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ORIGINAL ARTICLE – CANCER RESEARCH

FUT4 is involved in PD-1-related immunosuppression and leads to worse survival in patients with operable lung adenocarcinoma

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Abstract

Purpose As an important glycosyltransferase, fucosyltransferase IV (FUT4) is abnormally upregulated in different types of cancers, but its clinical role remains inexplicit. This work aimed to determine the predictive ability of FUT4 in lung adenocarcinoma (LUAD) after curative resection, as well as to explore the role of a possible FUT4 molecular mechanism on LUAD malignant behavior.

Methods A total of 273 LUAD patients after curative resection with complete clinicopathological and RNAseq data from The Cancer Genome Atlas (TCGA) cohort were collected. Correlation of FUT4 with overall survival (OS) was analyzed based on TCGA and further validated by online "Kaplan–Meier Plotter" database and IHC in 70 LUAD patients recruited in the First Hospital of China Medical University cohort. Multivariate Cox regression analysis and 1000 bootstrapping were performed to verify the predictive value of FUT4. Gene set enrichment assay (GSEA) was performed to investigate the biological characteristics. Correlation between PD-1 and FUT4 was analyzed based on TCGA cohort and validated by IHC on cohort from our hospital.

Results Increased FUT4 expression led to reduced overall survival (OS) of LUAD patients based on TCGA ($p=0.006$ and 0.001 for dichotomous and trichotomous modeling, respectively) and externally validated in KMPLOTTER ($p=0.01$) and by IHC based on cohort from our hospital ($p=0.005$ and $p=0.019$ for dichotomous and trichotomous modeling, respectively). FUT4 overexpression was an independent high risk factor for OS along with advanced pT stage and pTNM stage ($p=0.001$, $p=0.037$, and $p<0.001$, respectively). GSEA revealed that FUT4 overexpression might correlate with shortened survival of LUAD patients by promoting cell proliferation via ERBB signaling, and suppressing immune response-related pathways. FUT4 expression positively correlated with PD-1 in TCGA ($p=0.026$) and validated by IHC on cohort from our hospital ($p=0.029$).

Conclusions Increased FUT4 expression led to reduced OS in operable LUAD. FUT4 showed significant correlation with immune response and PD-1 expression.

Keywords FUT4 · Lung adenocarcinoma · PD-1 · Prognosis · Survival

Introduction

Non-small-cell lung cancer (NSCLC) ranks first in cancer mortality worldwide (Reck et al. 2013). Lung adenocarcinoma (LUAD) is the most common histological type of NSCLC that is responsible for more than 500,000 annual deaths globally (Imielinski et al. 2012). After radical resection, early stage LUAD patients could still relapse and die as a result of disease recurrence, with the relapse rate as high as 45% for stage I–II within 5 years of diagnosis (Huang et al. 2016a; Uramoto and Tanaka 2014; Crino et al. 2010). Therefore, the current prognosis prediction

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systems are deficient. Novel and well-validated molecular biomarkers that can predict the prognosis of LUAD need to be urgently explored.

Aberrant glycosylation plays a significant role in tumor progression. Fucosylation is one of the most important type of glycosylation. Alterations in the fucosylation of cancer cells closely correlate with proliferation, apoptosis, migration, invasion, angiogenesis and drug resistance in various cancers including LUAD (Shao et al. 2016; Honma et al. 2015; Chen et al. 2013; Huang et al. 2016b, St Hill et al. 2011; Liu et al. 2014, 2017; Kuo et al. 2012; Feng et al. 2016). FUT4 is a key enzyme for synthesizing tumor-associated carbohydrate antigen including Lewis A, B, X, and Y (Escrevente et al. 2006), which catalyzes the transfer of L-fucose from GDP-fucose to the substrates. FUT4 is highly expressed in several types of cancers, including leukemia, gastric and breast cancers, and is positively correlated with tumor progression (Taniguchi et al. 2000; Yan et al. 2015; Aziz et al. 2017; Stirewalt et al. 2008). Few evidence of the association between FUT4 and cancer prognosis has been reported. Early study reported that increased gene expression of FUT4 and/or FUT7 predicted shorter survival in lung cancer (Ogawa et al. 1996). However, both staging system and treatment strategies have undergone dramatical changes in the past two decades. Therefore, it is necessary to reevaluate the role of FUT4 in the prognosis of LUAD.

In the present study, prognostic value of FUT4 expression and its potential molecular mechanisms were investigated in LUAD by analyzing the cohort from TCGA and validated by IHC.

Materials and methods

TCGA data acquisition and screening

The TCGA project provided multimodal data on 522 LUAD cases, which can be accessed from the TCGA website (http://cancergenome.nih.gov/). The dataset was searched for LUAD cases based on the RNASeqV2 data. A total of 249 cases were excluded because of non-R0 excision (174 cases), neoadjuvant chemotherapy (2 cases), IIIb, IV or not staged (26 cases), with history of malignant tumor or multiple primary tumors (28 cases), and without expression profiles (19 cases). Thus, 273 cases with R0 excision and both clinical information and expression profiles were eligible (Fig. S1). The expression data of the FUT4 gene were collected for each eligible case and divided into high-, moderate- and low-expression groups. The cutoffs were set to 33% and 67%. The median follow-up time was 791 days (ranging from 44 to 7248 days).

Clinicopathological parameters (CPPs)

Gender, pT stage, pN stage and pTNM stage were considered as categorical variables. Age was measured as a continuous variable. Correlation of FUT4 with CPPs was evaluated by chi-square test or Spearman assay, as appropriate. Multiple imputation in tackling missing data of age was performed using R multivariate imputation by chained equation (MICE) package.

Survival analysis

Kaplan–Meier survival curves with log rank test were utilized to assess the association between FUT4 and overall survival. Univariate and multivariate analyses were performed by Cox proportional hazards regression models to estimate HR and 95% CI. Forward stepwise selection method was performed to construct the final multivariate model with Akaike information criterion. Internal validation of the Cox model was performed using bootstrapping with 1000 replications. Time-dependent receiver-operating characteristic (ROC) was constructed to determine the prognostic accuracy of FUT4 in the Cox model according to Heagerty et al. (2000). The AUC(t) curve based on the evaluation of the risk scores was plotted to illustrate time-dependent specificity and sensitivity for the corresponding ROC curve at each observed event time. CPPs found to be significantly associated with OS in the multivariate Cox regression model were identified and integrated as prognostic variables. A nomogram was established with the discrimination and calibration assessed by concordance index (c-index) and calibration curve, respectively.

External validation

To externally validate the prognostic value of FUT4, the Kaplan–Meier Plotter (KMPLOTTER) database analysis was used. OS was assessed in LUAD patients stratified by median FUT4 expression. All other parameters were at default settings, except for "treatment group", which was set as "only surgical margins negative", to maximally simulate the current cohort.

Patients and tissue specimens

Ethical Committee of the First Hospital of China Medical University approved human specimens used in this study. Clinical medical records and follow-up data of 70 primary LUAD patients (pathologic stages I–IIIA) received a complete resection between Jan 1st and Dec 31st 2010 were reviewed. Among the 37 patients with stage IB–IIIA, all patients received adjuvant chemotherapy as platinum-based

regimens. No patient was treated with EGFR-TKI. This report includes follow-up data until June 15, 2015, with a median follow-up period of 55.4 months (ranging from 2.6 to 64.7 months). Overall survival was defined on the period from the date of surgery to death or the last follow-up date.

Tumor tissue EGFR mutation detection

EGFR mutation analysis was carried out by the AmoyDx® Mutation Detection Test, a real-time PCR assay for qualitative detection of 26 hotspot mutations in EGFR, in which ADx-ARMS (amplification refractory mutation system) kit (Amoy Diagnostics, Xiamen, China) was used. All experiments were performed according to the manufacturer's instructions (Liu et al. 2013).

Staining and evaluation of immunohistochemistry

Formalin-fixed, paraffin-embedded primary LUAD tissues were sectioned into 4 μm thickness. The method of immunohistochemistry (IHC) was discussed in our previous study (Wen et al. 2017). IHC staining was performed using the following antibodies: anti-FUT4 antibody from Proteintech Group and anti-PD-1 antibody from Cell Signaling Technology (#86163s, clone name: D4W2J). All specimens were visualized and classified by two pathologists in a blinded manner based on the percentage of positive cells and the intensity of staining. The staining intensity of FUT4 was scored as 0, 1, 2, and 3 for negative, weak, moderate, and strong, respectively. The extent of staining was scored as 0, 1, 2, 3, and 4 for 0%, 1–25%, 26–50%, 51–75%, and 76–100%, respectively. The score of intensity and extent constitutes Staining score. Staining scored 0–1 was classified as low FUT4 expression, 2–4 as moderate expression, and 5–7 as high expression for trichotomous modeling or 0–4 as low FUT4 expression and 5–7 as high expression for dichotomous modeling. PD-1 was defined as negative or positive if extent of staining was $< 1\%$ or $\geq 1\%$ in T cells, respectively, according to reference (Muro et al. 2016).

Gene set enrichment analysis

The software Gene set enrichment assay (GSEA) v2.2.3 (http://www.broadinstitute.org/gsea) was used to perform Gene set enrichment analysis. FUT4 expression level was dichotomized as low and high categories to annotate phenotype, and KEGG gene sets from Molecular Signatures Database (MSigDB) were used as functional gene sets. $p < 0.05$ and FDR < 0.25 were utilized as threshold values to estimate the statistical significance.

Statistics

Statistical analyses were performed using R project version 3.2.3 (http://www.r-project.org/). Two-tailed $p < 0.05$ was considered to be statistically significant.

Results

Patient characteristics and association of FUT4 expression with CPPs in TCGA cohort

The TCGA cohort including 273 R0 patients (128 males, 145 females) with clinicopathological features representative of LUAD was used for analysis (Table 1). The median age at initial pathologic diagnosis was 66 years (range 33–84 years). The majority of patients had T2 (156 cases, 57.1%) and N0 (186 cases, 68.1%). pTNM stage I, II, and IIIA were 166 (60.8%), 69 (25.3%), and 38 (13.9%), respectively. As shown in Table 2, there was no statistical significance between patients with distinct FUT4 expression levels and gender, age, pT stage, pN stage, pTNM stage, EGFR and KRAS mutational status.

Table 1 Characteristics of the TCGA lung adenocarcinoma study cohort and cohort from the First Hospital of China Medical University

Characteristics	TCGA cohort ($N = 273$)		Cohort from the First Hospital of China Medical University ($N = 70$)	
	Number	%	Number	%
Age, median (range)	65	33–84	58	35–74
Gender				
Female	145	53.1	37	52.9
Male	128	46.9	33	47.1
T stage				
T1	93	34.1	45	64.3
T2	156	57.1	15	21.4
T3	24	8.8	10	14.3
N stage				
N0	186	68.1	37	52.9
N1 or x	87	31.9	33	47.1
TNM stage				
I	166	60.8	34	48.6
II	69	25.3	12	17.1
IIIA	38	13.9	24	34.3
EGFR status				
Wild	111	40.7	23	32.9
Mutation	30	11.0	47	67.1
N/A	132	48.4	0	0

Influence of FUT4 expression on LUAD survival based on online datasets

To investigate whether FUT4 expression was higher in tumors compared with adjacent normal tissues, RNA-sequencing data of LUAD were downloaded from TCGA database which totally contained 273 operable LUAD and 59 nontumorous tissues. As shown in Fig. 1a, b, FUT4 expression displayed borderlined significantly higher expression in paired LUAD tissues compared with adjacent normal tissues (samples from patients with tumor tissues and corresponding adjacent normal tissues) ($n=23$, $p=0.079$). While FUT4 expression displayed significantly higher expression in LUAD tissues ($n=273$) compared with normal tissues ($n=59$, $p=0.0169$).

To investigate whether FUT4 was correlated with prognosis in LUAD, we analyzed follow-up data of the TCGA cohort. As shown in Fig. 1c, d, patients with increased FUT4 expression had significantly shorter OS irrespective of the cutoff chosen (HR = 2.0 and 1.6, and log rank $p=0.006$ and 0.001 for dichotomous and trichotomous modeling of FUT4 expression, respectively). As external validation, a total of 204 margin-negative LUAD patients were included for survival analysis from the KMPLOTTER database. Patients with high level of FUT4 showed significantly shorter OS than those with low expression (HR = 2.68, $p=0.01$) (Fig. 1e). Moreover, the AUC of combination with FUT4 was improved comparing to including CPPs alone, which demonstrated that FUT4 expression enhance the predictive accuracy of the prognostic model (Fig. S2).

In univariate analysis using Cox proportional-hazard models, advanced pT stage, pN stage, pTNM stage, and higher FUT4 expression correlated with reduced OS ($p=0.001$, <0.001, <0.001, and <0.001, respectively). The multivariate Cox regression analysis identified FUT4 as an independent predictor of poorer OS as well as pT stage and pTNM stage (HR = 2.432, 95% CI 1.458–4.056, $p=0.001$; HR = 3.711, 95% CI 1.986–7.577, $p=0.037$; HR = 3.938, 95% CI 2.333–6.646 $p<0.001$, respectively). The final prognostic model was further validated using bootstrapping with 1000 replications (Table 3). A nomogram that predicts 1- and 3-year OS was constructed based on the final multivariate Cox regression model (Fig. S3A). The predictive accuracy for 1- and 3-year OS as examined by the concordance index (*c*-index) was 0.684 in the internal validation. The value indicated that the model was equipped with comparatively accurate ability of discrimination. The calibration plot for the probability of 1-year and 3-year (Fig. S3B, c) OS indicated favorable correlation between the predictive outcome and actual observation in the internal validation.

Table 2 The relationship between FUT4 expression and CPPs in LUAD patients

	FUT4 expression of cohort from TCGA, *n* (%)				FUT4 expression of cohort from our hospital, *n* (%)		
	Low	Moderate	High	*p* value	Low	High	*p* value
Age (years)				0.663			0.43
Median (range)	64 (33–84)	66 (39–84)	64 (38–84)		61 (35–74)	58 (35–73)	
Gender				0.136			0.142
Female	49 (54.4)	57 (61.3)	39 (43.3)		20 (62.5)	17 (44.7)	
Male	41 (45.6)	36 (38.7)	51 (56.7)		12 (37.5)	21 (55.3)	
T stage				0.276			0.309
T1	30 (33.3)	39 (41.9)	24 (26.7)		22 (68.8)	23 (60.5)	
T2	52 (57.8)	49 (52.7)	55 (61.1)		8 (25.0)	7 (18.4)	
T3	8 (8.9)	5 (5.4)	11 (12.2)		2 (6.3)	8 (21.1)	
N stage				0.633			0.019
N0	63 (70.0)	63 (67.7)	60 (66.7)		21 (65.6)	16 (42.1)	
N1 or x	27 (30.0)	30 (32.3)	30 (33.3)		11 (34.4)	22 (57.9)	
TNM stage				0.579			0.01
I	54 (60.0)	61 (65.6)	51 (56.7)		21 (65.6)	13 (34.2)	
II	23 (25.6)	23 (24.7)	23 (25.6)		4 (12.5)	8 (21.1)	
III	13 (14.4)	9 (9.7)	16 (17.8)		7 (21.9)	17 (44.7)	
EGFR status				0.738			0.796
Wild	32 (35.6)	40 (43.0)	39 (43.3)		10 (31.3)	13 (34.2)	
Mutation	12 (13.3)	6 (6.5)	39 (43.3)		22 (68.8)	25 (65.8)	
N/A	46 (51.1)	47 (50.5)	12 (13.3)		0 (0)	0 (0)	

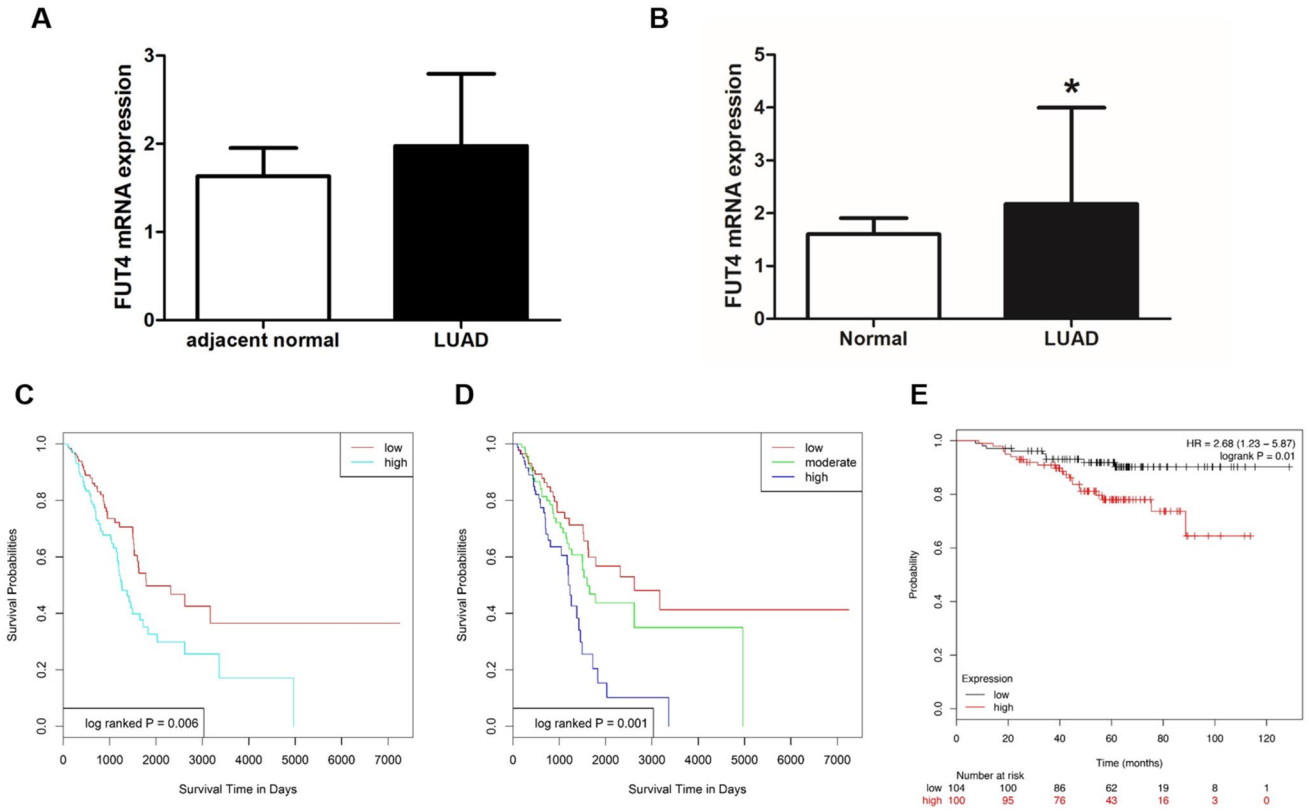

Fig. 1 Influence of FUT4 expression on LUAD survival based on online datasets. **a, b** Analysis of FUT4 expression in normal tissue and LUAD tissues by paired-sample *t* test or independent-sample *t* test. **c, d** Dichotomous and trichotomous modeling of Kaplan–Meier analyses for the OS of LUAD patients, respectively. **e** Kaplan–Meier analysis for the OS of LUAD patients in "Kaplan–Meier plotter" dataset according to distinct FUT4 expression level

IHC validation of FUT4 expression predicting poor survival in LUAD

The cohort from our own hospital including 70 R0 patients (33 males, 37 females) with clinicopathological features representative of LUAD was used for analysis (Table 1). The median age at initial pathologic diagnosis was 58 years (range 35–74 years). The majority of patients had T1 (45 cases, 64.3%) and N0 (37 cases, 52.9%). pTNM stage I, II, and IIIA were 34 (48.6%), 12 (17.1%), and 24 (34.3%), respectively. Among 70 patients, 47 (67.1%) patients demonstrated activating EGFR mutations.

The expression of FUT4 was detected in 70 LUAD tissues from our hospital by IHC. FUT4 expression was rare or very weak in the para-carcinoma tissues (data not shown), whereas cytoplasmically immunostained in LUAD cells (Fig. 2a–h). As shown in Fig. 2i, j, in line with relationship based on online datasets, patients with increased FUT4 expression had significantly shorter OS irrespective of the cutoff chosen (HR = 3.4 and 2.326, and log rank $p = 0.005$ and $p = 0.019$ for dichotomous and trichotomous modeling of FUT4 expression, respectively).

As shown in Table 2, there was no significant difference between patients with distinct FUT4 expression levels regarding patients' gender, age, pT stage or EGFR mutation status. However, enhanced FUT4 expression was significantly related to advanced pN stage ($p = 0.019$) and pTNM stage ($p = 0.01$).

GSEA predicts correlation of FUT4 expression with ERBB signaling and immune response

To investigate the biological characteristics shared by different FUT4 expression levels, we performed the GSEA assay. "KEGG_PATHWAYS_IN_CANCER", "KEGG_ERBB_SIGNALING_PATHWAY", "KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY", "KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY", "KEGG_PRIMARY_IMMUNODEFICIENCY" and "KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION" were significantly enriched in the high-FUT4-expression group (Fig. 3a–f). Suggesting the important role of ERBB signaling in LUAD cell proliferation, FUT4 expression was investigated in association with Ki67, which

Table 3 Univariate/multivariate Cox regression analysis for FUT4 expression and CPPs

Characteristics	No		Uni-variant analysis			Multi-variant analysis			
	Patients	Events	HR	95% CI	*p* value	HR	95% CI	*p* value	Bootstrapping 95% CI
Age (years)			0.983	0.664–1.456	0.933				
Gender			1.098	0.742–1.624	0.639				
Female	145	51							
Male	128	50							
T stage			1.849	1.288–2.654	0.001				
T1	93	24							
T2	156	63	1.416	0.885–2.268	0.147				
T3	24	14	3.972	2.024–7.796	< 0.001	3.711	1.986–7.577	0.037	1.046–4.455
N stage			2.261	1.527–3.347	< 0.001				
N0	186	52							
N1 or x	87	49							
TNM stage			1.990	1.558–2.542	< 0.001				
I	166	43							
II	69	35	2.398	1.530–3.759	< 0.001	2.207	1.401–3.476	0.002	1.209–3.127
III	38	23	3.800	2.272–6.355	< 0.001	3.938	2.333–6.646	< 0.001	1.871–5.761
FUT4			1.600	1.239–2.067	< 0.001				
Low	90	26							
Middle	93	35	1.410	0.846–2.351	0.187				
High	90	40	2.514	1.516–4.168	< 0.001	2.432	1.458–4.056	0.001	1.462–4.100

is one of the most extensive malignant cell proliferation markers. Ki67 expression was positively correlated with FUT4 in TCGA cohort ($p = 0.014$) (Table 4). These results suggested FUT4 may lead to poor prognosis of LUAD by promoting cell proliferation via activating EGFR signaling in LUAD. Results of "KEGG_PATHWAYS_IN_CANCER" and "KEGG_ERBB_SIGNALING_PATHWAY" inriched in FUT4-related poor prognosis in LUAD proved that GSEA results based on TCGA were highly reliable. Thus, the other category of GSEA results: the relationship between FUT4 and immune response needs point of concern.

Validation of the association of FUT4 expression with signaling pathways related to immune suppression and immune checkpoint PD-1/PD-L1

GSEA results showed that multiple signaling pathways related to immune response were significantly correlated with FUT4 expression (Fig. 3c–f). We wanted to understand whether FUT4 shortened survival of LUAD in an immunosuppressive manner. Hence, we investigated the correlation between FUT4 and immune checkpoint PD-1/PD-L1, as well as markers and corresponding effector-suppressing molecules of important immune suppressor cells: FoxP3 + regulatory T lymphocytes (Tregs) and myeloid-derived suppressor cells (MDSCs). FUT4 expression positively correlated with PD-1 ($p = 0.026$) (Table 5), and also significantly correlated with the surface markers of Tregs and FOXP3, and their corresponding effector-suppressing molecules (Table S1). These results indicated that high level of FUT4 probably reduced survival of LUAD by immunosuppression.

In LUAD tissues, PD-1 immunostaining was observed in immune cells infiltrating the tumor sites. Brown-yellow particles distributed in the membrane of immune cells were PD-1 staining. Out of 70 LUAD samples, 47.1% (33/70) showed PD-1 + staining and 52.9% (37/70) of patients had high expression of FUT4. Importantly, there was a significant positive correlation between expression of FUT4 and PD-1+ ($p = 0.029$) (Table 5). Figure 4 shows two representative patient sections of FUT4/PD-1 expression. It was demonstrated that cancer cells with high expression of FUT4 always had PD-1-positive immune cell infiltration (Fig. 4a, b, e, f). FUT4 in cancer cells was detected to have low expression, while the immune cells infiltrating always had a PD-1-negative phenotype (Fig. 4c, d, g, h).

Discussion

The current study investigated the clinical significance of FUT4 in LUAD, based on the RNAseq data from online database TCGA cohort. Survival analysis revealed that patients with higher FUT4 expression had shorter OS

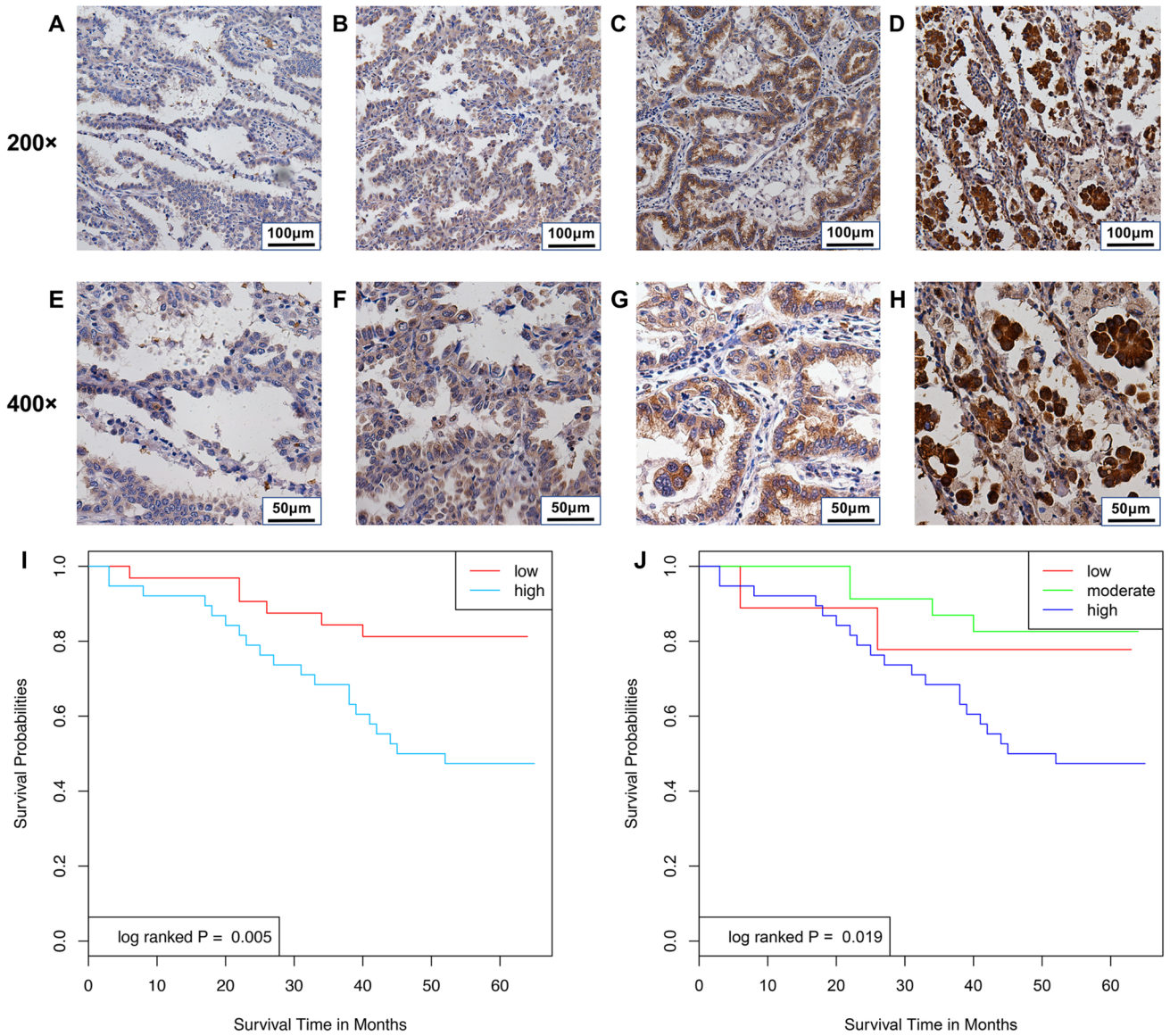

Fig. 2 **a–h** Representative images (×200; ×400) of negative, weak, moderate and strong were shown, respectively. **i, j** Dichotomous and trichotomous modeling of Kaplan–Meier analyses for the OS of LUAD patients with different levels of FUT4

compared to patients with lower FUT4 expression. Further detection by IHC based on LUAD specimens from our own hospital validated the positive relationship between FUT4 and poor prognosis. Based on the ROC curve, FUT4 strengthened the predictive efficacy of traditional clinicopathological features in LUAD. Nomogram, which predicts the prognostic risk of 1- and 3-year OS, verified the predictive value of FUT4. These results suggested the potential value of FUT4 as a biomarker for clinical prognostic prediction in LUAD.

Our findings are convincing and universally applicable based on the following evidences. First, FUT4 played a consistently poor prognostic role regardless of the cutoff value of FUT4 expression selected. Second, the independent prediction of FUT4 for OS was determined by the Cox regression model both in univariate and multivariate analyses, and further confirmed by 1000 internal bootstrap replications. Third, external validation based on another online database Kaplan–Meier Plotter obtained a concordant result. Consistent with early report that high FUT4 expression led to worse clinical outcome in lung cancer (Ogawa et al. 1996), our study performed a prognostic analysis specifically for LUAD based on a larger cohort from high-throughput RNA-sequencing dataset. Similar poor prognostic role of FUT4 was previously reported in mCRCs, whereas a cancer stem

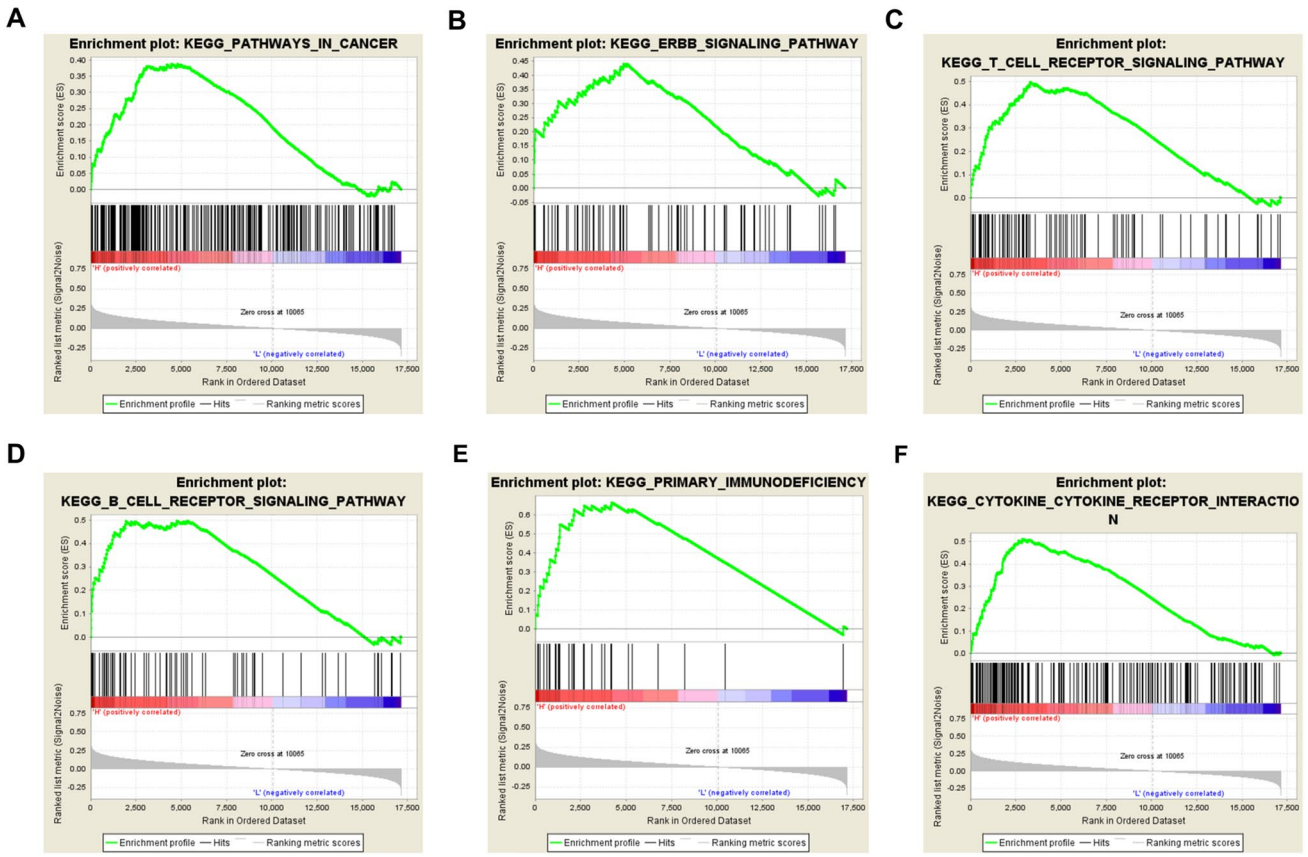

Fig. 3 FUT4-related signaling pathways leading to worse survival in LUAD. **a–f** GSEA results showed the correlation of FUT4- and LUAD-related gene sets in MSigDB

Table 4 The relationship between FUT4 expression and Ki67 and immunosuppression markers PD-L1/PD-1 in patients of TCGA cohort

	Expression of FUT4, *n* (%)			*p* value
	Low	Middle	High	
Ki67				0.017
Low	37 (41.1)	29 (31.2)	24 (26.7)	
Middle	28 (31.1)	37 (39.8)	28 (31.1)	
High	25 (27.8)	27 (29.0)	38 (42.2)	
PD-1				0.044
Low	41 (45.6)	18 (19.4)	31 (34.4)	
Middle	32 (35.6)	31 (33.3)	30 (33.3)	
High	17 (18.9)	44 (47.3)	29 (32.2)	
PD-L1				0.855
Low	36 (40.0)	23 (24.7)	31 (34.4)	
Middle	23 (25.6)	39 (41.9)	31 (34.4)	
High	31 (34.4)	31 (33.3)	28 (31.1)	

cell marker, CD15/FUT4 expression, was associated with shorter progression-free survival (Giordano et al. 2015).

Table 5 The relationship between FUT4 expression and PD-1 in LUAD patients by IHC

PD-1	FUT4, *n* (%)		*p* value
	High	Low	
Positive	22 (59.5)	11 (33.3)	0.029
Negative	15 (40.5)	22 (66.7)	

In this study, FUT4 was revealed statistically associated with pN and pTNM stage by IHC, which is consistent with the previously observed findings that overexpressed FUT4 positively correlates with tumor cell migration and invasion in lung and breast cancer (Tian et al. 2016; Yang et al. 2013). For N0 and N1 or x stage, TCGA cohort occupies more than 2/3 in N0 stage and less than 1/3 in N1 or x stage, while this ratio is almost half to half in cohort from our hospital. For pTNM stage, TCGA cohort had approximately 90% patients in stage I and II, while 2/3 patients in cohort from our hospital are in stage I and II. The different statistical results of the relationship between FUT4, and pN stage and pTNM stage, in all probability, might due to the different stage proportion between TCGA cohort and cohort from our hospital. Earlier

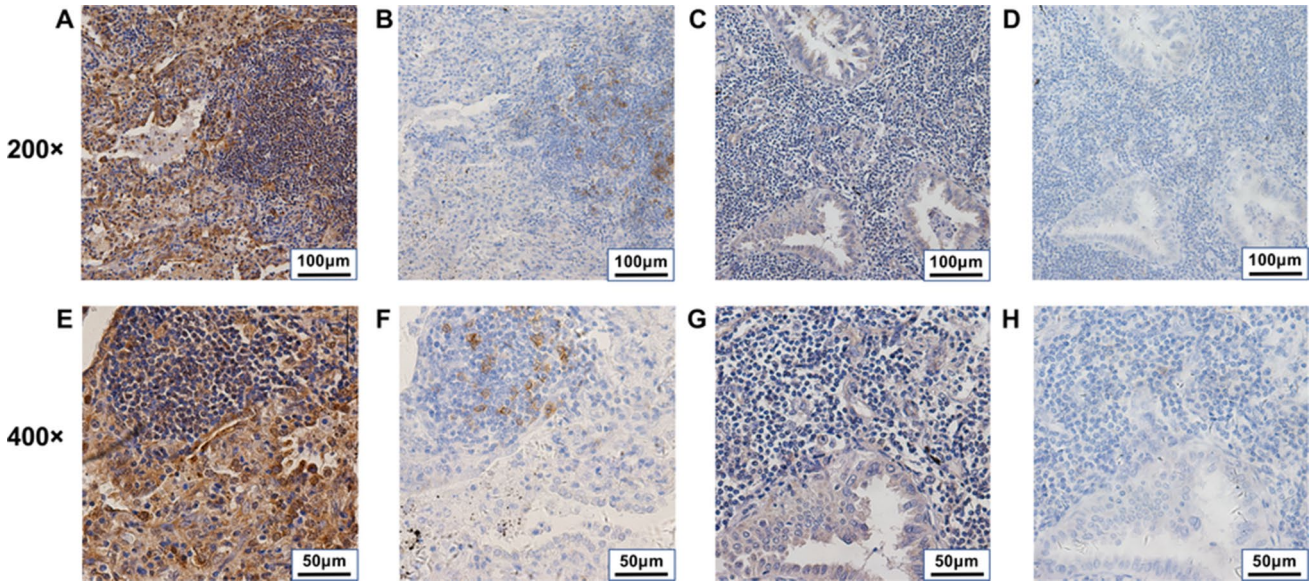

Fig. 4 The expression of FUT4 and PD-1 in LUAD tissues detected by IHC. **a** and **e**, and **c** and **g** showed the high and low expressions of FUT4 in tumor tissues (×200, ×400), respectively. **b** and **f**, and **d** and **h** showed the positive and negative expressions of PD-1 in the corresponding vision fields (×200, ×400), respectively

staging tendency of TCGA cohort may lead to the correlation between FUT4 expression and pN stage or pTNM stage to be ignored. The results need further validation of larger cohort.

As a marker of the proliferative activity of tumor cells, Ki67 carries valuable prognostic information (Gerdes et al. 1984; Scagliotti et al. 1993). Ki67 mRNA expression closely resembles the clinical characteristics of Ki67 labeling index determined by IHC (Varga et al. 2017; Muftah et al. 2017). In our analysis based on TCGA RNAseqV2, FUT4 exhibited a positive association with Ki67 expression. Besides, the GSEA results demonstrated that ERBB signaling and pathway in cancer were significantly enriched in response to FUT4 alteration in LUAD patients. Tumor-associated carbohydrate antigen Lewis Y oligosaccharide linked to EGFR plays a critical role in the activation of EGFR. As a key enzyme in the synthesis of Lewis Y, correlation of FUT4 expression with EGFR signaling pathway has been reported in several types of malignancies (Yang et al. 2010; Shan et al. 2015a). Previous reports demonstrated that suppression of FUT4 expression inhibited tumor cell proliferation via ERBB signaling in melanoma, epidermoid carcinoma, gastric and breast cancer (Shan et al. 2015b; Zhang et al. 2008; Aziz et al 2016; Yang et al. 2014). In lung cancer tissues, a recent study reported that the expression of FUT4 and EMT marker *N*-cadherin was consistently elevated, and downregulating FUT4 and LeY by ginsenoside Rg3 inhibited EMT and invasion by suppressing EGFR/MAPK and EGFR/NF-kB signal pathways (Tian et al. 2016). However, the proliferative ability of lung cancer cells was not investigated. Taken together, based on the prognostic correlation between FUT4 and Ki67, our GSEA results and previous studies of FUT4 functions in EGFR in other types of malignancies, FUT4 might lead to poor prognosis in LUAD by facilitating cell proliferation via ERBB signaling.

Notably, several signaling pathways associated with the immune system were involved in FUT4 expression differentiation in our GSEA results. During tumor progression, interactions between PD-1 and its ligands lead to T-cell apoptosis and cytokine secretion variations, which play a crucial role in tumor-mediated immune suppression and evasion (Freeman et al. 2000; Borghaei et al. 2015). In our analysis, FUT4 was positively correlated with PD-1 expression based on high-throughput RNA-sequencing data from TCGA and IHC results from LUAD patients of our hospital. PD-1 expression in tumor-infiltrating lymphocytes predicted poor prognosis in soft tissue sarcoma, pulmonary neuroendocrine tumor, follicular lymphoma, large B-cell lymphoma and renal cell carcinoma (Fan et al. 2016; Kim et al. 2013; Four et al. 2017; Smeltzer et al. 2014; Kang et al. 2013). Given the prognostic impact of infiltration of PD-1-positive immune cells in human cancer, PD-1 has been proposed as a novel target for immunotherapy of NSCLC (Topalian et al. 2012). Moreover, cytokines released by tumor cells may induce and recruit immunosuppressive cells, including regulatory T cells (Tregs) and myeloid-derived suppressive cells (MDSCs) in tumor microenvironment (Dunn et al. 2006). These cells exert their suppressive action through several mechanisms including upregulation of immunosuppressive factors such as inducible nitric oxide synthase (iNOS),

release of inhibitory cytokines such as IL-10 (O'Garra et al. 2008), and stimulation of inhibitory cell surface components on T cells such as PD-1 (Barber et al. 2006). High intratumoral MDSCs and/or Treg counts are related to poor prognosis of various human cancers (Gabrilovich et al. 2012; Diaz-Montero et al. 2009; Greten et al. 2011; Liotta et al. 2011; Bates et al. 2006; Ihara et al. 2017; Jiang et al. 2017), including NSCLCs (Vetsika et al. 2014, Uso et al. 2016; Zhao et al. 2016). Our supplementary results revealed that FUT4 was positively correlated with MDSC marker CD11b, CD33, Treg marker FOXP3, and their effector molecules iNOS and IL-10. IHC based on LUAD specimens from our own hospital confirmed there was a significant positive correlation between expression of FUT4 and PD-1+. Therefore, we deduced that the PD-1 pathway, MDSCs and/or Treg-mediated immunosuppression played a pivotal role in the adverse effect of FUT4 on LUAD prognosis. However, the actual contribution of the predicted mechanisms needs further clarification by cellular and molecular experiments.

We analyzed the mechanisms for the positive correlation of FUT4 with PD-1. First, FUT4 may regulate the expression of PD-1. It was indicated that the synthesis of cytokines and cytokine receptors can be regulated by FUTs (Tsou et al. 2013). The expression of PD-1 can be enhanced by local cytokines such as IL-2, -6, -7, -12, -15, -21, and VEGF-A (Wang et al. 2018; Bennett et al. 2003; Chinai et al. 2015; Kinter et al. 2008; Austin et al. 2014; Voron et al. 2015). Thus, it can be deduced that FUT4 may modulate the expression of PD-1 by facilitating or suppressing the expression of cytokines or cytokine receptors. Moreover, FUT4 is involved in synthesis of the common ligand of selectins, Lewis X, which mediates leukocyte chemotaxis and adhesion, and plays a key role in initiating tumor immunity (Julien et al. 2011). PD-1 is upregulated following T-cell receptor (TCR)-mediated activation and is readily observed on both activated and exhausted T cells (Barber et al. 2006). Therefore, PD-1 upregulation might be induced by Lewis X and selectin interaction. Second, PD-1 may regulate the expression of FUT4. Cytokines regulate FUT4 (Padró et al. 2011). The primary effect of PD-1 signaling is to inhibit TCR and essential costimulatory signals, and several inflammatory cytokines such as TNF-a, IFN-g and IL-2 were subsequently inhibited (Barber et al. 2006; Freeman et al. 2000; Latchman et al. 2001). Therefore, PD-1 may regulate the expression of FUT4 by influencing the cytokine synthesis. Third, based on the probability of cytokines regulating both FUT4 and PD-1, the same cytokine may influence the expression of FUT4 and PD-1 spontaneously.

In summary, based on high-throughput RNA-sequencing data from online datasets and validation of IHC from LUAD patients of our hospital, this study revealed that FUT4 is an independent poor prognostic marker for predicting clinical outcomes of LUAD patients. The potential mechanisms involved in the function of FUT4 in LUAD were predicted and validated elementarily. FUT4 may contribute to worse survival by PD-1-related immunosuppression and EGFR-mediated proliferation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethics Committee of the First Hospital of China Medical University approved the project and the study number of IRB approval is 2014-2014-25-2.

Informed consent Informed consent was obtained from all individual participants included in the study.

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