

IMP3 Predicts Invasion and Prognosis in Human Lung Adenocarcinoma

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Abstract

Purpose Insulin-like growth factor II mRNA-binding protein 3 (IMP3) is an oncofetal protein associated with several aggressive and advanced cancers. Whether IMP3 can predict invasion, and prognosis in patients with human lung adenocarcinoma (LAC) remains unclear.

Methods Ninety-five LAC and 75 non-tumor lung tissue samples were included in a tissue microarray. IMP3 expression was assessed by immunohistochemical examination. Correlation between IMP3 expression levels, clinicopathological characteristics, and overall prognosis was evaluated. In a separate in vitro study, RNA interference method was applied for knockdown of IMP3 gene in human LAC cell lines. Invasive potential of LAC cells was then evaluated by transwell migration assay.

Results IMP3 immunoreactivity was observed in 39 out of 95 (41.1 %) LAC patients, but not in non-tumor lung tissues. IMP3 expression levels were closely associated with histological grade ($P = 0.037$), TNM stage ($P = 0.034$), and

lymph node metastasis ($P = 0.011$). Patients presenting with positive IMP3 expression ($P = 0.000$), an advanced TNM stage ($P = 0.000$), and lymph node metastasis ($P = 0.001$) had a worse overall survival, compared to those lacking these characteristics. Both IMP3 expression (hazard ratio [HR], 2.310; 95 % confidence interval [CI] 1.192–4.476; $P = 0.013$) and TNM stage (HR 2.338; 95 % CI 1.393–3.925; $P = 0.001$) were independent predictors of poor prognosis. The invasive potential of LAC cells was significantly inhibited by IMP3 knockdown.

Conclusion IMP3 appears to play an important role in tumor invasion in patients with LAC and may serve as a useful prognostic biomarker in these patients.

Keywords Lung adenocarcinoma · IMP3 · Invasion · Prognosis · RNA interference

Introduction

Lung cancer is by far the most common cancer and the leading cause of cancer-related death worldwide [1]. The incidence of lung adenocarcinoma (LAC) has increased in most countries in recent decades. LAC has replaced squamous cell carcinoma as the most frequently occurring histological type [2–4]. The 5-year survival rate for patients with advanced LAC is of the order of 5–20 % [5]. A key contributor to the dismal prognosis is that the most patients are diagnosed at an advanced stage, often with metastatic lesions. In addition, LAC is a radiologically, histomorphologically, and clinically heterogeneous tumor with a wide variability in malignant potential. The prognosis of LAC is notoriously unpredictable; the TNM stage of the tumor at the time of diagnosis or surgery has a relatively low reliability [6, 7]. Identification of novel bio-markers

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that could help predict the tendency for invasive growth and metastasis, overall survival, as well as for aiding clinical decision-making is a key research imperative.

Insulin-like growth factor II mRNA-binding protein 3 (IMP3) is a member of the IMP family that consists of IMP1, IMP2, and IMP3. It is also referred to as K homology domain containing-protein over-expressed in cancer (KOC), IGF2BP3, and L523S [8, 9]. IMP3 binds to the 5' untranslated region of the IGF-II leader-3 mRNA as its translational activator, coding for IGF-II protein which regulates cell proliferation [9]. IMP3 is an oncofetal protein, which finds expression in developing epithelium, muscle tissue, and placenta during the early stages of human and mouse embryogenesis. Expression of IMP3 has not been demonstrated in normal adult human cells [10]. Recent studies have shown that IMP3 is an important cancer-specific gene associated with several types of cancers and that it is exclusively expressed in malignant tumors [11–15]. IMPs have been demonstrated to promote cell adhesion and invadopodia formation, suggesting its role in cell migration and invasion during cancer development and progression [16–18]. In several organ systems, IMP3 expression has been shown to correlate with a higher grade of tumor, increased risk of metastases, and poorer prognosis [19–22]. Our previous study also indicated that IMP3 was an independent prognostic factor in patients with cervical squamous cell carcinoma [15]. However, association of IMP3 expression in LAC has not been adequately researched. In previous studies, IMP3 expression was associated with histological differentiation, histological subtypes, and distant metastasis in LAC [19, 23, 24]. However, few studies have probed the association of IMP3 expression with survival. Further, the relationship between IMP3 expression and invasive potential of LAC cells is yet to be characterized at the cellular level.

In this study, we investigated IMP3 expression in human LAC tissue. The correlation between IMP3 expression, clinicopathological factors, and prognosis are examined. In a separate in vitro proof-of-concept study, we established cell sublines with stable knockdown of IMP3 gene to verify the role of IMP3 in tumor invasion.

Materials and Methods

Patients

IMP3 expression in 95 LAC and 75 non-tumor lung tissue samples was assessed by immunohistochemical (IHC) examination using commercially available tissue microarrays (Shanghai Outdo Biotech Co., Ltd. Shanghai, China). The operation time was from April 2004 to October 2007, and the follow-up time was July 2012. None of the patients

in this study received chemotherapy or radiotherapy prior to surgery. This study was conducted according to the regulations of the local ethical committee. Among the study subjects, 52 were male and 43 were female (male:female ratio: 1.21:1). The mean age was 58.9 years (range 20–84 years). Tumor nodes metastasis (TNM) stages were determined according to the Union for International Cancer Control (UICC) 2002 classification [25]. The number of patients having stage I, II, III, and IV LAC was 49, 14, 24, and 8, respectively. LAC tissues were histologically graded as moderately or poorly differentiated, as per the World Health Organization criteria [26].

Immunohistochemical Analysis

Immunohistochemical examination was performed as previously described [15]. IMP3 immunoreactivity generally manifested as concentrated cytoplasmic staining. Each sample was semiquantitatively scored for both intensity and proportion of IMP3 expression. Intensity of staining was classified as follows: 0: none, 1: weak, 2: moderate, and 3: strong; the percentage of positive cells was graded using the following 4 categories: 0: <5 %; 1: ≥ 5 to <25 %; 2: ≥ 25 to <50 %; and 3: ≥ 50 %. A final immunoreactivity score for each sample was computed by adding the two individual scores. A final score <2 was considered as negative, while ≥ 2 was considered as positive.

Cell Culture

The human immortalized bronchial epithelial cells, 16HBE, and LAC cells, including, A549, GLC-82, H358, H322, and SPC-A1, were cultured in RPMI-1640 (HyClone, Logan, UT) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco, Los Angeles, CA, USA). All cell lines were obtained from the Department of Pathology at the Southern Medical University. Cells were cultured under humidified air containing 5 % CO₂ at 37 °C.

Construction of IMP3 shRNA Lentiviral Vector and Transfection

Three target RNA interference (RNAi) sequences were designed based on the human IMP3 mRNA (GenBank Accession no. NM_006547) sequence and cloned into the pGC-LV-GFP vector (GeneChem, Shanghai, China). The RNAi sequence AGGAATTGACGCTGTATAA most effectively suppressed IMP3 mRNA in A549 and H322 cells, and was used in the subsequent experiments for endogenous IMP3 knockdown (A549-shIMP3, H322-shIMP3). Non-silencing (NS)-small interfering RNA (shRNA) (TTCTCCGAACGTGTCACGT) was also cloned into the pGC-LV-GFP vector and served as a negative control (A549-control, H322-control).

The recombinant virus was packaged in 293T cells using a lentivector expression system. For cellular transfection, A549 and H322 cells were subcultured at 1×10^6 /mL in 6-well culture plates and transfected with lentivirus-mediated IMP3-shRNA or NS-shRNA. The GFP expression level was detected via fluorescence microscopy (Olympus, Japan) to determine the infection efficiency at 72 h after transfection.

Western Blot and Real-Time Polymerase Chain Reaction

Western blot analysis, total RNA extraction, reverse transcription, and real-time PCR were carried out as previously described [15]. An anti-IMP3 antibody (Epitomics, USA) was diluted 1:3000. IMP3 forward and reverse primers were 5'-CGGAGACTGTTCATCTGTTTATCC-3' and 5'-TTCTGTTGTTGGTGCTGC TTT-3', respectively. GAPDH forward and reverse primers were 5'-GGAGCGAGATCCCTCCAAAAT-3' and 5'-GGCTGTTGTCATACTTCTCATGG-3', respectively. The relative levels of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method [27].

Transwell Migration Assay

A549 and H322 cells, with or without IMP3 knockdown, were plated at 1×10^6 /mL in 100 μ L serum-free medium in 24-well matrigel-coated transwell units with polycarbonate filters (8 μ m pore size, Corning, USA). The lower chamber was loaded with 500 μ L of RPMI-1640 containing 10 % FBS. After incubation for 6, 24, and 48 h under normal culture conditions, the top surface of the membrane was gently scrubbed with a cotton bud, fixed in 4 % paraformaldehyde, and stained with hematoxylin and eosin. The cells that had permeated through the membrane filters were counted using a light microscope and five microscopic fields ($\times 200$) were randomly selected to count the migrated cells.

Statistical Analysis

Statistical analysis was performed using SPSS software 13.0. Association between categorical variables was assessed using a χ^2 test. Correlation between IMP3 expression and the overall survival was evaluated using the Kaplan–Meier method and the log-rank test. A Cox regression proportional hazards model was used for multivariate analyses to determine the independent significance of relevant clinical covariates. The hazard ratio (HR) with 95 % confidence interval (CI) was measured to estimate the hazard risk for individual factors. Real-time PCR results were assessed by one-way analysis of variance (ANOVA). The results of transwell assay were assessed

using two sample *t* tests. Two-tailed *P* values of <0.05 were considered statistically significant.

Results

IMP3 Expression in LAC and Non-tumor Lung Tissue Samples

Immunostaining demonstrated that IMP3 expression was absent in all 75 non-tumor lung tissue specimens (Fig. 1a), while IMP3 expression was observed in 39 out of 95 (41.1 %) LAC tissue samples. The expression levels of IMP3 in LAC tissue samples varied between weak, moderate, and strongly positive (Fig. 1b–d).

Relationship Between IMP3 Expression and Clinicopathological Factors

Patients with LAC were categorized into IMP3 negative and IMP3 positive subgroups. IMP3 expression was present in 19.0 % (4/21), 42.6 % (20/47), and 55.6 % (15/27) of well, moderately, and poorly differentiated tumors, respectively. IMP3 expression increased significantly with histological grades ($P = 0.037$). Patients with stage III–IV LAC exhibited a greater IMP3 expression as compared to patients with I–II stages ($P = 0.034$). IMP3 expression significantly correlated with lymph node metastasis ($P = 0.011$). No significant correlation was observed between IMP3 expression and age ($P = 0.659$), gender ($P = 0.785$), and tumor size ($P = 0.489$) (Table 1).

Prognostic Significance of IMP3 Expression

The median survival time of LAC patients was 62.95 months and the 5-year survival was 54.7 %. Survival times were significantly decreased in LAC patients with IMP3 expression ($P = 0.000$), with advanced TNM stage ($P = 0.000$), and with lymph node metastasis ($P = 0.001$) (Fig. 2a–c) (Table S1). On univariate analysis, Cox proportional hazards model showed that advanced TNM stage ($P = 0.000$), lymph node metastasis ($P = 0.002$), and positive IMP3 expression ($P = 0.000$) correlated with a decreased overall survival (Table 2). However, multivariate analyses revealed that both TNM stage (HR 2.338; 95 % CI 1.393–3.925; $P = 0.001$) and IMP3 expression (HR 2.310; 95 % CI 1.192–4.476; $P = 0.013$) were significant independent predictors of survival (Table 2).

IMP3 Expression in LAC Cell Lines

IMP3 expression in 16HBE and LAC cells, including A549, GLC-82, H358, H322, and SPC-A1, were assessed

Fig. 1 IMP3 expression in non-tumor lung tissues (**a**), and LAC tissues: weak (**b**), moderate (**c**), and strong (**d**) positive by IHC staining, ($\times 400$)

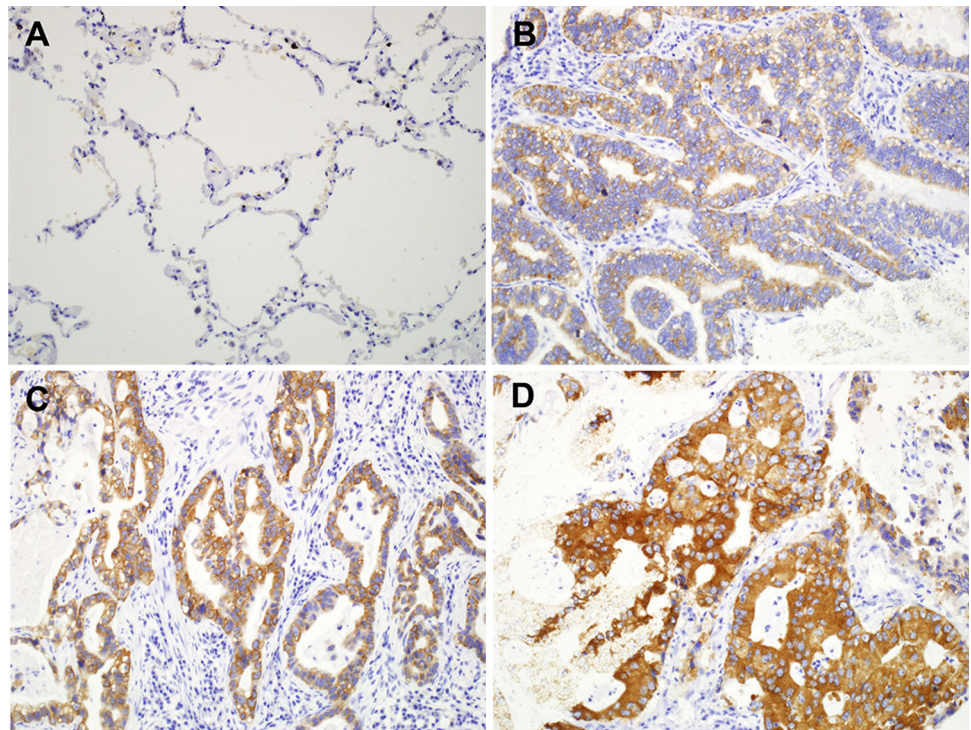


Table 1 Correlation between IMP3 expression and clinicopathological characteristics in 95 patients with LAC

Variables	Total	IMP3 expression (N/%)		χ^2	P
		Positive	Negative		
Total	95	39	56		
Age (years)					
<60	51	20 (39.2 %)	31 (60.8 %)	0.154	0.659
>60	44	19 (43.2 %)	25 (56.8 %)		
Gender					
Male	52	22 (42.3 %)	30 (57.7 %)	0.075	0.785
Female	43	17 (39.5 %)	26 (60.5 %)		
Tumor size (cm)					
≤ 3.0	43	16 (37.2 %)	27 (62.8 %)	0.480	0.489
> 3.0	52	23 (44.2 %)	29 (55.8 %)		
Histological grade					
Well-differentiated	21	4 (19.0 %)	17 (81.0 %)	6.593	0.037
Moderately differentiated	47	20 (42.6 %)	27 (57.4 %)		
Poorly differentiated	27	15 (55.6 %)	12 (44.4 %)		
TNM stage					
I	49	16 (32.7 %)	33 (67.3 %)	6.769	0.034
II	14	4 (28.6 %)	10 (71.4 %)		
III–IV	32	19 (59.4 %)	13 (40.6 %)		
Lymph node metastasis					
Yes	39	22 (56.4 %)	17 (33.6 %)	6.448	0.011
No	56	17 (30.4 %)	39 (69.6 %)		

by real-time PCR and Western blot analysis. As compared with 16HBE cells, significantly elevated IMP3 expression was observed in all LAC cell lines, except H358

($P = 0.001$) (Table S2, Fig. 3a, b). In addition, IMP3 expression in A549 and H322 cells was detected by immunocytochemical (ICC) staining. Consistent with the

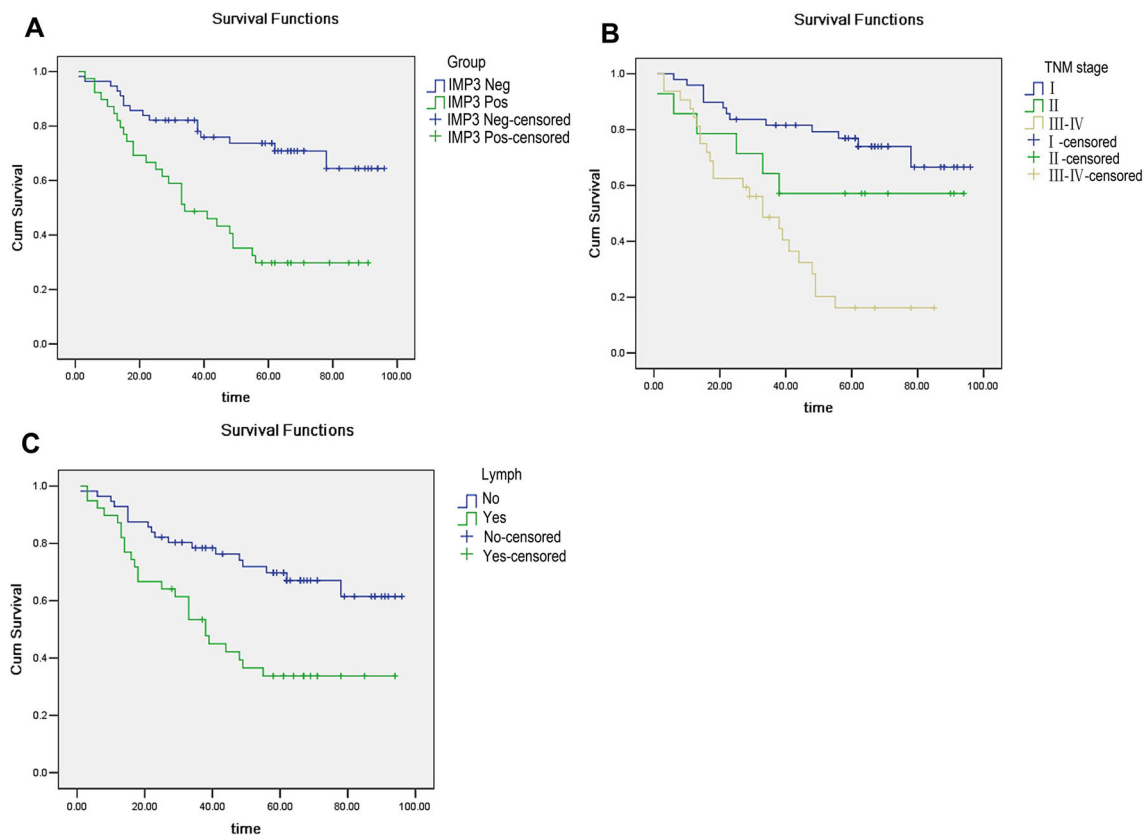


Fig. 2 Kaplan–Meier analysis of IMP3 expression. **a** TNM stage; **b** lymph node metastasis; **c** in patients with LAC

Table 2 Univariate and multivariate Cox regression analyses for prognostic factors

Variables	Univariate analysis HR (95 % CI)	Multivariate analysis HR (95 % CI)
Age (<60 vs. ≥60 years)	1.563 (0.856–2.855)	1.725 (0.899–3.311)
Gender (male vs. female)	0.861 (0.471–1.573)	0.699 (0.356–1.373)
Tumor size (≤3 vs. >3 cm)	1.128 (0.618–2.060)	0.537 (0.259–1.113)
Histological grade (well vs. moderate vs. poor)	1.495 (0.966–2.314)	1.107 (0.682–1.798)
TNM stage (I vs. II vs. III–IV)	2.178 (1.541–3.077)	2.338 (1.393–3.925)
Lymph node metastasis (no vs. yes)	2.608 (1.418–4.798)	0.827 (0.363–1.886)
IMP3 expression (– vs. +)	3.083 (1.657–5.736)	2.310 (1.192–4.476)

HR hazard ratio, CI confidence interval

results of immunostaining in tissue samples, ICC staining for IMP3 was concentrated cytoplasmic in LAC cells (Fig. 3c).

Establishment of Cell Sublines with Stable Knockdown of IMP3 Gene

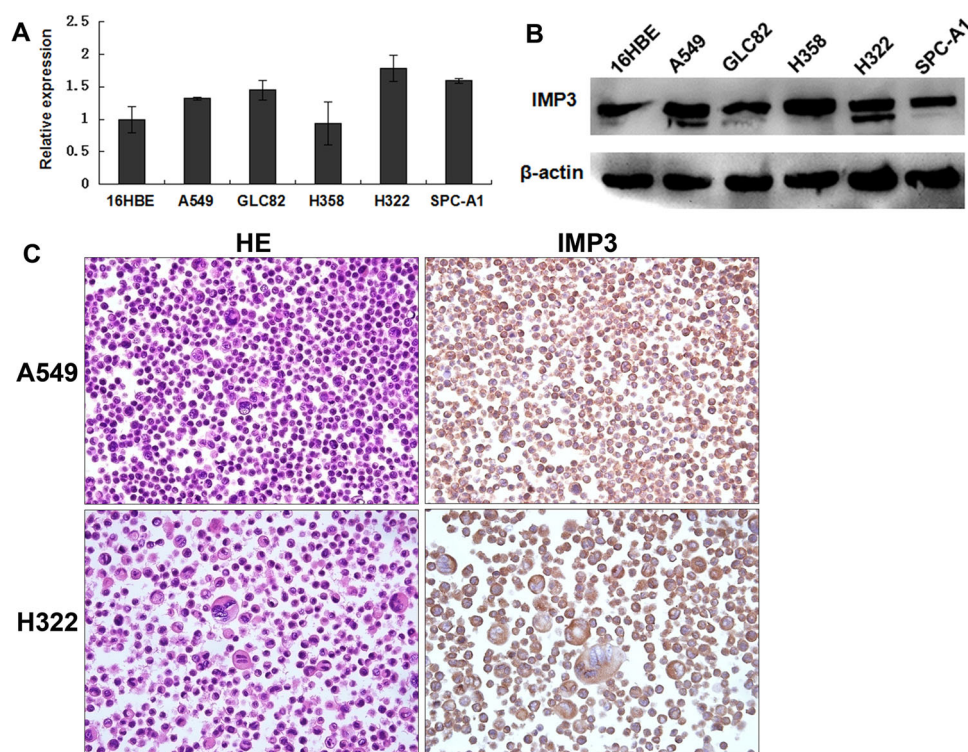
Lentiviral vectors, labeled with GFP, were stably transfected onto A549 and H322 cells for constitutive knockdown IMP3 gene. After transfection, GFP+ cells were observed by fluorescence microscopy (Fig. 4a) and were sorted by flow cytometry. The RNAi efficiency of IMP3

gene was measured by real-time PCR (Table S3, Fig. 4b, c) and Western blot analysis (Fig. 4d, e). Both mRNA and protein levels of IMP3 expression were significantly down-regulated in the A549- and H322-shIMP3 groups as compared to those in the control groups.

IMP3 Gene Knockdown Inhibits LAC Cell Invasion

Transwell migration assay was performed at 6, 24, and 48 h to assess the invasive characteristics of cell sublines. The upper chamber contained 100 μL cell suspension with serum-free medium; and the lower chamber was loaded

Fig. 3 IMP3 expression in LAC cell lines was assayed by real-time polymerase chain reaction (PCR). **a** Western blot analysis; **b** HE and ICC staining ($\times 400$) (C). *HE* hematoxylin and eosin



with 500 μ L of RPMI-1640 containing 10 % FBS (Fig. 5a). The number of migrated cells in A549-shIMP3 group was significantly lower than that in the A549-control group. Similarly, significant differences were observed between H322-shIMP3 and H322-control groups. A significant effect in terms of time, group, and time-group interaction of A549 and H322 cell sublines was observed (Table S4, S5) (Fig. 5b, c).

Discussion

The effect of IMP3 on multiple tumors has recently been investigated. IMP3 is a 580 amino acid oncofetal RNA binding protein that contains two RNA recognition motifs and 4 K homology domains [28], and controls translation of IGF-II mRNA during cell proliferation. In patients with endometrial serous carcinoma and renal cell carcinoma, IMP3 has been shown to be a prognostic biomarker and was reportedly associated with an increased likelihood of metastasis after surgery and a shorter metastasis-free survival [13, 29, 30].

In patients with colon cancer, IMP3 expression was shown to correlate with cancer progression and was associated with an increased risk of metastasis and recurrence after colonectomy [21, 31]. Some *in vitro* studies have also revealed the importance of IMP3 in cancer progression. Liao et al. demonstrated that IMP3 knockdown slowed cell

proliferation through an IGFII-dependent pathway in K562 human leukemia cells [32]. Depletion of IMP3 by RNA interference in hepatocellular carcinoma cell line, HA22T, decreased cell motility and attenuated the invasive characteristics as well as transendothelial migration. Microarray analysis revealed that IMP3 depletion was associated with downregulation of multiple genes involved in tumor invasion [33]. In a study by Vikesaa et al., IMPs appeared to induce cell adhesion and invasion by stabilizing CD44 mRNA [16]. However, in relation to human LAC, the prognostic value of IMP3 and its effect on tumor invasion remain unclear.

In the present study, IHC examination demonstrated IMP3 expression in 41.1 % (39/95) of LAC tissues, while it was absent in non-tumor lung tissue samples. Our findings are consistent with previous studies reporting IMP3 expression in 27–55 % of primary pulmonary adenocarcinoma cases [34]. Furthermore, we revealed demonstrated an association of IMP3 expression with histological grade and lymph node metastasis in LAC, which is also in line with results from earlier studies [19, 23, 24]. Of note, the expression level of IMP3, as determined by IHC analysis, increased with advancing disease state. The expression of IMP3 in patients with stage III–IV disease was significantly higher as compared to that of patients with stage I and II disease. Similar to our findings, Beljan et al. also reported evidence supporting the correlation of IMP3 expression with solid subtype and distant metastases, regardless of

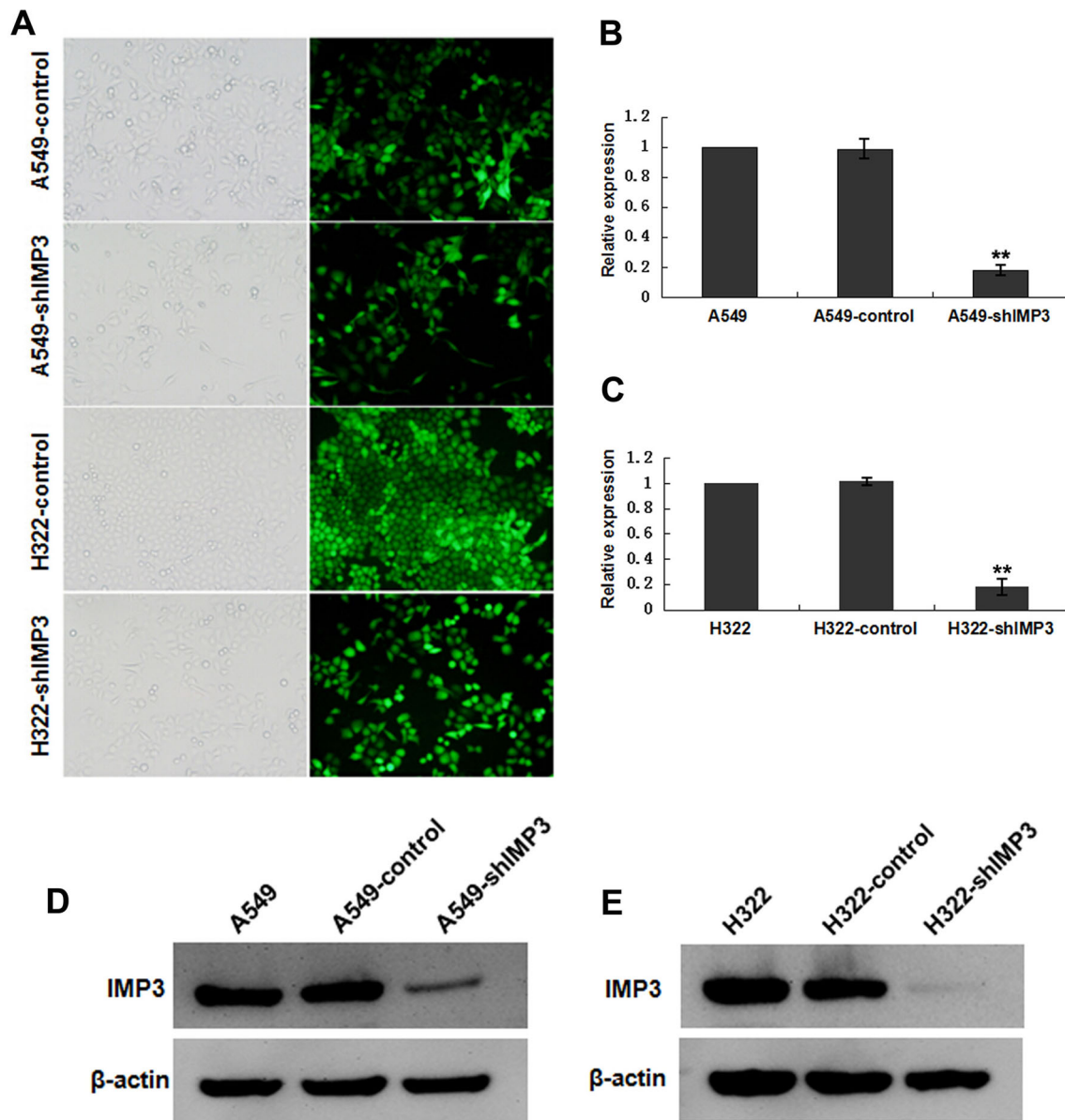


Fig. 4 Construction of cell sublines with stable knockdown of *IMP3* gene. Transfected cells with GFP+ were observed under fluorescence microscope (a). The RNAi efficiency of *IMP3* gene was measured by

real-time PCR (b, c) and Western blot (d, e). *PCR* polymerase chain reaction

histological subtype of LAC [24]. Our findings indicate a potential role of *IMP3* in the progression and invasion of LAC.

Early diagnosis of LAC is typically challenging, as early stages are often asymptomatic. LAC is a highly invasive malignancy, which spreads through blood and lymphatic vessels easily. The overall survival rate for patients with LAC continues to be poor in spite of the recent advances in its diagnosis and treatment. Therefore, the prediction of clinical prognosis for patient with LAC is very important, which may influence the choice of treatment strategies and outcomes.

In this study, we analyzed the correlation between clinicopathological factors and overall survival, and observed a significant correlation of *IMP3* expression with the overall survival. *IMP3* positive patients exhibited a poorer prognosis with a HR of 2.130 (95 % CI 1.192–4.476; $P = 0.013$) as compared to that of *IMP3* negative patients. Based on these results, *IMP3* expression appears to be a potential-independent biomarker for predicting poor prognosis of patients with LAC. There are two limitations identified in this study. First, the heterogeneity among LAC tissues may influence the result. The role of *IMP3* gene in LAC should therefore be further verified. Second, we did not investigate the

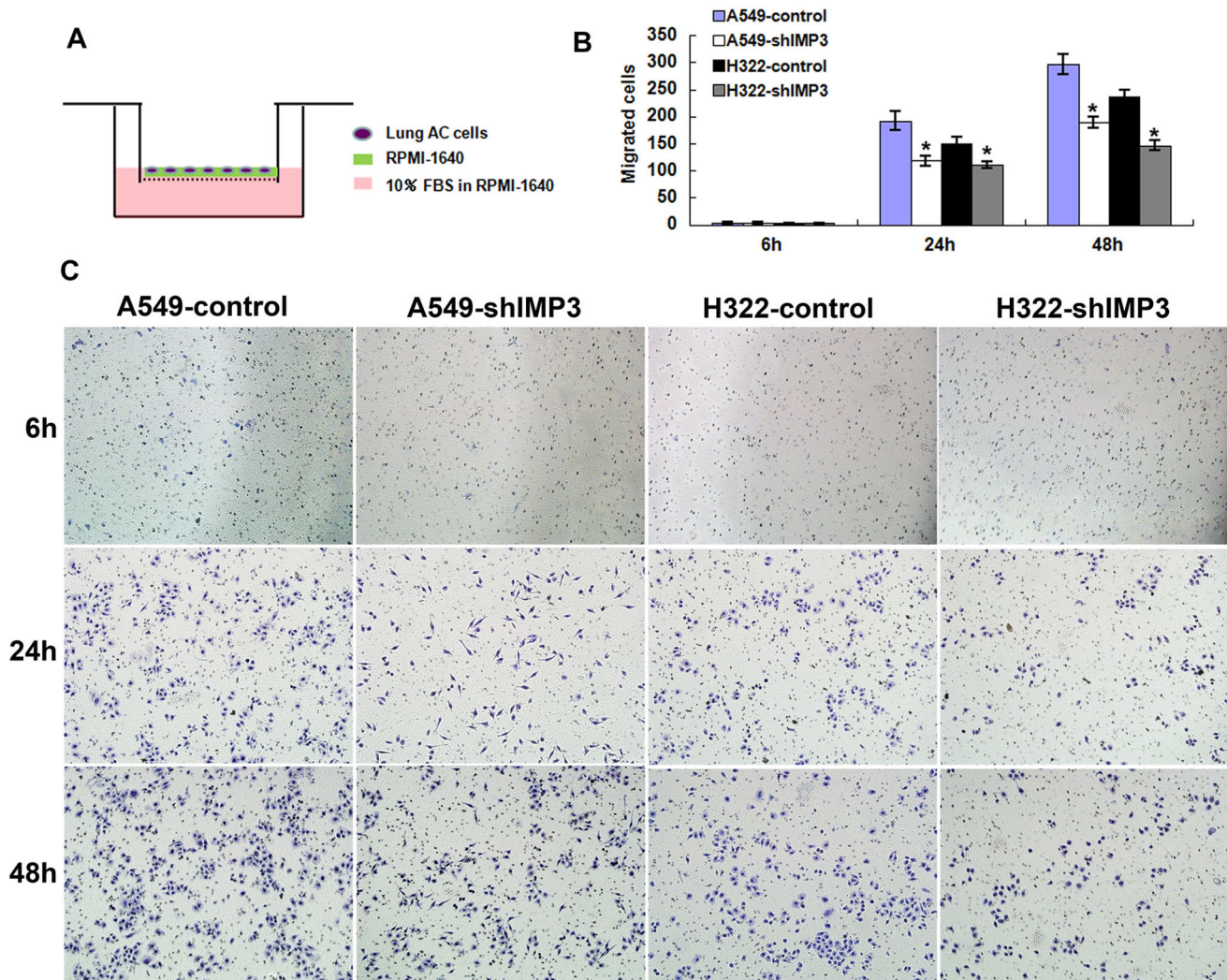


Fig. 5 Transwell migration assay of IMP3-shRNA transfected A549 and H322 cells. **a** The upper chamber contained 100 μ L cell suspension with serum-free medium; lower chamber was loaded with 500 μ L RPMI-1640 with 10 % FBS. **b, c** The migrated cells of all cell

sublines were detected by transwell migration assay at 6, 24, and 48 h. The cells permeating the membrane filters were counted using a light microscope. Five microscopic fields ($\times 200$) were randomly selected to count the cells. *FBS* fetal bovine serum

relationship between lung adenocarcinoma subtypes and IMP3 expression because the commercially available tissue microarrays were not able to discriminate subtypes. By collecting more specimens, the relationship between lung adenocarcinoma subclasses and IMP3 expression will be revealed in future studies.

Although the prognostic relevance of IMP3 in various malignancies has been confirmed, the mechanism underlying its correlation with tumor progression needs to be fully elucidated. Recent studies have shown that presence of IMP3 appeared to facilitate progression of pancreatic ductal adenocarcinoma by enhancing the pro-metastatic behavior of tumor cells [35]. Rivera et al. reported that IMP3 was capable of binding to the mRNAs of cyclin D1, cyclin D3, and cyclin G1, both in vivo as well as in vitro

[36]. In breast cancer cells, IMP3 promoted invasion and migration through the epithelial-mesenchymal transition (EMT) [37]. In a separate in vitro study, we investigated IMP3 expression in LAC cell lines to further verify the role of IMP3 in tumor invasion. Not surprisingly, IMP3 expression was up-regulated in most LAC cells except H358, but weak in immortalized bronchial epithelial cells. The heterogeneity of different origins or IMP3 expression heterogeneity may explain the different expression levels among LAC cell lines. Furthermore, invasiveness of LAC cells was significantly inhibited after knockdown of IMP3 gene using RNAi method. The results imply a potential association of IMP3 over-expression with tumorigenesis.

In a phase I clinical trial of a vaccine targeting L523S (IMP3) in thirteen patients with non-small cell lung cancer,

no dose-related toxic effects were observed. Although vaccination induced an immune response in only one patient, it is interesting to note that only two patients developed disease recurrence, while all remained alive during a median follow-up of 290 days. These findings support the case for further phase II investigation of the L523S vaccine [38]. Combined with the results in the current study, IMP3 may be a potential therapeutic target for LAC; however, further studies are warranted to provide more definitive evidence.

To conclude, IMP3 appears to play an important role in tumor invasion in patients with LAC and may serve as a prognostic indicator in these patients.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing interests.

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