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REVIEW ARTICLE

EZH2 is a biomarker associated with lung cancer diagnosis and immune infiltrates without prognostic specificity: a study based on the cancer genome atlas data*

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Abstract

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2). Dysregulation of EZH2 causes alteration of gene expression and functions, thereby promoting cancer development. Recent studies suggest that EZH2 has a potential prognostic role in patients with non-small cell lung cancer (NSCLC). However, the prognostic value of EZH2 expression levels in NSCLC is controversial. In this study, we evaluated the prognostic value in lung cancer (LC-LUAD/LUSC) based on data from The Cancer Genome Atlas (TCGA) database. Kruskal-Wallis test, Wilcoxon signed-rank test, and logistic regression were used to evaluate the relationship between EZH2 expression and clinicopathological features. Cox regression and the Kaplan-Meier method were adopted to evaluate prognosis-related factors. Gene set enrichment analysis (GSEA) was performed to identify the key pathways related to EZH2. The correlations between EZH2 and cancer immune infiltrates were investigated by single-sample Gene Set Enrichment Analysis (ssGSEA). EZH2 was found to be up regulated with amplification in tumor tissues in multiple LC cohorts. High EZH2 expression was associated with poorer overall survival (OS). GSEA suggested that EZH2 regulates innate immune system, ECM affiliated, matrisome, surfactant metabolism. Notably, ssGSEA indicated that EZH2 expression was positively correlated with infiltrating levels of Th2 cells and significantly negatively correlated with mast cell infiltration level. These results suggest that EZH2 is associated with LC immune infiltration and significantly over-expressed in lung cancer, and its diagnostic value is better than prognosis, which lays a foundation for further study of the immunomodulatory role of EZH2 in LC.

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Lung cancer is the second commonly diagnosed cancer (11.4% of total cases), closely to female breast cancer (11.7%). But, Lung cancer is the leading cause of cancer death (18.0% of the total cancer deaths) [1]. Non-small-cell lung cancer (NSCLC), which is the dominant form of lung cancer, is an early asymptomatic disease that constitutes a major global health problem. Non–small-cell lung cancer (NSCLC), including lung adenocarcinoma

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(LUAD), squamous cell carcinoma (LUSC), and large-cell carcinoma accounts for approximately 85% of all lung cancer cases [2]. Despite the development progressive of the clinical diagnosis and therapeutic strategies in lung cancer research, yet the overall five year survival rate is still less than 21% [3]. The lack of biomarkers to facilitate the diagnosis of early-stage disease and cancer metastasis remains one of critical challenges in NSCLC therapy [4]. Therefore, a profound understanding of the molecular mechanisms contributing to the development and progression of NSCLC is essential for developing specific diagnostic methods, as well as for designing individualized and effective physiological strategies.

With the development of molecular biology, researchers have found that in addition to changes in specific gene loci and activation of abnormal signaling pathways, there are also changes in some special epigenetic traits [5]. Abnormal epigenetic regulation of transcription plays an important role in carcinogenesis and cancer development [6]. A lot of evidence shows that epigenetic regulation plays an important role in the occurrence and development of tumors. Epigenetic modification can regulate chromatin status and gene expression through pathways such as DNA methylation and demethylation, histone modification, and chromatin remodeling without changing the DNA sequence [7, 8]. Human EZH2 gene is located on the long arm of chromosome 7 at 7q35. It contains 20 exons and encodes a protein composed of 746 amino acids [9]. Sequence analysis revealed that EZH family proteins contain four homologous domains [10]. Enhancer of zeste homolog 2 (EZH2) is a member of the family of polycomb group genes (PcGs), which is a group of important epigenetic regulators that repress transcription [6]. EZH2 is an enzymatic catalytic subunit of PRC2 that can alter gene expression by trimethylation of Lys-27 in histone 3 (H3K27me3) [11]. When the PRC2 complex is formed and recruited to the promoter region of the target genes, the SET domain of EZH2 catalyzes H3K27me3, leading to silencing its target genes involved in cell proliferation, cell differentiation, and cancer development [12]. Besides H3K27me3, PRC2 also methylates non-histone protein substrates, including the transcription factor GATA4 [13]. In addition, EZH2 methylates non-histone targets or directly interacts with other proteins to activate downstream genes in a PRC2-independent manner [14, 15]. Via the above 3 ways, EZH2 works as a master regulator of cell cycle progression [16], autophagy, and apoptosis [17], promotes DNA damage repair and inhibits cellular senescence [18] and plays an important role in cell lineage determination and relative signaling pathways [19]. Numerous studies have revealed that dysregulation of EZH2 is correlated with poor prognosis in solid tumor patients, in terms of higher tumor grade, distant metastases, and shorter disease-free survival [20], including breast cancer [21], liver cancer [22], lung cancer [23], ect.

EZH2 gene is highly expressed in NSCLC, and its expression level is closely related to the prognosis of patients. At the same time, studies have also confirmed that the application of EZH2 inhibitor can effectively reduce the invasion and migration ability of NSCLC and induce tumor cell apoptosis [24, 25]. However, based on their results, the prognostic value of EZH2 expression levels in LC is controversial. Therefore, we conducted a systematic bioinformatics analysis of EZH2 expression, diagnostic value, immune cell infiltration and prognosis in non-small cell lung cancer based on TCGA database. EZH2 is expected to be a new therapeutic target with a good application prospect and clinical diagnostic value in LC.

Materials and methods

Data collection and analysis

EZH2 expression profiles and TCGA and Genotype-Tissue Expression (GTEx) clinical pan-cancer data were downloaded from the University of California, Santa Cruz (UCSC) Xena database (https://xenabrowser.net/datapages/), including data on the 31 types tumor. To evaluate EZH2 expression, tumor tissues were obtained from TCGA, and normal tissues were obtained from TCGA and the GTEx database.

Data acquisition and preprocessing

The RNA-seq data of 1037 LC and 108 normal tissues and patient clinical information were downloaded from the Lung cancer (LUAD/ LUSC) Project of The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/) [26] until 30 August 2022 . Then, RNA-seq data in FPKM format were transferred to TPM (transcripts per million reads) format, retained, and further analyzed.

Differentially expressed gene analysis

We used the unpaired Student's t-test within the DESeq2 R package (3.6.3) [27] to compare the expression data (HTseq-Counts) between high- and low-expression groups according to the median EZH2 expression level. The thresholds for the DEGs were $|\log2\text{-fold change (FC)}| > 1.5$ and adjusted $P < 0.05$.

Enrichment analysis

Metascape (3.0) (http://metascape.org), a user-friendly, well maintained, free, gene list online analysis tool for gene analysis and annotation [28], was adopted to perform Gene Ontology (GO) analysis. Cluster Profiler package in R (3.6.3) [29] was used to perform Gene Set Enrichment Analysis (GSEA) and detect the correlation between EZH2 and the pathway. As a computational method,

GSEA determines whether a priori defined set of genes have statistical significance and concordant differences in two biological states. The samples were divided into high- and low-expression groups according to the median expression level of EZH2. DEseq was used to compare the different expressions between different groups. Gene set permutations were performed with 1,000 times random combinations for each analysis. In the whole process, the expression level of EZH2 was regarded as a phenotype. Additionally, the adjusted P and normalized enrichment score (NES) were utilized to sort the enriched pathways in each phenotype [30]. c2.cp.v7.0.symbols.gmt [Curated] in MSigDB collections was selected as a reference gene set. Gene sets with a false discovery rate (FDR) < 0.25 and adjusted $P < 0.05$ were considered significantly enriched.

Immune infiltration analysis by single-sample gene set enrichment analysis

ssGSEA classifies marker gene sets in a single sample with common biologic functions, chromosomal localization, and physiological regulation. In this study, the ssGSEA method was realized by the GSVA package [31] in R to analyze the immune infiltration for 24 types of immune cells and correlation between EZH2 and every immunocyte in LC samples according to the published literature [32]. The relative enrichment score of signature genes was quantified from the gene expression profile for each tumor sample. Spearman's correlation was adopted to analyze the correlation between EZH2 and 24 types of immune cells, and the Wilcoxon rank-sum test was adopted to analyze the infiltration of immune cells between the high-expression groups of EZH2.

Protein-protein interaction network

The Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org) [33] was applied to predict the PPI network. The combined score threshold of interaction was 0.4. Furthermore, visualize the PPI network by using Cytoscape (version 3.7.2).

Statistical analysis

The statistical data acquired from TCGA were merged and processed by R 3.6.3. The Wilcoxon rank-sum test and Wilcoxon signed-rank test were used for comparing the expression levels of EZH2 between LC and the control group. Kruskal–Wallis test, Wilcoxon rank-sum test, Wilcoxon signed-rank test, and Spearman's correlation were used to analyze the relation between EZH2 expression and grade of clinicopathological factors. Normal and adjusted Pearson's κ^2 test, Fisher's exact test, and univariate logistic regression were used to analyze whether the grade of clinicopathological factors affects EZH2 expression. Spearman's correlation and the Wilcoxon rank-sum test were adopted to analyze the infiltration of immunocytes between the high- and low expression groups of EZH2. Comparison of multiple groups was performed using a nonparametric Kruskal–Wallis test followed by a post hoc Dunn's test with Bonferroni correction for pairwise comparisons. Univariate Cox regression analysis and multivariate Cox regression analysis were used to evaluate the influence of EZH2 expression and other clinicopathological factors (age and gender) on survival. The significant variables in the univariate analysis ($P < 0.1$) were included into the multivariate analysis [34, 35]. The Kaplan–Meier curve was drawn to evaluate the prognostic value of EZH2. Hazard risk (HR) of individual factors was estimated by measuring the HR with a 95% confidence interval (CI).

Receiver operating characteristic (ROC) analysis was performed by the pROC package [36]. The calculated area under the curve (AUC) value ranges, which were from 0.5 to 1.0, indicated the discrimination ability of 50%–100%. We constructed a nomogram by the rms R package based on the results of the multivariate analysis. The predicted survival probability for 1, 3, and 5 years is visualized in the nomogram, which includes a calibration plot as well as significant clinical characteristics. All statistical tests were considered significant when two-tailed $P \leq 0.05$.

Results

Pan-cancer and lung cancer (TCGA-LUAD/LUSC) EZH2 expression analysis

We first evaluated EZH2 expression in TCGA and GTEx pan-cancer database, and found higher EZH2 expression in 29 tumors compared with the corresponding normal tissues, including: ACC, BLCA, BRCA, CESC, CHOL, COAD, DLBC, ESCA, GBM, HNSC, KICH, KIRP, LAML, LGG, LIHC, LUAD, LUSC, OV, PAAD, PRAD, READ, SKCM, STAD, TGCT, THYM, UCEC, and UCS. But, only 2 tumors were found that EZH2 expression was lower than that in the corresponding normal tissues, including: KRIC, THCA (Fig. 1a). Particularly, The Mann-Whitney U test results showed that the unpaired Tumor of lung cancer was higher than the Normal, and the median difference between the two groups was 1.868 (1.73-2.008), which was statistically significant ($P < 0.001$) (Fig. 1b). High EZH2 expression was observed in LUAD, LUSC in the TCGA cohort compared with the adjacent tissues. In LUAD, the median difference was 0.733 (0.616-0.848), which was statistically significant ($P < 0.001$) (Fig. 1c) and Weltch t' test showed that Tumor was higher than Normal average in LUSC, and the difference was 1.562 (1.454-1.669) ($t = 28.593$, $P < 0.001$) (Fig. 1d). Above all, suggesting that EZH2 may play a role in the pathogenesis and diagnosis of LC (LUAD/LUSC).

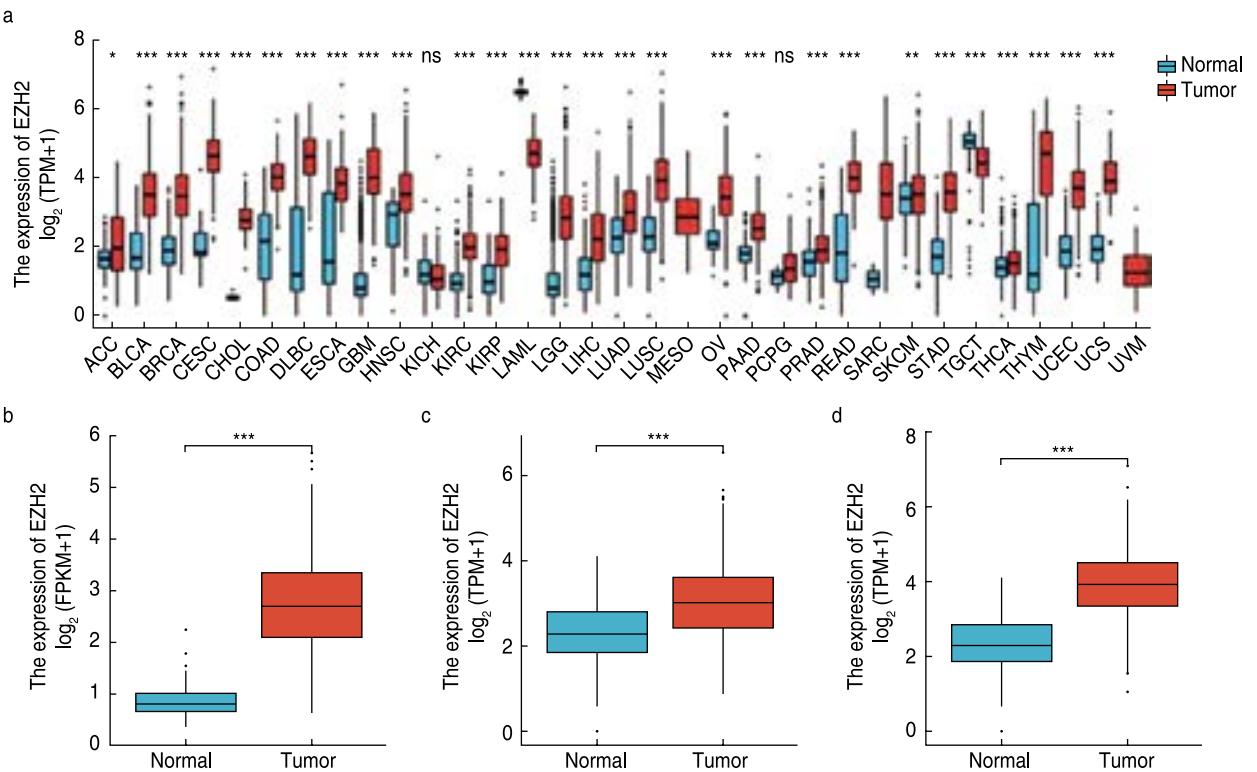

Fig. 1 Pan-cancer EZH2 expression analysis. (a) EZH2 expression in tumor and normal tissues in pan-cancer data of The Cancer Genome Atlas (TCGA) and GTEx; (b) EZH2 expression in tumor and normal tissues in LC from TCGA; (c) EZH2 expression in non-paired tumor and normal tissues in LUAD from TCGA; (d) EZH2 expression in non-paired tumor and normal tissues in LUSC from TCGA. Data were shown as mean ± SD. Identification of significance: ns, $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Clinical characteristics

The clinical data of 1037 Lung cancer patients included patient T stage, N stage, M stage, pathologic stage, gender, age, smoker, primary therapy outcome, anatomic neoplasm subdivision2, age median (IQR), residual tumor (Table 1). A total of 620 males and 417 females with a mean age of 67 years were analyzed in the present study, including 918 smoking patients and 93 non-smoking patients. The chi squared test showed that EZH2 was significantly correlated with T stage (P = 0.047), Gender (P = 0.01), Age (P = 0.019), Smoker (P < 0.01), and Anatomic neoplasm subdivision2 (P = 0.036). Fisher's exact test showed that EZH2 was not significantly correlated with Residual tumor (P = 0.111), N stage (P = 0.343). Wilcoxon rank sum test showed that EZH2 was significantly correlated with Age median (IQR) (P =0.011). EZH2 expression was not significantly correlated with other clinicopathological features, for instance: M stage (P = 0.334), Pathologic stage (P = 0.164), Primary therapy outcome (P = 0.399).

Identification of differentially expressed genes in lung cancer

Based on the cutoff criteria (|logFC| > 1.5 and adjusted P < 0.05), we identified a total of 1760 DEGs (1399 up-regulated and 361 down-regulated) after using the DESeq2 package in R [37] to analyze the HTSeq-count data from TCGA. DEG expressions were illustrated by a volcano plot (Fig. 2a). DEGs included 987 differentially expressed mRNAs (46 unprocessed pseudogenes, 2 unitary pseudogenes, 48 transcribed unprocessed pseudogenes, 41 TECs, 591 protein coding genes, 259 processed pseudogenes) which contained 42 up regulated unprocessed pseudogenes, 2 up regulated unitary pseudogenes, 24 up regulated transcribed unprocessed pseudogenes, 34 up regulated TECs, 340 up regulated protein coding genes, 274 up regulated processed pseudogenes and 4 unprocessed pseudogenes down regulated, 24 transcribed unprocessed pseudogenes down regulated, 7 TECs down regulated, 251 protein coding genes down regulated, 12 processed pseudogenes down regulated. DEGs included 773 differentially expressed RNAs (61 snRNA, 43 snoRNA, 4 Rrna pseudogene, 85 misc-RNA, 21 miRNA, 559 lncRNA) which contained 43 snoRNA upregulated, 4 rRNA pseudogene up regulated, 85 misc-RNA up regulated and 21 miRNAs (18 up regulated and 3 down regulated), 61 snRNA (60 up regulated and 1 down regulated), 559 lncRNAs (473 up regulated and 86 down regulated) (Fig. 2b). The heat map showed the correlation between high expression of

Table 1 Demographic and clinicopathological parameters of patients with Lung cancer in TCGA–LC (LUAD/LUSC)

Characteristics	Low expression of EZH2		High expression of EZH2		*P*
	n	%	*n*	%	
Total (*n*)	518		519		
T stage					0.47
T1	161	15.6	128	12.4	
T2	272	26.3	311	30.1	
T3	59	5.7	61	5.9	
T4	25	2.4	17	1.6	
M stage					0.343
N0	334	32.9	334	32.9	
N4	104	10.2	122	12	
N1	62	6.1	52	5.1	
N3	2	0.2	5	0.5	
M stage					0.334
M0	379	47.1	394	48.9	
M1	19	2.4	13	1.6	
Pathologic stage					0.164
I	279	27.2	260	25.4	
II	128	12.5	157	15.3	
III	83	8.1	85	8.3	
IV	20	2	13	1.3	
Gender					0.001
Female	234	22.6	183	17.6	
Male	284	27.4	336	32.4	
Age					0.019
≤ 65	202	20	244	24.2	
> 65	298	29.5	265	26.3	
Smoker					< 0.01
No	63	6.2	30	3	
Yes	439	43.4	479	47.4	
Primary therapy outcome					0.399
PD	59	7.3	43	5.3	
SD	29	3.6	25	3.1	
PR	5	0.6	6	0.7	
CR	315	39	325	40.3	
Anatomic neoplasm subdivision					0.036
Central lung	89	20.7	120	28	
Peripheral lung	117	27.3	103	24	
Age (years), median (IQR)	68 (60–74)		66 (60–73)		0.111
Residual tumor					0.111
R0	361	45.9	393	49.9	
R1	17	2.2	8	1	
R2	5	0.6	3	0.4	

TOP10 differentially expressed genes, low expression of TOP10 differentially expressed genes, high expression of TOP10 differentially expressed lncRNAs, and low expression of TOP10 differentially expressed lncRNAs and EZH2 gene co-expression (Fig. 2c, 2d).

Functional enrichment analysis of differentially expressed genes

We used Metascape to perform GO enrichment analyses of the functions of EZH2-associated DEGs in LC. The GO results displayed that EZH2-associated DEGs had significant regulation on metabolic process, cellular process, response to stimulus, biological regulation, localization, biological process involved in interaction between organisms, multicellular organismal process, negative regulation of biological process, immune system process, signaling, regulation of biological process, reproductive process, locomotion, and positive regulation of biological process (Fig. 3a). A network of EZH2 and its potential co-expression genes in EZH2-related DEGs are shown in Fig. 3b.

As many pathways contribute to tumor formation,

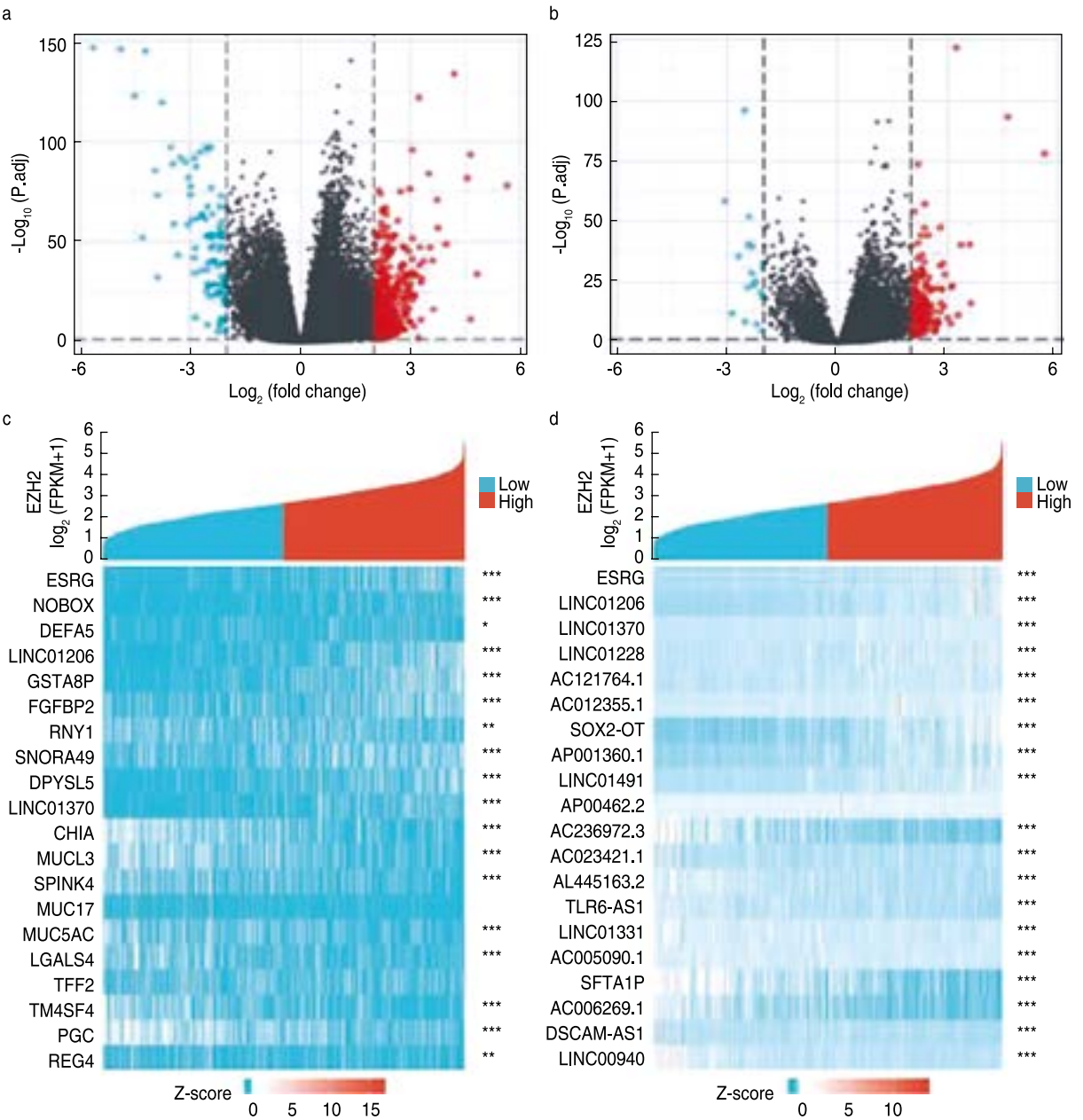

Fig. 2 Results of differentially expressed gene (DEG) analysis. (a) Volcano plot of DGEs; (b) Volcanco plot of differentially expressed lncRNAs; (c) Heat map analysis of co-expression of TOP10 high and low DEGs and EZH2 gene; (d) Heat map analysis of co-expression of TOP10 high and low lncRNAs and EZH2 gene

high EZH2 expression associated with poor survival may be related to active signaling pathways in LC. We performed GSEA of differences between low- and high–EZH2 expression data sets to identify the key signaling pathways associated with EZH2. A total of 11 pathways showed significant differences (False discovery rate (FDR. q value) < 0.25, p. adjusted < 0.05) in the enrichment of the MSigDB collection (c2.cp.v7.2.symbols.gmt). The most significantly enriched signaling pathways based on their NES. In particular, EZH2 was related to innate immune system, ECM affiliated, matrisome, surfactant metabolism, neutrophil degranulation, diseases of metabolism, metabolism of lipids, SLC mediated trans membrane transport, post translational protein modification, disease (Fig. 3c–3f).

The correlation between EZH2 expression and immune infiltration

We employed Spearman's correlation to show the association between the expression level (TPM) of EZH2

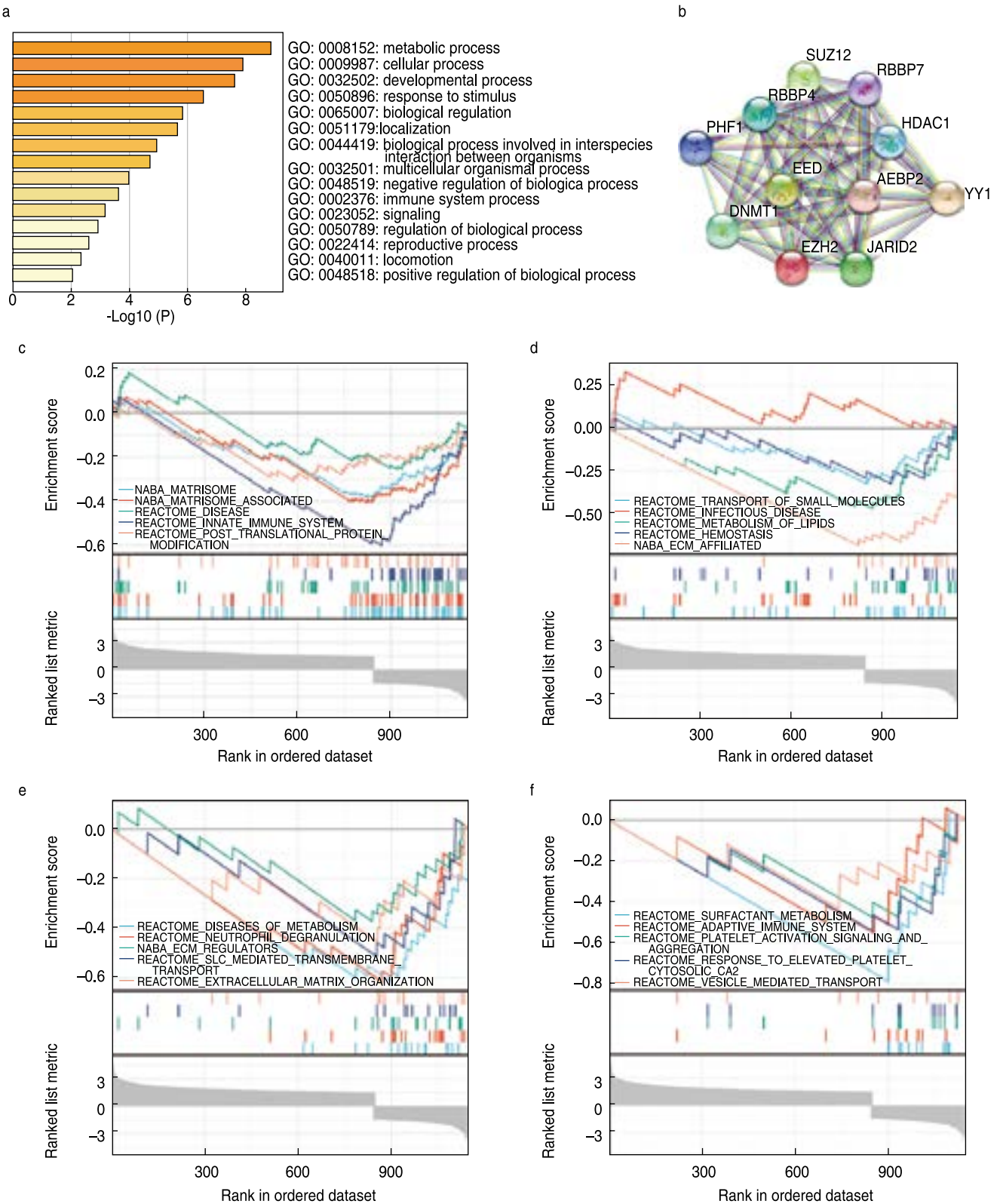

Fig. 3 Enrichment analysis of EZH2 in LC. (a) Top 15 biological process enrichment related to EZH2-related genes with enrichment heat-map; (b) Network of EZH2 and its potential co-expression genes in DNTTIP1-related DEGs; (c -f) Results of enrichment analysis from GSEA

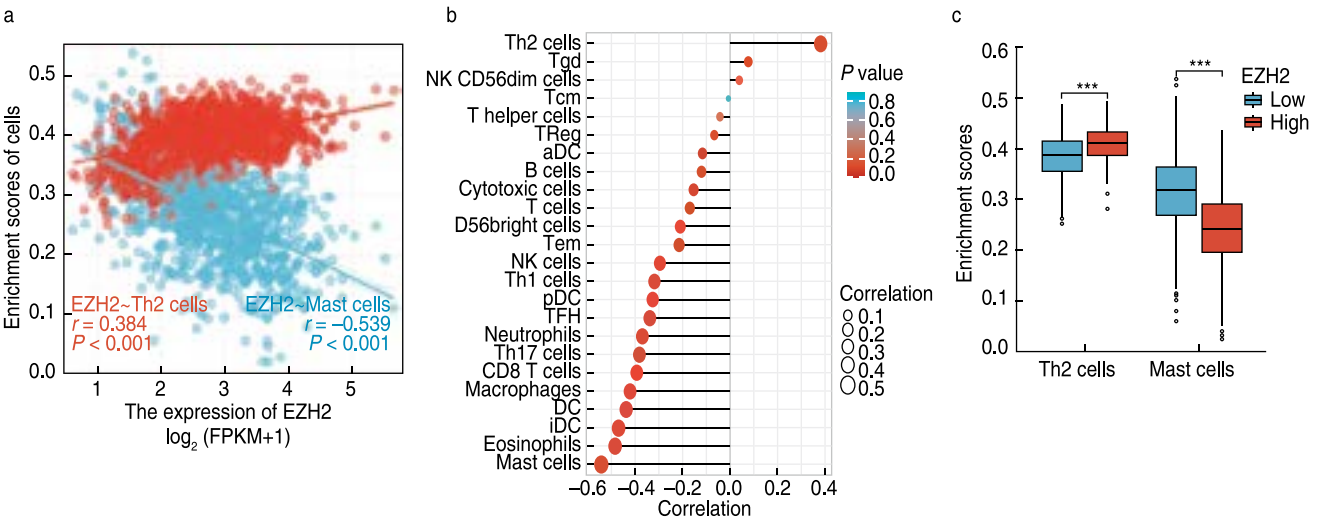

Fig. 4 Results of analysis between EZH2 expression and immune infiltration. (a) Th2 cells were significantly positively correlated with EZH2 expression and mast cells were significantly negatively correlated with EZH2 expression; (b) Correlation between the relative abundances of 24 immune cells and EZH2 expression level; The color of dots shows the absolute value of Spearman R; (c) Th2 cells and mast cells infiltration level in different EZH2 expression groups

and immune cell infiltration level quantified by ssGSEA in the LC (LUAD/ LUSC) tumor microenvironment. Th2 cells were significantly positively correlated with EZH2 expression (Spearman R = 0.384, $P < 0.001$) and Mast cells were significantly negatively correlated with EZH2 expression (Spearman R = -0.539, $P < 0.001$) (Fig. 4a). Other immune cell subsets, including Eosinophils, iDC, DC, Macrophages, CD8 T cells, Th17 cells, Neutrophils, TFH, pDC, NK cells, Th1 cells, were also correlated with EZH2 expression (Fig. 4b). The Th2 cell infiltration level in the EZH2 high expression group was significantly different from that of the low expression group ($P < 0.001$), and the Mast cell infiltration level in the EZH2 low expression group was significantly different from that of the high expression group ($P < 0.001$) (Fig. 4c).

Associations between EZH2 expression and clinico-pathologic variables

The Kruskal-Wallis test revealed that the between-group differences in Race, T stage, N stage, Pathologic stage, Residual tumor, Primary therapy outcome of LC (Fig. 5g-l), and Wilcoxon rank-sum test revealed that expression of EZH2 was significantly correlated with Age ($P < 0.001$), Gender ($P < 0.001$), Smoker ($P < 0.01$), Number pack years smoked ($P < 0.001$), Anatomic neoplasm subdivision ($P < 0.001$) and Anatomic neoplasm subdivision ($P < 0.01$) (Fig. 5a-f). Bonferroni correction was applied to the p value of Dunn's test to correct for multiple comparisons within the Race, T stage, N stage, Pathologic stage, Residual tumor, Primary therapy outcome of LC (Fig. 5). Logistic regression analysis showed that EZH2 was significantly correlated with T stage ($P = 0.022$), residual tumor ($P = 0.039$), age ($P = 0.016$), smoker ($P < 0.001$), and gender ($P = 0.001$) (Table 2).

EZH2 is highly expressed in paired LC samples compared with normal tissues from TCGA (Fig. 6a). The area under the curve (AUC) of EZH2 was 0.987, which indicated that EZH2 might be a potential diagnostic molecule in lung cancer patients (Fig. 6b).

In the Cox regression model, variables with $P < 0.1$ in univariate Cox regression were included in multivariate Cox regression. The variables that met this threshold were Age ($P = 0.022$), pathologic stage ($P < 0.001$), T stage ($P < 0.001$), M stage ($P < 0.001$), residual tumor ($P < 0.001$) (Table 3). Furthermore, multivariate Cox regression showed that residual tumor ($P = 0.006$) was independent prognostic factors for overall survival ($P < 0.05$).

The Kaplan-Meier survival curve drawn by the survminer package in R was used to evaluate the prognostic value of EZH2 in overall survival of LC. The prognostic values of EZH2 in relation to the overall survival under different subgroups of LC in TCGA are shown in Fig. 7a. The Kaplan-Meier survival curve of the EZH2 in overall survival in different subgroups of LC in TCGA-LC (LUAD/ LUSC). Gene expression values were divided into high- and low-expression groups according to the median value. The expression of EZH2 had significant effect in N1 & N2 & N3 subgroups of N stage, and stage II & stage III & stage IV subgroups of pathologic stage. High expression of EZH2 was associated with poor overall survival in LC N stage (N1 & N2 & N3) (HR = 0.70 (0.52-0.95), $P = 0.023$) and Pathologic stage (II & III & IV) (HR = 0.74 (0.57-0.96), $P = 0.024$) (Fig. 7b). EZH2 expression has no prognostic value in LC overall

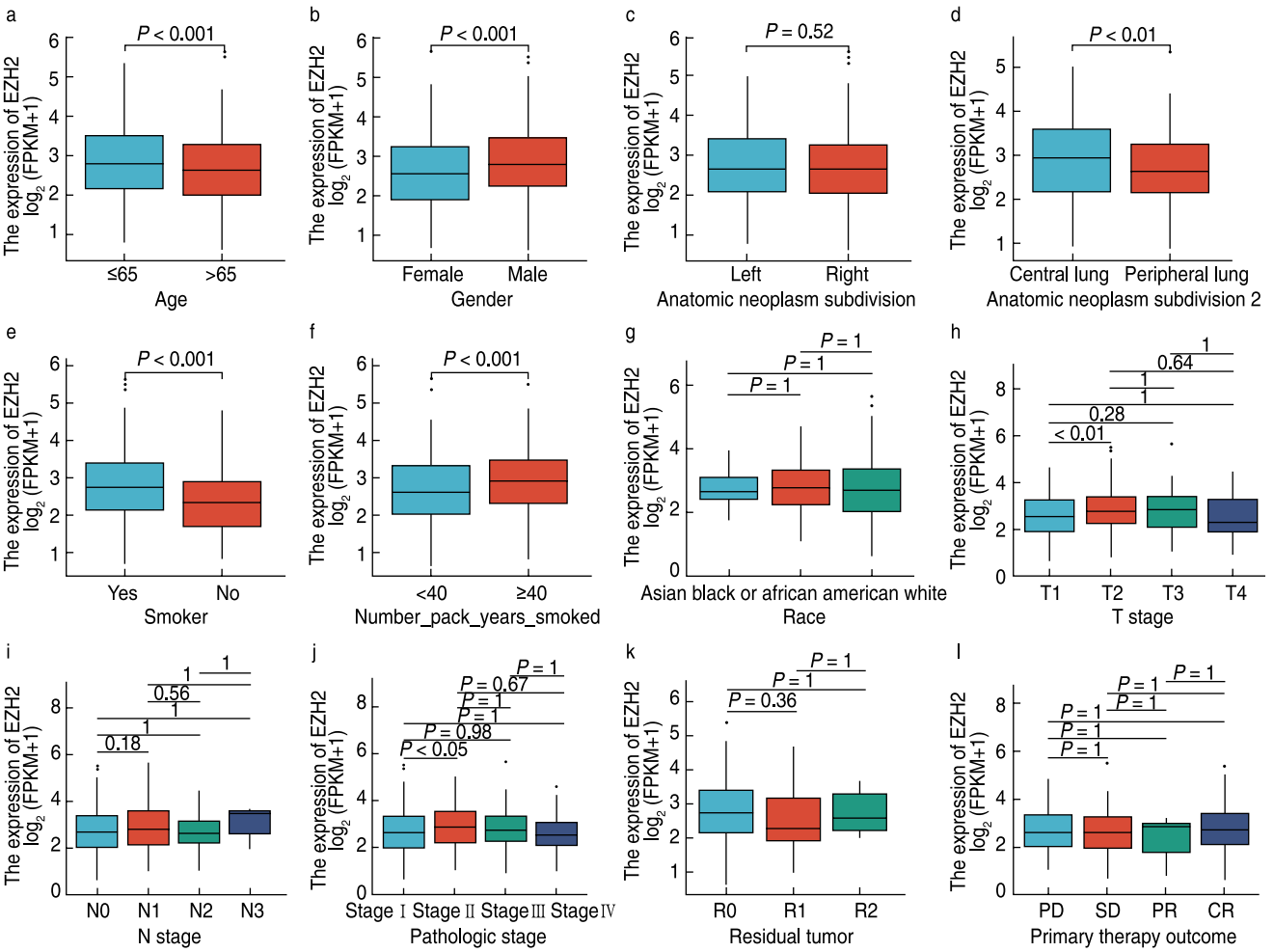

Fig. 5 Association between the EZH2 expression and different clinicopathological characteristics. (a) Association between the EZH2 expression and Age of LC; (b) Gender; (c) Anatomic neoplasm subdivision; (d) Anatomic neoplasm subdivision 2; (e) Smoker; (f) Number-pack-years-smoked; (g) Race; (h) T stage of LC; (i) N stage of L; (j) pathologic stage of LC; (k) Residual tumor; (l) Primary therapy outcome

Table 2 EZH2 expression associated with clinicopathological characteristics (logistic regression)

Characteristics	Total (*n*)	Odds Ratio (OR)	*P* value
T stage (T2&T3&T4 *vs.* T1)	1,034	1.374 (1.047–1.808)	0.022
N stage (N1&N2&N3 *vs.* N0)	1,015	1.065 (0.822–1.382)	0.632
M stage (M1 *vs.* M0)	805	0.658 (0.313–1.340)	0.254
Pathologic stage (Stage IV & Stage III *vs.* Stage I & Stage II)	1,025	0.929 (0.682–1.264)	0.638
Residual tumor (R1&R2 *vs.* R0)	787	0.459 (0.212–0.941)	0.039
Age (>65 *vs.* ≤65 years)	1,009	0.736 (0.573–0.944)	0.016
Smoker (Yes *vs.* No)	1,011	2.291 (1.468–3.650)	< 0.001
Gender (Male *vs.* Female)	1,037	1.513 (1.179–1.943)	0.001

survival (OS) (HR = 0.92 (0.76-1.12), $P = 0.427$), disease specific survival (DSS) (HR = 0.88 (0.66-1.16). $P = 0.348$), progress free interval (PFI) (HR = 0.84 (0.68-1.04), $P = 0.106$) (Fig. 7c). The lower part of these figures is shown in the risk table which records the number of people still under follow-up at each time point. The prognosis data are derived from an article published in Cell [38].

Discussion

The polycomb group (PcG) proteins were first identified in Drosophila as regulatory factors that

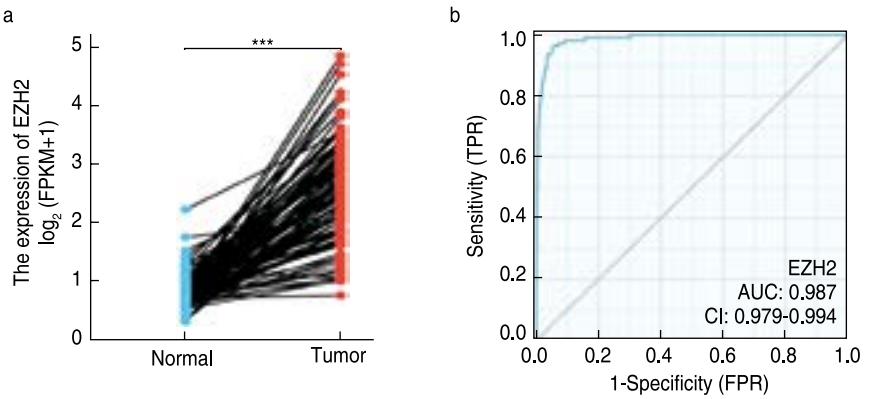

Fig. 6 Prognostic value of EZH2 in LC (LUAD/ LUSC). (a) EZH2 was also highly expressed in paired LC samples compared with paired normal tissues from TCGA; (b) ROC curve indicates that EZH2 is a potential diagnostic marker in LC patients

Table 3 Univariate and multivariate analyses of clinicopathological parameters in patients with lung cancer in TCGA-LC (LUAD/ LUSC)

Characteristics	Total (*n*)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age (years)					
> 65	561	Reference			
≤ 65	445	0.791 (0.646-0.967)	0.022	0.794 (0.603-1.045)	0.099
Gender					
Female	410	Reference			
Male	612	1.164 (0.949-1.428)	0.145		
Pathologic stage					
I	533	Reference			
III, IV, II	477	1.913 (1.566-2.337)	< 0.001	1.488 (0.995-2.225)	0.053
T	1019				
T1	289	Reference			
T2&T3&T4	730	1.574 (1.239-1.999)	< 0.001	1.229 (0.876-1.722)	0.233
N stage					
N0	660	Reference			
N1	222	1.541 (1.225-1.938)	< 0.001	0.930 (0.619-1.397)	0.726
M stage					
M0	760	Reference			
M1	32	2.269 (1.439-3.577)	< 0.001	1.533 (0.841-2.796)	0.163
Residual tumor					
R1&R2	32	Reference			
R0	742	0.339 (0.212-0.541)	< 0.001	0.429 (0.233-0.788)	0.006
Smoker					
No	90	Reference			
Yes	906	0.883 (0.617-1.263)	0.496		
EZH2					
Low	509	Reference			
High	513	0.902 (0.741-1.097)	0.301		

transcriptionally silence the expression of the bithorax homeobox (Hox) gene cluster [39]. It has been reported that PcG proteins interact with each other and generate two major complexes, polycomb repressive complexes 1 and 2 (PRC1 and PRC2) [40]. PRC2 is a highly conserved complex in many species of plants and animals. It is mainly composed of four subunits: EZH2, EED, SUZ12, and RbAp46/48 (Fig. 8a) [41]. Enhancer of zeste homolog 2 (EZH2), the core catalytic subunit of the PRC2 complex, and its SET domain at the C- terminus provides the histone methyltransferase activity (Fig. 8b), plays a critical role in regulating a wide range of biological processes, including tumor development and malignancy, stem cell renewal and development, immune response, and cell senescence [42]. Aberrant epigenetic regulation plays a critical role in tumorigenesis by altering genome-wide gene expression.

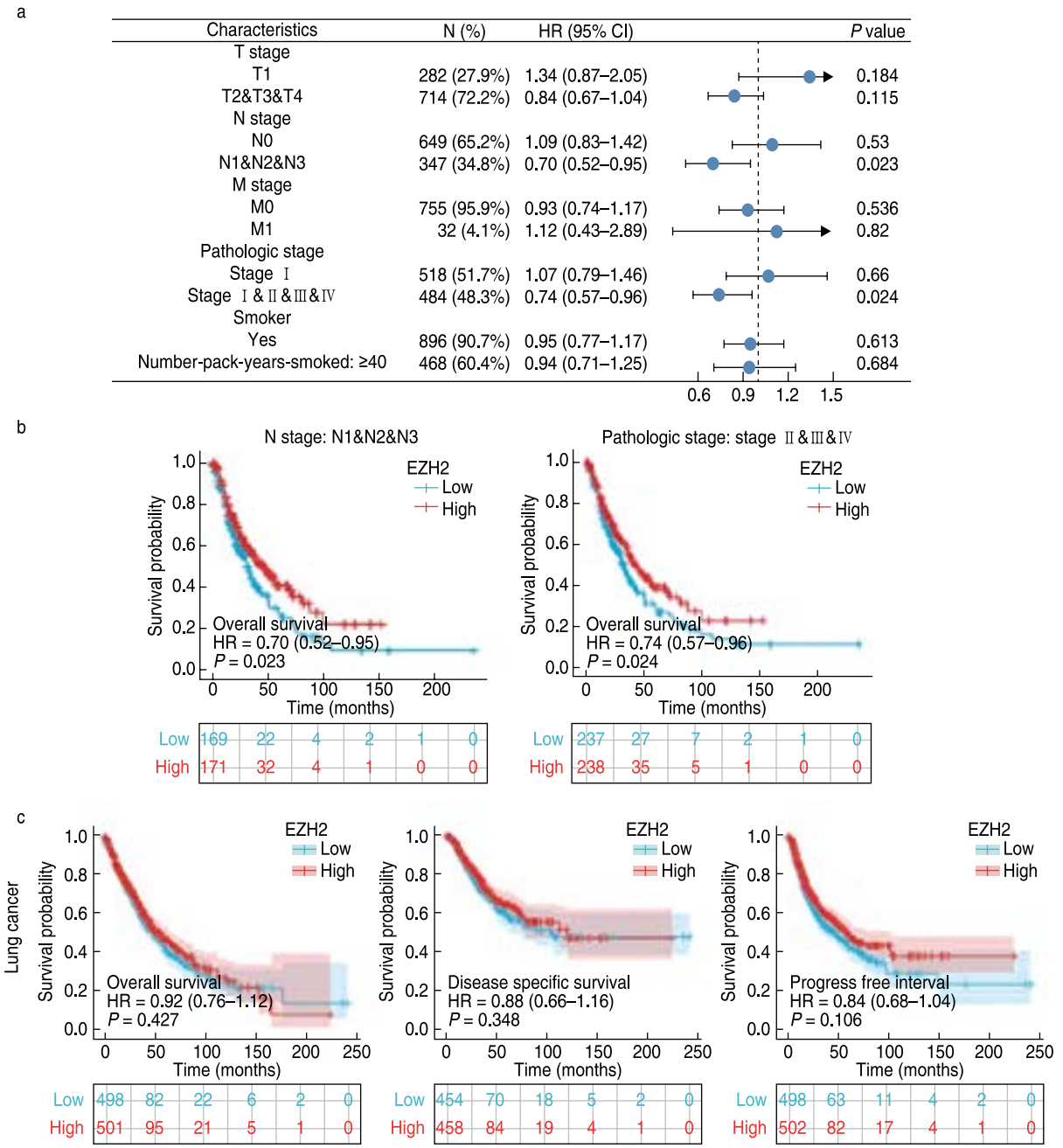

Fig. 7 Prognostic value of EZH2 in different analyses. (a) Forest plot of prognostic value of EZH2 in overall survival of LC; (b) The Kaplan–Meier survival curve of the EZH2 in overall survival in different subgroups of LC in TCGA–LC (LUAD/ LUSC). High expression of EZH2 was associated with poor overall survival in LC N stage (N1 & N2 & N3) and Pathologic stage (II & III & IV) subgroups; (c) EZH2 expression has no prognostic value in LC (LUAD/ LUSC) Overall Survival (OS), Disease Specific Survival (DSS), and Progress Free Interval (PFI)

In this study, we comprehensively investigated the expression, immune infiltration, diagnosis and prognostic value of epigenetic regulatory genes such as EZH2 in LC (LUAD/ LUSC) tissues using public databases.

In this study, bio-informatics analysis of sequencing data from TCGA was performed to gain a deeper understanding of the potential function of EZH2 in LC and guide future research in LC. Elevated EZH2 expression in LC was associated with advanced clinicopathological features (Age, Gender, Anatomic neoplasm subdivision2, Smoker, Number_pack_years_smoked, T1 vs T2 stage, pathological stage I vs II), poor prognosis, and survival time. Furthermore, in univariate and multivariate Cox regression analyses, we found that after removing

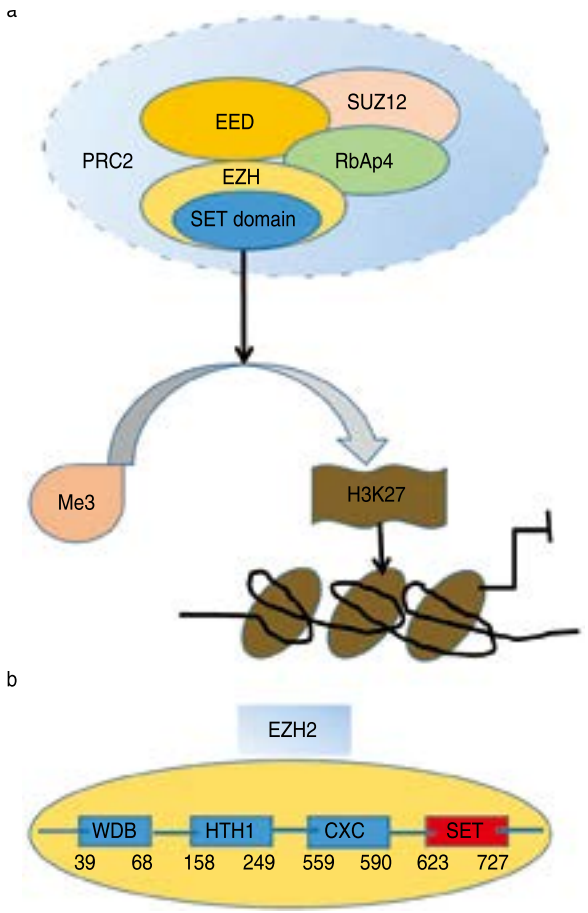

Fig. 8 The polycomb repressive complex 2 (PRC2) complex composition, regulation function, and schematic diagram of the enhancer of zeste homolog 2 (EZH2) domains. (a) The PRC2 complex contains four core subunits which include EZH2, EED, SUZ12, and RbAp46/48. PRC2 induces target genes' transcriptional repression through EZH2- mediated H3K27 trimethylation. (b) EZH2 has four domains: WD- binding domain; PRC2 HTH 1 domain, CXC domain, and SET domain

confounding factors, EZH2 was not an independent prognostic factor, which is not showed a higher prognostic value than many other clinical variables. Our results suggested that EZH2 is a potential prognostic and diagnostic marker deserving further clinical validation.

The PPI network indicates that EZH2 can interact with several histone deacetylase members such as HDAC1, EED, RBBP4, SUZ12, RBBP7, AEBP2, etc. HDAC1 are key enzymes that maintain the acetylation balance of nucleosomes in basic units of chromosomes. Their catalytic histone deacetylation is closely related to the inhibition of gene transcription [43]. The Polycomb Repressive Complexes (PRCs) are large protein multimers that modify lysine residues on histones. The two primary PRCs noted in mammals are PRC1, formed by several distinct proteins but most notably RING1A/B and BMI1, and PRC2. The core subunits of PRC2 are enhancer of zeste homolog 1/2 (EZH1/EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 (SUZ12) [44]. Within the PRC2 complex, EZH2 (and to a lesser extent, EZH1) is the catalytic subunit responsible for methyltransferase activity. Both EZH proteins were observed to be phosphorylated, although EZH1 phosphorylation was shown to lead to degradation, while EZH2 phosphorylation led to reduced function [45, 46].

GSEA showed that innate immune system, ECM affiliated, Matrisome, Surfactant metabolism activation in LC were enriched in the EZH2 high-expression phenotype. These findings indicated that EZH2 might participate in the regulation of metastasis and immune response in the tumorigenesis of LC. ssGSEA and Spearman's correlation were adopted to uncover connections between EZH2 expression and immune infiltration levels in LC. Our results demonstrated that EZH2 expression was significantly positively correlated with Th2 cells. Our results suggest a possible mechanism where EZH2 regulates the balance of Th1/Th2 in LC. The Th2 cells produce IL-4 and IL-10 and inhibit the host immune system, hence having a role in promoting tumor growth [47, 48]. This indicates that over-expression of EZH2 promotes Th2 cell immune response and infiltration in tumor progression. Th1/Th2 balance can be regulated to inhibit tumor progression. A global Th1/Th2-like cytokine shift (a decrease in Th1 and an increase in Th2 cytokines) can be induced to promote HCC metastasis [49]. On the other hand, there was significantly an inverse correlation between Mast cells and EZH2. Furthermore, there was a strong-to-moderate correlation between DC, macrophages, Th17 cells, *et al.* and EZH2 expression. The NSCLC cells could release CCL5 and recruit MCs to the tumor micro-environment. Moreover, the MCs-derived factors were responsible for tumor growth. When NSCLC cells were activated, MCs produced various factors that induced EMT and migration [50]. Interestingly, EZH2 gene has a significant negative correlation with MC. High expression of EZH2 in NSCLC has a lower MC infiltration, which may be an important mechanism for the better prognosis of cancer patients. Due to the role of DCs in initiating anti-tumor immunity, there is a negative selective pressure hampering the accumulation of DCs by tumor secreted mediators that inhibit dendropoiesis, promote DC apoptosis [51], and accelerate DC turnover. The suppression of DCs is normally found in tumors and may facilitate LC progression. Th17 immune cells were associated with both good and bad prognoses [52]. Th17 cells can also drive anti-tumor immune responses by recruiting immune cells into tumors, activating effector CD8+ T cells, or even directly by converting toward Th1 phenotype and producing IFN-γ [53]. The down regulation of Th17 caused by EZH2 over-expression may affect h1/h2 balance, leading to a bad prognosis. All findings according to ssGSEA support that EZH2 has a role in

regulating and recruiting immune infiltrating cells in LC. However, more trials are needed to accurately understand the relationship between EZH2 and Th1/Th2 balance in vivo.

The high expression of EZH2 is closely related to a poor prognosis in many tumors, but its value in the prognosis of NSCLC is still controversial [54, 55]. To our best knowledge, previous meta analyses did not specifically explain the prognostic relationship between EZH2 and lung cancer [56], or no bioinformatics evidence was used to support it [57]. Therefore, we searched TCGA database and Kaplan-Meier plotter database for large-scale global lung cancer database mining to find EZH2 potential correlation with LC. In our study, EZH2 expression was significantly higher in LC tissues compared to the normal lung tissues. Our study demonstrates that higher EZH2 expression correlates with poorer OS, DSS and PFI in LC patients. In addition, the Kaplan-Meier survival curves with high HR for poor OS, DSS and PFI when EZH2 was highly expressed in LC, however, there was no statistically significant difference in the low expression of EZH2 group, suggesting that EZH2 has certain prognostic guiding value but does not have specificity as a prognostic biomarker in LC. Moreover, Kaplan-Meier survival analysis was performed in LC patients according to their EZH2 expression levels, stratified by clinicopathological characteristics. A high level of EZH2 expression was associated with poor prognosis of LC patients with N stage (N1 & N2 & N3) and Pathologic stage (II & III & IV), thus indicating that the high association between EZH2 expression level and survival may be influenced by the degree of invasion and metastasis.

As single-stranded RNA molecules with a length of 200 to 100,000nt, long-chain noncoding RNAs have multiple interactions with EZH2. Single genetic variance analysis showed that the total number of lncRNA ID is 14077, among them, meet | log2 (FC) | > 1.5 & p.adj < 0.05 threshold of lncRNA has 559, under the threshold, the High expression in the High group (logFC is) as the number of 473,The number of Low expression (logFC negative) in Low group was 86;The heat map correlation analysis of EZH2 co-expression showed that AP000462.2 had no significant correlation (Spearman P = 0.289). Long intergenic non-coding RNA 00467 promotes lung adenocarcinoma proliferation, migration and invasion by binding with EZH2 and repressing HTRA3 expression [58]. LncRNA MSTO2P promotes proliferation and autophagy of lung cancer cells by up-regulating EZH2 expression [59]. lncRNA PVT1 Promotes Metastasis of Non-Small Cell Lung Cancer Through EZH2-Mediated Activation of Hippo/ NOTCH1 Signaling Pathways [60]. lncRNA ZEB2-AS1 Aggravates Progression of Non-Small Cell Lung Carcinoma via Suppressing PTEN Level [61]. This shows that, through the up-regulation of the EZH2 expression, long noncoding RNA silences tumor suppressor genes and promotes lung cancer invasion and migration, which are associated with poor prognosis. This is consistent with our findings. However, the high expression of EZH2 is not only related to the regulation of long noncoding.

Although our investigation of the relationship between EZH2 and LC furthered our understanding of the vital role of EZH2 in LC, some limitations remained. First, cell experiments and clinical samples should be used to verify the correlation between EZH2 mRNA and protein expression. In the present study, we only used mRNA levels to predict protein expression [62]. Second, clinical factors such as the details of patient treatment should be sufficiently considered to clarify the specific role of EZH2 in the development of LC. Third, while multicenter research based on public databases intends to overcome the shortage of single-center studies, retrospective studies have two major shortages. One is missing variables. In our study, to clarify the specific role of EZH2 in the development of LC comprehensively, more clinical factors should be taken into consideration such as the detail of treatments for every single patient involved. However, the information of treatments was often inconsistent or even lacking in public databases; the other is sample size imbalance. We have a smaller number of healthy samples in our control group than that of LC patients in our study; the sample size imbalance may lead to statistical bias. Therefore, future prospective studies are needed to reduce analysis bias. Finally, we cannot illustrate the expression of LC from the protein level and also can not evaluate the direct mechanisms of EZH2 involved in LC progression. Consequently, further studies are needed to clarify the direct mechanisms of EZH2 in LC.

EZH2 is not only related to LC, but it is also closely related to the occurrence and development of other tumors. In diffuse large B cell lymphoma, it is found that inhibition of EZH2 activity may provide a promising treatment for EZH2 mutant lymphoma [63]. However, Another study found that diffuse large B cell lymphomas based on EZH2 mutations and BCL2 translocation genetic subtypes have higher survival rates than other subtypes [64]. This also suggests to some extent that EZH2 not only promotes tumor progression but may play a benefical role in certain tumors or certain tumor subtypes. Depending on the cellular environment and the activated oncogenic pathways, the changes in epigenetic modifications caused by EZH2 defects may lead to tumor progression through various mechanisms.

In summary, Unlike genetic mutations, epigenetic aberrations are reversible so targeting the relevant epigenetic factors by small molecules is potentially an efficient approach to “fix” dysregulated gene/ chromosome-regulatory systems caused by epigenetic changes in cancer. Additional experiments are needed to

evaluate the relationship between EZH2 expression and clinical features, LC stage, and prognosis using additional clinical data, which might facilitate the identification of new markers for evaluating tumor stage, aiding drug development, and improving treatment efficiency.

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Conflicts of interest

The authors indicated no potential conflicts of interest.

Author contributions

Conceptualization, writing-original draft, and supervision: Xiaokun Wang, Min Qi and Gaoyang Lin; methodology and formal analysis: Xu Zhu, Zhengtong Zhao, Gaoyang Lin, Yufeng Cao, and Y-FT; resources: Fuman Wang and Daijun Xing; validation and visualization: Xiaokun Wang, Min Qi, Xu Zhu, Zhengtong Zhao, Yufeng Cao, and Gaoyang Lin; writing-review and editing: Xiaokun Wang and Gaoyang Lin; funding acquisition: Gaoyang Lin and Yufeng Cao. All authors contributed to the article and approved the submitted version.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

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