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## The HIF-1 $\alpha$ /PKM2 Feedback Loop in Relation to EGFR Mutational Status in Lung Adenocarcinoma

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### ABSTRACT

**Objective:** Gene mutations in tumor cells can lead to several unique metabolic phenotypes, which are crucial for the proliferation of cancer cells. EGFR mutation (EGFR-mt) is the main oncogenic driving mutation in lung adenocarcinoma (LUAD). HIF-1  $\alpha$  and PKM2 are two key metabolic regulatory proteins that can form a feedback loop and promote cancer growth by promoting glycolysis. Here, the linkage between EGFR mutational status and HIF-1 $\alpha$ /PKM2 feedback loop in LUAD were evaluated.

**Methods:** Retrospective study were performed on LUAD patients ( $n=89$ ) undergoing first-time therapeutic surgical resection. EGFR mutation was analyzed by real-time PCR. Immunohistochemistry was used to measure the expressions of HIF-1 $\alpha$  and PKM2.

**Results:** We found that the protein expressions of HIF-1 $\alpha$  and PKM2 were significantly higher in LUAD than normal lung tissues. In adenocarcinomas, the two protein expressions were both correlated with worse pTNM stage. Moreover, the correlation between the proteins of HIF-1 $\alpha$ /PKM2 feedback loop and the EGFR mutational status were also analyzed. We found that EGFR-mt tumors showed higher HIF-1 $\alpha$  and PKM2 proteins compared to tumors with EGFR wild-type. Meanwhile, HIF-1 $\alpha$  expression was significantly correlated with higher pTNM stage, and PKM2 showed a similar trend, only in EGFR-mutated tumors. The expression of HIF-1 $\alpha$  was positively correlated with PKM2 in LUAD, furthermore, this correlation was mainly in patients with EGFR-mt.

**Conclusion:** Different expression and clinical features of HIF-1 $\alpha$ /PKM2 feedback loop was existed between LUAD and normal lung tissues, especially in EGFR mutational tumors, supporting the relationship between EGFR mutation and the key related proteins of aerobic glycolysis (HIF-1 $\alpha$  and PKM2) in lung adenocarcinomas.

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lung adenocarcinoma; glycolysis; HIF-1 $\alpha$ ; PKM2; EGFR

### Introduction

Lung cancer is the main cause of cancer death in the world.<sup>1</sup> Lung adenocarcinoma (LUAD) accounts for over 40% of lung cancer and is the most common histological subtype of lung cancers.<sup>2</sup> Despite recent progress in lung cancer research, the prognosis of LUAD patients has not significantly improved.<sup>3</sup> Thus, it is urgent to understand the potential molecular mechanisms underlying the pathogenesis of LUAD and discover new prognostic biomarkers and therapeutic targets for LUAD.

Glycolysis, also namely the “Warburg effect,” refers to the conversion of glucose into lactate even aerobic environments, which plays a key role in the growth and metastasis of cancer cells by producing large amounts of metabolic intermediates.<sup>4</sup> Pyruvate kinase M2 (PKM2) is a key enzyme in the glycolytic pathway, and its upregulation was found in various of cancers, including bladder cancer and hepatocellular carcinoma.<sup>5,6</sup> By

increasing the glucose consumption and lactate production,<sup>7</sup> PKM2 significantly promoting the development and progression of cancer. Guo et al have shown that increased expression of PKM2 contributes to therapeutic resistance of lung adenocarcinoma (LUAD) stem cells, indicating that PKM2 is a potential therapeutic target for LUAD.<sup>8</sup> Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is an important transcriptional factor of glycolysis. HIF-1 $\alpha$  can activate pkm2 gene transcription,<sup>9</sup> while PKM2 participates in a positive feedback loop that promotes HIF-1 transactivation, thereby enhancing the response of cells to hypoxia or oncogene activation.<sup>10</sup> Amounts of data indicate that HIF-1 $\alpha$  and PKM2 promote tumor growth by facilitating the Warburg effect.<sup>11,12</sup> However, there is still limited research on the relationship between PKM2/HIF-1 $\alpha$  feedback loop and the progression of lung adenocarcinoma.

Many studies have found that tumor related gene mutations are closely related to the changes in metabolic activity, genes

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that was involved like isocitrate dehydrogenase (IDH), BRAF and EGFR.<sup>13,14</sup> In the study of Babic et al, they found that the EGFR mutation enhanced glycolysis by promoting glycolytic gene expression in brain cancer.<sup>15</sup> And Nilsson et al found that in non-small cell lung cancer cells, the mutation status of EGFR is correlated with high HIF-1 $\alpha$  level even under hypoxic conditions, implying that cells with EGFR mutation may have a significant regulatory effect on the expression of HIF-1 $\alpha$ .<sup>16</sup> A recent study showed that the EGFR signaling activation caused nuclear translocation of PKM2, which in turn activates cyclin D1 and Myc.<sup>17</sup> While, the association between EGFR-mt and the protein expressions of PKM2/HIF-1 $\alpha$  feedback loop in LUAD still need to be clarified.

In our present study, we found that the expression levels as well as the clinical and pathological characteristics of HIF-1 $\alpha$ /PKM2 feedback loop protein were different in LUAD and normal lung tissues, and these differences were also existed between EGFR-mt and EGFR-wt LUAD groups. The above results supporting the relationship between oncogenic EGFR mutation and the key related proteins of aerobic glycolysis (HIF-1 $\alpha$  and PKM2) in lung adenocarcinomas.

## Material and Methods

### Patients and Samples

A total of 89 patients who underwent surgical resection of the primary LUAD in the Second Hospital of Hebei Medical University, and had complete clinical data, were reviewed in this retrospective analysis. All patients provided available tissues, including primary tumors and matched normal lung tissues. All patients did not receive any chemotherapy or radiotherapy before the study. TNM system was used for the staging of cancers and the WHO system for the morphologic classification.

### Detection of EGFR Gene Mutation

Genomic DNA was extracted from LUAD tissues sampled from surgically resected specimens. EGFR mutation status was measured by the Stratagene Mx3000p Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA) as previously described.<sup>18</sup> Two professional analysts were separately responsible for the assessment of the results.

### Immunohistochemistry

4 $\mu$ m serial sections were cut from formalin-fixed, paraffin-embedded LUAD tissues. The sections were conventional dewaxing to water and performed antigen retrieval using EDTA, after incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 min, sections were blocked with 1% bovine serum albumin (BSA) for 10 min, and incubated with HIF-1 $\alpha$  (1:200) and PKM2 (1:400) at 4°C overnight. The slides were incubated with anti-rabbit/mouse IgG antibody the next day, after incubating with reaction enhancer, the slides were colored with DAB and counterstained with hematoxylin. Negative control was performed by the absence of the primary antibody.

## Immunohistochemical Staining Evaluation

We quantitatively scored the sections through the percentage of positive cells and the staining intensity as previously.<sup>19,20</sup> The effect that brown-yellow particles were observed in the nucleus or the cytoplasm was considered as HIF-1 $\alpha$  and PKM2 positive. Then percentage and intensity of positive cell were both recorded. The percentage scores of the stained area: 0:  $\leq$ 5%; 1: 6–25%; 2: 26–50%; 3: 51–75%; 4:  $\geq$ 76%. The staining intensity: 0: no staining; 1: light yellow; 2: brown yellow; 3: brown. Finally, multiply the two scores to get the final score: negative expression: score < 8; positive expression: score  $\geq$  8.

## Statistical Analysis

Statistical analyses were done with SPSS16.0 Software (SPSS Inc., Chicago, IL, USA).

The Chi-squared test and Fisher exact test were used to evaluate the comparison of PKM2 and HIF-1 $\alpha$  protein expression between LUAD and corresponding normal tissue, as well as between EGFR-mt and EGFR-wd groups. The correlation among PKM2 and HIF-1 $\alpha$  was analyzed by Pearson's correlation test.  $p < 0.05$  was considered to indicate a statistically significant difference.

## Results

### EGFR Mutation Status

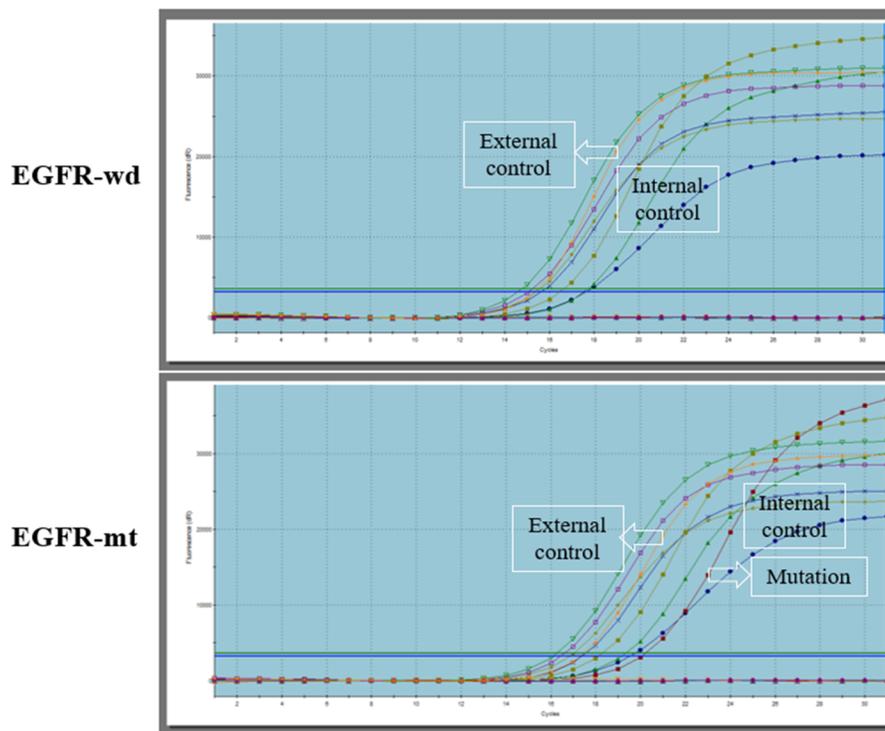
Histologically confirmed that all 89 (49 females, average age 61 years) included cases were lung adenocarcinoma (LUAD). EGFR mutations were found in 44 (49.4%) out of 89 patients included in this study (Figure 1). Among patients with EGFR mutations, the proportion of females and never smoking was significantly higher (34 out of 44 patients,  $p < 0.001$ ) (36 out of 44 patients,  $p < 0.001$ ). No significant difference was found in TNM staging between patients with different EGFR mutational status ( $p = 0.964$ ,  $p = 0.979$ ) (Table 1).

### The Protein Expressions of HIF-1 $\alpha$ and PKM2 in Lung Adenocarcinoma Tissues

Immunohistochemical analysis of LUAD tumor tissues showed that HIF-1 $\alpha$  and PKM2 were both mainly expressed in the cytoplasm or nucleus of tumor cells. Semiquantitative scoring was utilized to calculate the result. The scores of different groups were compared with Chi-squared test. As shown in Figure 2 and Table 2, compared to non-tumor tissues, HIF-1 $\alpha$  and PKM2 protein levels significantly increased in LUAD tissues ( $p = 0.024$ ,  $p = 0.001$ ). In addition, spearman correlation analysis showed that the expression of HIF-1 $\alpha$  was positively correlated with the expression of PKM2 in LUAD tissues ( $p = 0.003$ ,  $r = 0.307$ ) (Table 3).

### Relationship between Expressions of PKM2, HIF-1 $\alpha$ and Clinical Features of Lung Adenocarcinoma

We explored the relationship between the two proteins of HIF-1 $\alpha$ /PKM2 feedback loop and the clinical pathologic



**Figure 1.** EGFR mutational status of lung adenocarcinoma (LUAD). Abbreviation: EGFR-mt, EGFR mutation adenocarcinoma; EGFR-wd, EGFR wild type adenocarcinoma.

**Table 1.** The clinicopathological features of 89 LUAD patients.

Clinical characteristics	Overall n	EGFR-mt n (%)	EGFR-wd n (%)	P value
All	89	44	45	
Age				0.310
<60 years	33	14	19	
≥60 years	56	30	26	
Gender				<0.001
Male	40	10	30	
Female	49	34	15	
Smoking				<0.001
Never	55	36	19	
ever	34	8	26	
Stage				0.964
I	49	24	25	
II	17	8	9	
III	15	10	5	
IV	8	2	6	

Abbreviation: EGFR, epidermal growth factor receptor; LUAD, lung adenocarcinoma; EGFR-mt, EGFR mutation lung adenocarcinoma; EGFR-wd, EGFR wild type lung adenocarcinoma.

features of LUAD in Table 4, and we observed that HIF-1α and PKM2 expressions were both correlated with pTNM stage ( $p=0.023$ ,  $p=0.023$ ). But there was no significant of HIF-1α, PKM2 expression correlation with age, gender and smoking history. Although not statistically significant, HIF-1α and PKM2 had a trend to be highly expressed in lymph node metastases group ( $p=0.736$ ,  $p=0.117$ ) (Table 4).

**HIF-1α Correlated with PKM2 Proteins in EGFR-Mutated LUAD Tissue**

EGFR-mutated tumors showed higher HIF-1α protein ( $p=0.001$ ) and higher PKM2 protein expression ( $p=0.033$ ), compared to tumors with EGFR wild-type (Figure 3 and

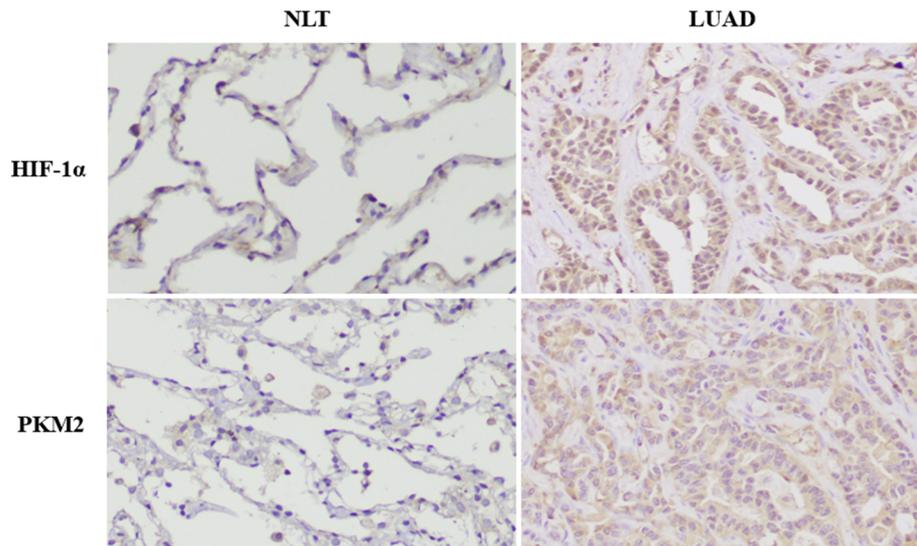
Table 5). Spearman correlation test revealed that the expression of HIF-1α were positively correlated with PKM2 in EGFR-mt ( $r=0.379$ ,  $p=0.011$ ) but not in EGFR-wd ( $r=0.149$ ,  $p=0.327$ ) LUAD tissues (Table 6). The above data showed that HIF-1α correlated with PKM2 and increases PKM2 expression in EGFR-mt LUAD.

**HIF-1α was Correlated with pTNM Stage Only in EGFR-mt LUAD Tissues**

Subsequently, Chi-squared test was used to detect the relationship between PKM2, HIF-1α expressions and clinical characteristics in both EGFR-mt and EGFR-wd groups. We found that high expression of HIF-1α was obviously correlated with pTNM stage only in EGFR-mt group ( $p=0.030$ ) but not EGFR-wd group ( $p=0.227$ ), and PKM2 showed a similar trend though without statistical significance ( $p=0.062$ ) (Table 7).

**Discussion**

HIF-1α and PKM2 are two key metabolic regulatory proteins, they enhance each other’s expression by forming feedback loop, thus playing an important role in the occurrence and development of various human tumors.<sup>11,12</sup> Nevertheless, the effect of this feedback loop in lung adenocarcinoma and their relationship with EGFR mutational status is still unclear. This study compared the expression and clinical pathological significance of PKM2, HIF-1α proteins in LUAD and normal lung tissue. We found that the two proteins were both highly expressed in LUAD tissues and related to higher pTNM stage of LUAD. Furthermore, the



**Figure 2.** Different expressions of HIF-1 $\alpha$  and PKM2 in normal lung tissue (NLT) and lung adenocarcinoma (LUAD).

**Table 2.** Expressions of HIF-1 $\alpha$  and PKM2 in normal lung tissue and lung adenocarcinoma.

	HIF-1 $\alpha$		PKM2	
	+	-	+	-
NLT	41	48	28	61
LUAD	56	33	50	39
<i>P</i> value	0.024		0.001	

Abbreviation: NLT, normal lung tissue; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; PKM2, Pyruvate kinase M2.

**Table 3.** Relationship between the expression of HIF-1 $\alpha$  and PKM2 in lung adenocarcinoma.

		HIF-1 $\alpha$	
		+	-
PKM2	+	38	12
	-	18	21

$p=0.003, r=0.307$

**Table 4.** Relationships of HIF-1 $\alpha$ /PKM2 feedback loop proteins with clinical pathological characteristics in adenocarcinomas of lung.

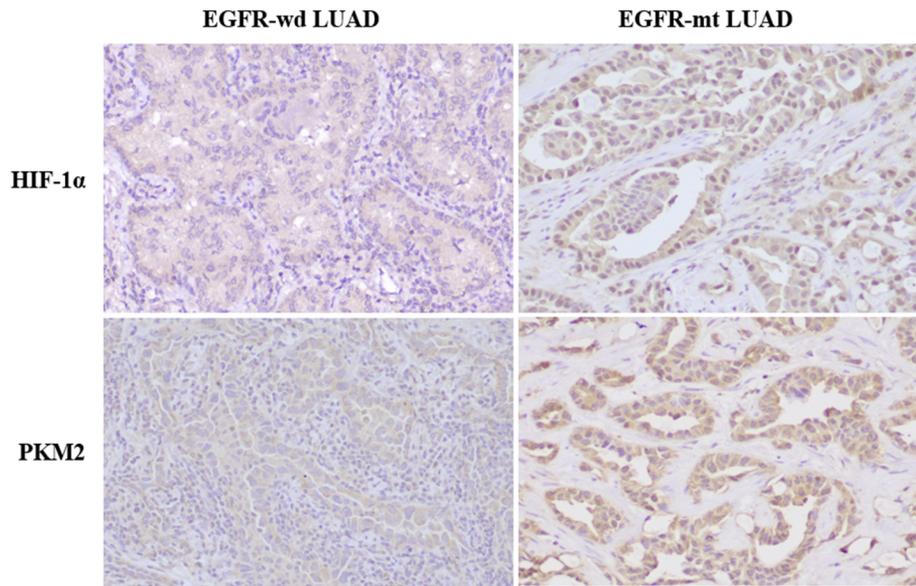
Clinical characteristics	overall	HIF-1 $\alpha$		<i>P</i> value	PKM2		<i>P</i> value
		+	-		+	-	
All	89	56	33		50	39	
Age							
<60 years	33	20	13	0.728	17	16	0.496
$\geq 60$ years	56	36	20		33	23	
Gender							
Male	40	22	18	0.162	25	15	0.278
Female	49	34	15		25	24	
Smoking							
Never	55	37	18	0.280	32	23	0.628
Ever	34	19	15		18	16	
Stage							
I	49	26	23	0.023	22	27	0.023
II	17	11	6		12	5	
III	15	13	2		10	5	
IV	8	6	2		6	2	

relationship between HIF-1 $\alpha$ , PKM2 expressions and the EGFR mutational status were also analyzed, and our findings indicated higher expressions of HIF-1 $\alpha$  and PKM2 in

EGFR mutation tumors relative to EGFR wild-type tumors. Meanwhile, the two proteins indicated a higher relationship with higher pTNM stage only in EGFR mutation tumors. A positive association was found between HIF-1 $\alpha$  and PKM2 in LUAD, and this correlation was mainly shown in patients with EGFR mutation. Our results firstly revealed that the expressions and clinical pathological significance of HIF-1 $\alpha$ , PKM2 were different in LUAD and normal lung tissues, and this difference as more obvious in EGFR mutational tumors, supporting the relationship between EGFR mutation and HIF-1 $\alpha$ , PKM2 in lung adenocarcinomas.

Cancer cells reprogram their glucose metabolism from oxidative phosphorylation to glycolysis to accelerate growth and proliferation. This phenomenon caused the “Warburg Effect” and was first described by Otto Warburg in the 1920s<sup>21</sup> and has been documented for over 90 years. Glycolysis was modulated by many enzymes of the glycolytic pathway. Pyruvate kinase M2 (PKM2), a glycolytic rate-limiting enzyme, catalyzes the final step of glycolysis, and was revealed to play a crucial role in tumorigenesis by promoting the metabolic reprogramming. It is reported that PKM2 level increases in cancers of hepatocellular, prostate and bladder, and has made an important contribution to the development and metastasis of tumors.<sup>9,22,23</sup> Consistent with previous reports, in this study, we found higher expression of PKM2 in LUAD tissue and PKM2 higher expression was correlated with higher pTNM stage in LUAD patients.<sup>24</sup> Though with no statistical significance, we found an increasing trend of PKM2 expression in tumors with lymph node metastasis, which may indicate poor clinical outcomes in LUAD patients.

It has been shown that the gene transcription of PKM2 is activated by hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ). While in turn, PKM2 is an essential co-activator that stimulates the transactivation of HIF-1 $\alpha$  in tumor cells. Thus, PKM2 and HIF-1 $\alpha$  constitute a positive feed-forward loop to reprogram glucose metabolism in cancer cells.<sup>25</sup> HIF-1 $\alpha$  abnormal expression is increasingly known as a critical step in cancer progression. It has been reported that the deletion of HIF-1 $\alpha$  markedly impair metastasis of breast cancer in a mouse



**Figure 3.** Different expressions of HIF-1α and PKM2 in EGFR wild type (EGFR-wd) and EGFR mutation (EGFR-mt) lung adenocarcinomas.

**Table 5.** HIF-1α and PKM2 protein expressions in EGFR wild-type versus EGFR-mutated lung adenocarcinoma.

	HIF-1α		PKM2	
	+	-	+	-
EGFR-mt	35	9	30	14
EGFR-wd	21	24	20	25
<i>P</i> value	0.001		0.033	

Abbreviation: EGFR-mt, EGFR mutation lung adenocarcinoma; EGFR-wd, EGFR wild type lung adenocarcinoma.

**Table 6.** Relationship between the expression of HIF-1α and PKM2 protein in EGFR wild-type versus EGFR-mutated lung adenocarcinoma.

		HIF-1α			
		EGFR-mt		EGFR-wd	
		+	-	+	-
PKM2	+	27	3	11	9
	-	8	6	10	15
		<i>p</i> =0.011, <i>r</i> =0.379		<i>p</i> =0.327, <i>r</i> =0.149	

Abbreviation: EGFR-mt, EGFR mutation lung adenocarcinoma; EGFR-wd, EGFR wild type lung adenocarcinoma.

model.<sup>26</sup> Jacoby et al found that inhibition of HIF-1α with PX-478 suppressed the progression and spread of small cell lung cancer and lung adenocarcinoma in mice.<sup>27</sup> And in our present study, we also found an increased expression of HIF-1α in LUAD tissues, the higher expression of HIF-1α was correlated with higher pTNM stage, suggesting a major role of HIF-1α in the pathogenesis of LUAD. Moreover, through Spearman correlation test of IHC results, we found the expression of PKM2 was significantly correlated with HIF-1α, indicating that PKM2 and HIF-1α form a feedback loop in LUAD.

The energy metabolism of cancer cells is mainly relay on aerobic glycolysis rather than mitochondrial oxidative phosphorylation. Many oncogenes, including EGFR, may facilitate the acquisition of the glycolytic phenotype.<sup>14,15</sup> The association between glucose metabolism and EGFR mutation in lung cancer has been studied before, with some controversial results. Most of the studies reported that EGFR-mt was associated with low glucose metabolism,<sup>28-30</sup> while

**Table 7.** Relationships of HIF-1α/PKM2 feedback loop proteins with clinical pathological characteristics in EGFR wild-type versus EGFR-mutated lung adenocarcinoma.

Clinical characteristics	Overall	EGFR-mt						EGFR-wd					
		HIF-1α			PKM2			HIF-1α			PKM2		
		+	-	<i>P</i>	+	-	<i>P</i>	+	-	<i>P</i>	+	-	<i>P</i>
All	89	35	9		30	14		21	24		20	25	
Age													
<60 years	33	11	3		9	5		9	10		8	11	
≥60 years	56	24	6	1.000	21	9	0.975	12	14	0.936	12	14	0.787
Gender													
Male	40	7	3		9	1		15	15		16	14	
Female	49	28	6	0.685	21	13	0.194	6	9	0.526	4	11	0.090
Smoking													
Never	55	29	7		24	12		8	11		8	11	
ever	34	6	2	1.000	6	2	0.970	13	13	0.600	12	14	0.787
Stage													
I	49	16	8		14	11		10	15		8	17	
II	17	7	1		5	3		4	5		7	2	
III	15	10	0		9	1		3	2		1	4	
IV	8	2	0	0.030	2	0	0.062	4	2	0.227	4	2	0.123

Abbreviation: EGFR-mt, EGFR mutation adenocarcinoma; EGFR-wd, EGFR wild type adenocarcinoma.

others considered that EGFR-mt was associated with increased glucose metabolism,<sup>31,32</sup> and some studies found that there was no statistical difference of glucose metabolism between EGFR-mt and EGFR-wt groups.<sup>33,34</sup> Therefore, more research still needed to elucidate the association. NSCLC may merge with various driving mutations other than EGFR that can affect glucose metabolism, and this may be the reason for the controversial results of glucose metabolism in EGFR-mt patients. In our present study, we found higher expressions of HIF-1 $\alpha$  and PKM2 in EGFR mutation tumors relative to EGFR wild-type tumors. These results were supported by Nilsson et al, who recently demonstrated that the expression of EGFR mutations is correlated with higher HIF-1 $\alpha$  expressions in NSCLC and NIH-3T3 cells, which means that cells carrying EGFR activated mutations may has a regulatory effect on HIF-1 $\alpha$  expression.<sup>16</sup> While the relationship of PKM2 and EGFR signaling pathway had already been elucidated, Yang et al revealed that cytosolic PKM2 stabilizes mutant EGFR protein expression through regulating HSP90-EGFR association.<sup>17</sup> The positive correlation of HIF-1 $\alpha$  and PKM2 expressions in EGFR mutation tumors emphasized the relationship between oncogenic mutated EGFR signaling and HIF-1 $\alpha$ , PKM2 in lung adenocarcinomas.

Taken together, our study demonstrated that different expression and clinical features of the proteins of HIF-1 $\alpha$ /PKM2 feedback loop was existed between lung adenocarcinoma and normal lung tissues, and this difference was also found in EGFR-mutation and EGFR-wild type tumors, indicating that the EGFR mutation was correlated with the key related proteins of aerobic glycolysis (HIF-1 $\alpha$  and PKM2) in lung adenocarcinomas.

## Disclosure Statement

No potential conflict of interest was reported by the author(s).

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