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ORIGINAL ARTICLE

p100 functions as a metastasis activator and is targeted by tumor suppressing microRNA-320a in lung cancer

Aimin Xing, Li Pan & Jingzhen Gao

Respiratory Department, Tianjin 4th Center Hospital, Tianjin, China

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Correspondence
Aimin Xing, Respiratory Department, Tianjin 4th Center Hospital, No.1 Zhongshan Road, Hebei District, Tianjin 300140, China.
Tel: +86 22 2624 9176
Fax: +86 22 2624 9121
Email: xingaimin1234@sina.com

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Abstract

Background: Lung cancer is among the most frequently diagnosed types of cancer worldwide, with high morbidity and mortality. Metastasis accounts for the deadliest and most poorly understood feature of lung cancer. Herein, we demonstrate that SND1 (also known as p100) acts as a candidate metastasis activator and is targeted by microRNA-320a (miR-320a) in lung cancer cells.

Methods: p100 expression in lung cancer cell lines and tissues was determined by quantitative real time-PCR and Western blot. RNA interference was applied to investigate the functions of p100 in lung cancer cell migration, reflected by wound healing and transwell assays. Luciferase reporter assay, quantitative real time-PCR, and Western blot were finally used to examine miR-320a targeting of p100 in lung cancer cells.

Results: p100 expression was significantly higher in lung cancer cell lines and tissues compared to normal human bronchial epithelial cells and matched normal lung tissues. Downregulation of p100 by RNA interference obviously inhibited lung cancer cell migration in vitro. Moreover, we validated p100 as a direct target of miR-320a, a tumor suppressing microRNA repressing lung cancer cell migration. Finally, we showed an inversely expressed correlation between p100 and miR-320a in tested lung cancer tissues and cell lines, both of which acted as important prognostic factors in lung cancer.

Conclusion: Our findings identify that p100, targeted by tumor suppressing miR-320a, is a key metastasis activator in lung cancer, and both p100 and miR-320a could be considered as biomarkers for prognosis of lung cancer patients.

Introduction

Lung cancer, classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), is the leading cause of cancer-related death worldwide, with NSCLC comprising over 80% of all lung cancer cases.[1,2] The present standard therapeutics, including surgery, chemotherapy, and radiotherapy, have limited outcomes and the overall five-year survival rate is as low as 15%.[3,4] Most deaths from lung cancer are caused by metastasis.[5] Tumor cells have already spread to distant organs upon diagnosis, leading to poor prognosis.[6] Until now, metastasis has been the most poorly understood aspect of cancer pathogenesis. Understanding the molecular mechanisms governing lung cancer metastasis in depth is a critical requirement to identify novel therapeutic targets.

SND1 (also known as p100) is a multifunctional and evolutionary conserved protein that regulates gene expression at both transcriptional and translational levels.[7] p100 was originally identified as an interactor of EBNA2 and facilitates its transcriptional activity.[8] However, later studies have considered p100 a transcription co-activator, interacting with c-Myb, STAT5, and STAT6.[9–11] Several reports have suggested that p100 is associated with tumorigenesis.[12,13] Only one report on lung cancer has characterized the upregulation and chemoresistance features of p100 in NSCLC cells.[14] Knowledge of the biological function of p100 in lung cancer remains largely elusive. A recent intriguing finding suggested that p100 is involved in cancer cell metastasis as a candidate metadherin-interacting protein.[15] Based on these conclusions, we hypothesized that

p100 overexpression in lung cancer has a metastasis-promoting effect.

Therefore, we examined the expression and role of p100 in pro-metastasis processes of lung cancer cells and identified the specific upstream microRNA (miRNA) regulator of p100 – miR-320a. We further determined whether the two factors could predict survival in these lung cancer patients.

Methods

Patients and tissues

Forty lung cancer patients hospitalized at the Tianjin 4th Center Hospital from January 2002 to June 2016 were included in the study. Patient information and prognosis data were collected for serial years. Lung cancer and paired normal tissues were collected during surgery and were immediately frozen in liquid nitrogen and stored at −80°C until used. Informed consent was obtained from all patients according to the guidelines of the ethics committee of Tianjin 4th Center Hospital.

Cell culture

Human normal bronchial epithelial Beas-2B cells and human lung cancer cells SPC-A-1, NCI-H1299, NCI-H292, and NCI-H661 were cultured in RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics. All cells were maintained at 37°C, with 5% CO_2 in 95% air-humidified conditions.

Small interfering RNAs, microRNAs, and transfections

The p100 small interfering RNAs (siRNAs), miR-320s mimics, miR-320a inhibitor, and controls were all obtained from GenePharma (Shanghai, China). The cells were seeded in six-well plates and grown to 50% confluence prior to transfection. The cells were then transfected with Lipofectamine 2000 Reagent (Life Technologies) according to the manufacturer's instructions.

Reverse transcription and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions; 2μg of total RNA was used for real time (RT)-PCR using M-MLV reverse transcriptase (TaKaRa, Dalian, China). Complementary DNA synthesis was performed using oligo $(dT)_n$ primers (TaKaRa) for messenger RNAs (mRNAs) and specific primers (RiboBio, Guangzhou, China) for miRNAs. Quantitative (q) RT-PCR was performed using the SYBR Green Master Mix (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 was used as internal controls for genes or microRNAs, respectively. The specific primer sequences for the genes are as follows:
SND1-F: GTGGAGTGAGGGGAACGGT;
SND1-R: TAATCTGGGAGGAGCAGGG;
GAPDH-F: CACCCACTCCTCCACCTTT;
GAPDH-R: CTTCCTCTTGTGCTCTTGC.

Western blot

Cells were transfected with siRNAs or miRNAs, washed twice with ice-cold PBS, and lysed with RIPA buffer (Beyotime Biotechnology, Shanghai, China). Cell lysates were separated in 6% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk in PBS with Tween 20 buffer, and were incubated with primary antibodies for p100 (Abcam, Cambridge, UK) and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime), according to the manufacturer's instructions. The protein of interest was visualized using electrochemiluminescence Western blotting substrate (Pierce Biotechnology, Rockford, IL, USA).

Transwell assay

Transwell assays were performed as per the manufacturer's protocol. Briefly, H1299 cells were seeded in the upper chamber without FBS, while the lower chamber was filled with culture medium containing FBS. Cells that migrated through the membrane after 24 hours were fixed with paraformaldehyde, stained with crystal violet and photographed under a microscope.

Wound healing assay

SPC-A-1 cells were seeded in a six-well plate until they reached 100% confluence. A 200ml pipette tip was used to scratch a straight line to generate a wound. The cells were washed with PBS and cultured in full medium for 72 hours. SPC-A-1 cell migration into denuded areas was monitored and visualized using a 40× magnification phase contrast microscope (Olympus Corporation, Tokyo, Japan). Accurate wound measurements were performed at 0 and 72 hours to calculate the migration rate.

Dual-luciferase 3′-untranslated region reporter assay

Dual-luciferase 3′ untranslated region (UTR) reporter assay was conducted to validate p100 as a direct target of

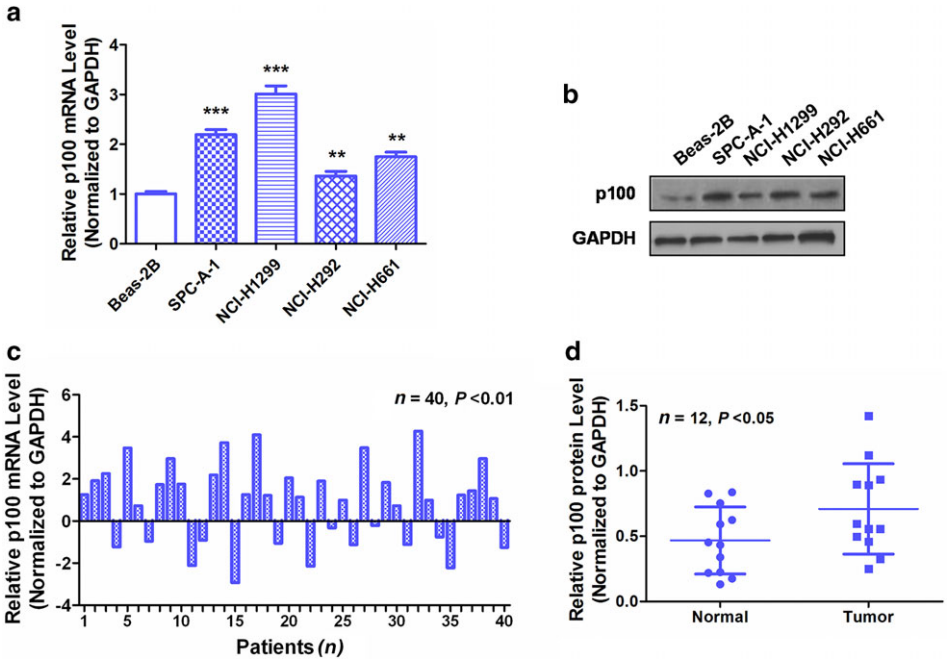

Figure 1 p100 is upregulated in human lung cancer cell lines and tissues. (**a**) Quantitative real time (qRT)-PCR data of p100 messenger RNA (mRNA) expression and (**b**) Western blot data of p100 protein expression in human normal bronchial epithelial Beas-2B cells and human lung cancer SPC-A-1, NCI-H1299, NCI-H292, and NCI-H661 cells. (**c**) qRT-PCR data of p100 mRNA expression in 40 paired lung cancer and adjacent non-cancerous tissues. (**d**) Western blot data of p100 protein expression in 12 paired lung cancer and adjacent non-cancerous tissues. $^{**}P < 0.01$; $^{***}P < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

miR-320a. Wild type or mutant forms of 3′ UTR sequences of p100 were inserted into the pmirGLO vector (Promega, Madison, WI, USA). H1299 cells were transfected with miR-320a mimics or control mimics with wild type or mutant p100 3′-UTR plasmids. Forty-eight hours later, the cells were collected according to the manufacturer's protocol (Promega). Firefly and Renilla luciferase activity was detected using Dual-luciferase Reporter Assay System Kits (Promega). Normalized luciferase activity was expressed as a ratio of firefly luciferase to Renilla luciferase units.

Statistical analysis

Statistical analyses were performed using a two-tailed Student's *t*-test with Microsoft Excel 2013. All figures were constructed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). The correlation between p100 and miR-320a was identified by linear regression analysis. Kaplan–Meier survival analysis was performed by log-rank test using original collected data. Differences of $P < 0.05$ were considered statistically significant. Data were represented as mean ± standard deviation.

Results

p100 is increased in human lung cancer cell lines and tissues

To investigate the function of p100 in lung cancer, p100 mRNA and protein expression levels were assessed in a panel of NSCLC cell lines and Beas-2B cells. Both qRT-PCR and Western blot revealed that p100 expression was increased (around twofold) in lung cancer cell lines compared to normal epithelial cells (Fig 1a,b). Moreover, qRT-PCR analysis showed marked upregulation of p100 mRNA in tested lung cancer tissues: 22 out of 40 lung cancer cases expressed a twofold or greater increase compared to adjacent normal tissues (Fig 1c). Western blot analysis of p100 protein expression showed similar results (Fig 1d). These results suggest that upregulation of p100 in lung cancer cells may contribute to malignancy.

Knockdown of p100 represses migration of lung cancer cells in vitro

p100 is a candidate metadherin-interacting protein with metastasis-promoting functions in breast cancer.[15] Together with our observation that higher p100 expression correlates with lung cancer, these factors predict the involvement of p100 in lung cancer cell metastasis. In order to characterize this effect, we performed knockdown assay to inhibit the endogenous expression of p100 by employing two independent siRNAs. As shown in Figure 2a,b, p100 was efficiently silenced in siRNA-transfected H1299 and SPC-A-1 cells. We then evaluated metastasis-related functions by conducting wound healing and transwell assays. The transwell assays verified that H1299 cells silencing p100 harbored a significantly lower rate of migration than control (scrambled non-targeting siRNA) transfected cells (Fig 2c). The level of p100 was

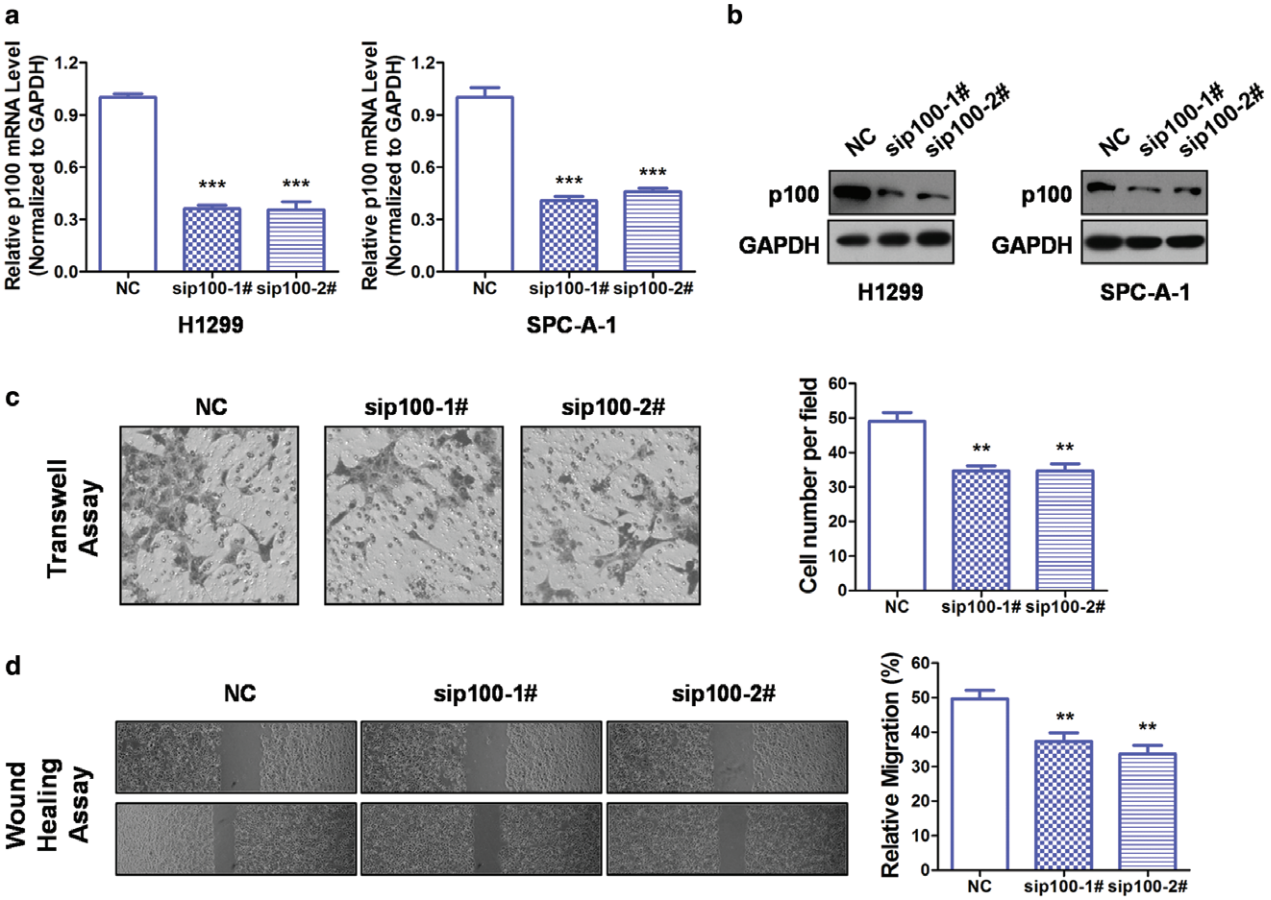

Figure 2 Knockdown of p100 represses lung cancer cell migration in vitro. (**a**) Quantitative real time (qRT)-PCR and (**b**) Western blot data of H1299 and SPC-A-1 cells after knockdown of p100 with two independent small interfering RNAs (siRNAs). (**c**) H1299 cells and (**d**) SPC-A-1 cells were transfected with two independent siRNAs or control siRNAs. Twenty-four hours later, transwell and wound healing assays, respectively, were performed. $^{**}P < 0.01$; $^{***}P < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, normal control.

positively associated with the rates of wound healing: lower levels of p100 expression correlated with slower healing (Fig 2d) in SPC-A-1 cells. These findings strongly indicate that p100 represses lung cancer cell migration in vitro.

Tumor suppressing miR-320a directly targets p100

As is well known, miRNAs function as key regulatory molecules of intracellular gene expression, and their deregulation is associated with the carcinogenesis of various human cancers, including lung cancer tumorigenesis.[16,17] The possible upstream miRNAs that target p100 were predicted using the Targetscan program (http://www.targetscan.org/vert_70/). Among the searched items, miR-320a attracted our attention because p100 3′-UTR contains a putative target sequence for miR-320a (Fig 3a), and several significant reports have found that miR-320a effectively suppresses lung cancer cell growth and metastasis.[18–20] We used the dual-luciferase reporter system to validate miR-320a targeting of p100 in lung cancer cells. Overexpression of the miR-320a mimics obviously repressed the firefly luciferase reporter activity of the wild-type p100 3′-UTR, but did not impact activity of the mutant 3′-UTR constructs (Fig 3b), indicating that miR-320a directly targeted p100 by binding to its 3′-UTR. Subsequent qRT-PCR and Western blot analysis affirmed that overexpression of miR-320a mimics inhibited p100 expression; conversely, inhibition of miR-320a led to elevated p100 expression (Fig 3c,d).

p100 and miR-320 predict lung cancer survival

Having identified p100 as a target of miR-320a in lung cancer cells, we then determined whether expression of the two molecules correlated with each other in lung cancer tissues. qRT-PCR analysis showed marked downregulation of miR-320a in 40 pairs of lung cancer tissues: 18 out of

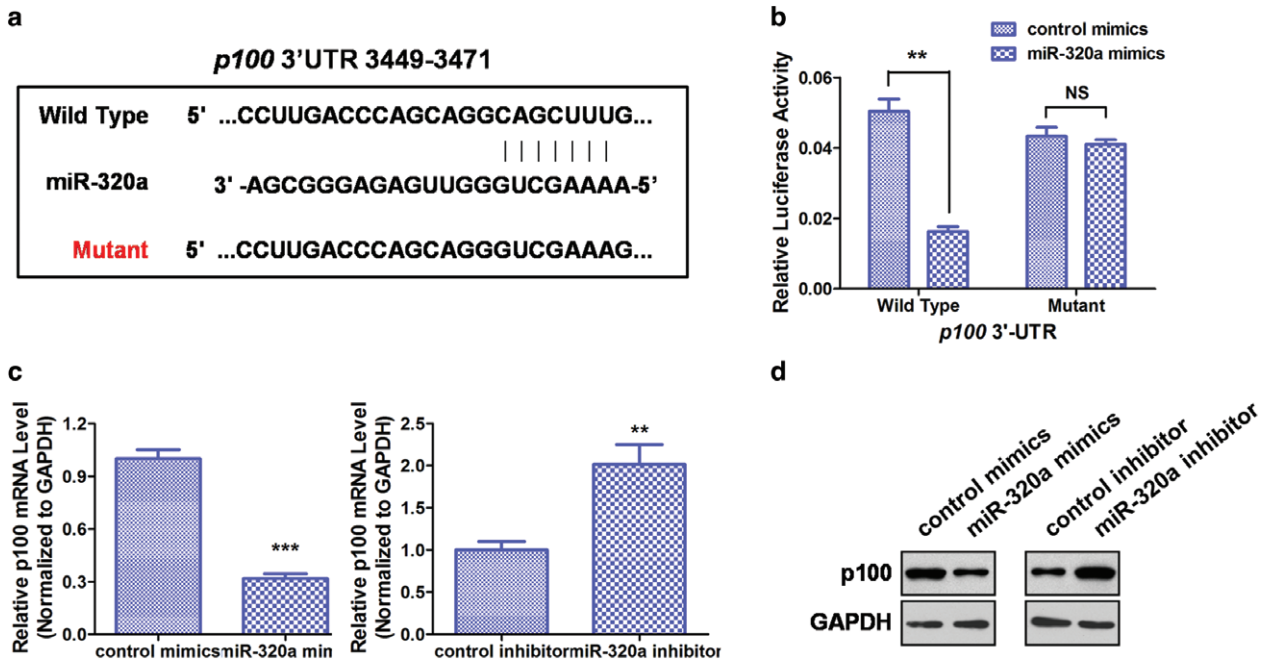

Figure 3 microRNA-320a (miR-320a) directly targets p100 in lung cancer cells. (**a**) Schematic representation of the luciferase reporter plasmids containing the p100 3′-untranslated region (UTR) and putative wild type or mutant miR-320a binding sequence in the 3′-UTR of p100 messenger RNA (mRNA). (**b**) H1299 cells were co-transfected with miR-320a mimics or control mimics with wild type or mutant p100 3′-UTR plasmid. Forty-eight hours later, dual luciferase reporter assay was performed. Effects of miR-320a dysregulation on endogenous p100 expression analyzed by (**c**) quantitative real time-PCR and (**d**) Western blot. $^{**}P < 0.01$; $^{***}P < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

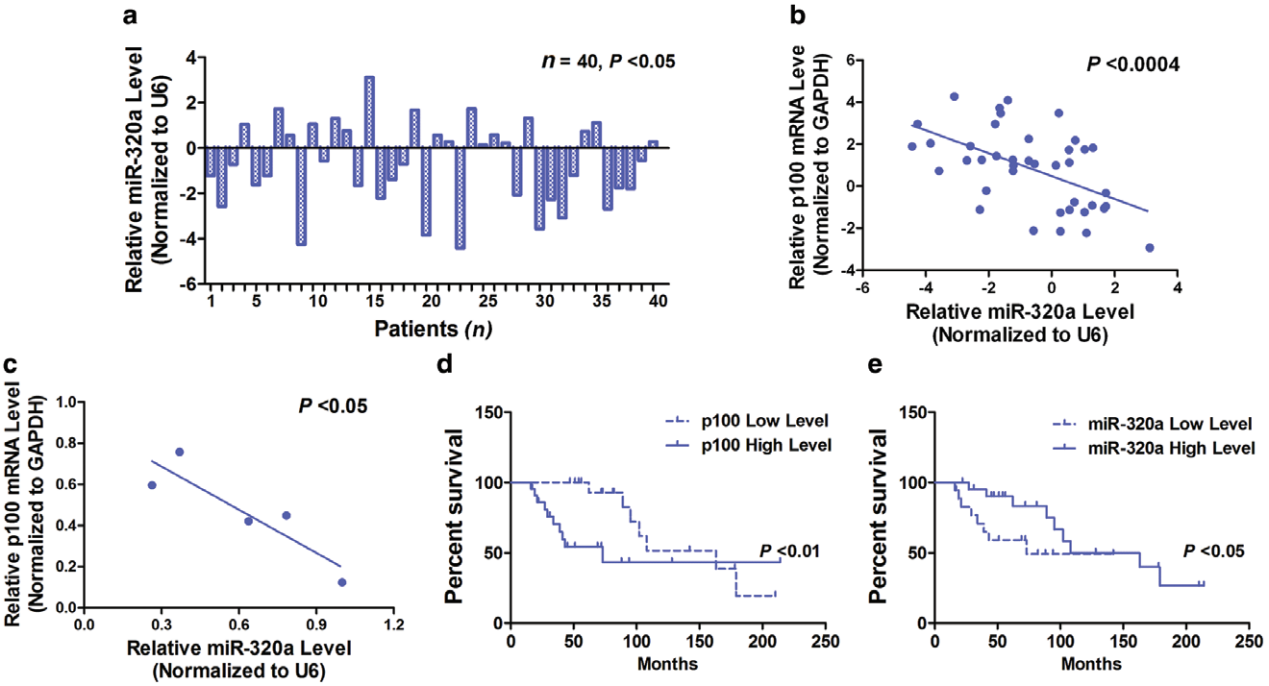

Figure 4 The correlation between p100 and microRNA-320 (miR-320) predicts lung cancer survival. (**a**) Quantitative real time (qRT)-PCR data of miR-320a expression in 40 paired lung cancer and adjacent non-cancerous tissues. The correlation between miR-320a and p100 messenger RNA (mRNA) expression in (**b**) 40 human lung cancer samples and (**c**) human lung cancer cell lines using qRT-PCR. Spearman's correlation was analyzed. (**d**) Higher p100 levels and (**e**) lower miR-320a levels predicted significantly poorer overall survival. Kaplan–Meier survival curves were derived from the original survival data of the 40 lung cancer patients. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

40 lung cancer cases expressed a twofold or greater decrease compared to the adjacent normal tissues (Fig 4a). As shown in Figure 4b,c, we found a significant converse correlation between miR-320a and p100 levels in 40 lung cancer specimens and cell lines. Furthermore, Kaplan–Meier analysis showed that postoperative overall survival of lung cancer patients with higher p100 or lower miR-320a expression levels was significantly shorter (Fig 4d,e). These data indicate that both p100 and miR-320a can be considered vital prognostic biomarkers of lung cancer.

Discussion

Despite achievements in cancer therapy made during the last decade, intrinsic and acquired tumor resistance remains a core issue for patients, clinicians, and researchers. Identifying the candidate molecular biomarkers associated with tumorigenesis to treat tumors such as lung cancer remains a great challenge.

p100 is a multifunctional transcription co-activator with high conservation from yeast to humans. However, the comprehensive role of this protein in most human cancers, including lung cancer, remains unclear. In this study, we firstly showed that p100 expression was significantly increased in lung cancer cell lines and tissues. Through cellular function assays, we verified that knockdown of p100 by two different siRNAs obviously inhibited lung cancer cell migration and invasion, although in vivo data was missing from the present study. To identify the upstream miRNA that targeted p100, bioinformatic prediction was applied. We considered p100 a direct target of miR-320a, a tumor suppressing miRNA in lung cancer cells, via the repression of cell proliferation and invasion. Moreover, there was an inverse correlation between p100 and miR-320a expression in lung cancer tissues, both of which could serve as vital prognostic factors in lung cancer.

It is still unknown how p100, possibly cooperating with miR-320a, achieves metastatic promotion in lung cancer cells. Likely possibilities are via transcriptional co-activation or post-transcriptional modulation of RNAs. Alternatively, as a component of the RNA-induced silencing complex, p100 is also involved in the degradation of edited double-strand RNA molecules.[21] Therefore, further study is needed to test these possibilities and further explore the mechanism of p100 in promoting lung cancer metastasis.

Herein, we report for the first time that p100, directly targeted by tumor suppressing miR-320a, inhibits lung cancer cell migration in vitro. Functional activation of p100 might be a novel mechanism underlying the early carcinogenesis of lung cancer and miR-320a could be used to treat this disease by targeting p100; both may serve as prognostic biomarkers for lung cancer patients.

Disclosure

No authors report any conflict of interest.

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