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ITCH is a Prognostic Biomarker and Immune Infiltrates in Lung Squamous Cell Adenocarcinoma

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Abstract

Background: The treatment of lung squamous cell carcinoma is limited and shows resistance to various treatment methods.

Methods: TCGA-LUSC data set was obtained from the TCGA website, and use different R packets to analyze the difference in the expression of ITCH in lung squamous cell carcinoma, its role in the prognosis, the difference in the distribution of immune cells and the calculation of immune score. Online websites such as GSCA, UALCAN and TIMER were used for ITCH expression analysis and pathway enrichment analysis. In vitro, RT-PCR and Western blot were used to detect changes in ITCH mRNA and protein levels. Wound healing experiments and 40 transwell experiments were used to detect changes in cell metastasis ability.

Results: Using online data sets and in vitro experiments, it was confirmed that ITCH was highly expressed in lung squamous cell carcinoma patients and the prognosis of lung squamous cell carcinoma patients with high expression of ITCH was poor. This abnormal expression could be caused by the reduction of the methylation level of the ITCH promoter site and the increase of the copy number. Pathway enrichment analysis showed that ITCH might promote the progression of lung squamous cell carcinoma by promoting the metastasis of lung squamous cell carcinoma. In vitro experiments also confirmed that ITCH could indeed promote the metastasis of lung squamous cell carcinoma cells. In addition to its effect on tumor cells, ITCH reconstructs the infiltration of tumor immune cells. According to this feature, it can provide a basis for the application of immunosuppressive agents for lung squamous cell carcinoma.

Conclusion: ITCH is a poor prognostic factor for lung squamous cell carcinoma and a potential therapeutic target.

Keywords: Lung squamous cell carcinoma; ITCH; prognosis; Metastasis; Immune microenvironment.

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Introduction

Lung cancer is the most common cancer in the world, and lung squamous cell carcinoma (LUSC) is a major subtype of non-small cell lung cancer with extremely poor prognosis [1,2]. Although the treatment of lung cancer has made rapid progress, the treatment of lung squamous cell carcinoma is limited, and the drive gene mutation rate of lung squamous cell carcinoma is low [3]. In addition to 61 increasing the burden of patients, gene testing for patients with squamous cell carcinoma has no prominent significance in guiding clinical treatment. Therefore, the guidelines do not recommend gene testing as a routine examination for patients with squamous cell carcinoma. In addition to routine surgery and chemotherapy, immune checkpoint inhibitors have become an available treatment for advanced squamous cell carcinoma of the lung [4]. Therefore, there is an urgent need to find possible target genes and efficacy evaluation indicators of immunotherapy.

The ubiquitin E3 ligase ITCH, also named atrophin-1 interacting protein 4 (AIP4), is a member of the NEDD4 HECT-type family E3 ligases [5], which is an important member of the classic E3 ubiquitination process. In past studies, the role of ITCH in cancer has been gradually revealed.

Depending on the difference of its ubiquitination substrate, it may play a different role in cancer.

For example, ITCH overexpression has been observed in some human cancers, including anaplastic thyroid carcinoma [6], breast cancer and ovarian cancer [7]. However, in other studies, ITCH plays an anti-tumor role, for example, its ubiquitin PD-L1 promotes its interpretation in melanoma [8], which may be due to the wide substrate differences of ITCH. In addition to its effect on tumor cells, ITCH also plays a regulatory role on immune cells and tumor-related immune microenvironment. For example, ITCH has played an active role in promoting the differentiation of Treg [9,10]. However, clinical studies have shown that tumor infiltrating Tregs are usually associated with poor prognosis of cancer, including lung cancer[11]. In addition to its effect on Treg cells, ITCH can also inhibit the activation of T cell antigen receptor (TCR), which is an important way for T cells to exert anti-tumor effect [12]. In lung cancer, previous studies have shown that in lung adenocarcinoma, ITCH promotes the metastasis of lung cancer cells under hypoxia [13], but 82 there is no report of ITCH function in lung squamous cell carcinoma.

In this study, we first found that the expression of ITCH in lung squamous cell carcinoma was significantly higher than that in paracancerous tissue, and the number of ITCH copies in cancer tissue was significantly increased, and its expression gradually increased with the increase of stage, and the prognosis of lung squamous cell carcinoma patients with high expression of ITCH was poor. Based on these results, we further analyzed the correlation between ITCH and the clinicopathological characteristics of lung squamous cell carcinoma, and found that it was significantly related to "aneuploid" and "Hyposia", which may be the reason why ITCH promoted the progression of lung squamous cell carcinoma. In addition, we found that the methylation of 91 ITCH promoter increased significantly, which may be an important reason for the high expression of ITCH. Through pathway enrichment analysis, we explored the reason why ITCH promoted the progression of lung squamous cell carcinoma. The results showed that ITCH was closely related to the metastasis process, and the results of in vitro experiments also confirmed that ITCH could promote the metastasis of lung squamous cell carcinoma cell lines. In addition to its effect on lung cancer cells, ITCH can reshape the tumor microenvironment, induce the infiltration of M2 macrophages and neutrophils, and has a high immune score in patients with low expression of ITCH lung squamous cell carcinoma, and is sensitive to the effect of immune checkpoint 99 inhibitors. These results preliminarily reveal the role of ITCH in lung squamous cell carcinoma, and provide a basis for clinical prognosis and drug use.

Materials and methods

Data download and processing

TCGA database is an extremely important cancer genomics project. We download sequencing data and clinical data of lung squamous cell carcinoma through TCGA database (www.tcga-data. nci.nih. gov/tcga) [14]. The selected samples include ITCH gene expression data and relevant clinical data, including age, sex, T, N, M stages, pathological stages, survival status, etc. Since all data are downloaded from the public database, the study does not require the approval of the Ethics Committee.

Gene expression analysis of ITCH

ITCH mRNA expression level in different cancer types were obtained from TIMER (http://timer.comp-genomics.org/) database [15]. ITCH protein expression in different patient was also obtained from HPA database (https://www.proteinatlas.org/) [16]. In order to detect the difference in the expression of ITCH in different Stages, N stages, and whether smokers or not, the TCGA-LUSC data set was selected for detection using the UALCAN online data set (https://ualcan.path.uab.edu/) [17]. Tnmplot online data set (https://www.tnmplot.com/) [18] was used to detect the expression level of ITCH in tumor, adjacent tissues and metastatic lesions.

Prognostic analysis

First of all, R package was used to carry out univariate analysis and umultivariate analysis on ITCH and pathological parameters, and the analysis results are shown in heat map and forest map. Kaplan-Meier curve was further used to analyze the difference in survival rate between high and low expression of ITCH.

The nomogram was established by R “rms” and “survival” packages. Calibration curves were further used to assess the accuracy of nomograms in differentiating patient groups.

Tumor Mutational Burden (TMB)

Download the RNA sequencing data, gene mutation data, and clinical data of LUSC from the TCGA database (https://gdc-portal. nci.nih.gov/). Data is stored in mutation annotation format and processed with VarScan software. Use the "maftools" package to analyze and summarize these mutation data [19]. After obtaining the TMB value, we further analyzed the correlation between different ITCH expressions and the TMB value of each sample.

Correlation analysis of tumor immune infiltrating cells

CIBERSORT algorithm is used to quantify the proportion of immune cells in the mixed cell population [20,21]. RNA-Seq (FPKM format) sample analysis can obtain each example of 22 immune cell abundance matrix, including macrophages (macrophages M1,

M2 macrophages and M0 macrophages), T cells (T cell helper (Tfh) cells, memory CD4+T cells, and activated memory CD4+T cells, γδ T cells, CD8+T cell subsets, and CD4+T cells), resting natural killer (NK) cells, activated NK cells, mast cells, activated memory B cells, resting dendritic cells, activated dendritic cells, naïve B cells, monocytes, plasma cells, neutrophils, and eosinophils [21]. The CIBERSORT result of the sample ($p<0.05$) shows that the immune cell group fraction generated by CIBERSORT is accurate and can be used for further analysis. Normalize the CIBERSORT output estimation and sum the immune cell type score to 1. Using the normalized gene expression data of TCGA-LUSC data set, the relative proportion of 22 immune cell subtypes was determined using the R "CIBERSORT" package to evaluate the correlation between the expression of ITCH in TCGA-LUSC samples and the immune infiltrating cells.

Immunotherapy response prediction

Immune cell abundance identifier (ImmuCellAI) is a calculation method, which was released in 2020 and is used to predict the response to immune checkpoint blockade based on the abundance of immune cells (especially different T cell subsets) [22]. The abundance of infiltrating immune cells was calculated by ImmuCellAI and used to establish a reaction prediction model. The radial basis function kernel support vector machine (RBF kernel support vector machine) was used to establish the immune treatment response prediction model. The Cancer Immunome Atlas (TCIA) provides the comprehensive immune genome analysis results of the next generation sequencing data (NGS) of 20 solid cancers from the cancer genome atlas (TCGA) and other data sources. The cancer immunity atlas (TCIA) online toolis used to predict the response of immunochemotherapy [23]. The quantitative score of tumor immunogenicity ranges from 0 to 10, which is called immunophenotypic score (IPS). IPS can be used to predict the reaction of immunosuppressive agents at immune checkpoints [23].

CNV and promoter methylation prediction

GSCA online portal (http://bioinfo.life.hust.edu.cn/GSCA/#/) [24] was used to detect the change of ITCH gene CNV and the difference of promoter methylation sites, and detect the correlation with the expression of ITCH mRNA, selecting TCGA-LUSC data set.

ITCH co-expression networks and pathway enrichment

The LinkedOmics database (http://www.linkedomics.org/login.php) [25] was used to determine the ITCH co-expression genes by using Pearson's correlation coefficient, filtering by TCGA-LUSC dataset, the results were showed via volcanic map and heat maps. Then, the Gene Ontology Biological Process (GO_BP), and KEGG pathways of ITCH and its co-expression genes by using gene set enrichment analysis (GSEA).

Cell lines and culture condition

The human lung cancer cell line H1563 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). H1563 was maintained in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) under a mixture of 95% air and 5% CO_2. According to the cell 179 doubling time, the average passage is 3-4 days.

SiRNA transfection

SiRNAs and negative control (NC) were purchased from View Solid Biotech (Beijing, China). All siRNAs were transfected into cells using jet PRIME reagent (Polyplus) according to the manufacturer's protocol.

RNA extraction and quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

Total RNA extracted as described above [26]. Primer sequences for ITCH: Forward 189 (5'-GACCGGCTGCCATCTTAGTC-3'), Reverse (5'-GGGTTAAGGCGTTGTCTCCA-3').

Western blotting analysis

Western blotting was performed as our previously described [26]. The primary antibodies: Rabbit monoclonalanti-ITCH (ab108515,1:500) was purchased from Abcam (USA). Rabbit monoclonal anti-GAPDH (5174S,1:2000) was purchased from Cell Signaling Technology (USA).

Migration and invasion assay

Transwell assays were performed to assess cell migration and invasion ability as described [26]. In general, we knock down ITCH in lung cancer cells, and the 24-wellchamber and polycarbonate inserts with 8-µmpore size membranes (Corning, USA) was used to carry outmigration assays.

The cells (7×10^4 cells/well) transfed with siRNA were loaded into the upper chamber with 200 µl serum-free RPMI 1640 medium. The lower chambers contained 500 µl of RPMI 1640 with 10% FBS. After 48 hours, the migrated cells on to the outer side of the membrane were stained with 0.1% Giemsa solution for 1h. Five different fields were captured and counted at ×20 magnificationper well.

Wound healing assays

The treated cells are inoculated into a six hole plate. When the cell confluence reaches about 90%, 200 µl pipette tip was used to scratch a straight line. After that, the supernatant was replaced with 209 fresh medium without FBS twice. The migration images were taken 0 and 24 hours after the 210 scratch. All experiments were repeated three times. Image J software was used to analyze and quantify blank areas.

Statistical analysis

R software (https://r-forge.r-project.org/3.6.3) was used for statistical analysis. The survival curve was compared by Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of the survival rate among the groups. For unmatched samples, the comparison between the two groups was analyzed by Mann-Whitney test or Student's t test. For paired samples, Wilcoxon rank sum test was used. Kruskal Wallis rank sum test was used for multiple comparisons to compare the differences between groups. Pearson correlation analysis is used to measure the degree of correlation between some variables, and the correlation strength is determined by the value of correlation coefficient r. In order to estimate the influence of single continuous gene expression variable and independent prognostic factors on prognosis, single factor and multiple factor Cox regression analysis were performed respectively. P value <0.05 is considered

statistically significant.

Results

ITCH is highly expressed in lung squamous cell carcinoma

First, the TIMER online data set was used to detect the expression level of ITCH gene in various tumors and paracancerous tissues. The results showed that the expression level of ITCH gene in lung cancer tissue was significantly higher than that in paracancerous tissues. Both lung adenocarcinoma and lung squamous cell carcinoma had statistical significance, but the expression difference in lung squamous cell carcinoma was greater (Figure 1A). We further downloaded TCGA-LUSC to further verify the difference between its expression in cancer and paracancerous tissues. The results were consistent with our expectations. The expression of ITCH in lung squamous cell carcinoma was significantly higher than that in paracancerous tissues (Figure 1B), and the expression was higher in metastatic lesions (Figure 1C). Further analysis of the correlation between its expression and clinicopathological parameters showed that the expression of ITCH was statistically different only from T1 to T2 in T stage. Although the expression of ITCH was 239 higher in T4, there was no statistical difference compared with T1, which suggested that ITCH might play an important role in the formation of early local lesions (Figure 1D).

In the process of lymph node metastasis, compared with normal tissues, cancer tissues expressed higher expression of ITCH regardless of lymph node metastasis. However, there was no significant difference in the expression level of ITCH under different lymph node metastasis states, indicating that ITCH may not participate in the process of lymph node metastasis (Figure 1E). This trend also exists in the overall stage. Even in stage 1, the expression of ITCH is significantly higher than that of adjacent cancer, but this trend does not increase with the increase of stage. Although the expression in stage 4 is significantly increased, the number of samples in this stage is small (Figure 1F). In addition, we examined the correlation between smoking and the expression of ITCH, the pathogenic factor of lung cancer, and found that smoking and non-smoking did not significantly affect the expression of ITCH (Figure 1G). Further, we carried out a more comprehensive correlation analysis of clinical features (Figure 2A). The results showed that ITCH was not only related to T stage, but also significantly related to gene aneuploid score, sex and hypoxia. Figures 2B, 2C, 2D shows the difference level caused by this difference in expression of ITCH, which may indicate that ITCH may exist in the initial development and distant metastasis of lung squamous cell carcinoma. Using HPA online data, the expression level of ITCH protein in lung squamous cell carcinoma and adjacent tissues was displayed (Figure 2E).

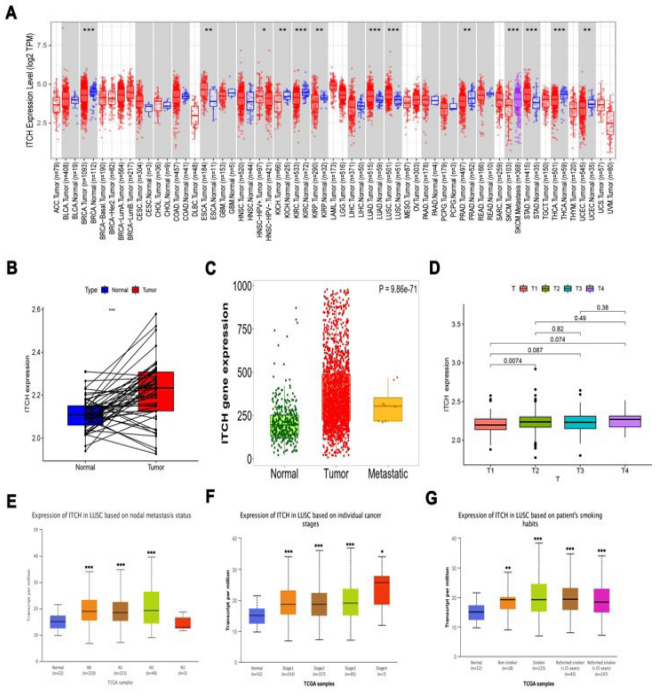

Figure 1: ITCH is highly expressed in lung squamous cell adenocarcinoma.
(A) Expression status of the ITCH gene in different cancer was analyzed by TIMER database. **(B)** Expression status of the ITCH gene in normal and primary tissues of lung squamous cell adenocarcinoma. **(C)** Expression status of the ITCH gene in normal, primary tissues and metastatic tissues of lung squamous cell adenocarcinoma. **(D,E,F,G)** Expression status of the ITCH gene in different T stage **(D)**, nodal metastasis status **(E)**, individual cancer stages **(F)** and smoking564 status **(G)** of lung squamous cell adenocarcinoma. (*p<0.05,**p<0.01,***p<0.001).

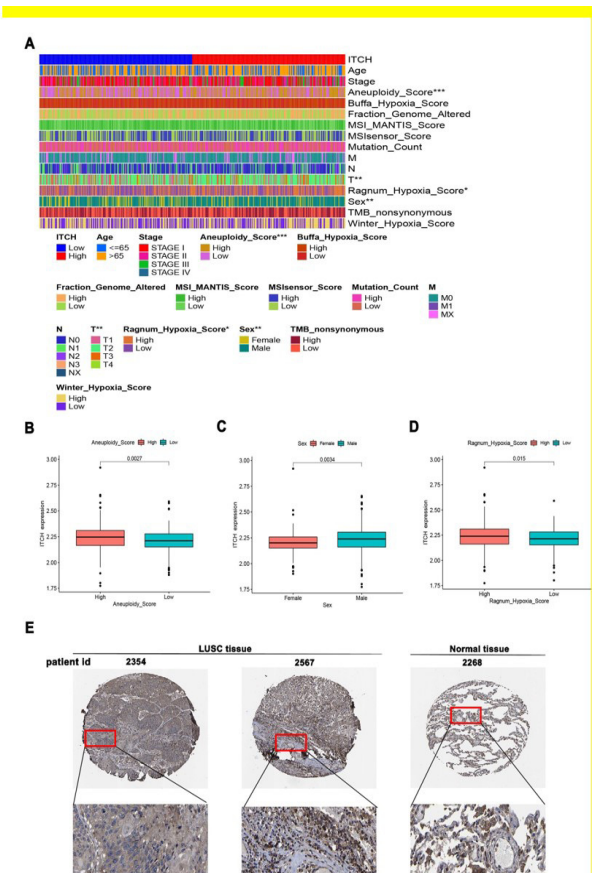

Figure 2: Correlation between ITCH and clinicopathologic features of lung squamous cell 567 carcinoma.
(A) An overall analysis of the correlation between ITCH and clinicopathologic features of lung squamous cell carcinoma. **(B,C,D)** The correlation between ITCH expression and aneuploidy score **(B)**, sex **(C)** and Hyposia score **(D)**. **(E)**The expression level of ITCH protein in patient is derived from HPA database. (*p<0.05,**p<0.01).

Increased copy number and decreased promoter methylation of ITCH in lung squamous cell carcinoma

We further explored the factors that increase the expression of ITCH in lung squamous cell carcinoma. At the same time, we analyzed the two genes that have the highest correlation with ITCH, SERNIC3 and MAPRE1. The results showed that the amplification of these three genes is the main change in lung squamous cell carcinoma. Among them, hete.amp is the important way, homo.amp accounts for a small percentage, and the deletion part accounts for a small part (Figure 3A). Figures 3B and 3C show the details of different amplification methods. Figure 3E shows the specific changes of copy types of these three genes.Further, we evaluated whether this copy number amplification led to the change of mRNA expression. The results showed that the change of copy number of ITCH and MAPRE1 was significantly positively correlated with mRNA 269 expression, with a correlation coefficient greater than 0.5, but this correlation was less obvious on the SERNIC3 gene, with a correlation coefficient of 0.48 (Figures 3D,F). Figure 3F shows the specific correlation coefficients and statistical differences. In addition, we detected the promoter methylation status and mRNA expression of these three genes, and found that the methylation level of ITCH and MAPRE1 promoters was significantly negatively correlated with their mRNA expression. Figure 3G shows the specific correlation coefficient and statistical difference. These results led us to speculate that the high expression of ITCH may be due to the abnormal amplification of its copy number and the reduction of methylation in its promoter.

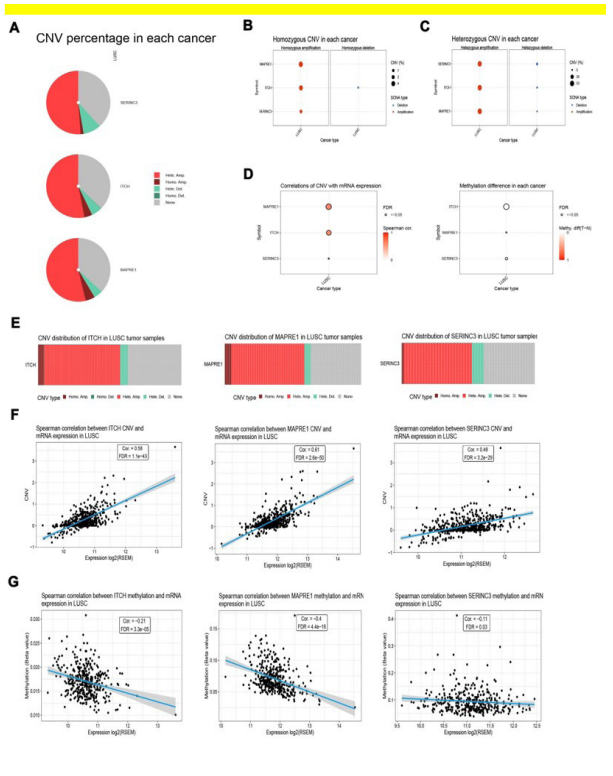

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Figure 3: The copy number and promoter methylation of ITCH in lung squamous cell carcinoma.
(A) The overall CNV changes of ITCH, SERINC3 and MAPRE1 in LUSC data set. **(B)** Homozygous level of ITCH, SERINC3 and MAPRE1 in LUSC data set. **(C)** Heterzygous level of ITCH, SERINC3 and MAPRE1 in LUSC data set. **(D)** The correlation between the CNV, methylation level of ITCH, SERINC3 and MAPRE1 promoters and their mRNA expression. **(E)** The CNV changes of ITCH, SERINC3 and MAPRE1 in LUSC data set. **(F)** The correlation between the CNV change of ITCH, SERINC3 and MAPRE1 and their mRNA expression. **(G)** The correlation between the methylation level of ITCH, SERINC3 and MAPRE1 promoters and their 581 mRNA expression.

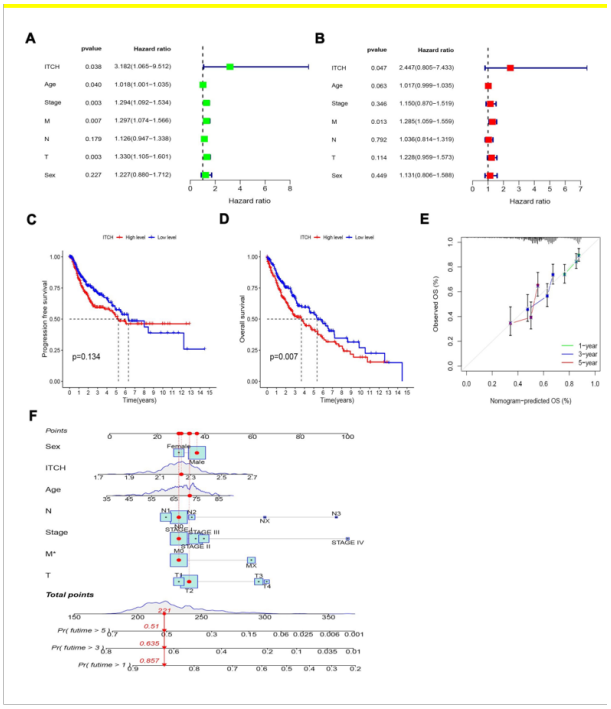

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Figure 4: The prognosis of lung squamous cell carcinoma patients with high expression of ITCH is 583 poor.
(A) Univariate analysis of prognostic factors. **(B)** Multivariate analysis of prognostic indicators. **(C,D)** Association of ITCH expression with PFS **(C)** and OS **(D)** of patients with lung squamous cell carcinoma. **(E)** The calibration curves for predicting patient OS at a 1- year, 3-year and 5-year in the internal verification. **(F)** Nomogram model predicting the 1- year, 3-year and 5-year OS in patients with lung squamous cell carcinoma. PFS: progression-free survival.OS: overall survival.

ITCH is a prognostic indicator of lung squamous cell carcinoma

Next, we focus on evaluating the prognostic predictive role and applicability of ITCH in lung 280 squamous cell carcinoma. Based on univariate analysis (Figure 4A) of prognostic factors and umultivariate analysis (Figure 4B), we found that ITCH is a stable adverse prognostic factor for lung squamous cell carcinoma. In univariate analysis, age/stage/T/M stage and the expression of ITCH were both adverse prognostic factors of lung squamous cell carcinoma, and both were statistically significant. However, in multivariate analysis, only M stage and the expression of ITCH were statistically significant, indicating that ITCH was a more stableindependent prognostic indicator of lung squamous cell carcinoma than clinicopathological parameters. Finally, we examined the role of ITCH in the prognosis of lung squamous cell carcinoma, and found that patients with high expression of ITCH had poor overall prognosis (Figure 4D). But PFS has no statistical significance (Figure 4C). Based on this result, we built a diagnostic nomogram predicting the 1 -, 3 -, and 5-year OS (Figure 4F). Draw calibration curve to prove the prediction effectiveness of OS in the constructed nomograph (Figure 4E). These results suggest that ITCH is a relati-

vely stable independent prognostic factor for lung squamous cell carcinoma and can help predict survival time.

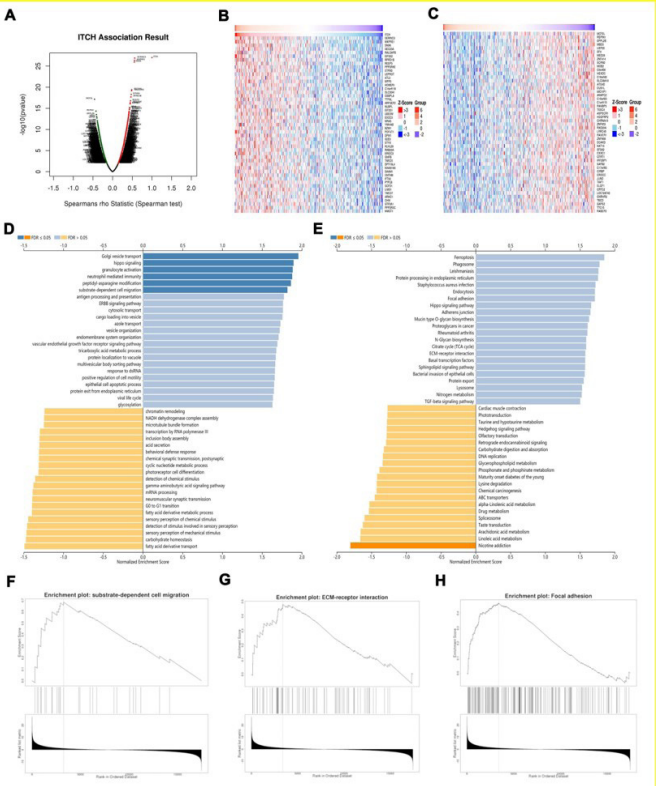

Figure 5: ITCH co-expression Networks and pathway enrichment. **(A)** Volcano map of ITCH-related genes in LUSC dataset. **(B,C)** Top 50 Positive **(B)** and negative **(C)** correlation genes with ITCH in LUSC dataset. **(D,E)** GO annotations analysis **(D)** and KEGG pathway analysis **(E)**.

ITCH participates in the metastasis of lung squamous cell carcinoma

In order to understand the biological function of ITCH in lung squamous cell carcinoma, we explored the co-expression pattern of ITCH in TCGA-LUSC by applying the LinkFinder module in LinkedOmics online website. First of all, ITCH-related genes in lung squamous cell carcinoma were detected and expressed by volcanic map (Figure 5A). Figures 5B and 5C show the heat map of the first 50 genes with positive and negative correlation with ITCH respectively. In addition, based on these differentially expressed genes, the GO and KEGG analysis was carried out to evaluate the possible way of ITCH to play its biological function. The results showed that the GO term annotation (Figure 5D) showed that the co-expressed genes of ITCH were mainly involved in "substrate-dependent cell migration" (Figure 5F). KEGG pathway analysis (Figures 5E) shows that ITCH may participate in the "ECM-receptor interaction" (Figure 5G) and "Focal adhesion" (Figure 5H) pathway. These results showed that ITCH expression network had a great influence on cell adhesion and metastasis of lung squamous cell carcinoma. These results suggest that ITCH may participate in the process of cell metastasis of lung squamous cell carcinoma, thus promoting the progression of lung squamous cell carcinoma. Therefore, we will test the metastasis effect of ITCH on lung squamous cell carcinoma cells *in vitro*. Figures 6A and 6B show the changes of mRNA and protein levels of ITCH knockdown in lung cancer cells. The ability of cell migration and invasion was significantly down-regulated after the knockout of ITCH (Figure 6C), and the wound healing experiment also showed that the ability of cell migration was also down-regulated after the knockout of ITCH (Figure 6D). This is consistent with our previous online analysis results, indicating that ITCH is involved in the metastasis process of lung squamous cell carcinoma, thus promoting the progression of lung squamous cell carcinoma and affecting its prognosis.

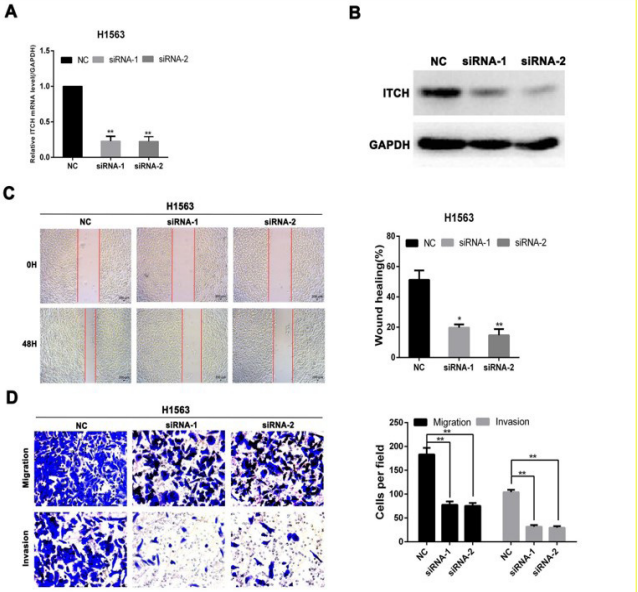

Figure 6: ITCH promotes lung cancer cell metastasis in vitro.
(A,B) RT-PCR **(A)** and western blot assay **(B)** were used to detect the expression changes of ITCH.
(C) Wound healing assays was used for the evaluation of migration ability of ITCH of lung cancer cells. Original magnification, 50×. **(D)** Transwell migration and invasion assays experiment were used to detect the effect of ITCH gene on the metastatic ability of lung cancer cells. Original magnification, 200×. Scale bar = 100μm. All the data were expressed as mean ± SD, based on 602 Student's t-test. (*p<0.05, **p<0.01).

The correlation between ITCH and immune cell infiltration and the efficacy of guiding immune checkpoints

We further tested the role of ITCH in the infiltration of immune cells. The results showed that the high expression of ITCH was in direct proportion to the infiltration of M2 macrophages and neutrophils and had statistical significance, while it was in inverse proportion to CD8+T cells, Tregs cells and mono cells and had statistical significance (Figure 7A). However, the tumor mutation load (TMB) related to the efficacy index of the current immune checkpoint inhibitor has no obvious correlation (Figure 7B). The single-cell data further refined the cell composition of the sample, and found that the largest component of cells is macrophages, of which M2 and M0 type account for the majority (Figure 7C). In addition, we analyzed the prognosis of these immune cell types with strong correlation, and found that the difference in infiltration of other immune cell subtypes, except neutrophils, would cause the difference in prognosis, of which the more obvious impact is macrophages, indicating that ITCH may further promote the progression of lung squamous cell carcinoma through the infiltration and polarization of macrophages cells (Figure 7D). Based on the correlation between ITCH and immune cells, we further tested

the differences of IPS scores based on PD1 and CTLA4 expression in different expression of ITCH samples. The results showed that compared with the low expression group, IPS scores in the high expression of ITCH group were lower than those in the low expression group, which indicated that the efficacy of using immune checkpoint inhibitors in the high expression of ITCH lung squamous cell carcinoma might be average (Figure 7E), which provided basic guidance for clinical guidance of the application of immune checkpoint inhibitors.

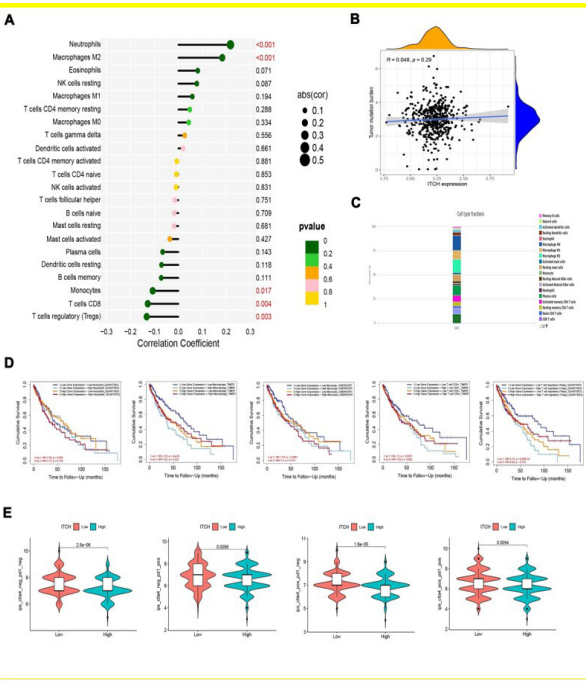

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Figure 7: ITCH promotes lung cancer cell metastasis *in vitro*.
(A,B) RT-PCR **(A)** and western blot assay **(B)** were used to detect the expression changes of ITCH.
(C) Wound healing assays was used for the evaluation of migration ability of ITCH of lung cancer cells. Original magnification, 50×. **(D)** Transwell migration and invasion assays experiment were used to detect the effect of ITCH gene on the metastatic ability of lung cancer cells. Original magnification, 200×. Scale bar = 100μm. All the data were expressed as mean ± SD, based on 602 Student's t-test. (**p*<0.05, ***p*<0.01).

Discussion

Most of the functions of ITCH depend on ITCH-deficient mice, which play an important role in the regulation of immune system as ubiquitin ligase. Lack of ITCH in mice has been proved to cause serious autoimmune disease [27]. In human body, ITCH can also cause immune deficiency syndrome [28]. This may be due to the fact that ITCH down-regulates the inflammatory signal pathway through ubiquitin, especially the T cell-mediated immune response [29,30]. However, in tumors, ITCH inhibits the activation of T cell antigen receptor (TCR), thus inhibiting T cell activity [12,31]. In our study, we also reached a similar conclusion that high expression of ITCH is negatively correlated with CD8+T cell infiltration, whichmay a mechanism of ITCH promoting tumor progression. However, ITCH is inversely correlated with M2 type macrophages and Treg cells. There is a significant positive correlation with M2 type macrophages that promote cancer, but there is a negative correlation with Treg cells that promote cancer [32,33]. This may be related to different inflammation-related ubiquitination substrates of ITCH. For example, recent studies have found that ITCH binds, ubiquitinates and downregulates PD-L1/L2 on the tumor surface in human melanoma cells treated with MAPKi, So as to promote T cell activation [8]. This may be caused by the difference of expression position of ITCH in cells, but the specific mechanism needs further exploration. In addition, the prognosis analysis based on different immune cell infiltration showed that macrophages, CD8+T and Treg cells had a significant impact on the prognosis, indicating that ITCH might promote tumor progression by reshaping the immune microenvironment and readjusting the proportion of immune-related cell infiltration. Based on this, we further analyzed the efficacy of immune checkpoint inhibitors, and found that the immune score in the low expression ITCH group was higher, and it might be easier to benefit from immune checkpoint inhibitors.

For the evaluation of efficacy sensitivity of immune checkpoints, we also evaluated the relationship between the expression of ITCH and aneuploid score. Aneuploid is an imbalance in the number of chromosomes or chromosome arms, which is an almost universal feature of human cancer. Some studies have revealed that the negative effect of tumor aneuploidy on anti-tumor immunity may be through the mechanism of immune evasion, including down-regulation of PD-L1 expression and inhibition of CD8+T cell reaction in tumor [34,35]. In addition, previous work has determined that the increase of aneuploidy in tumor is a marker of low overall survival rate, and suggested that aneuploidy be used as a biomarker of clinical results [36]. Recently, it has also been found that aneuploidy is a powerful predictor of survival in patients with non-small cell lung cancer receiving immunotherapy [37], which is also consistent with our results. In lung squamous cell carcinoma with high expression of ITCH, the aneuploid score is higher, which indicates a poor prognosis. Research shows that patients with high aneuploid scores may be more likely to benefit from immune checkpoint inhibitors [38,39]. Combined with our analysis results, that is, patients with lung squamous cell carcinoma with high expression of ITCH are likely to benefit from immune checkpoint inhibitors, but this may contradict the efficacy indicators of immune checkpoint inhibitors obtained from the expression of PD-1 and CTLA4. The other approved indicator that can be used to guide the application of immunosuppressive agents at immune detection points is TMB [40]. Our research shows that there is a weak positive correlation between the expression of TMB and the expression of ITCH. Previous literature also shows that the correlation between TMB and aneuploid score is very small [39]. Therefore, this may also explain why the expression of ITCH is significantly correlated with aneuploid score, but the correlation with TMB is not very obvious, which also shows that the prognostic significance of TMB in guiding immune checkpoint therapy in lung squamous cell carcinoma is very limited. As for the contradictory view that the immune efficacy score shows that patients with low expression of ITCH lung squamous cell carcinoma may benefit from immunotherapy while the aneuploid score shows that patients with high expression of ITCH lung squamous cell carcinoma may benefit from immunotherapy, we prefer the prediction result of aneuploid score, Because more and more studies have shown that ITCH directly regulates the expression of PD-L1 in tumor cells [8], it may cause a deviation in the score. In addition to its role in immunity, the role of ITCH in tumor cells has been gradually revealed.

For example, in breast cancer, ITCH can negatively regulate tumor suppressor LATS1 through ubiquitination, thereby increasing YAP activity, and activating Hippo kinase pathway, leading to increased cell proliferation, decreased apoptosis, and promoting tumor cell EMT [41]. In triple negative breast cancer (TNBC), the nuclear localization of ITCH makes TNBC cells have DDR inhibition to counteract replication stress and increase the survival rate and growth potential of cancer cells [42]. In lung adenocarcinoma, ITCH promotes the metastasis of lung cancer cells under hypoxia [13]. In this study, we focused on the treatment resistance subgroup of lung squamous cell carcinoma. The results showed that ITCH was highly expressed in lung squamous cell carcinoma, and the prognosis of patients with high expression of ITCH was poor. In terms of mechanism, our results were consistent with previous research results. ITCH could promote the metastasis of lung squamous cell carcinoma cells. In the analysis of pathway enrichment, we also found that ITCH could regulate the Hippo pathway in lung squamous cell carcinoma, This is consistent with previous research results [43,44]. However, whether it can promote cancer through the same pathway 407 in lung squamous cell carcinoma has not been verified, which is also our next experimental plan.

In view of the important role of ITCH in lung squamous cell carcinoma, we also explored the possible reasons for the imbalance of ITCH expression in lung squamous cell carcinoma. The 410 analysis results showed that the mRNA expression level of ITCH was significantly correlated with the copy number (CNV) and methylation level. The increase of the amplified copy number led to the increase of ITCH expression, but methylation often inhibited the expression of target genes [45].

This trend also existed in this study, The expression of ITCH is significantly related to the level of methylation, which makes us speculate whether the decreased methylation of the promoter site of ITCH leads to a significant increase in the expression of ITCH, which needs further validation *in vitro* and *in vivo*.

Conclusion

Based on these results, we conclude that the expression of ITCH is increased in lung squamous cell carcinoma, and promotes the metastasis of lung squamous cell carcinoma, leading to poor prognosis. At the same time, the highly expressed ITCH regulates the redistribution of tumor immune cells by reshaping the tumor microenvironment, and promotes the immune escape of tumor cells. In addition, its high expression may be due to the methylation imbalance of its promoter site. More importantly, ITCH can be used as an important indicator of the benefits of clinical application of immune checkpoints, providing a basis for clinical guidance of drug use.

Declarations

Conflict of interests: The authors have declared that no competing interest exists.

Data availability statement: The data that support the findings of this study are available from the First author, upon reasonable request.

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Author contributions: G-LB and G-JF conceived and designed the study. Online data download and analysis by G-JF. G-LB did the experiments and analyzed results, and written the manuscript. G-LB and G-JF conducted experimental guidance. G-JF revised the manuscript.

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