein level between cancerous and adjacent normal tissues.

ESCC is a phenotype resulted from alterations in multiple genes with their proteins involved in a complex and interactive networks. Few studies have focused on molecular epidemiology of *PLCE1* in ESCC and, in particular, *PLCE1* expression and its possible role in Kazakh ESCC have not been reported thus far. Given the discrepant reports of *PLCE1* expression in ESCC in other Chinese ethnic groups (6, 22), the present study aimed to investigate the expression of *PLCE1* using a combination of immunohistochemistry (ICH) and real-time polymerase chain reaction (PCR) and to determine the relationship between the expression of *PLCE1* and the clinical features in ESCC of Kazakh origin, an ethnic group exhibiting high incidence and familial clustering of ESCC.

MATERIALS AND METHODS

Patients and tissue specimens

Tissue microarrays (TMAs) were used for immunostaining of PLCE1 in 110 Kazakh patients with ESCC collected between year 1984 and 2011 from the First University Hospital, Shihezi University School of Medicine, Xinjiang Yili Prefecture Friendship Hospital and the People's Hospital of Xinjiang Uyghur Autonomous Region. There were no restrictions regarding age, sex, or disease stage. None of the patients received prior surgery other than diagnostic biopsies, chemotherapy, or radiation therapy. Clinical data were collected on clinicopathologic variables, such as tumor sites, invasion depth, and lymph node metastasis. All cases with pathologic diagnoses for tumor-node-metastasis (TNM) stages were evaluated according to Cancer Stage Manual, 7th Edition, issued in 2009 by the American Joint Committee on Cancer (AJCC/UICC). Of the 110 ESCC specimens, 50 matched adjacent non-malignant tissues were available as controls. All patients were enrolled by written informed consent, and the study was approved by the Institutional Ethical

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Review Board (IERB) at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

PLCE1 expression detected by IHC using TMAs

Paraffin-embedded materials were sampled from 110 formalin-fixed esophageal cancer tissues and 50 adjacent normal tissue samples with 0.6-mm-diameter tissue cores using a tissue arrayer (ALPHELYS, Plaisir, France). Slides were stained according to manufacturers' protocols for PLCE1 (HPA015598; Sigma-Aldrich Co., St. Louis, MO, USA). Briefly, paraffin-embedded sections with 4 μm were baked at 65 °C for 30 min and then rehydrated through graded alcohols, as previously described. Each 4-μm tissue section was deparaffinized, then rehydrated and incubated with fresh 3% H_2O_2 in methanol for 10 min at room temperature. Then, the sections were autoclaved in 1 mM Ethylenediaminetetraacetic acid buffer (pH 8.0) at 100 °C for 5 min for anti-PLCE1, then cooled to 30 °C for 30 min. Tissue sections were then incubated at 4 °C overnight with anti-PLCE1 rabbit polyclonal antibody at a dilution of 1:50 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, then washed in PBS and incubated with secondary antibody for 30 min at room temperature. Subsequently, 3,3-Diaminobenzidine was employed to visualize PLCE1 antibody binding and then the tissue sections were counterstained with hematoxylin.

Semi-quantitiative assessment and scoring

The expression of PLCE1 was scored semiquantitatively according to the percentage of positive cells and the cytoplasmic staining intensity. The percentage of positive staining cells: 0 (<5% positive cells), 1 (6–25% positive cells), 2 (26–50% positive cells), 3 (51–75% positive cells), or 4 (>75% positive cells). The cytoplasmic staining intensity: categorized as 0 score, negative; 1 score, buff; 2 score, yellow; 3 score, brown. The percentage of positivity of epithelial cells and staining intensities were then multiplied to generate the 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 $3 \times 4 = 12$. Five random fields were selected for scoring from each slide, and the mean score for each slide was used for the final analyses. As a result, the IS range was from 0 to 12. Optimal cut-off values for this assessment system were identified as follows: high expression of PLCE1 was defined as an expression index score of ≥ 4, and low expression of PLCE1 was defined as an expression index score of <4. Immunohistochemical scoring was performed independently by two observers and the inter-observer variability was <3%.

Cell lines and culture conditions

Five human ESCC cell lines (TE1, EC109, EC9706, KYSE450, KYSE150) were obtained from Shanghai Fuxiang Biotechnology Co., Ltd. (Shanghai, China). All the selective cell lines were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin. ESCC cell lines were compared to normal esophageal tissues (NETs, three NETs were randomly selected from the 50 matched adjacent non-malignant tissues as mentioned above). The cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

cDNA synthesis and real-time PCR

RNA was extracted from normal and tumor tissue of esophageal samples using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription of total RNA with oligo dT was performed by QuantiTect Rev. Transcription Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocols. cDNA was amplified with specific primer sets: *PLCE1* (Hs_*PLCE1*_1_SG QuantiTect Primer Assay, QT00095935); ACTB (Hs_ACTB_2_SG QuantiTect Primer Assay, QT01680476) in Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA, USA). Quantitative Real-time PCR was carried out using SYBR green PCR Master Mix (Qiagen, Hilden, Germany), containing ROX as a reference dye. The following thermal cycling program was applied: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 57 °C, and 45 s at 72 °C. Data were normalized for beta-actin (*ACTB*) expression using comparative threshold cycle method. The PCR efficiency for *ACTB*, *PLCE1* was measured using standard curves generated by serial dilution of cDNA. All experiments were performed in triplicates. Polymerase chain reaction product quality was monitored using post-PCR melt-curve analysis. Cycle threshold (C_t), the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined. ΔC_t values were calculated by subtracting the *ACTB* C_t values from the *PLCE1* C_t values ($\Delta C_t = C_t$ (*PLCE1* in ESCC/adjacent normal sample) $- C_t$ (*ACTB* gene in ESCC/adjacent normal sample). Relative expression level was determined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ (ESCC sample) $- \Delta C_t$ (adjacent normal sample). $2^{-\Delta\Delta Ct}$ indicates the fold change in ESCC samples relative to adjacent normal samples.

Statistical analysis

All statistical analyses were performed with SPSS software (SPSS Standard version 13.0, SPSS, Chicago, IL, USA). The χ^2 test or the Fisher's exact test was

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used to evaluate differences in protein expression of PLCE1 between normal and ESCC groups as well as the correlation between PLCE1 protein expression and clinicopathologic characteristics. Moreover, independent sample t test was also applied to assess the correlation between PLCE1 IS and clinicopathologic characteristics. Paired-sample T test was used to evaluate differences in mRNA expression of *PLCE1* between ESCC groups and adjacent normal tissues. Independent sample t test and the χ^2 test were applied to compare mRNA expression levels of *PLCE1* between different categorical data. Correlation between *PLCE1* mRNA expression and IS in ESCC was assessed by Pearson's correlation analysis. Differences with a p value of <0.05 were considered statistically significant.

RESULTS

Overexpression of PLCE1 protein in Kazakh ESCC tissues

Using IHC approach and anti-PLCE1 antibody, Fig. 1A–D showed that diffused and strong IS for PLCE1 (7.727 ± 3.461) was recorded in the cytoplasm of carcinoma cells, especially in the peripheral layers of cancer cell nests (Fig. 1E–F). Of 110 surgical ESCC specimens, 79.1% (87 of 110) of them were highly stained for PLCE1. However, only 38% (19 of 50) of adjacent non-malignant tissues were defined as having high IS for PLCE1 expression and 62% (31 of 50) of the remaining non-malignant tissues were scored as having no or low PLCE1 expression (Fig. 1G) with an average IS of 4.739 ± 3.289. As shown in Fig. 1H, the IS of PLCE1 in the ESCC specimens were significantly higher than those in the non-malignant specimens ($p < 0.001$).

Correlation of PLCE1 protein expression with clinicopathologic characteristics

We further analyzed expression strength of PLCE1 in 110 ESCC cases against their clinical characteristics. These cases were divided into two groups based on their IS of PLCE1 staining: cases with a score of ≥ 4 were categorized as high expression group and cases with a score of <4 were categorized as low expression group. It appeared that, as shown in Table 1, high PLCE1 expression cases was significantly correlated with lymph node metastasis ($p = 0.003$) and advanced TNM stage ($p = 0.015$). These data suggest that upregulated expression of PLCE1 may be involved in tumor metastasis and aggressiveness. There were no correlations found between PLCE1 expression and gender ($p = 0.311$), age ($p = 0.366$), tumor location ($p = 0.802$), tumor size ($p = 0.511$), histologic grade ($p = 0.363$), and the depth of tumour invasion ($p = 0.448$).

PLCE1 mRNA expression and its concordance with the expression by IHC

We compared mRNA expression levels of *PLCE1* in 50 tumor specimens to their paired (NETs) using real time PCR. To accurately quantify mRNA expression of *PLCE1*, a housekeeping gene (*ACTB*) was similarly amplified as an internal control (see Materials and Methods) to normalize the results. In NETs, *PLCE1* mRNA levels ranged from 0.0007 to 0.4829 with a median of 0.107. In ESCC samples, *PLCE1* mRNA levels ranged from 0.0001 to 0.945 with a median of 0.139. As a whole, the mean mRNA level of *PLCE1* in ESCC samples was 1.85-fold higher compared with those in corresponding normal esophageal epithelia, as shown in Fig. 2A (0.259 ± 0.038 vs 0.140 ± 0.018, $p = 0.0012$). Furthermore, we also found a moderately high expression of *PLCE1* mRNA in ESCC cell lines (TE1, EC109, EC9706, and KYSE450) compared to NETs (Fig. 2B).

The ratio of *PLCE1* mRNA expression levels in primary tumors and corresponding NETs were calculated for all cases (Fig. 2C). This ratio of *PLCE1* ranged from 0.02 to 10.69, with a median of 2.72 (SE, 0.33). By receiver operating characteristic analyses, we obtained a cut-off value of 2.00. We observed that 28 tumors (56% Fig. 2C, solid columns) had *PLCE1* mRNA levels greater than those observed in matching NETs. On the other hand, 22 tumors (44% Fig. 2C, open columns) had reduced levels of *PLCE1* mRNA. The results further confirmed that expression of *PLCE1* was significantly increased in Kazakh ESCC.

Moreover, the levels of *PLCE1* mRNA expression in tumors showed a moderately positive correlation with IS scores (Spearman

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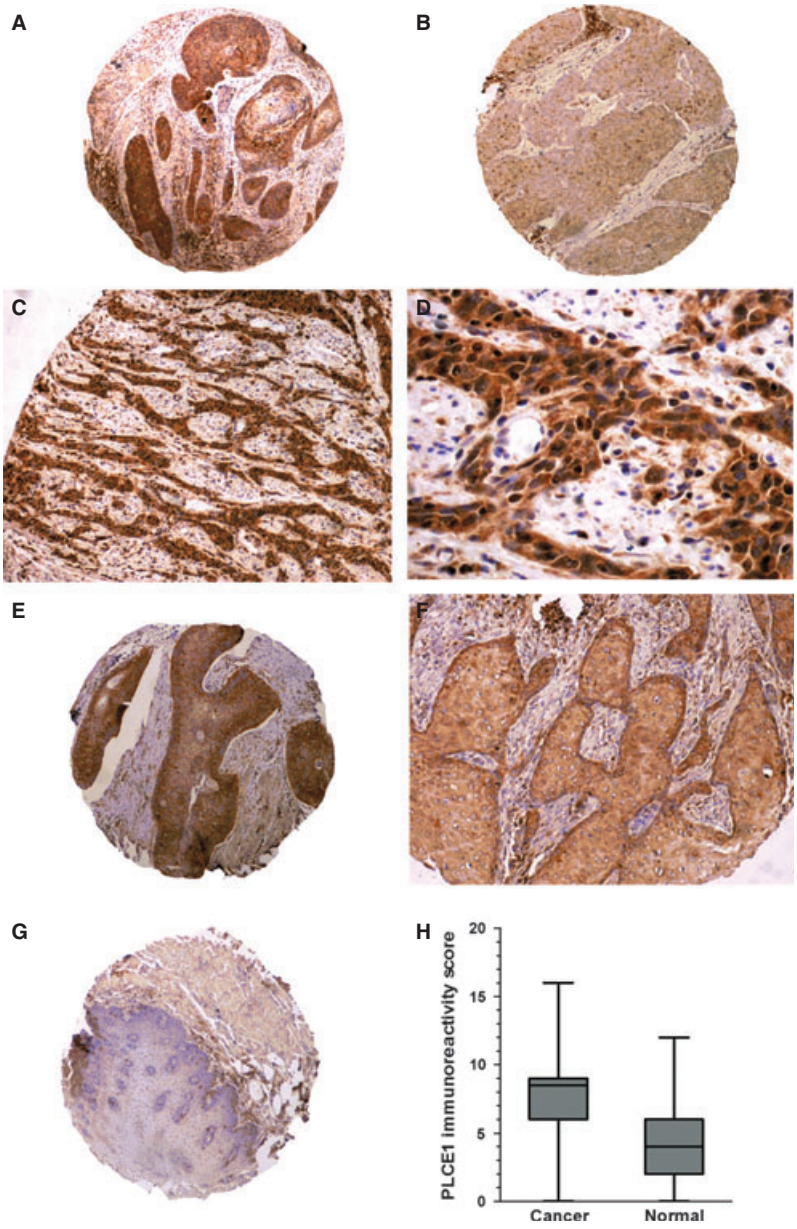

Fig. 1. Immunohistochemical staining of *phospholipase C epsilon 1* (PLCE1) protein expression in esophageal cancers and adjacent normal tissues. Esophageal squamous cell carcinoma (ESCC) tissues show a strong positive (A) and weak positive (B) expression of PLCE1 protein (×40). (C) Lots of scattering small clusters of invasive cancer cells show strong PLCE1 protein staining (×100). (D) High power view (×400) shows that strong cytoplasm staining is observed in the cytoplasm of cancer cells. (E–F) The strong expression of PLCE1 protein is particularly seen in these cells located in the peripheral layers of cancer nests. (G) shows the low expression of PLCE1 was detected in a normal esophageal tissues (NETs) (×40). (H) Boxplot shows that PLCE1 expression levels in ESCC are significantly higher than that in normal esophageal squamous epithelium ($p < 0.001$).

correlation coefficient (ρ) = 0.297, $p = 0.040$, Fig. 2D). In addition, a significant difference was observed in mRNA expression levels between the low IS group (<4) and the high IS group (≥ 4) (0.3451 ± 0.0410 vs 0.2085 ± 0.0249, $p = 0.007$, Fig. 2E).

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Table 1. The correlations between phospholipase C epsilon 1 (PLCE1) protein expression and clinicopathologic factors

Parameters	PLCE1 protein expression			
	Total (%)	High (%) n = 87	Low (%) n = 23	p-value[1]
Gender				0.311
Male	72 (65.5)	59 (67.8)	13 (56.5)	
Female	38 (34.5)	28 (32.2)	10 (43.5)	
Age(years)[2]				0.366
≤ 55	39 (35.5)	29 (33.3)	10 (43.5)	
> 55	71 (64.5)	58 (66.7)	13 (56.5)	
Tumor location				0.802
Cervical	31 (28.2)	25 (28.7)	6 (26.1)	
Thoracic	79 (71.8)	62 (71.3)	17 (73.9)	
Tumor size (cm)[3]				0.511
≤ 6	46 (41.8)	35 (40.2)	11 (47.8)	
> 6	64 (58.2)	52 (59.8)	12 (52.2)	
Histologic grade[4]				0.363
G_1	30 (27.3)	22 (25.3)	8 (34.8)	
G_{2-4}	80 (62.7)	65 (65.5)	15 (52.2)	
Invasion depth				0.448
T1–T2	45 (40.9)	34 (39.1)	11 (47.8)	
T3–T4	65 (59.1)	53 (60.9)	12 (52.2)	
Lymphatic invasion				0.003
Negative	61 (55.5)	42 (48.3)	19 (82.6)	
Positive	49 (44.5)	45 (51.7)	4 (17.4)	
TNM stage[5]				0.015
I/II	78 (53.6)	57 (65.5)	21 (91.3)	
III/IV	32 (46.4)	30 (34.5)	2 (8.7)	

[1]Chi-square test.
[2]Mean age.
[3]Mean tumor size.
[4]Histologic grade was with reference to WHO classification published in 2009.
[5]TNM stage was based on the UICC criteria published in 2009.

Association of *PLCE1* mRNA expression with clinicopathologic variables

To evaluate the clinicopathologic impacts of *PLCE1* mRNA expression on ESCC, we analyzed the correlation of various clinicopathologic variables with expression patterns (Table 2) and mRNA levels of the *PLCE1* (Fig. 3) respectively. *PLCE1* overexpression was significantly associated with lymph node metastasis. *PLCE1* overexpression was observed in 75.0% (21 of 28) of cases with lymph node metastasis compared with low expression of *PLCE1* in 59.1% (13 of 21) of cases without lymph nodes metastasis ($p = 0.015$). In cancer TNM stage analyses, overexpression of *PLCE1* was observed in 67.9% of cases (19 of 28) with advanced stages of tumor (III/IV), while only 27.3% (6 of 22) of cases in lower TNM stages showed overexpressed *PLCE1* ($p = 0.004$). Independent sample t test was also applied to determine the correlation of *PLCE1* mRNA mean relative expression (MRE) with different histopathologic features. The mRNA levels of *PLCE1* were also significantly correlated with lymph node metastasis of Kazakh ESCC (0.5181 ± 0.0594 vs 0.1144 ± 0.0245, $p < 0.001$, Fig. 3). Furthermore, advanced TNM stages III/IV of ESCC were correlated with higher levels of *PLCE1* mRNA expression (0.3432 ± 0.0515 vs 0.1570 ± 0.0545, $p = 0.013$, Fig. 3). In both methods of analysis, no association was observed between *PLCE1* expression and the remaining clinicopathologic features described above.

DISCUSSION

It has been reported that the function of *PLCE1* is related to cellular differentiation and apoptosis through its coaction with Ras family

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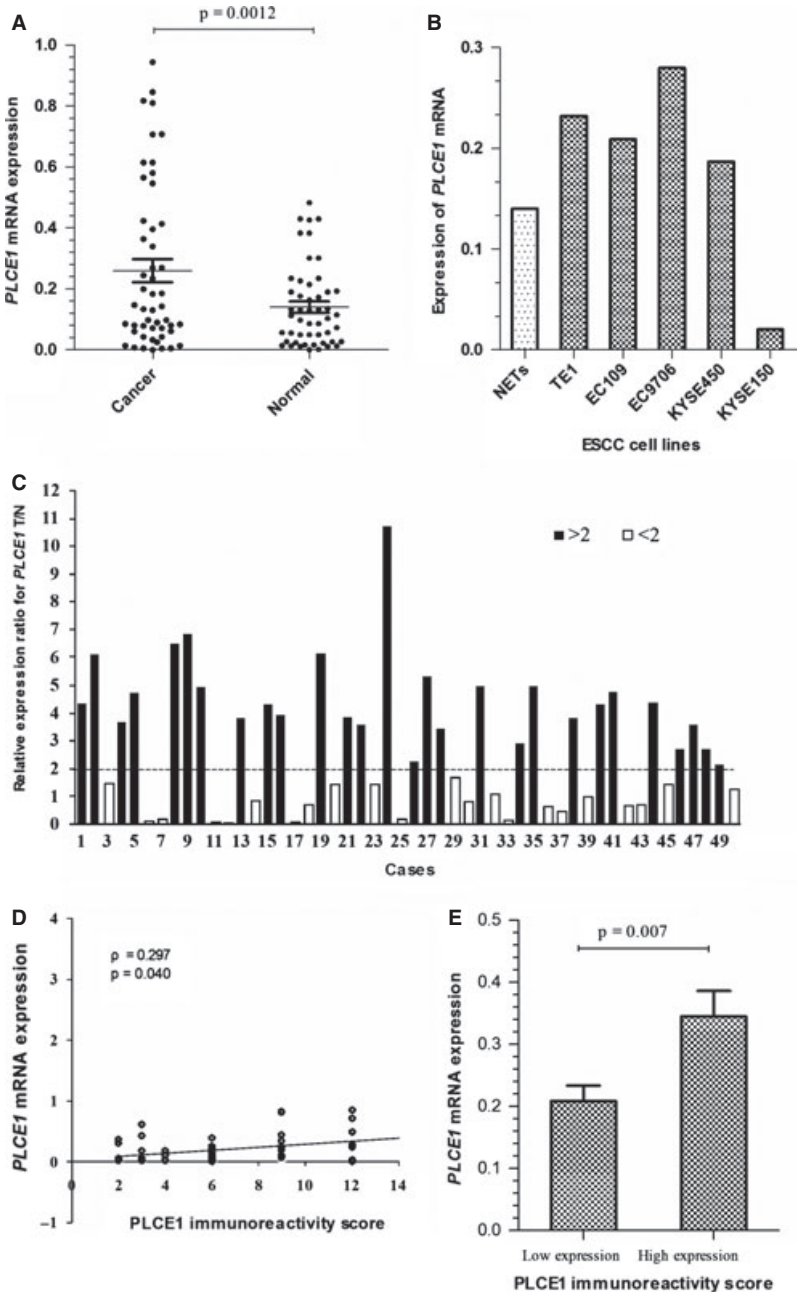

Fig. 2. Expression level of *PLCE1* mRNA in esophageal squamous cell carcinoma (ESCC) in comparison with NETs. (A) Expression level of *PLCE1* mRNA in ESCC compared with NETs (0.259 ± 0.038 vs 0.140 ± 0.018, $p = 0.0012$). (B) Expressions of *PLCE1* mRNA in five ESCC cell lines (TE1, EC109, EC9706, KYSE450, and KYSE150). The expression of phospholipase C epsilon 1 (*PLCE1*) in ESCC cell lines was compared to three normal esophageal tissues (NETs) randomly selected from the 50 matched NETs as mentioned in Materials and Methods section. (C) Relative mRNA expression radios for *PLCE*1 in samples from tumor (T) and adjacent normal (N) tissues. Dotted line denoted the cut-off value 2.00 for *PLCE1*. Solid and open columns represented the above or below the cut-off value of fold differences. (D) In tumors, the level of *PLCE1* mRNA expression showed a moderate positive correlation with immunoreactivity score (IS) ($\rho = 0.297$, $p = 0.040$). (E) A significant difference was observed in mRNA expression levels between the low IS group (< 4) and the high IS group (≥ 4) ($p = 0.007$).

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Table 2. Correlation of *phospholipase C epsilon 1* (*PLCE1*) mRNA expression with different clinicopathologic features

	PLCE1 mRNA expression		
	High n = 28 (%)	Low n = 22 (%)	p-value[1]
Invasion depth			0.585
T1–T2	11 (39.3)	7 (31.8)	
T3–T4	17 (60.7)	15 (68.2)	
Lymphatic invasion			0.015
Negative	7 (25.0)	13 (59.1)	
Positive	21 (75.0)	9 (40.9)	
TNM Stage[2]			0.004
I/II	9 (32.1)	16 (72.7)	
III/IV	19 (67.9)	6 (27.3)	

[1]Chi-square test.
[2]TNM stage was based on the UICC criteria published in 2009.

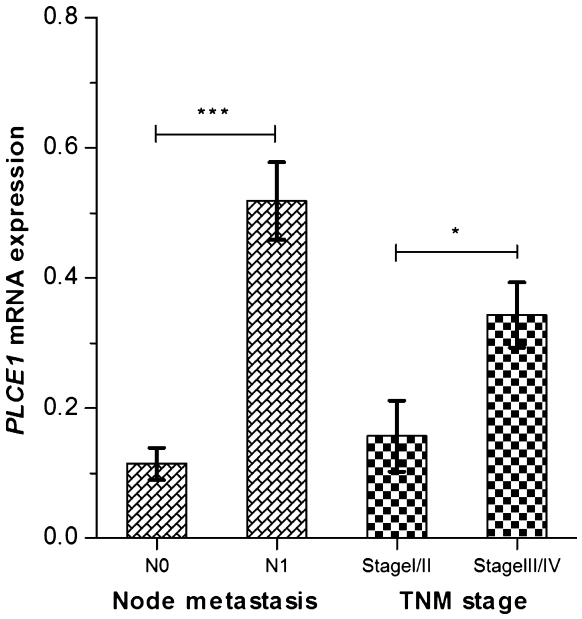

Fig. 3. Association of *PLCE1* mRNA expression and clinicopathologic features. Error bars represents standard error of mean (SEM) and asterisks represents statistically significant difference ('*' means $p < 0.05$, '***' means $p < 0.001$). Mean relative expression (MRE) *phospholipase C epsilon 1* (*PLCE1*) of mRNA was significantly higher in patients with lymph nodes metastasis ($p < 0.001$). In advanced TNM stages of tumor (stage III/IV), a significantly higher MRE of *PLCE1* mRNA was observed ($p = 0.013$).

proteins (23, 24), which is different from other molecules of the PLC family (25). Although recent epidemiologic and etiologic studies have suggested that the carcinogenesis of ESCC involves multiple factors, stages, and alterations in gene expression (26, 27), the precise mechanism(s) responsible for the development of ESCC are largely unknown. Most recently, three independent research teams identified *PLCE1* gene as a susceptibility gene for ESCC in Chinese Han populations (5–7). However, there is limited information in *PLCE1*expression studies, and the two documented reports have shown conflicting results in this regard (6) (22). Conclusive results are needed to demonstrate associations of this susceptibility gene with ESCC at the expression level.

PLCE1 gene encodes a novel ras-related protein (R-Ras) effector, a multiple-function protein with a complex structure, mediating the cellular differentiation and apoptosis (23, 24). There is evidence that *PLCE1* may be linked to carcinogenic processes. For example, mutations in the *PLCE1* are known to cause nephrotic syndrome characterized by diffuse mesangial sclerosis and focal and segmental glomerulosclerosis (28). *PLCE1* knockout mice are resistant to intestinal tumor formation (13, 29). Using IHC analyses on tissue microarrays, we have demonstrated a significantly higher level of PLCE1 expression in the cytoplasm of cancer cells, especially in the peripheral layers of cancer cell nests. Using a different method on the same specimens, we have further shown that the mRNA expression levels of *PLCE1* are also increased in ESCC. Moreover, IHC and real-time PCR analyses have independently shown the correlations of overexpressed PLCE1, at both protein and mRNA levels, with lymph node metastasis and advanced TNM stage (Tables 1 and 2). These findings suggest that *PLCE1* may play an important role in the pathogenesis of ESCC, promising PLCE1 as a potential candidate biomarker in diagnosis and treatment of ESCC.

Our observations of upregulated mRNA and protein expression of *PLCE1* in Kazakh ESCC tissues are in agreement with the findings in a Chinese Han population by Wang et al. (6), but differ from those by Hu et al.(22), who found no difference in PLCE1 IHC scores between ESCC and matching adjacent normal tissues. This discrepancy may be

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resulted from several factors, of which sample size may be one as Hu et al. used a smaller sample size (n = 39) (22) than those by Wang et al. (n = 188) (6) and by ours in this study (n = 110). The other possible factors resulting in the discrepancy may be population heterogeneity (Chinese Han ethnic groups by Wang et al. vs Hu et al.) and genetic backgrounds of different ethnicities(our Kazakh ethnicity vs Hu's Han ethnicity), which needs to be further clarified in studies using uniform ethnic groups with larger sample size.

Gene expression is modulated by both genetic and epigenetic mechanisms. Hu et al. (22) has reported that the functional variation of *PLCE1* SNP (rs2274223 A > G) might reduce gene expression in NETs in eastern Chinese populations. Interestingly, the SNP, rs2274223, which is located on 10q23 (Chr10:96044913–96070375), is found within the area between two recombination hot spots where NOC3L is located. NOC3L has been linked to the control of DNA replication during mitotic clonal expansion (30), which may have a role in regulating *PLCE1* expression. In addition, using Gene Set Enrichment Analysis, Danielsen et al. (31) have found that gene expression, especially 'phosphatidylinositol signaling network', that is *PLCE1* and *PLCD1*, is significantly downregulated in both sKyoto Encyclopedia of Genes and Genomes database in Colorectal Cancer. Furthermore, they also found one of 19 colon cancer cell lines showing promoter methylation for *PLCE1* gene. For this reason, whether the epigenetic changes of *PLCE1* play an important role in regulation of the abnormal expression model between the Kazakh ESCC and normal tissues is a critical need to be determined.

The detailed biologic significance of altered *PLCE1* expression in cancers remains poorly understood. Our results indicate that the association of the lymph node metastasis activity and TNM stage of tumor to *PLCE1* overexpression is higher than those without *PLCE1* overexpression. Invasion of cancer cells into blood and lymphatic vessels is critical point for cancer metastasis. Indirect evidence for this is provided by a report showing that *PLCE1* plays an oncogenic role in intestinal carcinogenesis through augmentation of inflammatory signaling pathways and angiogenesis (29). One of the vital mechanisms of angiogenesis promoted by *PLCE1* seems to be relevant to the *PLCE1*'s role in the induction of VEGF expression, which is one of the important angiogenic factors and necessary constituents for tumorigenesis and metastasis (32, 33). Additionally, another study has reported that the aberrant activation of *PLCE1* induces the *PLCE1*-Ca^{2+} signaling pathway, which causes the up-regulation of Ca^{2+}/calmodulin-dependent kinase II, leading to phosphorylation of the cytoskeletal protein filamin. This process reduces its interaction with filamentous actin and promotes tumor cell migration in head and neck squamous cell carcinoma progression by binding Ras family small GTPases (20). These findings, overexpression of *PLCE1* may alter the motility of ESCC cells through the same signaling pathway, appear to provide reasonable explanations for our observations that overexpressed *PLCE1* may be involved in metastasis and aggressiveness, which warrants detailed investigations *in vitro*.

In conclusion, this is the first study showing that both protein and mRNA of *PLCE1* are overexpressed in Kazakh patients with ESCC, which correlate with lymph node metastasis and advanced TNM stages of ESCC. These findings may potentiate the gene as a candidate biomarker for cancer metastasis and aggressiveness in ethnic Kazakh patients with ESCC. A further prospective study is needed to investigate whether the *PLCE1* is a poorer prognosis factor for Kazakh ESCC. Although the mechanisms on why the *PLCE1* is in overexpression and how it contribute to lymph node metastasis of Kazakh ESCC are unknown, the findings of our study provide the prerequisite for a further study of the mechanism related to carcinogenesis in Kazakh ESCC.

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