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ORIGINAL ARTICLE

BIN1 reverses PD-L1-mediated immune escape by inactivating the c-MYC and EGFR/MAPK signaling pathways in non-small cell lung cancer

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Non-small cell lung cancer (NSCLC) is one of the most common and malignant carcinoma worldwide, and the incidence and mortality are increasing rapidly. Immunotherapy targeting programmed death 1/programmed death ligand 1 (PD-L1) signaling has shown prominent clinical effects in treating NSCLC; however, a poor understanding of the associated regulating molecular mechanisms of PD-L1 has become one of the biggest obstacles for further improving efficacy. Bridging integrator-1 (BIN1) can regulate numerous cancer-related molecules to exert multiple tumor-suppressing effects by either interacting or not interacting with c-MYC. In the present study, we observed that there exists a negative correlation between the expression of PD-L1 and BIN1 in NSCLC tissues. The expression levels of BIN1 and PD-L1 were significantly related to the tumor, lymph node and metastasis grade (TNM) stage, invasion range and lymph node metastasis. Simultaneously, for NSCLC patients, the expression statuses of BIN1 and PD-L1 might be independent prognostic factors. Furthermore, the expression of tumor-infiltrating lymphocytes was positively associated with BIN1 expression and negatively related to PD-L1 expression in NSCLC tissues. Importantly, we showed that PD-L1 was under the control of BIN1. In addition, the overexpression of BIN1 could inhibit the c-MYC and epithelial growth factor receptor (EGFR)-dependent PD-L1 expression and reverse the suppressive immuno-microenvironment *in vivo*. Taken together, our findings indicated that BIN1 restoration in NSCLC could reverse PD-L1-mediated immune escape by inactivating the c-MYC and EGFR/mitogen-activated protein kinase pathways.

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INTRODUCTION

Immune escape is one of the hallmark features of primary malignant lung cancers, of which more than 85% are classified as non-small cell lung cancer (NSCLC).[1–3] Although conventional treatments have improved the treatment effects of NSCLC patients, the unsatisfied overall curative effects have directed the attention of medical researchers to immunotherapy. Immune checkpoint blockade, especially programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) blockade, is one of the most effective immunotherapies in treating NSCLC. However, the objective responses have been manifested in a fraction of patients. Therefore, understanding the relative regulatory mechanisms of PD-L1 becomes important for establishing effective therapies.

PD-L1 is overexpressed in multiple human cancers, such as lung, pancreas, ovary and colon cancer and melanoma.[4,5] The primary function of PD-L1 is to transmit inhibitory signals that could increase apoptosis of antigen-specific human T-cell clones and induce differentiation of naive $CD4^+$ T cells into regulatory T cell and maintains regulatory T cell-suppressive functions via interacting with PD-1.[6–8] Considering the critical functions of PD-L1 in immune suppression, demonstrating the relative regulatory mechanisms is essential to establish new therapies. Recent studies have demonstrated that MYC inactivation could enhance the antitumor immune response by downregulating the expression of CD47 (Cluster of Differentiation 47) and PD-L1 in mouse cancers and human tumors.[9] Importantly, increasing studies have verified that PD-L1 expression is related to mutation status of epithelial growth factor receptor (EGFR), which has been proven to occur frequently in lung adenocarcinoma.[10–12] Numerous studies have indicated that EGFR-tyrosine kinase inhibitors (TKIs) including Gefitinib and Erlotinib can reverse apoptosis of T cells by inhibiting PD-L1 expression.[11] However, the synergistic effect of EGFR-TKIs and anti-PD-1 antibody in NSCLC remains unclear.[12] Thus, clarifying the related regulatory mechanisms of PD-L1 is essential for exploring effective treatment methods for NSCLC.

Bridging integrator-1 (BIN1) is a MYC-interacting adaptor protein that has features of a tumor suppressor.[13] BIN1 is frequently attenuated or even silenced in human tumors including melanoma, breast, prostate, bladder and lung cancers.[14,15] Multiple researches have indicated that losing of BIN1 has great significance in driving progression of cancers.[16–19] However, whether loss of BIN1 has effect on the progression of NSCLC remains unclear. BIN1 functionally correlates with c-MYC in tumors and suppresses its tumorigenesis and transactivation characteristics in a binding domain-dependent manner.[20–22] Recently, numerous researches have indicated that MYC regulates the expression of CD47 and PD-L1.[9] These researches raised the

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possibility that BIN1 involved in the mechanisms of PD-L1 regulation. To the best of our knowledge, no research has focused on the functions of BIN1 in the regulation of PD-L1.

Here we observed that PD-L1 was negatively related to BIN1 expression, and that they were both significantly associated with clinicopathological parameters of NSCLC patients. At the same time, we found that the expression statuses of BIN1 and PD-L1 could affect the infiltration of tumor-infiltrating lymphocytes (TILs) in NSCLC tissues. Importantly, BIN1 exerted its PD-L1 down-regulating effect by inactivating the c-MYC and EGFR signaling pathways, whose functions have been intensively studied in NSCLC. Furthermore, overexpression of BIN1 could reverse immune escape in an NSCLC-bearing mouse model by suppressing PD-L1 expression. Our results revealed a novel effect of BIN1 on the expression of PD-L1 in NSCLC and laid a foundation for establishing a BIN1-based therapeutic strategy for reversing the suppressive immuno-microenvironment.

RESULTS

Correlation between BIN1 and PD-L1 expression with NSCLC clinicopathological parameters and cancer-specific survival for NSCLC patients

To reveal the expression pattern of BIN1 and PD-L1, we detected their expressions in 179 NSCLC tissues using immunohistochemistry. BIN1 staining in cell nuclei was rare in cancer cells, whereas PD-L1 staining on cytomembranes and in the cytoplasm was mainly present in cancer cells and infiltrating immune cells (Figures 1a and b). As shown in Table 1 and Figure 1c, there was a negative association between the expression of PD-L1 and BIN1 in NSCLC tissues at both the protein and mRNA level ($P < 0.001$).

Then we evaluated the potential effects of BIN1 and PD-L1 in NSCLC tumorigenesis by analyzing their relationships with NSCLC clinicopathological parameters. The expression statuses of BIN1 and PD-L1 were closely related to the tumor, lymph node and metastasis grade (TNM) stage, invasion range and lymph node metastasis, but not related with gender or age, indicating that BIN1 low expression and PD-L1 high expression might account for the progression of NSCLC (Table 2). Furthermore, by using univariate analysis, we found that BIN1 expression, PD-L1 expression, TNM stage, invasion depth and lymph node metastasis were the factors significantly related to survival, (all $P < 0.05$), whereas gender and age were not (all $P > 0.05$). Kaplan–Meier analysis indicated that BIN1 low expression and PD-L1 high expression were correlated with worse overall survival, respectively (log-rank test: both $P < 0.001$; Figures 1d and e). The median survival times for patients with low and high BIN1 expression were 15 and 25 months, respectively, which meant that comparing with the NSCLC patients with high BIN1 expression had better prognosis than those with low expression (unadjusted hazard ratio: 0.404, 95% confidence interval: 0.284–0.574, $P < 0.001$). Contrastively, for the patients with high and low PD-L1 expression, the median survival times were 16 and 22 months, respectively, indicating that the high PD-L1 expression was significantly correlated with shorter overall survival of NSCLC patients (unadjusted hazard ratio: 2.717, 95% confidence interval: 1.905–3.873, $P < 0.001$). In conclusion, these results displayed that BIN1 low expression and PD-L1 high expression were both related to poor prognosis of NSCLC.

The relationship between BIN1, PD-L1 and TILs in NSCLC

To confirm the association between BIN1, PD-L1 and TILs in NSCLC, we explored the expression statuses of $CD3^+$ TILs and $CD8^+$ TILs in 179 NSCLC tissues using immunohistochemistry (Figures 2a and b). We scored a sample as $CD3^+$ TIL and $CD8^+$ TIL positive when at least 30% of tumor cells showed specific staining.[23] Using this cutoff, we found that 92 samples (51.4%) were $CD3^+$ TILs cell positive, 57 samples (61.96%) of $CD3^+$ TILs cell-positive tissues were BIN1 positive and 41 samples (44.57%) were PD-L1 positive; as shown in Table 3, there was a positive association between the expression of BIN1 and $CD3^+$ TILs in NSCLC tissues and a negative correlation between the expression of PD-L1 and $CD3^+$ TILs in NSCLC tissues. For the expression status of $CD8^+$ TILs cells, 72 samples (40.2%) were $CD8^+$ TILs cell positive, 47 samples (65.28%) of $CD8^+$ TILs cell-positive tissues were BIN1 positive and 32 samples (44.44%) were PD-L1 positive. The results demonstrated that the expression of $CD8^+$ TILs was positively associated with BIN1 and negatively related to PD-L1 in NSCLC tissues (Table 4). In conclusion, high expression of BIN1 was significantly related to higher percentage of TILs and high expression of PD-L1 was associated with lower proportion of TILs, raising a possibility that the overexpression of BIN1 could promote infiltration of TILs.

BIN1 inhibited PD-L1 expression in NSCLC cell lines

We detected the expression levels of BIN1 and PD-L1 in NSCLC cell lines and the human embryo lung cell line 2BS. Compared with 2BS, BIN1 was attenuated or lost in H1975, A549, HCC827, H1299 and H1650 cells (Figures 3a and b, $P < 0.05$); inversely, PD-L1 was highly expressed in H1975, HCC827, H1299 and H1650 cells (Figures 3a and b, $P < 0.05$). Then, to validate the possible regulating function of BIN1 in PD-L1 expression, cell lines that stably overexpressed BIN1 was constructed. BIN1-overexpressed H1975 and HCC827 cells revealed a notable reduction of PD-L1 expression (Figures 3c–f, $P < 0.05$). Furthermore, we transfected BIN1-small interfering RNA (siRNA) or empty vector into H460 and H1299 cells, which had high BIN1 expression. Knockdown of BIN1 caused a notable upregulated expression of PD-L1 (Figures 3g–j, $P < 0.001$). Consequently, our results demonstrated that BIN1 could inhibit the expression of PD-L1 in NSCLC cell lines. Besides, we also found that BIN1 overexpression suppressed the malignant biological behaviors of NSCLC cells (Supplementary Figures 1 and 2, $P < 0.05$).

BIN1 overexpression inhibited the expression of PD-L1 by inactivating c-MYC

The most primary function of BIN1 is to suppress the activation of c-MYC; however, the effect of BIN1 on c-MYC activation in NSCLC remains unclear. As shown in Figure 4a, BIN1 and c-MYC were mainly located in the nucleus with little distribution in the cytoplasm and cytomembrane ($P < 0.05$). Double immunofluorescence clearly showed that the expression of c-MYC in the nucleus was reduced in BIN1-overexpressed H1975 cells (Figure 4a, $P < 0.05$). These results demonstrated that BIN1 could inhibit the activation of c-MYC.

As MYC could regulate the expression of PD-L1, we explored whether c-MYC was the only mechanism of PD-L1 regulation when c-MYC was 'off.' In the BIN1-overexpressed H1975 cells, siRNA knockdown of c-MYC caused a reduction in the level of PD-L1 mRNA and protein (Figures 4b and c, $P < 0.001$), but PD-L1 was still expressed. Together, we concluded that BIN1 could regulate the expression of PD-L1 in other c-MYC-independent pathways.

BIN1 overexpression suppressed the expression of PD-L1 by inactivating the EGFR/MAPK signaling pathway in NSCLC cells

To explore the potential effect of the EGFR/mitogen-activated protein kinase (MAPK) pathway on PD-L1 expression in NSCLC cells, we first characterized whether PD-L1 expression could be increased after activation of the EGFR/MAPK signaling pathway. Treated with EGF stimulant in low PD-L1-expressing A549 cells increased the phosphorylation and activation of EGFR and ERK, indicating that EGFR/MAPK signaling was activated (Figure 5a,

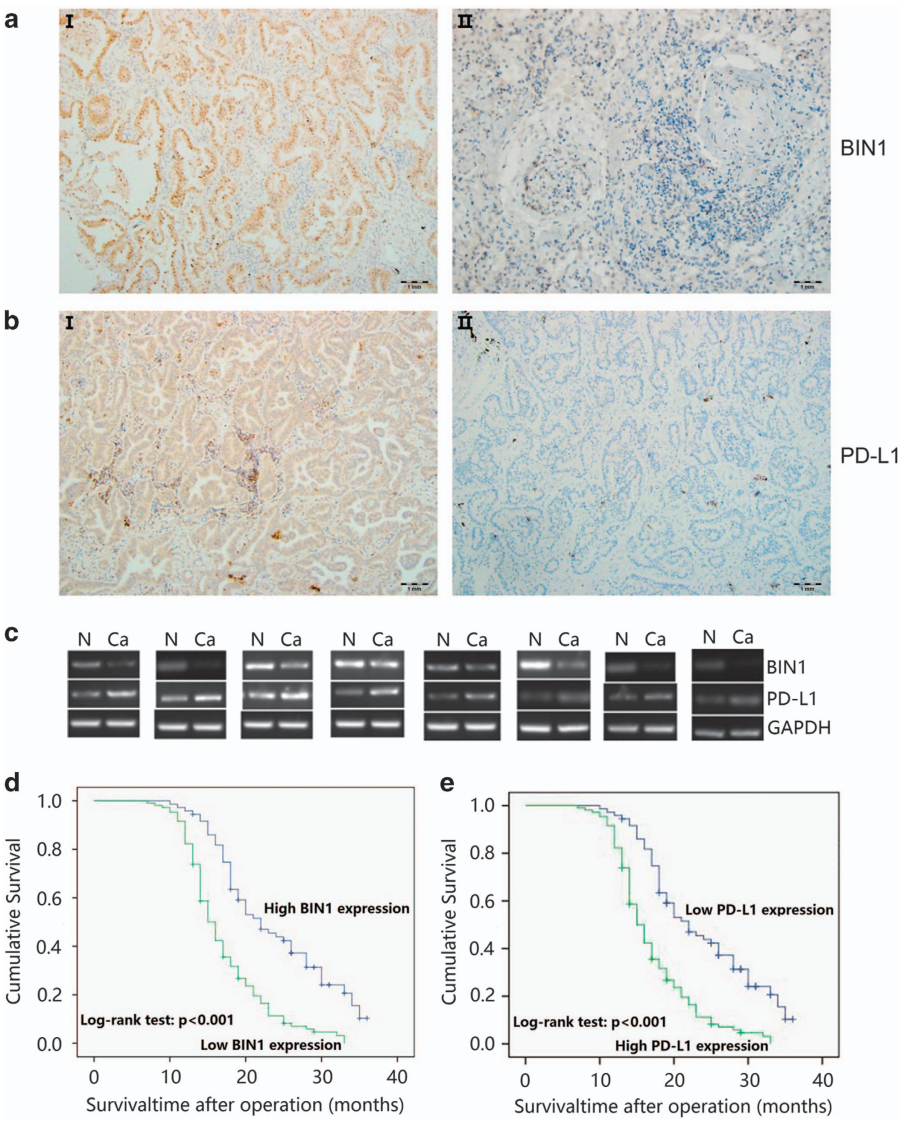

Figure 1. Expression statuses of BIN1 and PD-L1 in NSCLC tissues. (**a**) BIN1 protein expression in NSCLC tumor tissues (SP × 100). I: Positive staining of BIN1 in NSCLC tissue; II: Negative staining of BIN1 in NSCLC tissue. (**b**) PD-L1 protein expression in NSCLC tumor tissues (SP × 100). I: Positive staining of PD-L1 in NSCLC tissue; II: Negative staining of PD-L1 in NSCLC tissue. (**c**) The gene expression of *BIN1* and *PD-L1* in normal tissues and corresponding NSCLC tumor tissues. N for normal lung tissues, and Ca for NSCLC tissues. (**d**) Correlation between low BIN1 expression and poor patient survival. (**e**) Correlation between high PD-L1 expression and poor patient survival.

Table 1. Correlation between the expression of BIN1 and PD-L1 in NSCLC tissues

PD-L1	*BIN1*			*Spearman's correlation*	
	High	*Low*	*Total*	*r*	P-*values*
High	17	90	107	−0.555	< 0.001
Low	51	21	72		
Total	68	111	179		

Abbreviations: BIN1, bridging integrator-1; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1.

$P < 0.001$). At the same time, we found that PD-L1 expression was highly upregulated compared with control cells (Figure 5a, $P < 0.001$). Treatment with EGFR inhibitor Icotinib in PD-L1 high expressing H1975 cells led to a slightly decrease in the levels of p-EGFR and p-ERK, and reduced the expression of PD-L1 protein (Figure 5b; $P < 0.001$). Then, we used the MEK inhibitor GSK1120212 to further determine the effect of MAPK signaling activation on the expression of PD-L1. The addition of MEK inhibitor significantly suppressed the phosphorylation and activation of ERK, as expected, and inhibited the expression of PD-L1 in NSCLC cells (Figure 5c, $P < 0.001$).

Furthermore, we assessed the effect of BIN1 overexpression on the activation of EGFR/MAPK signaling pathway. A survey of molecules involved in EGFR/MAPK signaling pathway further demonstrated the decreased activation and phosphorylation of EGFR, MEK and ERK in BIN1-overexpressed H1975 cells when compared with empty vector group and the control group (Figure 5d, $P < 0.001$).

BIN1 overexpression could inhibit the tumorigenesis of NSCLC and associated mechanisms

To clarify the function of BIN1 in tumorigenesis *in vivo*, we injected BIN1-overexpressed H1975 cells into nude mice and the average

Table 2. Associations of BIN1 and PD-L1 in NSCLC tissues with patient clinical parameters at protein level

	n	*Expression of BIN1* (n)		P-*values*	*Expression of PD-L1* (n)		P-*values*
		High	*Low*		*High*	*Low*	
Gender							
Male	117	43 (36.75%)	74 (63.25%)	0.493	72 (61.54%)	45 (38.46%)	0.326
Female	62	25 (40.32%)	37 (59.68%)		35 (56.45%)	27 (43.55%)	
Age (years)							
⩽60	113	44 (38.94%)	69 (61.06%)	0.834	68 (60.18%)	45 (39.82%)	0.767
>60	66	24 (36.36%)	42 (63.64%)		39 (59.09%)	27 (40.91%)	
TNM stage							
I+II	100	60 (60%)	40 (40%)	< 0.001	41 (41%)	59 (59%)	0.001
III	79	8 (10.13%)	71 (89.87%)		66 (83.54%)	13 (16.46%)	
Invasion range							
T1+T2	68	43 (63.24%)	25 (36.76%)	<0.001	20 (29.41%)	48 (70.59%)	0.010
T3	111	25 (22.52%)	86 (77.48%)		87 (78.38%)	24 (21.62%)	
Lymph node metastasis							
Negative	89	57 (64.04%)	32 (35.96%)	<0.001	30 (33.71%)	59 (66.29%)	<0.001
Positive	90	11 (12.22%)	79 (87.78%)		77 (85.56%)	13 (14.44%)	

Abbreviations: BIN1, bridging integrator-1; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1.

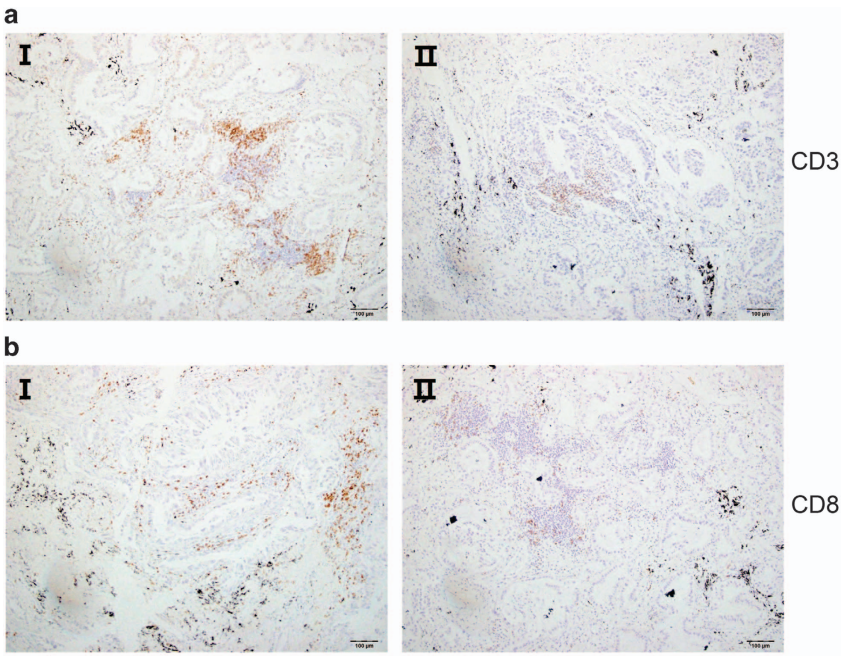

Figure 2. Representative views of TILs expression in NSCLC tissues. (**a**) $CD3^+$ TILs expression in NSCLC tumor tissues (SP × 100). I: Positive staining of $CD3^+$ TILs in NSCLC tissue; II: Negative staining of $CD3^+$ TILs in NSCLC tissues. (**b**) $CD8^+$ TILs expression in NSCLC tumor tissues (SP × 100). I: Positive staining of $CD8^+$ TILs in NSCLC tissue; II: Negative staining of $CD8^+$ TILs in NSCLC tissues.

volume of cancers formed in BIN1-overexpressed mice was notably smaller when compared with control cells (Figure 5e, $P < 0.05$), which demonstrated that BIN1 could inhibit the tumorigenesis of NSCLC. In addition, to identify the mechanisms of immune regulation by BIN1 *in vivo*, we detected the expression alterations of immunosuppressive related factor PD-L1 and major molecules involved in EGFR/MAPK signaling pathway (p-EGFR) by flow cytometry with nude mice tumor tissues and the results demonstrated that BIN1-transfected mice had a reduction of p-EGFR and PD-L1 expression compared with control groups (Figure 5f, $P < 0.001$). These results indicated that restoration of BIN1 expression could inhibit immune escape by inactivating the EGFR/MAPK/PD-L1 axis *in vivo*.

DISCUSSION

In this study, we investigated a potential role of BIN1 in inhibiting tumor immune escape in NSCLC. The current study provides evidence indicating that BIN1 can reverse the suppressive immuno-microenvironment of NSCLC, and that the underlying

Table 3. Correlation between the expression of BIN1, PD-L1 and $CD3^+$ TILs in NSCLC tissues

$CD3^+$ TILs	*BIN1*			*Spearman's correlation*		*$CD3^+$ TILs*	*PD-L1*			*Spearman's correlation*	
	High	*Low*	*Total*	*r*	P-*values*		*High*	*Low*	*Total*	*r*	P-*values*
High	57	35	92	0.508	<0.001	High	41	49	92	−0.292	<0.001
Low	11	76	87			Low	66	23	87		
Total	68	111	179			Total	107	72	179		

Abbreviations: BIN1, bridging integrator-1; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; TIL, tumor-infiltrating lymphocyte.

Table 4. Correlation between the expression of BIN1, PD-L1 and $CD8^+$ TILs in NSCLC tissues

$CD8^+$ TILs	*BIN1*			*Spearman's correlation*		*$CD8^+$ TILs*	*PD-L1*			*Spearman's correlation*	
	High	*Low*	*Total*	*r*	P-*values*		*High*	*Low*	*Total*	*r*	P-*values*
High	47	25	72	0.461	<0.001	High	32	40	72	−0.256	0.001
Low	21	86	107			Low	75	32	107		
Total	68	111	179			Total	107	72	179		

Abbreviations: BIN1, bridging integrator-1; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; TIL, tumor-infiltrating lymphocyte.

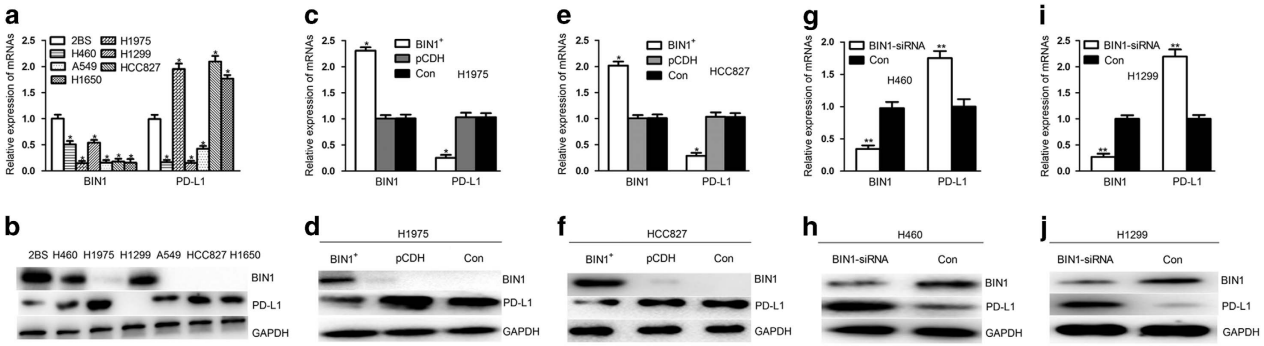

Figure 3. BIN1 overexpression inhibited the expression of PD-L1. (**a**) The mRNA expression of *BIN1* and *PD-L1* in NSCLC cell lines and embryo lung cells detected by quantitative reverse-transcription PCR (qRT–PCR). (**b**) The protein expression of BIN1 and PD-L1 in NSCLC cell lines and embryo lung cells detected by western blotting. (**c**) Overexpression of *BIN1* could inhibit *PD-L1* expression at gene level in H1975. (**d**) Overexpression of BIN1 could inhibit PD-L1 expression at protein level in H1975 cells. (**e**) Overexpression of *BIN1* could inhibit *PD-L1* expression at gene level in HCC827 cells. (**f**) Overexpression of BIN1 could inhibit PD-L1 expression at protein level in HCC827 cells. (**g**) Knockdown of *BIN1* expression significantly increased *PD-L1* expression at gene level in H460 cells. (**h**) Knockdown of BIN1 expression significantly increased PD-L1 expression at protein level in H460 cells. (**i**) Knockdown of *BIN1* expression significantly increased *PD-L1* expression at gene level in H1299 cells. (**j**) Knockdown of BIN1 expression significantly increased PD-L1 expression at protein level in H1299 cells. GAPDH served as an internal control. Significant *P*-values marked by asterisk: $*P<0.05$ and $**P<0.001$.

mechanisms might involve PD-L1, c-MYC and the EGFR/MAPK signaling pathways. Our results first demonstrated that in tumor tissues of NSCLC patients, low expression of BIN1 was observed, which was negatively correlated with PD-L1 expression. In addition, the expression statuses of BIN1 and PD-L1 were both associated with poor prognosis, at the same time, survival analysis results indicated that they might be independent prognostic factors for NSCLC patients. Furthermore, we found that the expression statuses of BIN1 and PD-L1 could affect the ratio of TILs in NSCLC tissues. In particular, we reported for the first time that BIN1 could downregulate the expression of PD-L1 by inactivating the c-MYC and EGFR/MAPK signaling pathways. Taken together, our study indicated that BIN1 could act as a tumor suppressor by reversing the suppressive immuno-microenvironment of NSCLC.

BIN1 is a crucial adaptor protein in many cytobiological activities such as cell cycle progression, apoptosis and immune regulation. Low expression of BIN1, which was frequent in numerous tumor types, had been reported to contribute to cancer progression. Our previous study demonstrated that BIN1 expression was low in ESCC tissues, and that this low expression was negatively related to the prognosis of ESCC patients.[18] In the current study, we found that BIN1 was also attenuated in NSCLC and loss of BIN1 was related to poor NSCLC clinicopathological parameters including high TNM stage (stage III), wide tumor invasion (T3) and positive lymph node metastasis. Importantly, Muller *et al.*[24] demonstrated that BIN1 could enhance T-cell-dependent antitumor activity by inhibiting indoleamine 2, 3-dioxygenase (IDO) expression in mouse tumors, which extended the function of BIN1 to tumor immunology.[25] In the present study, we found that PD-L1 expression was negatively correlated with BIN1 expression in NSCLC tissues and high expression of PD-L1 was associated with high TNM stage (stage III), wide tumor invasion (T3) and positive lymph node metastasis of NSCLC patients. After multivariate analysis, the aberrant expression statuses of BIN1 and PD-L1 were found to be predictors of poor prognosis in NSCLC patients. Multiple researches have

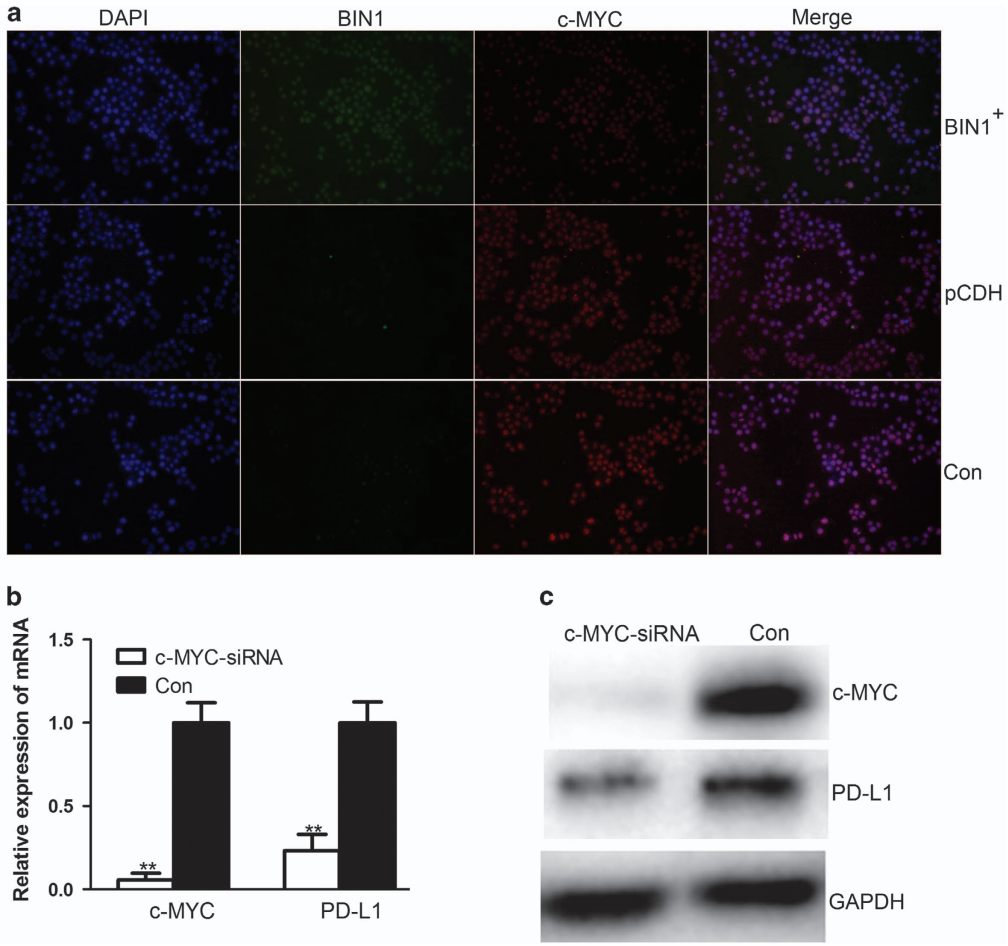

Figure 4. BIN1 inhibited the expression of PD-L1 via interacting with c-MYC *in vitro*. (**a**) Double immunofluorescent microscopy to identify BIN1 and c-MYC in BIN1-overexpressed H1975 cells and that of control cells. (**b**) Knockdown of c-MYC expression significantly decreased PD-L1 expression at mRNA level in H1975 cells. Data shown are means ± s.d. ($n \geqslant 3$, $^{**}P < 0.001$). (**c**) Knockdown of c-MYC expression significantly decreased PD-L1 expression at protein level in H1975 cells. GAPDH was used as an internal control.

demonstrated that high presence of TILs was related to better clinical outcome.[26,27] The TILs, especially $CD8^+$ T cells have been reported to be a good prognostic factors in many tumors, including NSCLC.[28–30] In our study, we also found that the expression of TILs was positively associated with BIN1 and negatively related to PD-L1 in NSCLC tissues. More importantly, BIN1 overexpression could inhibit PD-L1 expression in NSCLC cells and BIN1 overexpression could inhibit the proliferation, cell cycle arrest, migration and invasion of NSCLC cells. BIN1 was initially identified as a tumor suppressor directly interacting with the N-terminal of c-MYC protein.[24,31] Several studies have demonstrated that attenuation of BIN1 had great significance in driving progression of cancers, whereas its overexpression in cancers could inhibit cell proliferation, the cell cycle, cell migration and induce apoptosis via MYC-dependent or MYC-independent mechanisms.[32,33] The present study demonstrated that BIN1 could suppress the activation of c-MYC. At the same time, our research also demonstrated that BIN1 overexpression could suppress the activation of the EGFR/MAPK signaling pathways, but those specific mechanisms remain to be further explored.

PD-L1, an important immunosuppressive factor, has a key role in immune escape by interacting with PD-1.[34–37] Recent studies showed that blocking the PD-1/PD-L1 pathway had great clinical significance.[38] Our results demonstrated that overexpression of BIN1 could inhibit the expression of PD-L1. Recently, increasing studies have demonstrated that PD-L1 expression is related to MYC and aberrant activation of EGFR.[9–12,39] Significantly, MYC inactivation in mouse tumors was able to downregulate CD47 and PD-L1 expression, and enhance the antitumor immune response.[9] Lastwika *et al.* indicated that activation of the AKT-mTOR but not MAPK pathway promoted immune escape by driving expression of PD-L1.[40] However, Ota *et al.*[41] showed that both EML4-ALK and mutant forms of EGFR modulated PD-L1 expression via common downstream pathways mediated by MEK-ERK and PI3K-AKT in NSCLC. In the current study, we found that knockdown of c-MYC could inhibit the expression of PD-L1, whereas the EGFR/MAPK pathway could induce the expression of PD-L1 in NSCLC. In addition, the MEK inhibitor GSK112012 could inhibit PD-L1 expression. Akbay *et al.*[11] proposed that EGFR-TKIs could inhibit PD-L1 expression, and Chen *et al.*[12] demonstrated that apoptosis of T cells could be reversed after EGFR-TKI treatment. Our studies indicated that Icotinib could also suppress the expression of PD-L1.

Although multiple researches have focused on PD-L1 expression and its immunosuppressing effect in NSCLC, we are the first to report its expression and relation to BIN1 in NSCLC patients, to determine prognostic significances. Our studies indicated that the expression statuses of BIN1 and PD-L1 were closely associated with clinical pathological characteristics and the proportion of TILs. Meanwhile, we are the first to discover that BIN1 can inhibit c-MYC/PD-L1 and the EGFR/MAPK/PD-L1 axis. In addition, restored expression of BIN1 was able to suppress the malignant biological functions of NSCLC cells. Thus, these results demonstrated that

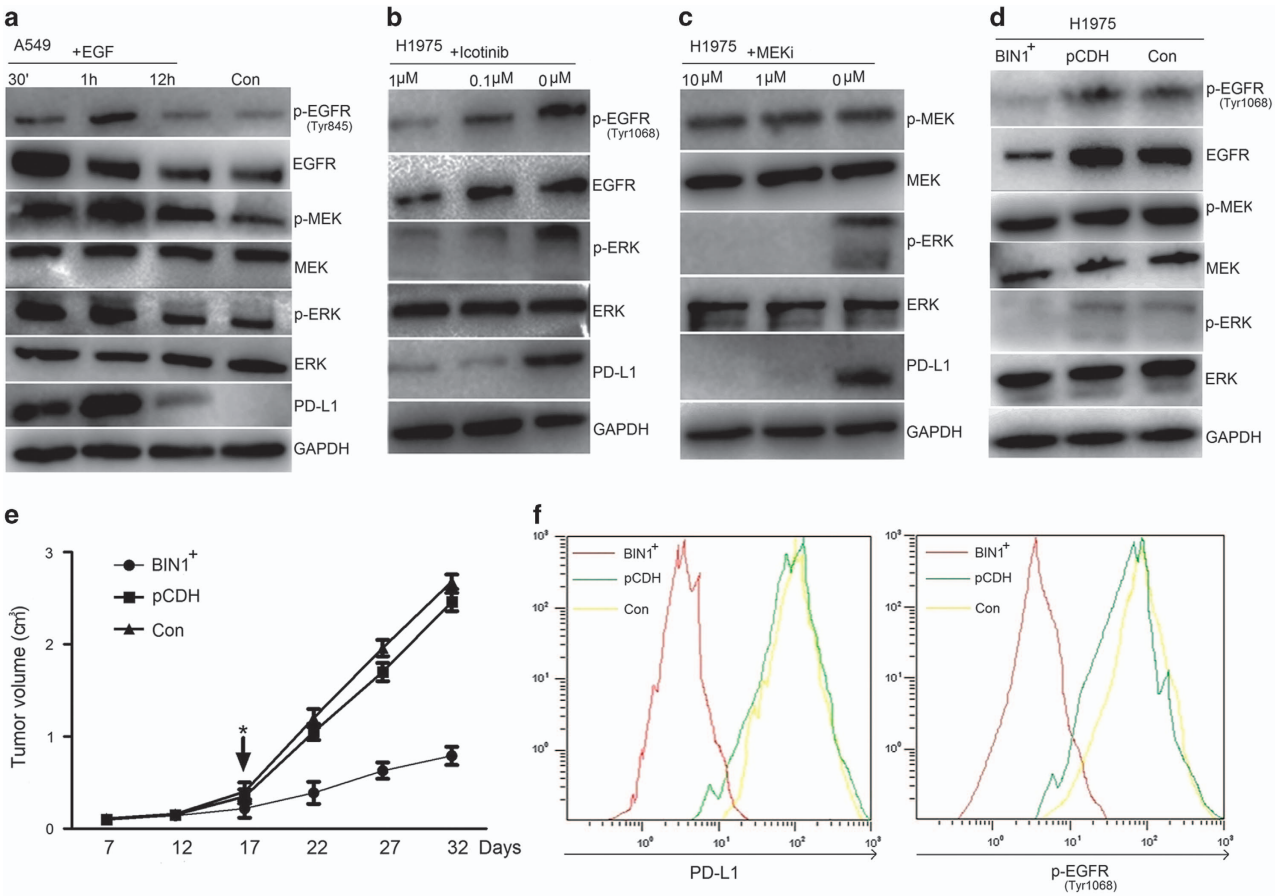

Figure 5. EGFR/MAPK signaling pathway induced the expression of PD-L1 and the effect of BIN1 on NSCLC tumorigenesis. (**a**) EGF-upregulated PD-L1 protein expression. A549 cells were either left untreated or treated with EGF (10 ng/ml) for 30 min, 1 and 12 h. Cells were collected and PD-L1 expression was determined by western blotting. GAPDH was used as an internal control. (**b**) Icotinib reduced PD-L1 protein expression in H1975 cells. Cells were either left untreated or treated with Icotinib (1 μM or 0.1 μM) for 24 h. Cells were collected and PD-L1 expression was determined by western blotting. GAPDH was used as an internal control. (**c**) MEK inhibitor GSK112012 significantly inhibited the activation of ERK and suppressed PD-L1 expression in H1975 cells. Cells were treated with MEK inhibitor (MEKi, 1 μM or 0.1 μM) for 48 h and PD-L1 expression level was determined by western blotting. (**d**) The protein levels of EGFR/MAPK signaling pathway-related proteins EGFR, p-EGFR, ERK, p-ERK, MEK and p-MEK in H1975 cells were detected by western blotting. (**e**) The tumor volume of nude mice with or without BIN1 vector-transfected H1975 cells. Data shown are means ± s.d. ($n \geqslant 3$, $^*P < 0.05$). (**f**) Flow cytometry (FCM) assay showing expression levels of PD-L1 and p-EGFR in tumor tissues in three groups' tumor. GAPDH was used as an internal control.

BIN1 was a potential protein regulator of PD-L1, which could reverse the suppressive immuno-microenvironment of NSCLC.

MATERIALS AND METHODS

Materials

Antibodies to total PD-L1 (E1L3N), MEK (D2R10), p-MEK (166F8), ERK (D3I5V), p-ERK (197G2), EGFR (15F8) and p-EGFR (1H12) were all purchased from Cell Signaling Technology, Inc. (Boston, CA, USA). Antibodies to total BIN1 (EPR13463-25), c-MYC (9E10), GAPDH (ab9485), p-EGFR (EPR2149Y), CD3 (PS1) and CD8 (144B) were purchased from Abcam, Inc. (Cambridge, MA, USA). The BIN1-expressing lentivirus vector pCDH-BIN1 (pCDH-CMV-MCS-EF1-puro-BIN1) and the control vector pCDH (pCDH-CMV-MCS-EF1-puro) and c-MYC-siRNA were purchased from GenePharma Company (Shanghai, China). Go Taq qPCR Master Mix was purchased from Promega (Madison, WI, USA). RevertAid First Strand cDNA Synthesis Kits was purchased from MBI Fermentas (Hanover, MD, USA). Annexin V phycoerythrin and 7-aminoactinomycin D (7-AAD) double stain was purchased from BD Pharmingen (San Diego, CA, USA). EGF stimulant was obtained from PROSPEC (cyt-217, East Brunswick, CA, USA). EGFR TKIs (Icotinib) and MEK inhibitor (GSK1120212) were all purchased from Selleckchem (Shanghai, China).

Patients and specimens

The standard of tissues collection for our study was similar as previously described.[18]

Silencing BIN1 expression in H460 cells and knockdown c-MYC expression in H1975 cells

The siRNA sequences of c-MYC were as follows: c-MYC siRNA (target sequence: 5′-AACGUUAGCUUCACCAACAUU-3′) and control siRNA (target sequence: 5′-AAUUCUCCGAACGUGUCACGU-3′). The siRNA sequences of BIN1 and the transient transfection procedure was similar as previously described.[42]

RNA extraction and quantitative reverse-transcription PCR

Reverse-transcription PCR primers for human *BIN1* mRNA on the basis of the sequence in NCBI (accession number U68485). *BIN1* forward 5′-C AAGTCCCCATCTCAGCCAG-3′, reverse 5′-GGATCACCAGCACCACATCA-3′, *GAPDH* forward 5′-ACCACAGTCCATGCCATCACT-3′, reverse 5′-TCCACCACCC TGTTGCTGTA-3′. *PD-L1* forward 5′-CCTACTGGCATTTGCTGAACGCAT-3′, reverse 5′-ACCATAGCTGATCATGCAGCGGTA-3′. The experiments were triplicated.

Immunohistochemical assay for detection of BIN1, PD-L1 and TILs protein levels

The protein levels of BIN1, PD-L1 and TILs in NSCLC tissues were analyzed by immunohistochemistry as previously described.[18]

Western blotting assays for detection of BIN1 and PD-L1 protein levels

The protein levels of BIN1 and PD-L1 in NSCLC cells were analyzed by western blotting as previously described.[42]

Immunofluorescence

Immunofluorescence assay was performed to identify the location of BIN1 and c-MYC in H1975 cells and the effect of BIN1 overexpression on c-MYC activation, as previously described.[42]

In vivo tumor growth assay

BALb/c nude mice were used for tumor growth assay *in vivo* and the assay was performed as previously described.[43]

Flow cytometry assay

The mechanisms of immune regulation by BIN1 *in vivo* were assessed by flow cytometry analysis. Briefly, mice were killed and whole tumor tissues were cut into small pieces and prepared in collagenase containing buffer, and 10% FBS in RPMI1640 medium for 45 min. Then, RBC lysis buffer was administrated to cells and debris was removed through a cell strainer. For flow cytometry analysis, the cell pellet was dissolved by 2% fetal calf serum in Hank's balanced salt solution. Effect of BIN1 on cell cycle were performed as previously described.[43]

Cell viability assay and colony formation assay

The effect of BIN1 on NSCLC cell viability and colony formation was determined by MTT reduction (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and colony-forming assay, as previously described.[44]

Tumor cell migration and invasion assays

Wound-healing and transwell assay experiments were performed as previously described.[45]

Statistical analysis

All statistical analyses were performed with SPSS statistics software, version 20.0 (SPSS, Chicago, IL, USA). For NSCLC, Spearman's rank correlation was used to analyze the association of BIN1, PD-L1 and TILs expression at protein level. The methods of survival analysis was similar as previously described.[18] A P-value < 0.05 was considered to be statistically significant and all P-values were two-tailed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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