

## Decreased Expression of PDHE1 $\alpha$ Predicts Worse Clinical Outcome in Esophageal Squamous Cell Carcinoma

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**Abstract.** *Background: Aerobic glycolysis is an important hallmark of cancer cells. Deficiency of pyruvate dehydrogenase component  $\alpha$  subunit (PDHE1 $\alpha$ ) leads to mitochondrial dysfunction and promotes glycolysis. Therefore, studies on the expression of PDHE1 $\alpha$  in cancer are warranted. Patients and Methods: The PDHE1 $\alpha$  protein was immunohistochemically investigated and analyzed in 157 samples of surgically dissected esophageal squamous cell carcinoma (ESCC) and 21 'normal' esophageal epithelia tissues. Results: PDHE1 $\alpha$  protein expression was lower in ESCC. Absence of PDHE1 $\alpha$  protein expression in tumor cells was found in 77.1% of cases. Negativity for PDHE1 $\alpha$  protein in tumor cells was correlated with poor tumor differentiation ( $p < 0.05$ ) and shorter overall survival ( $p < 0.05$ ). Conclusion: Lack of PDHE1 $\alpha$  protein expression in ESCC is associated with poor prognosis. Our findings also verify the existence of aerobic glycolysis switch in ESCC, and strongly advocate novel therapeutic strategies in reversing the Warburg effect in tumors.*

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**Key Words:** PDHE1 $\alpha$ , esophageal squamous cell carcinoma, immunohistochemistry, Warburg effect, overall survival.

Human esophageal squamous cell carcinoma (ESCC) ranks as the most malignant lesion in the world, especially in some areas in China, South Africa, France and Italy (1). Current therapies for ESCC are far from satisfactory. Despite numerous studies on cancer genomics, it is hard to discover and develop therapies targeting the thousands of genes which are mutated in ESCC.

Tumor cells preferentially metabolize glucose through glycolysis and produce excessive lactate, even in the presence of oxygen, known as the Warburg effect or 'aerobic glycolysis', which has been attracting general attention in cancer research. Increasing evidence indicates that enzymes involved in this metabolic switch are potential targets in cancer therapy (2-4). Reports on this metabolic reprogramming in ESCC are few.

Pyruvate dehydrogenase complex (PDHC), composed of E1, E2, E3 and E2/E3 binding proteins, plays a key role in the control over mitochondrial metabolic pathways by catalyzing the irreversible oxidation of glucose-derived pyruvate to acetyl-CoA. PDHC activity deficiency leads to dysfunction of mitochondria and promotes glycolysis in cells (5). Importantly, lack of PDHC component expression, or decreased activity of PDHC has been found in lung cancer, hepatoma and cutaneous carcinoma in comparison to their normal counterpart tissues (6-8). The PDHC component E1 (PDHE1) is a heterotetramer of two E1 $\alpha$  and two E1 $\beta$  subunits. The E1 $\alpha$  subunit contains the E1 active site and plays a key role in the function of the PDHC. Some evidence suggests that defects of PDHE1 $\alpha$  is the main reason of PDHC dysfunction (5, 9-11).

There are studies which directly or indirectly suggest the existence of a glycolytic switch in human ESCC (12-14), implying a possibility of reduced PDHE1 $\alpha$  protein expression. In the present study, we immunohistochemically examined the expression of PDHE1 $\alpha$  in human ESCC tissues and explored its relationship with conventional clinicopathological characteristics in a series of 157 patients with ESCC from Anyang, P. R. China with long-term follow-up.

## Patients and Methods

This project was carried out by Anyang Tumor Hospital in collaboration with the Norwegian Radium Hospital, Norway. The Ethics Committee of Anyang Tumor Hospital and Anyang Hygiene Bureau approved the study. All the patients involved provided written informed consent.

In total, 157 surgically dissected ESCC samples were included in this study. All patients underwent operation at Anyang Tumor Hospital, Henan, P. R. China between 1989 and 1994. The detailed clinicopathological features are summarized in Table I. All patients were followed up from the confirmed date of diagnosis until death or 31 May 2004, except for 16 patients lacking follow-up information. Tumors were classified in terms of the International Union against Cancer (UICC) 2003 standard (15). Two pathologists at the Department of Pathology of the Anyang Tumor Hospital and two pathologists at The Norwegian Radium Hospital reviewed the type and grade of histology of the specimens. In addition to these 157 ESCC specimens, 21 samples of 'normal' esophageal squamous epithelia adjacent to tumor collected from Anyang Tumor Hospital were also studied.

**Immunohistochemistry (IHC).** The formalin-fixed, paraffin-embedded sections were obtained from the Department of Pathology, Anyang Tumor Hospital, Henan, P. R. China. Dako EnVision™ Flex+ (K8012; Dako, Glostrup, Denmark) was applied for IHC staining. De-paraffinization and unmasking of epitopes were performed in PT link with low pH target retrieval solution (Dako), followed by blocking of peroxidase for 5 min with DAKO blocking buffer. The slides were then incubated with the following reagents: rabbit monoclonal antibody against human PDHE1 $\alpha$  subunit (C54G1, 1:400; Cell Signaling, Danvers, MA, USA) at 4°C overnight, EnVision™ Flex+ rabbit linker for 15 min and EnVision™ Flex/HRP enzymes for 30 min at room temperature. All sections were stained with 3,3'-diaminobenzidine tetrahydrochloride for 5 min and counterstained with hematoxylin. All slides were finally dehydrated and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Scientific, Waltham, MA, USA). Smooth muscle tissue is always positive for PDHE1 $\alpha$  staining and was used as positive control in this study. Negative controls were produced using the same concentration of non-immune rabbit IgG instead of the rabbit antibody to human PDHE1 $\alpha$ .

**IHC scoring system.** Immunostaining was evaluated by two pathologists from The Norwegian Radium Hospital. Slides were classified as positive if more than 10% of tumor cells were stained. Similarly, slides were classified as positive if more than 10% of squamous epithelium cells of adjacent noncancerous tissues were stained.

**Statistical analyses.** Statistical analyses of the data were processed using the SPSS17.0 statistical software package (SPSS, Chicago, IL

Table I. Clinicopathological information of patients with esophageal squamous cell carcinoma and their relationship with expression of pyruvate dehydrogenase component  $\alpha$  subunit (PDHE1 $\alpha$ ).

Variable	Total	PDHE1 $\alpha$ expression		p-Value
		Negative	Positive	
Age (years)				
<50	45	35	10	0.815
50-60	54	40	14	
>60	58	46	12	
Tumor size (mm)				
<30	34	25	9	0.824
30-60	109	85	24	
>60	14	11	3	
TNM stage				
I/II	103	79	24	0.644
III/IV	54	42	12	
Lymph node metastasis				
No	95	73	22	0.687
Yes	62	48	14	
Differentiation grade				
High	56	36	20	0.012
Middle	51	43	8	
Low	50	42	8	

USA). Chi-square tests (Pearson and linear-by-linear as appropriate) were performed for analyzing the associations of PDHE1 $\alpha$  expression and clinicopathological variables. Survival curves were plotted through the Kaplan-Meier method and compared with the use of the two-sided log-rank test. A p-value of less than 0.05 were regarded as statistically significance.

## Results

**PDHE1 $\alpha$  protein expression in normal esophageal squamous epithelium.** We first analyzed PDHE1 $\alpha$  expression in the 21 samples of adjacent non-cancerous epithelia of ESCC. In these epithelia, the basal cells were always positive for PDHE1 $\alpha$  expression. The suprabasal cells in these epithelia stained more positively than did basal cells (Figure 1A). In general, 80-100% of the cells in the basal and suprabasal layers stained positively with the antibody against PDHE1 $\alpha$ . As shown in Figure 1A, smooth muscle cells, fibroblasts and endothelial cells were also positive for PDHE1 $\alpha$  protein expression. All positive staining was confined to the cytoplasm.

**Decreased expression of PDHE1 $\alpha$  protein in ESCC.** PDHE1 $\alpha$  expression was detected in a series of 157 ESCC samples. Representative PDHE1 $\alpha$  antibody-positive and -negative tumors are shown in Figure 1B-D. It was discovered that a large number of tumors were negative for expression of this protein. Out of the 157 tumors, only 36 (22.9%) expressed the PDHE1 $\alpha$  protein, while all the other 121 tumors (77.1%)



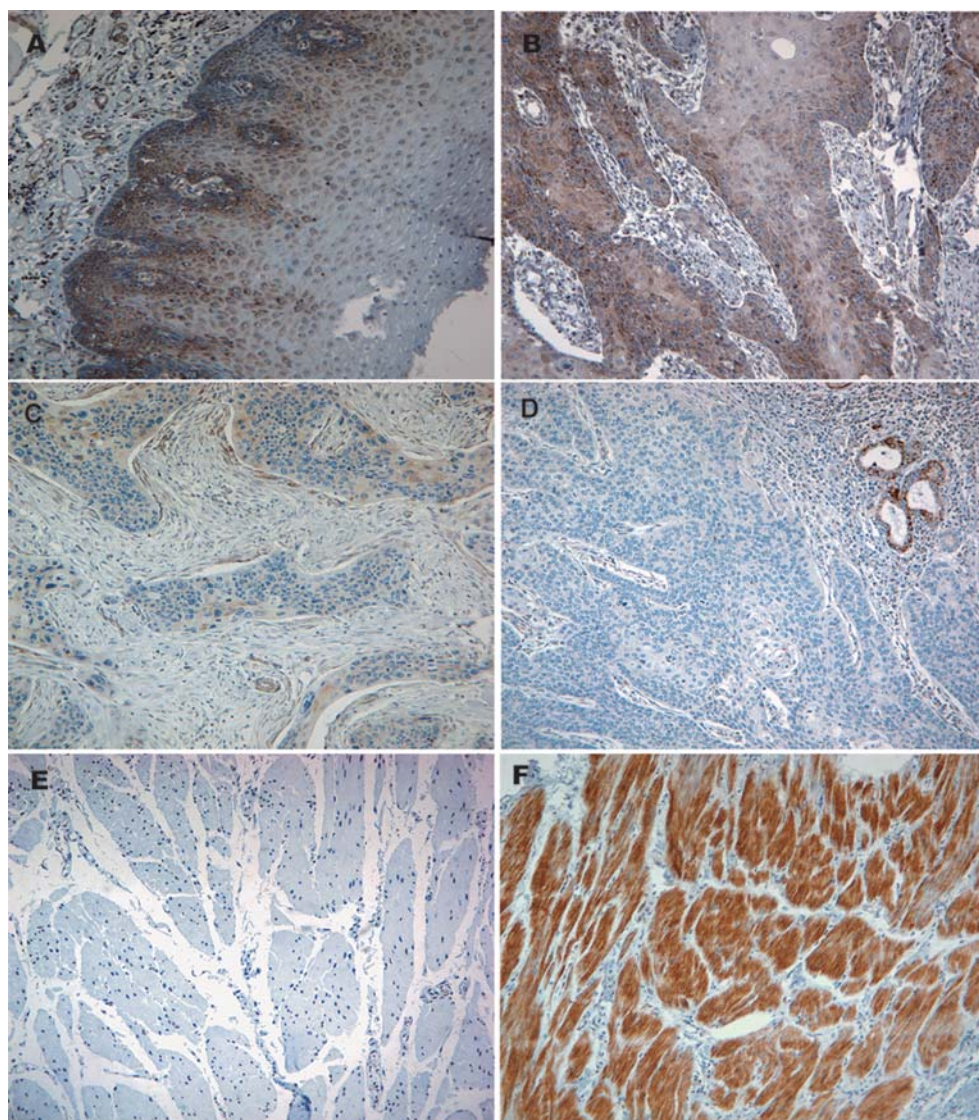


Figure 1. Expression of pyruvate dehydrogenase component  $\alpha$  subunit (PDH E1 $\alpha$ ) protein in normal epithelia adjacent to esophageal squamous cell carcinoma (ESCC) and ESCC samples. Positive cytoplasmic PDHE1 $\alpha$  protein expression can be seen in the basal cells and suprabasal cells of the 'normal' esophageal squamous epithelium (A), and a well-differentiated ESCC shows strong cytoplasmic PDHE1 $\alpha$  protein expression (B). Tumor cells in a moderately-differentiated ESCC can be seen to be partly positive (35%) for PDHE1 $\alpha$ , while the endothelial cells and fibroblasts in this tumor are also positive for PDHE1 $\alpha$  protein expression (C). Tumor cells in a poorly-differentiated ESCC tumor are negative for the antibody staining, but the neighboring normal glandular epithelial cells are positive for the PDHE1 $\alpha$  protein expression (D). E and F show negative and positive smooth muscle cells of esophageal tissues, respectively. All images were taken at  $\times 200$  magnification.

were negative for PDHE1 $\alpha$  protein expression. Similarly to normal epithelium, immunoreactivity to PDHE1 $\alpha$  antibody was also confined to the cytoplasm of tumor cells. The positivity in the PDHE1 $\alpha$  protein-positive tumors ranged from 10% to 90% tumor cells (median=30%). In addition to this, PDHE1 $\alpha$  protein expression was also found in stromal fibroblasts, endothelial cells, smooth muscle cells and infiltrating lymphocytes inside the tumors.

*Decreased expression of PDHE1 $\alpha$  protein correlates with pathological differentiation of ESCC.* The association between PDHE1 $\alpha$  protein expression and the clinicopathological features were analyzed. As summarized in Table I, PDHE1 $\alpha$  expression was significantly associated with higher histological grade of ESCC ( $p < 0.05$ ). In other words, PDHE1 $\alpha$  protein expression in ESCC samples was significantly associated with better tumor differentiation. As

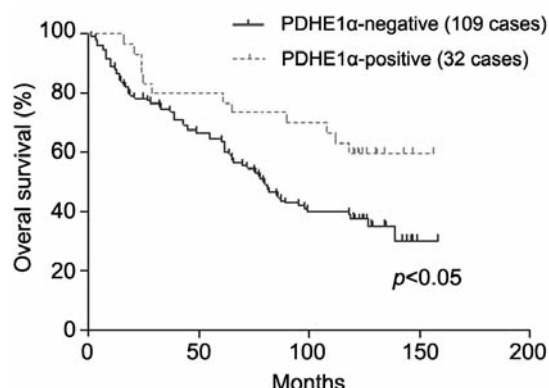


Figure 2. Overall survival analyses based on pyruvate dehydrogenase component  $\alpha$  subunit (PDHE1 $\alpha$ ) protein expression. Negative PDHE1 $\alpha$  protein expression in tumor cells was found to be significantly associated with poor overall survival in patients with esophageal squamous cell carcinoma ( $p < 0.05$ ).  $p$ -Values were determined by the log-rank test.

shown in Table I, PDHE1 $\alpha$  protein expression was noted in 20/56 (35.7%) samples of highly differentiated ESCC, but only in 8/50 (16%) samples of poorly differentiated ESCC. No significant association was found between the PDHE1 $\alpha$  protein expression and other clinical parameters such as age, tumor size, TNM stage and lymph node metastasis.

**Decreased PDHE1 $\alpha$  protein expression is associated with poor overall survival.** To evaluate the association of PDHE1 $\alpha$  expression and survival in ESCC, the overall survival curve was calculated by the Kaplan–Meier method and compared using the log-rank test. Since the follow-up data of 16 patients were missing, 141 cases were enrolled in the survival analysis, among which 32 cases expressed PDHE1 $\alpha$  protein. As shown in Figure 2, PDHE1 $\alpha$  protein expression in these ESCC tumors was significantly associated with better overall survival, in other words, a lack of PDHE1 $\alpha$  protein expression in ESCC was significantly associated with poor overall survival ( $p < 0.05$ ).

## Discussion

To the best of our knowledge, this is the first study to investigate the relationship of PDHE1 $\alpha$  with ESCC. We evaluated PDHE1 $\alpha$  protein IHC in a series of 157 ESCC samples and 21 esophageal epithelia of tumor-adjacent non-cancerous tissues. We showed that PDHE1 $\alpha$  expression is significantly lower in human ESCC tissues compared to its expression in the tumor-adjacent 'normal' esophageal epithelial cells. Furthermore, a lack of PDHE1 $\alpha$  protein expression in tumors was significantly associated with poor histological differentiation and shorter overall survival.

In our present study, the rabbit IgG monoclonal antibody, raised against PDHE1 $\alpha$  from Cell Signaling, was used. To verify its specificity and reactivity, optimization of this antibody was extensively performed in our laboratory using both positive and negative controls. While consistent PDHE1 $\alpha$  protein expression was revealed in the tumor-adjacent 'normal' esophageal epithelial cells, the majority of tumors (77%) were negative for PDHE1 $\alpha$  protein expression. This finding is in accordance with IHC results for PDHE2 and PDHE2/E3 binding protein(7), in which PDHE2 and PDHE2/E3 binding protein expression was strongly and consistently confined to the cytoplasm of normal bronchial and alveolar cells, while in 59 non-small cell lung carcinomas, the expression of PDHE2 and PDHE2/E3 binding protein was not expressed in a large proportion of cancer cells, and negative PDHE2 and PDHE2/E3bp expression was verified in about half of the cases. Similarly, our present findings are also in line with the results from studies on skin cancer and hepatomas (6, 8) where PDH levels were significantly decreased in cancer compared to their normal counterparts.

We did not divide the PDHE1 $\alpha$ -positive tumors into separate groups. The main reason for this was due to the small number of positive tumors; moreover, most positive tumors exhibited only up to 50% positivity in the tumor cells, and only two tumors were homogeneously positive (Figure 1B).

We showed that negative PDHE1 $\alpha$  protein expression in ESCC tumors is significantly associated with poor differentiation and shorter overall survival ( $p < 0.05$ ). This result seems to contrast with the finding in a non-small cell lung cancer study by Koukourakis *et al.*, where a small sub-group of clinically-aggressive tumors maintained coherent expression of PDH(7). However, it is not clear from that study how the expression of PDHE2/E3 binding protein and PDHE2 alone predict survival in their patients. Also their study included only 42 cases for survival analyses.

Together with the literature, our current finding of decreased PDHE1 $\alpha$  protein expression in tumors may indicate its involvement in tumorigenesis of ESCC. Low levels of PDHE1 $\alpha$  expression should result in reduced PDHC activity and thus, in part, be expected to promote a metabolic shift towards aerobic glycolysis and decreased dependence on mitochondrial oxidative phosphorylation. Such metabolic shift may confer cells with a selective advantage for survival, increasing their life-span, and may make them more vulnerable to carcinogenesis, since they will face more mutational insults than other normal cells. If this is the case, extensive molecular studies of PDHE1 $\alpha$  are warranted in ESCC.

Aerobic glycolysis, or the Warburg effect, is a robust metabolic feature of many tumors (16). Such deregulated metabolism is suggested to be due in part to functional



attenuation of mitochondrial oxidative phosphorylation in cancer cells (17). Several mitochondrial enzymes are often defective and involved in the metabolism of mitochondrial tricarboxylic acid cycle in cancer (18-20). Our present results may add PDHE1 $\alpha$  to this suppressor list, with the same fundamental function in energy and metabolic homeostasis.

PDHC serves as the gatekeeper of the mitochondrial tricarboxylic acid cycle by catalyzing the irreversible oxidation of pyruvate to acetyl-CoA. PDHE1 $\alpha$ , as the catalytic component of PDHC, is the most important component of PDHC, since PDHC is regulated by phosphorylation of PDHE1 $\alpha$  (21). A large amount of evidence suggests that absence of PDHE1 $\alpha$  directly reflects a deficiency in PDHC functionality. In many cases, defects in PDHE1 $\alpha$  lead to dysfunction in mitochondria and are involved in shifting metabolism from oxidative phosphorylation to glycolysis (5, 9). Most of the studies on PDHE1 $\alpha$  deficiency in humans are on mitochondrial oxidative phosphorylation disorders, leading to lactic acidosis, a similar phenomenon in tumors with aerobic glycolysis features (5, 22, 23).

It is known that PDHC activity is inactivated by pyruvate dehydrogenase kinases (PDKs), which phosphorylate PDHE1 $\alpha$ , and is restored by pyruvate dehydrogenase phosphatases (PDPs), which then dephosphorylate PDHE1 $\alpha$  (24). Increased PDK1 expression has been reported in head and neck squamous cancer and acute myeloid leukemia (25, 26). It is documented that PDP1, when inhibited by oncogenic signals, promotes tumor growth by promoting the Warburg effect (27). All these findings may point to a common possibility, *i.e.* decreased level of PDHE1 $\alpha$  subunit or decreased PDHC enzyme activity, plays an important role in ESCC

Only the protein expression of this enzyme subunit was studied here. For PDHE1 $\alpha$ -positive tumors, decreased PDHC activity cannot be ruled out, since enzyme activity may also be influenced by other factors. Thus, systemic studies of PDHE1 $\alpha$ , both at the gene and protein level, and of enzymatic activity, will shed light on ESCC, in consideration of their disordered metabolic activities. It is interesting that deacetylase sirtuin-3 (SIRT3) interacts with PDHE1 $\alpha$  and increases PDHC enzymatic activity via changes in protein acetylation and reverses the Warburg effect, in turn protecting against cancer formation (28).

In summary, our findings, for the first time, reveal decreased PDHE1 $\alpha$  protein expression in human ESCC, and such a lowered expression is significantly associated with poor histological differentiation and significantly shorter overall survival, a strong verification of the existence of the aerobic glycolytic switch in ESCC. Therefore, we strongly advocate the development of novel therapeutic strategies in inhibiting or reversing the Warburg effect in tumors.

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