Original Article

Applications of novel monoclonal antibodies specific for Ciz1 in evaluating its levels in cancer tissues and plasma from lung cancer patients

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Abstract: Backgrounds: Cip1-interacting zinc-finger protein1 (Ciz1) is a nuclear matrix-associated DNA replication factor which has been found to be overexpressed in several types of cancers. Ciz1 is also negatively correlated with the prognosis of patients with colorectal cancer. However, the relationship between Ciz1 and prognosis of lung cancer patients remains elusive. This study was designated to examine Ciz1 expression pattern and its potential as a biomarker of prognosis in lung cancer. Methods: We generated monoclonal antibodies (mAbs) against Ciz1 using hybridoma fusion technology. With one mAb, we detected the levels of Ciz1 in lung cancer cell lines as well as tissues and plasma from lung cancer patients by Western blot. In addition, we examined Ciz1 levels in the human lung adenocarcinoma tissue microarray by immunohistochemical staining. We also investigated the prognostic value of Ciz1 in lung cancer by Kaplan-Meier method. Results: Six mAbs against Ciz1 were generated. Ciz1 protein levels in plasma from lung cancer patients were found to be higher than those from benign patients (P = 0.0016). Ciz1 was also highly expressed in lung adenocarcinoma compared with its corresponding adjacent tissues (P = 0.0017). Importantly, high expression of Ciz1 was negatively correlated with the prognosis of lung adenocarcinoma patients (P = 0.0298), which was further confirmed by network database analysis. Conclusion: Our results suggested that Ciz1 may be involved in lung cancer progression and could be utilized as a novel prognostic factor for lung adenocarcinoma patients.

Keywords: Ciz1, monoclonal antibody, lung cancer, immunoassay

Introduction

Ciz1 (Cdkn1A-interacting zinc finger protein1) was originally identified in the yeast two hybrid system to screen proteins capable of interacting with CDK2 inhibitor p21Cip1/Waf1 [1]. The C-terminal domain of Ciz1 is attached to nuclear matrix, and resides within foci that partially colocalize with sites of DNA replication [2, 3]. Ciz1 participates in the assembly of DNA pre-replication by binding to cyclinE and recruiting cdc6 in the G1 phase and cooperates with cyclin A and CDK2 to promote mammalian DNA replication [4-6]. In addition, Ciz1 may be involved in DNA damage repair as the substrate of ATM kinase [7]. Based on these studies, Ciz1 is proposed to function as a “mediator” that bridges cell cycle regulators during cell cycle progression. Considering that deregulated cell cycle is tightly associated with several diseases, it is tempting to evaluate Ciz1’s roles in disease progression.

Recently, Ciz1 is reported to be associated with Alzheimer’s disease [3, 8], dystonia [9], and rheumatoid arthritis [10]. The expression of Ciz1 is increased in several types of cancers, including Ewing’s tumor [11], prostate carcinoma [12], and gastric cancer [13]. Alternative splicing of Ciz1 transcript leads to at least 22 variants, most of which are not characterized [14]. Abnormal alternative splicing is the basis for its association with a range of diseases, including pediatric tumors, breast cancer, lung
Ciz1 as a novel prognostic factor for lung cancer

cancer [15] as well as neurological abnormalities [16]. Ciz1 is upregulated in gallbladder cancer, in which it can interact with TCF4 and positively regulates Wnt signaling, thereby promoting the proliferation and migration of cancer cells [17]. In breast cancer, Ciz1 binds to estrogen receptors and increases the expression of estrogen downstream genes, causing the genesis of breast cancer [3]. The mRNA and protein levels of Ciz1 in colorectal cancer tissues are highly expressed and negatively correlated with the prognosis of patients [18]. However, the prognostic role of Ciz1 in other cancers remains unclear. In lung cancer, an alternative splicing variant of Ciz1 (Ciz1b) can classify 98% of patients of lung cancer from normal controls. By detecting Ciz1b, 95% sensitivity was achieved to distinguish non-small cell lung cancer and benign lung nodules [15]. However, there is no anti-Ciz1 monoclonal antibody (mAb) available and the previous studies on detecting Ciz1 protein levels utilized anti-Ciz1 polyclonal antibodies [11, 12, 14, 15, 17-19].

In this study, we generated a panel of Ciz1-specific mAbs. With these novel Ciz1 mAbs, we found elevated levels of Ciz1 in lung cancer cell lines, plasma samples and tissues from lung cancer patients and further revealed its negative correlation with the prognosis of lung adenocarcinoma patients. Our results validate the clinical applications of these mAbs and suggest that Ciz1 is a potential biomarker for lung cancer.

Materials and methods

Antigens

Ciz1 upstream fragment (Ciz1U) is 450 bp in length located at 1056~1505 bp of human Ciz1 gene and encodes the 352aa~501aa region of Ciz1 protein (NM_012127.2). Ciz1U gene was cloned from an A549 cDNA library and inserted into pGEX4T1 vector to generate pGEX4T1-Ciz1U. The antigen GST-Ciz1U was purified by Glutathione-Sepharose beads from induced bacteria transformed with pGEX4T1-Ciz1U.

Generation and purification of anti-Ciz1 mAbs

To generate mAbs to human Ciz1, BALB/c mice (Vital River Laboratory, Beijing, China) were immunized with purified GST-Ciz1U protein (50 μg/mouse) emulsified in complete Freund’s adjuvant (Sigma) and were boosted every 4 weeks with the same amount of antigen in incomplete adjuvant. Seven days after the last injection, spleen cells from one immunized BALB/c mouse were fused with Sp2/0 cells. Culture supernatants were screened for the presence of specific anti-Ciz1 antibodies by indirect ELISA as described below. The stable hybridomas were expanded as ascitic fluids in BALB/c mice and the mAbs were purified by protein-A/G chromatography from the ascitic fluids. The purity and activity of the mAb preparations were monitored by SDS-PAGE and ELISA, respectively. Antibody concentrations were determined by measuring the absorbance at 280 nm (C_{mg/mL} = OD_{280} * 0.6768) and BSA as a protein standard on SDS-PAGE. Isotype determination of mAbs was done with the mouse mAb isotyping kit (Hycult Biotechnology BV).

Cell lines and plasmids

SP2/0 myeloma cells, lung cancer cell lines H1299, PC9, A549, H460 and PG, mouse embryonic fibroblast cell line NIH3T3 were obtained from ATCC. All the cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen).

Full-length human Ciz1 gene with myc-tag was synthesized by GENEWIZ (Suzhou, China) and inserted into the pcDNA3.0 vector. Plasmids were transiently transfected into cells cultured in 60 mm plates with Lipofectamine 2000 reagent (Thermo Fisher Scientific).

Enzyme-link immunosorbent assay (ELISA)

Microtiter plates (Costar) were coated with 5 μg/mL of GST or GST-Ciz1U fusion proteins at 4°C overnight. After washing, hybridoma supernatants were incubated for 1 h. Horseradish peroxidase-labeled goat anti-mouse immunoglobulin (1:2000) was used as the secondary antibody. Plates were incubated for another 1 h, washed and substrate solution (0.01% (w/v) 3,3′,5,5′-tetramethylbenzidine, 0.003% (v/v) H$_2$O$_2$, pH 5.0) was added. Substrate conversion was stopped by the addition of 2 mol/L H$_2$SO$_4$ and the absorbance was measured at 450 nm with a microplate reader (Bio-Rad, model 550).
Knockdown of Ciz1

For transient knockdown of Ciz1 in A549 and H1299 cells, following siRNAs (synthesized by GenePharma, Shanghai, China) were used: si-Ciz1-1#: 5'-GCAAGGACUGGACCAGUUUTT-3', si-Ciz1-2#: 5'-GGACACACCGAAAGACCAATT-3', si-Ciz1-3#: 5'-CCGGAUGACAGUACCGAAATT-3', NC: 5'-UUCUCCGAACGUGACACGU-3'. Cells were plated at a density of 2 × 10⁵ cells/well in six-well plates. 50 nM Ciz1 or NC siRNA was added to the cells by using Oligofectamine (Thermo Fisher Scientific) according to the manufacturer's protocol. After 48 h, the cells were collected and subjected to Western blot.

Western blot analysis and antibodies

To detect endogenous or exogenous Ciz1, cells were directly homogenized in 2 × loading buffer to prepare whole cell extracts. Fresh human lung cancer tissues (provided by the Tissue Bank of Peking University Cancer Hospital & Institute, Beijing, China) were homogenized in RIPA buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS) and 1 × protease inhibitor cocktail. Alternatively, plasma samples from patients with lung cancer or lung benign diseases (provided by the Department of Biochemistry, Chinese PLA General Hospital, Beijing, China) were diluted 5 times with PBS and denatured in 2 × loading buffer. Protein samples were fractionated on 12% SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes. Pre-stained molecular weight markers (Bio-Rad) were run in parallel. The blotted membranes were blocked by 5% non-fat milk in PBST for 1 h and incubated with antibodies at room temperature (RT) for 1 h. After washing, the membranes were probed with goat anti-mouse antibody conjugated with horseradish peroxidase (1:2000) for 1 h and washed three times with PBST. Bound antibodies were visualized using enhanced chemiluminescence (ECL) detection system.

Anti-GAPDH (10494-1-AP) was from Proteintech. Anti-myc-tag (AB103) and anti-GST-tag (AB101) were from TianGen (Beijing, China). HRP-anti-mouse (ab6789) was obtained from Abcam and used as secondary antibody.

Immunoprecipitation

A549 cells were homogenized in ice-cold lysis buffer containing 50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, and 1 × protease inhibitor cocktail. After 20 min incubation on ice, the cell lysates were centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatants were recovered. Indicated antibodies (2 μg) were co-incubated with 500 μg cell lysates and 20 μl protein G agarose (Thermo Fisher Scientific) for 12 h at 4°C. The normal mouse IgG (2 μg) was used as control. The precipitates were washed four times with lysis buffer, once with PBS and eluted in 2 × loading buffer. Protein samples were subjected to Western blot.

Immunohischemistry

Paraffin sections were deparaffinized with xylene. Following rehydration in distilled water, antigen was retrieved by heating in EDTA (pH 8.0, Zymed). Endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide at RT for 10 min. Non-specific binding was blocked with PBST containing 10% goat serum and 3% skimmed milk for 2 h at RT. Anti-Ciz1 mAb 1C12 (0.25 μg/μL) was added to slides and incubated at 4°C overnight. Following three washes, slides were incubated with Envision (DAKO) for 20 min at RT. Diaminobenzidine was used as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted. Evaluation of immunohistochemical slides was done with a Nikon Eclipse E800 microscope. Samples were evaluated under light microscopy independently by two pathologists without prior knowledge of the patients' clinical data. The intensity of the staining was scored on a scale of 0 to 3, where 0, 1, 2, and 3 represents no staining, weak staining, moderate staining and strong staining, respectively.

The lung adenocarcinoma tissue microarray (array number: XT16-022; batch number: HL-ugA180Su02) was purchased from OUTDO BIOTECH (Shanghai, China). The array contained 93 cases of lung adenocarcinoma and some adjacent tissues with 5-10 years of follow-up (operation time was between July, 2004 and June, 2009, follow-up was ended in August, 2014). The survival rate between phase 1, phase 2, phase 3 and phase 4 was significantly different (P < 0.0001).
Ciz1 as a novel prognostic factor for lung cancer

Figure 1. The characterization of anti-Ciz1 mAbs. A. SDS-PAGE of induced GST and GST-Ciz1U fusion proteins in crude E. coli lysates. B. SDS-PAGE of purified GST and GST-Ciz1U. C. ELISA for the specificity of anti-Ciz1 mAbs. A 96-well plate was coated with GST or GST-Ciz1U (50 μL/well) and probed with indicated Ciz1 mAbs. D. Western blot analysis for specificity of anti-Ciz1 mAbs. GST-Ciz1 (10 ng) and GST (10 ng) were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membrane. Respective blots were cut and the blots were probed with indicated Ciz1 mAbs. Anti-GST mAb was used as a positive control. E. Western blot analysis for specificity of anti-Ciz1 mAbs by detecting lysates from PG cells transfected with pcDNA3.0-myc-Ciz1 or pcDNA3.0-myc. 20 μg cell lysates were resolved by SDS-PAGE and the blots were probed with indicated Ciz1 mAbs. Anti-myc mAb was used as a positive control. F. Immunoprecipitation for comparing the affinity of anti-Ciz1 mAbs. Total cell lysates were isolated from A549 cells, and equal amounts of protein were subjected to immunoprecipitation (IP) with indicated mAbs and normal IgG. The immunoprecipitates were analyzed for Ciz1 after Western blot using anti-Ciz1 1C12. Input, 20 μg A549 cell lysate.

Statistical analysis

Data analysis was performed using SPSS 12.0 or Graph Pad Prism 5. P value less than 0.05 was considered statistically significant. The analysis of Ciz1 protein levels in lung cancer tissues and its adjacent normal tissues used paired t-test. Two-tailed student’s t-test was
Ciz1 as a novel prognostic factor for lung cancer

used to analysis differential Ciz1 protein levels in plasma samples from patients with lung cancer and benign lung lesions. Chi-square analysis is used for differential expression of Ciz1 in lung cancer tissue microarrays. The relationship between survival time and the expression of Ciz1 used Kaplan-Meier survival analysis.

Online clinical data analysis was performed via KM plotter (http://kmplot.com/). Network database information (including the level of Gene Expression spectrum data and the corresponding clinical information) came from NCBI’s Gene Expression Omnibus website (https://www.ncbi.nlm.nih.gov/geo/), containing CAARRAY (n = 504), GSE14814 (n = 90), GSE19188 (n = 157), GSE29013 (n = 55), GSE30219 (n = 307), GSE31210 (n = 246), GSE3141 (n = 111), GSE31908 (n = 40), GSE37745 (n = 196), GSE43580 (n = 150), GSE50081 (n = 130), and GSE8894 (n = 13) data sets.

Results

Generation of anti-Ciz1 mAbs and characterization of their specificities

By using purified GST-Ciz1U (Figure 1A, 1B) as the antigen, we immunized mice to generate antibodies. After fusing spleen cells from the immunized mice with SP2/0 myeloma cells, we obtained 6 hybridoma clones stably secreting specific anti-Ciz1 mAbs, i.e. 1C12 (IgG1), 149# (IgG2b), 1E2 (IgG2b), 3E6 (IgG2a), 5A4 (IgG1), 9E8 (IgG2b). Results from ELISA (Figure 1C) and Western blot (Figure 1D) showed that these anti-Ciz1 mAbs specifically recognized GST-Ciz1U but not GST. The Ciz1 mAbs were further assessed for their specificities with cell lysates derived from PG cells which were transfected with pcDNA3.0-myc-Ciz1 or pcDNA3.0-myc. These results showed that the 1C12, 149# and 1E2 distinctively recognized exogenous Ciz1 (Figure 1E). Next, we tested the affin-
Ciz1 as a novel prognostic factor for lung cancer

Next, we tested the expressions of endogenous Ciz1 in cultured cells. In Western blot analysis (Figure 2A), protein signals at about 100 kDa corresponding to the endogenous Ciz1 protein were revealed in 5 lung cancer cell lines, but not in NIH3T3 cells. Ciz1 was expressed in A549 and H1299 cells at a high level, but at a low level in PC9, H460 and PG cells. There were two bands recognized by 1C12, likely due to post-translational modification. The expressions of Ciz1 was knocked down by siRNAs in A549 and H1299 cells (Figure 2B), which further validated the specificity of 1C12 mAb. Next, 8 lung cancer tissues and their corresponding adjacent tissues were detected by Western blot (Figure 2B). Quantification of protein bands showed significantly higher expressions of Ciz1 in cancer tissues than in adjacent tissues (P = 0.0218) (Figure 2C). This result demonstrated that Ciz1 is highly expressed in lung cancer tissues.

Ciz1 expressions in plasma samples from lung cancer patients

It is reported that variant Ciz1 is a circulating biomarker for early lung cancer [15]. By Western blot, we detected Ciz1 protein in the plasma samples from 54 lung cancer patients (adenocarcinoma, n = 29; squamous cell carcinoma, n = 12; small cell carcinoma, n = 12; unknown, n = 1) and 54 benign lung diseases patients (Figure 3A). Through quantification of protein bands, it was demonstrated that plasma Ciz1 of patients with cancer was higher than those with benign diseases (P = 0.0016) (Figure 3B). According to the histological classification of different lung cancer types, it was further found that the high expression of Ciz1 in adenocarcinoma, but not in squamous or small cell carcinoma, was statistically significant compared with patients with benign lung lesions (P = 0.0026) (Figure 3C). These results confirm Ciz1 as a circulating biomarker for lung adenocarcinoma.

Ciz1 expressions in tissues from lung cancer patients and its prognostic value

To investigate the clinical significance of Ciz1 in lung cancer, we performed immunohistochemical analysis to examine Ciz1 expression in lung adenocarcinoma tissue microarray with follow-
Ciz1 as a novel prognostic factor for lung cancer

The expression of Ciz1 protein in lung adenocarcinoma tissue was significantly higher than that in the adjacent tissues \((P = 0.0017)\), and percentage of weak (+), moderate (++), and strong (+++) intensity of Ciz1 immunostaining was 21%, 28%, and 8% in adenocarcinoma tissues, whereas 23%, 11%, and 0% in adjacent tissues \((P = 0.0017)\). For this cohort, Kaplan-Meier survival analysis showed that the expression of Ciz1 at high level could predict poor survival rate \((P = 0.0298)\). To further validate the correlation between Ciz1 and lung cancer prognosis, several large-scale lung cancer datasets were integrated, and the relationships between Ciz1 and OS (Overall Survival), FP (First Progression) and PPS (Post Progression Survival) were analyzed by KM plotter. The results showed that Ciz1 was negatively correlated with OS and FS, but irrelevant with PPS \((P = 0.0298)\). After stratification based on histological classification, it was shown that these two inverse correlations were evident in adenocarcinoma \((P = 0.0298)\). However, Ciz1 was only negatively correlated with FP, but not OS, in squamous cell carcinoma \((P = 0.0298)\). Therefore, Ciz1 may be an indicator for evaluating the survival of lung cancer patients, especially those with lung adenocarcinoma.

Discussion

In this study, we first generated six anti-Ciz1 mAbs recognizing both exogenous and endogenous Ciz1. The mAb 5A4 could not detect exogenous and endogenous Ciz1 because of its poor affinity. Other five anti-Ciz1 mAbs can be applied to ELISA, Western blot, immunoprecipitation, and the mAb 1C12 could be utilized in immunohistochemistry.

Next, we sought to demonstrate Ciz1’s roles in lung cancer. In the detection of plasma sam-
Ciz1 as a novel prognostic factor for lung cancer

In immunohistochemical staining of lung adenocarcinoma tissue microarray, positive expression rate of Ciz1 in lung adenocarcinoma (57%) was higher than in normal adjacent tissues (34%). We found that patients with high expression of Ciz1 had a poor prognosis, which was confirmed by network database analysis. Our present study is the first to explore the relationship between Ciz1 and the prognosis of lung adenocarcinoma. Considering that Ciz1 is involved in cell cycle control [6, 20] and DNA

Figure 5. Dataset-based Kaplan-Meier survival analysis of lung cancer according to Ciz1 mRNA levels. A. The relationship between Ciz1 mRNA levels and Overall Survival (OS), First Progression (FP) and Post Progression Survival (PPS) of lung cancer. B. The relationship between Ciz1 mRNA levels and OS, FP and PPS of lung adenocarcinoma. C. The relationship between Ciz1 mRNA levels and OS, FP and PPS of lung squamous cell carcinoma.

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Ciz1 as a novel prognostic factor for lung cancer

replication initiation [4], the abnormal expression of Ciz1 and its alternative splicing may contribute to the malignant phenotype and genomic instability of lung cancer cells [21, 22]. In addition, Ciz1 is also involved in several lung cancer-related signaling pathways. According to reports, Ciz1 can regulate estrogen receptors downstream genes [3] and interact with transcription factor 4 to induce transcription of Wnt signaling genes [17]. The Wnt pathway [23] and estrogen receptors are both activated in non-small cell lung cancer [24, 25], suggesting that Ciz1 may promote lung cancer progression through multiple pathways.

In summary, we generated novel anti-Ciz1 mAbs which specifically recognize exogenous and endogenous Ciz1 protein and are suitable for detecting Ciz1 in various biological samples such as cell lysates, tissues, and plasma samples. We found elevated plasma Ciz1 in lung cancer patients and overexpressed Ciz1 in lung cancer tissues. Moreover, our data suggested that Ciz1 may be an indicator for evaluating the survival of lung cancer patients, especially those with lung adenocarcinoma. Future studies will delineate the detailed mechanisms underlying Ciz1-regulated lung cancer progression.

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Disclosure of conflict interest

None.

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References


Ciz1 as a novel prognostic factor for lung cancer


