Lamin B1 Is a Novel Therapeutic Target of Betulinic Acid in Pancreatic Cancer
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Abstract

Purpose: Betulinic acid, a naturally occurring pentacyclic triterpenoid, exhibits potent antitumor activities, whereas the underlying mechanisms remain unclear. In the current study, we sought to determine the role and regulation of lamin B1 expression in human pancreatic cancer pathogenesis and betulinic acid–based therapy.

Experimental Design: We used cDNA microarray to identify betulinic acid target genes and used tissue microarray to determine the expression levels of lamin B1 in pancreatic cancer tissues and to define their relationship with the clinicopathologic characteristics of pancreatic cancer. We also used in vitro and in vivo models to determine the biologic impacts of altered lamin B1 expression on and mechanisms underlying lamin B1 overexpression in human pancreatic cancer.

Results: We found that lamin B1 was significantly downregulated by betulinic acid treatment in pancreatic cancer in both in vitro culture and xenograft models. Overexpression of lamin B1 was pronounced in human pancreatic cancer, and increased lamin B1 expression was directly associated with low-grade differentiation, increased incidence of distant metastasis, and poor prognosis of patients with pancreatic cancer. Furthermore, knockdown of lamin B1 significantly attenuated the proliferation, invasion, and tumorigenicity of pancreatic cancer cells.

Conclusions: Lamin B1 plays an important role in pancreatic cancer pathogenesis and is a novel therapeutic target of betulinic acid treatment. Clin Cancer Res; 19(17); 1–11. ©2013 AACR.

Introduction

Pancreatic cancer is currently the fourth leading cause of cancer-related deaths worldwide. The median survival duration from diagnosis to death is about 6 months, and the overall 5-year survival rate is less than 5% (1–3). Although surgery remains the best choice for pancreatic cancer treatment, most cases are diagnosed at an advanced stage, making patients poor candidates for surgical resection (4–6). Gemcitabine, the first-line agent for advanced pancreatic cancer currently used in clinic, provides only a marginal survival benefit (7). Furthermore, pancreatic tumor is highly resistant to chemotherapy and radiation therapy. Development of more effective therapeutic agents is urgent.

Betulinic acid is a phytochemical triterpenoid acid from bark extracts and exhibits significant cytotoxic effects on pancreatic cancer cells (8, 9). A few molecular targets of betulinic acid have been documented including ErbB2, NF-κB, STAT3, HIF-1α, etc. (10–12). Recent reports have shown that degradation of transcription factor Sp1 is a critical mechanism mediating the antitumor activity of betulinic acid and that overexpression of Sp1 renders cancer cells resistant to betulinic acid (8, 9, 13). However, knockdown of Sp1 expression does not drastically affect the growth of pancreatic cancer cells in vitro, whereas reduced expression of Sp1 attenuates tumor growth and angiogenesis in animal models (14, 15). In contrast, betulinic acid exhibits direct cytotoxic effects against tumor cells in vitro (8). Thus, molecular mechanisms other than interfering with Sp1 signaling should exist underlying the antitumor effects of betulinic acid.

Lamin B1 is an important member of the lamin protein family (16). Lamins are conserved proteins making up nuclear matrix and involved in a list of fundamental cellular functions including nuclear stability, chromatin structure,
Translational Relevance

We have used pancreatic cancer tissue microarray and molecular biology and animal models to evaluate the role and regulation of lamin B1 as a therapeutic target of betulinic acid in human pancreatic cancer. Our clinical and mechanistic findings indicate that lamin B1 is a novel therapeutic target of betulinic acid and that increased lamin B1 expression significantly associated with the clinicopathologic characteristics of and predicted poor prognosis for patients with pancreatic cancer. Moreover, lamin B1 positively regulates pancreatic cancer cell migration, invasion, and growth, suggesting a novel cellular basis for the critical role of lamin B1 expression in pancreatic cancer development and progression, and the deregulated lamin B1 signaling could be a promising new molecular target for designing novel preventive/therapeutic strategies to control this malignancy. Therefore, our findings may have a significant effect on clinical management of patients with pancreatic cancer.

Cell culture and betulinic acid treatment

The human pancreatic adenocarcinoma cell lines AsPC-1, CaPan-1, CaPan-2, MiaPaCa-2, BxPC-3, Hs766T, PANC-1, and PA-TU-8902 were purchased from the American Type Culture Collection. The pancreatic cancer cell lines MDAPanc-28 and MDAPanc-48 were gifts from Dr. Paul J. Chiao (The University of Texas MD Anderson Cancer Center, Houston, TX). All of these cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated in a 37°C, 5% CO2 humidified atmosphere. FG human pancreatic adenocarcinoma cells were used as reported previously (32).

Total RNA extraction and quantitative RT-PCR

Total RNA from each sample was isolated by TRIzol Reagent (Invitrogen Co.). The cDNA was synthesized by using a RT-PCR Kit (TAKARA). Quantitative reverse transcription PCR (RT-PCR) was carried out using 2 μL of cDNA product and the real-time PCR Master Mix (Toyobo) and an ABI 7500 sequence detection system (Applied Biosystems). The PCR primers used were: LMNB1, 5’-ttgccagcttgtgaatac-3’ (forward) and 5’-ctgcagacctccagtcagg-3’ (reverse); ACTIN, 5’-caccatggcaatgagcggttc-3’ (forward) and 5’-aggttggatgctgagtct-3’ (reverse); CDDN1, 5’-ctgatcacaustctcaagta-3’ (forward) and 5’-tgcagactctcagcatca-3’ (reverse); and CDK4, 5’-cagcactagactatctgg-3’ (forward), 5’-ctggtagttgtagttaacaa-3’ (reverse).

Sample preparation and Affymetrix exon array analysis

PANC-1 cells were treated with 50 μmol/L betulinic acid or DMSO for 48 hours. Total RNA was isolated from betulinic acid- or dimethyl sulfoxide (DMSO)-treated PANC-1 cells using a Qiagen kit (Qiagen, Inc.). To analyze gene expression patterns in betulinic acid- or DMSO-treated PANC-1 cells on a whole-genome scale, 1 μg of total RNA from betulinic acid- or DMSO-treated PANC-1 cells was processed and labeled using the GeneChip Whole Transcript Sense Target Labelling Assay (Affymetrix, Inc.) and then hybridized to Affymetrix GeneChip Human Exon 1.0 ST Arrays (Affymetrix, Inc.). The array was scanned and processed by Shanghai Life Technologies Corporation.

Western blot analysis

Standard Western blotting was conducted using 35 μg whole-cell protein lysates with primary antibodies against lamin B1 (sc-6216, Santa Cruz Biotechnology) or Sp1 (sc-59, Santa Cruz Biotechnology) and proper secondary antibodies (anti-rabbit IgG and anti-goat IgG, Santa Cruz Biotechnology). Equal protein sample loading was monitored using an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (G9545, Sigma-Aldrich).
Transgenic transfection and siRNA sequences
Transfection of siRNAs into pancreatic cancer cells was conducted using Lipofectamine 2000 CD (Invitrogen). In brief, the cells were seeded in 6-well plates at a confluence of 30% to 50% and were subsequently transfectected with siRNA at different doses as indicated for 48 hours before functional assays were carried out. The final concentration of siRNA was 40 or 80 nmol/L. The siRNAs sequences were: CGCGCUUUGGUGAGAGGUGAdTdT (targeting LMNB1; ref. 33) and GAAUUUGUGCCUGCUUGCGtCtTdT (targeting Sp1).

Cell wound-healing assay
Forty-eight hours after siRNA transfection, a wound was generated by scraping with a 10-μL tip and maintained at 37°C. After 20 hours, the cells in the wounded monolayer were photographed, and cell migration was assessed by measuring gap sizes at multiple fields.

Cell invasion assay
Cell invasion assay was conducted using a specialized Chemicon invasion chamber, which included a 24-well tissue culture plate with 12 cell culture inserts (Millipore). The inserts contained an 8-μm pore size polycarbonate membrane with a precoated thin layer of basement membrane matrix (ECMatrix). Ten percent FBS-containing medium was placed in the lower chambers to act as a chemottractant. A total of 5 × 10^4 PANC-1 and 3 × 10^5 AsPC-1 in a 300 μL volume of serum-free medium were placed in the upper chambers and incubated at 37°C for 24 hours. Invasive cells on the lower surface of the membrane, which had invaded the ECMatrix and had migrated through the polycarbonate membrane, were stained by the staining solution and counted under a microscope in 5 randomly selected fields at a magnification of x100.

Cell proliferation assay
AsPC-1 and PANC-1 cells were transfected with siLMNB1 or siCtrl. After 48 hours, 6-well plates were seeded with 2.5 × 10^4 AsPC-1 cell per well and 1 × 10^4 PANC-1 cells per well. Cell counting was conducted daily for 6 days.

Pancreatic cancer xenograft models and drug treatment
Pathogen-free male athymic BALB/C nude mice were purchased from the Institute of Animal Center, Chinese Academy of Sciences, Shanghai, China. Tumor xenografts were established by subcutaneous inoculation of 1 × 10^6 PANC-1 or AsPC-1 cells into the right scapular region of nude mice. Ten days after tumor injections, betulinic acid (25 or 50 mg/kg) was mixed in corn oil and administered via oral gavages 3 times a week. Corn oil was administered as control. The medication was given for a total of 30 days starting 10 days after implantation of the primary xenograft. Then, the mice were sacrificed and the xenografts were harvested for further study. All procedures were conducted in accordance with Institution Guidelines and were approved by the Committee of Shanghai Changhai Hospit-
Fig. S3A, lamin B1 was mostly detected on the nucleus envelop of certain acinar cells in the tumor adjacent normal tissues. In a tissue microarray (TMA), no cytoplasmic aggregation of lamin B1 was observed (Supplementary Fig. S3B). Moreover, very little lamin B1 was detected in the islet regions (Supplementary Fig. S3A).

**Lamin B1 overexpression and its direct association with pancreatic cancer clinicopathologic characteristics**

To determine the effect of lamin B1 on pancreatic cancer development and progression, we investigated the expression of lamin B1 protein in a TMA containing primary pancreatic cancers and paired tumor adjacent normal pancreatic tissues (Supplementary Table S2). Lamin B1 expression was significantly increased on the nuclear envelope of pancreatic cancer cells ($P < 0.001$; Fig. 2A). Presence of aberrant upregulation of lamin B1 expression at both mRNA and protein levels were further confirmed in 5 pancreatic cancer and paired normal pancreatic tissue specimens by using real-time PCR and Western blotting (Fig. 2B). Because a larger nucleus presents more DNA and proteins involved and usually suggests a more malignant phenotype, we determined the lamin B1 expression intensity among cells with different nucleus sizes. As shown in Supplementary Fig. S4, larger nucleus cells exhibited high lamin B1 expression when compared with those smaller ones. Those results suggested that lamin B1 expression correlate with increased pancreatic malignancy.

We further analyzed the relationship between clinicopathologic parameters and lamin B1 expression levels in pancreatic cancer tissues (Supplementary Table S2; Fig. 2C...
and D). High lamin B1 staining was inversely correlated with the overall survival duration in Kaplan–Meier survival analysis; 60 patients with high lamin B1 expression had a median survival duration of only 9.75 months, whereas the 82 patients with low lamin B1 expression had a median survival duration of 12.4 months \( (P = 0.007; \text{Fig. 2D}) \).

Multivariate analysis showed that high level of lamin B1 expression (lamin B1-high) was associated with shorter survival \( (P = 0.04) \) independent of human gender, age, tumor differentiation, metastasis (Supplementary Table S3).

Furthermore, increased lamin B1 expression was correlated with decreased levels of tumor differentiation and significant difference was detected between well (grade I) and poorly differentiated (grade III) tumors (Fig. 3A). Lamin B1 expression was significantly upregulated in late stage of pancreatic cancer (Supplementary Table S2). In addition, lamin B1 expression in specimens with metastasis was significantly higher than that in those without metastasis (Fig. 3B). These data strongly suggested that lamin B1 expression plays critical roles in pancreatic cancer and be a valuable prognosis biomarker for this disease.

**Inhibition of pancreatic cancer growth in vitro and animal models by knockdown of lamin B1 expression**

To determine the role of lamin B1 in pancreatic cancer cell proliferation, we treated AsPC-1 and PANC-1 cells with lamin B1 siRNA. Efficiency of knockdown against lamin
B1 was confirmed using Western blotting (Fig. 4A). Knockdown of lamin B1 significantly inhibited the growth of cancer cells (Fig. 4A). To investigate the mechanism by which lamin B1 affects pancreatic cancer growth, we tested the effects of altered lamin B1 expression on cell-cycle distribution. As shown in Supplementary Fig. S5, knockdown of lamin B1 induced G1 arrest in both AsPC-1 cells and PANC-1 cells and this cell-cycle arrest directly correlated with a significant suppression of the mRNA expression levels of cyclin D1 and CDK4.

Because increased lamin B1 expression correlated with pancreatic cancer metastases (Fig. 3B), we investigated the role of lamin B1 in pancreatic cancer cell migration and invasion. Wound-healing assay showed that silencing lamin B1 in AsPC-1 and PANC-1 cells significantly attenuated the migration and invasion ability of pancreatic cancer cells \( (P < 0.05; \text{Fig. 4B and C}) \). Consistently, silencing lamin B1 significantly inhibited pancreatic tumor growth \( \text{in vivo} \) (Fig. 5A). Immunohistochemical analysis validated the knockdown efficiency against lamin B1 in the xenograft tumors (Fig. 5B). Thus, overexpression of lamin B1 contributes to pancreatic cancer proliferation and invasion.

**Suppression of lamin B1 by betulinic acid is independent of Sp1**

Previous studies have implied that Sp1 be an important transcription factor that regulates the expression of lamin B1 (30, 34) and that multiple Sp1-binding sites were identified within human lamin B1 promoter region (Fig. 1D). To determine whether suppression of lamin B1 by betulinic acid was dependent on Sp1 signaling, we first quantitated the expression level of Sp1 in the TMA using immunohistochemistry. Consistent with our previous results, Sp1 expression is significantly higher in pancreatic cancer tissues than in the normal control. Sp1 is also overexpressed in xenograft tumors sampled from nude mice (Fig. 6A). There was a direct correlation between lamin B1 and Sp1 expression levels, whereas no statistical significance was observed (Fig. 6A, \( P = 0.11 \)). We also evaluated Sp1 and lamin B1 expression levels in 11 pancreatic cancer cell lines (Fig. 6B), showing no linear correlation \( \left( r^2 = 0.145, P = 0.248; \text{Fig. 6B} \right) \). Furthermore, knockdown of Sp1 did not cause a drastic reduction of lamin B1 expression (Fig. 6C). Interestingly, mithramycin A, a commonly used Sp suppressor, showed inhibitory effects on lamin B1 (Fig. 6C), which suggested that suppression of betulinic acid to lamin B1 did not work...
through Sp1, but other members of the Sp family proteins. Finally, knockdown of lamin B1 attenuated pancreatic cancer cell proliferation in vitro, whereas knockdown of Sp1 has little effect on pancreatic cancer growth in vitro (Fig. 6D), suggesting that Sp1 signaling was dispensable for lamin B1 expression and function.

Discussion

In current study, we discovered 4 lines of evidence supporting the critical roles of lamin B1 in mediating antitumor activities of betulinic acid in pancreatic cancer. First, betulinic acid treatment significantly suppressed lamin B1 expression in pancreatic cancer cells in culture and in animal models. Second, an elevated expression of lamin B1 directly correlated with the progression of pancreatic cancer. Increased expression of lamin B1 predicted poor differentiation, high metastatic potential, and short patient survival duration. Third, knockdown of lamin B1 attenuated the growth and migration and invasion of pancreatic cancer in vitro and in animal models. Fourth, consistent with that LMNB1 is a Sp1 downstream gene as predicted by promoter sequence analysis (30, 31), Sp1 might regulate the expression of lamin B1. However, inhibition of lamin B1 by betulinic acid appeared to be independent of Sp1 signaling, suggesting that lamin B1 be a novel target critically mediating the anti-cancer effects of betulinic acid.

Betulinic acid has significant antitumor activities (13, 35, 36) and is undergoing development with assistance from the Rapid Access to Intervention Development program of the National Cancer Institute. Of particular interest is its direct and relatively selective cytotoxic effect on tumor cells versus normal or nonneoplastic cells (36). However, the molecular basis of betulinic acid antitumor activity is unclear. Recent studies have identified Sp1 as a target molecule mediating the antitumor activity of betulinic acid (9, 13), and its overexpression renders pancreatic cancer cells resistant to betulinic acid cytotoxicity (8). However, Sp1 knockdown does not drastically affect the growth of pancreatic cancer cells in vitro (14). A study using genetic mouse model also indicates that Sp1 is dispensable for cell growth and differentiation (37). Thus, molecular mechanisms other than Sp1 should exist underlying the antitumor
effects of betulinic acid. Our current study has shown for the first time that betulinic acid downregulated the expression of lamin B1 protein, which plays important roles in cellular functions, including DNA replication, the formation of the mitotic spindle, chromatin organization, and regulation of gene expression, and is essential for cell survival. Thus, we have uncovered a novel mechanism underlying the antitumor effects of betulinic acid, and our study may help develop potentially more effective betulinic acid–based targeted therapeutics.

The role of lamin B1 in cancer development and progression is unclear. Apparently, different tumor types exhibited distinct patterns of lamin B1 expression (29). Among all the epithelial neoplasms, there are no consistent patterns of lamin B1 expression (21). A reduced expression of lamin B1 occurs frequently in various carcinomas, including gastric cancer, colon cancer, squamous and adenocarcinoma of the esophagus, cervical and uterine body cancers, breast cancer, and also bronchial carcinoma. In contrast, an increased expression pattern was found in other cancers, including prostate cancer and liver cancer (22–24). In pancreatic cancer, the expression levels of lamin B1 increased in 2 of 3 cases examined (21). In our current study of 142 pancreatic cancer specimens and matched normal pancreatic specimens, we determined the clinical significance of lamin B1 expression. We observed that lamin B1 expression was significantly higher in pancreatic cancer tissues than in the corresponding tumor adjacent normal tissues. Higher lamin B1 expression was significantly associated with poor prognostic factors, such as the presence of distant/lymph node metastasis and low grade of differentiation. More importantly, lamin B1–positive staining was
associated with poor overall survival rates. Therefore, we provided the first evidence that lamin B1 can be used as a novel biomarker for outcome in patients with pancreatic cancer. In another word, at the time of initial diagnosis of pancreatic cancer, lamin B1 expression can be used not only to design optimal, individualized treatment but also to distinguish patients who would benefit from close monitoring after surgery from those who would not. Our notion is further supported by a recent publication, which has shown that lamin B1 mRNA was present in plasma of patients with hepatocellular carcinoma (HCC) and was able to detect early-stage HCCs with a sensitivity of 76% and a specificity of 82% (24). Therefore, lamin B1 may be potentially used for early detection of pancreatic cancer.

Because a modulation of “biomarker” expression can usually produce therapeutic effects (38), we further evaluated the biologic functions of lamin B1 within pancreatic cancer. Our work showed that knockdown of lamin B1 significantly attenuated the proliferation, invasion, and tumorigenesis of pancreatic cancer cells. These findings are consistent with our clinical data, which showed that lamin B1–positive expression closely associated with poor differentiation and high metastatic potential. Thus, targeted inhibition of lamin B1 expression and/or function may constitute a novel therapeutic strategy for pancreatic cancer.

Given that the fundamental roles of lamin B1 in physiology and cancer biology remain controversial, further investigation into the interactions between lamin B1 signaling and other major oncogenic signaling pathways is warranted. Prior studies have also shown that Sp1 plays critical roles in pancreatic carcinogenesis (14, 15) and also identified multiple Sp1-binding sites within the promoter region of lamin B1. Both Sp1 and lamin B1 proteins were knocked down by betulinic acid treatment, suggesting that lamin B1 might be a further downstream target of Sp1. However, no significant correlations between Sp1 and lamin B1 proteins...
(neither cell lines nor tissue specimens) were identified in the present study. Furthermore, Sp1 knockdown using siRNA has only marginal impacts on lamin B1 expression, whereas mithramycin A, a commonly used Sp1-targeted drug, did inhibit lamin B1 expression. All these data established the notion that overexpression of lamin B1 in pancreatic cancer appeared to be independent of Sp1 signaling but other protein members of Sp family, suggesting a unique role of lamin B1 in pancreatic tumorigenesis. Thus, betulinic acid may simultaneously suppress both Sp1 and lamin B1 and generate synergistic antitumor effects in pancreatic cancer.

In summary, we have shown that betulinic acid targeted both Sp1 and lamin B1, which appeared to be distinct from each other in signaling, thus uncovering a novel mechanism underlying the potent antitumor effects of betulinic acid in pancreatic cancer. We have also shown that lamin B1 expression closely correlated with the clinical feature of patients with pancreatic cancer and regulated the proliferation, invasion, and metastasis of pancreatic cancer cells, thus providing critical insight into the role of lamin B1 in the progression of pancreatic cancer. Therefore, lamin B1 expression in pancreatic cancer highlights its potential as a novel molecular biomarker and therapeutic target. The role and regulation mechanisms of lamin B1 in pancreatic cancer development and progression warrant more studies to ensure an eventual translation into clinical uses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: L. Li, X. Kong, Z. Jia, J. Gao, L. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Li, Y. Du, X. Kong, Z. Li, Z. Jia, J. Cui, J. Gao, L. Wang, K. Xie
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