LASP1 Is a HIF1α Target Gene Critical for Metastasis of Pancreatic Cancer

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Abstract

LASP1 is an actin-binding protein associated with actin assembly dynamics in cancer cells. Here, we report that LASP1 is overexpressed in pancreatic ductal adenocarcinoma (PDAC) where it promotes invasion and metastasis. We found that LASP1 overexpression in PDAC cells was mediated by HIF1α through direct binding to a hypoxia response element in the LASP1 promoter. HIF1α stimulated LASP1 expression in PDAC cells in vitro and mouse tumor xenografts in vivo. Clinically, LASP1 overexpression in PDAC patient specimens was associated significantly with lymph node metastasis and overall survival. Overall, our results defined LASP1 as a direct target gene for HIF1α upregulation that is critical for metastatic progression of PDAC.

Introduction

Despite recent advances in the diagnosis and therapeutic modalities, pancreatic ductal adenocarcinoma (PDAC) remains the digestive tract tumor with the poorest prognosis (1). PDAC is the fourth highest cause of cancer-related deaths in the United States, with a 5-year survival rate lower than 5% (2). The aggressive course of PDAC progression is mostly due to delayed diagnosis, resistance to conventional chemotherapies, and development of metastasis (3). The underlying mechanisms that trigger PDAC invasion and metastasis remained largely unknown, although it is believed that the hypoxic microenvironment and hypoxia-induced factors are critical drivers of PDAC metastasis and progression (4).

LIM and SH3 protein 1 (LASP-1) is an actin-binding protein containing an N-terminal LIM domain and two actin-binding domains in the core of LASP-1 protein (5). LASP-1 was first identified from a cDNA library of metastatic axillary lymph nodes of patients with breast cancer, and the gene was mapped to human chromosome 17q21 (6, 7). LASP-1 interacts with the actin cytoskeleton at the site of cell membrane extensions, but not along actin stress fibers (5, 8–10). The SH3 domain at the C-terminus is involved in interactions with zyxin, pallidin, lipoma-preferred partner, and vasodilator-stimulated phosphoprotein (9, 11). LASP-1 reportedly localizes within multiple sites of dynamic actin assembly, such as focal contacts, focal adhesions, lamellipodia, membrane ruffles, and pseudopodia, and is involved in cell proliferation and migration (12). It was previously reported that LASP-1 overexpression induced cell proliferation and migration in human breast cancer, ovarian cancer, colorectal cancer, malignant childhood medulloblastoma, and hepatocellular carcinoma (5, 13–16). However, the role of LASP-1 in PDAC progression has not been examined.

In this study, we aim to investigate the role of LASP-1 in PDAC progression. Our data showed that LASP-1 was overexpressed in PDAC, and LASP-1 overexpression promoted PDAC cell migration and invasion in vitro and metastasis in xenograft mouse models. LASP-1 overexpression in PDAC is mediated by HIF1α, which directly binds to and transactivates the LASP-1 promoter. Our findings indicate that LASP-1 is a novel direct HIF1α target gene that promotes PDAC metastasis and progression.

Materials and Methods

Cell culture and hypoxic treatment

Human PDAC cell lines, CFPAC-1, BxPC-3, and Panc-1 were obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and MIA-PaCa-2 was obtained from the ATCC. All the cell lines were obtained in 2013, and recently authenticated in August 2014 through the short tandem repeat analysis method. These cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2 using DMEM with 10% FBS. For hypoxic treatment, cells were placed in an incubator (Thermo Electron Co.) in an atmosphere consisting of 95.5% N2, 5%CO2, and 1.5% O2.

Western blot analysis

Whole-cell extracts were prepared by lysing cells with RIPA lysis buffer supplemented with a protease inhibitor cocktail (Sigma). Protein concentrations were quantified using Pierce Protein Assay Kit (Pierce). Protein lysates (20 μg) were separated by SDS-PAGE,
Zhao et al.

and target proteins were detected by Western blot analysis with antibodies against HIF1α (1:1,000), LASP-1 (1:2,000), and β-actin (1:1,000; Supplementary Table S1). Specific proteins were visualized using an Enhanced Chemiluminescence Detection Reagent (Pierce).

Reverse transcription PCR

Total RNA was isolated from transfected cells with TRIzol Reagent (Invitrogen) and used for first-strand cDNA synthesis using the First-Strand Synthesis System for reverse transcription PCR (RT-PCR, Takara). Each sample was processed in triplicate, and β-actin was used as a loading control. Each experiment was repeated independently at least three times. PCR primers used are indicated in Supplementary Table S1.

Immunofluorescence

To assess LASP-1 and F-actin distribution, human PDAC cells were seeded onto glass slides for different treatments. The cells were then washed once with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.1% Triton X-100 in PBS for 30 minutes at room temperature, and blocked for 1 hour with 3% BSA in PBS. Then cells were stained with anti-LASP-1 antibody (1:200 dilution, overnight at 4°C). F-actin was stained with phallolidin-FITC (Beyotime Biotechnology). Cells were mounted with DAPI Fluoromount-G media with DAPI nuclear stain (Southern Biotech). Slides were viewed with Olympus confocal microscopy.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed using a commercial kit (Upstate Biotechnology) according to the manufacturer’s instructions. Primers flanking the hypoxia response elements (HRE) of the VEGF promoter were used as a positive control (17, 18). The PCR primers are indicated in Supplementary Table S1.

siRNA duplexes, plasmid constructs, transient transfection, and stable transfection in pancreatic cancer cells and luciferase assay

siRNAs against LASP-1 and HIF1α were designed and synthesized from GenePharma (Supplementary Table S1). The human LASP-1 cDNA was cloned into the pcDNA3.1 plasmid expression vector. The pcDNA3.1-HIF1α plasmids were prepared as previously described (17, 18). LASP-1 overexpression in Panc-1 cells, lentivirus-mediated plasmid was done using the pLV-CDNA system (Biosettia) following the manufacturer’s instructions. Lentivirus encoding DNA were packaged as previously described (19). After transfection, the medium containing lentivirus was collected, filtered, and transferred onto Panc-1 cells. Infected cells were selected with puromycin (1 μg/mL) for 7 days.

Genomic DNA fragments of the human LASP-1 gene, spanning from +1 to −2,000 relative to the transcription initiation sites were generated by PCR and inserted into pGL3-Basic vectors (denoted as pGL3-LASP-1). All constructs were sequenced to confirm their identity. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) as previously described (17, 18). For transfection, cells were plated at a density of 5 × 10^4 cells/well in 6-well plates with serum-containing medium. When the cells were 80% confluent, the siRNA duplexes or overexpression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) for 48 hours. The cells were collected for cell migration and invasion analysis, Western blot analysis, RT-PCR, and immunofluorescence, etc.

Wound healing and cell migration assay

A wound-healing assay was performed according to published protocol (20). Invasion assays were performed with 8.0-μm pore inserts in a 24-well Transwell. For this assay, 1 × 10^5 cells were isolated and added to the top chamber of a Transwell with DMEM. The invasion assay was performed using 1/6 diluted Matrigel (BD Biosciences) coated filters. DMEM with 10% FBS was added to the bottom chamber and the cells were allowed to incubate for 24 hours. Cells that had migrated to the bottom of the filter were stained with a three-step stain set (Thermo Scientific). All experiments were repeated independently at least three times.

Animal studies and measurement of metastasis in orthotopic pancreatic cancer mouse model

Female 4-week-old nude nu/nu mice were maintained in a barrier facility on high-efficiency particulate air (HEPA)-filtered racks. All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. Digoxin and saline for injection were obtained from Tianjin Medical University Cancer Institute and Hospital (Shanghai, China). Cells were harvested by trypsinization, washed in PBS, resuspended at 10^5 cells/mL in a 1:1 solution of PBS/Matrigel, and injected subcutaneously into the right flank of 16 nude nu/nu mice. Primary tumors were measured in three dimensions (a, b, c), and volume was calculated as abc × 0.52 (21). Primary tumors were harvested from the flank of nude nu/nu mice. Part of the tumor was fixed by formalin and embedded using paraaffin, and the rest of the tumor was used for protein extraction.

For orthotopic tumor cell injection, 18 nude nu/nu mice were divided into three groups (Panc-1/pLV Vector + saline, Panc-1/pLV Vector + digoxin, and Panc-1/pLV LASP-1 + digoxin, respectively, 6 mice each). A total of 2.0 × 10^6 cells were injected into the carefully exposed pancreas of nude mice. The pancreas was then returned to the peritoneal cavity; the abdominal wall and the skin was closed with skin clips. Six weeks later, the number of visible metastatic lesions in the gut, mesentery, and liver was determined (22).

Immunohistochemistry

With approval from the Ethics Committee, PDAC samples were obtained from 91 patients (aged 36–83 years) undergoing surgical resection with histologic diagnosis of PDAC at the Tianjin Cancer Institute and Hospital (Shanghai, China). Immunohistochemistry for HIF1α and LASP-1 of PDAC patient tissues was performed using a DAB Substrate Kit (Maxin). Immunoreactivity was semiquantitatively scored according to the estimated percentage of positive tumor cells as previously described (18). Staining intensity was scored 0 (negative), 1 (low), 2 (medium), and 3 (high). Staining extent was scored 0 (0% stained), 1 (1%–25% stained), 2 (26%–50% stained), and 3 (51%–100% stained). The final score was determined by multiplying the intensity scores with staining extent and ranged from 0 to 9. Final scores (intensity score × percentage score) less than 2 were considered as negative staining (−), 2–3 were low staining (+), 4–6 were medium staining (++) and >6 were high staining (+++).
Statistical analysis

The Student t test for paired data was used to compare mean values. ANOVA is used to analyze the data of two groups with continuous variables. Nonparametric data were analyzed with the Mann–Whitney U test. The categorical data were analyzed by either Fisher exact or χ² method. Each experiment was conducted independently at least three times, and values were presented as mean ± SD, unless otherwise stated. Analyses were performed using SPSS18.0 statistical analysis software.

Results

LASP-1 overexpression promoted PDAC cell migration and invasion

To determine the role of LASP-1 in PDAC progression, we compared its expression levels in PDAC specimens and paired adjacent normal pancreatic tissues from PDAC patients. Despite interindividual variations, LASP-1 protein (Fig. 1A) and mRNA (Fig. 1B) levels were found to be evidently upregulated in PDAC samples when compared with adjacent normal pancreatic tissues, suggesting that LASP-1 was activated at transcriptional levels during PDAC progression. Furthermore, LASP-1 signal measurement by immunohistochemistry was detected in most (87.9%) of PDAC tissues (Fig. 1C). Intriguingly, in pancreas tumors other than malignant PDAC, such as serous cystadenoma and neuroendocrine tumors, LASP-1 expression was negative (Fig. 1C).

Next, we set out to examine the effects of LASP-1 overexpression on PDAC cell migration and invasion. To investigate the role of LASP-1 in the aggressive phenotypes of PDAC cells in vitro, we used siRNA transfection to knock down LASP-1 expression in two PDAC cell lines with high endogenous LASP-1 levels (CFPAC-1 and MIA-PaCa-2; Fig. 2A). Out of the three pairs of siRNA, siRNA #3 most efficiently knocked down LASP-1 expression by more than 70% (Fig. 2B and C), and was used in the subsequent functional studies. Cell migration and invasion analysis using Transwell assay suggested that LASP-1 depletion in CFPAC-1 and MIA-PaCa-2 cells evidently reduced cell migration and invasion (Fig. 2D). In the wound-healing assays, the migratory activity of CFPAC-1 and MIA-PaCa-2 cells was also inhibited by LASP-1 silencing (Fig. 2E). Immunofluorescence analysis confirmed regional colocalization between LASP-1 and F-actin (Fig. 2F). Interestingly, compared with CFPAC-1/siNC and MIA-PaCa-2/siNC cells, the morphology of CFPAC-1/siLASP-1 and MIA-PaCa-2/siLASP-1 cells lacked thin and long pseudopods (Fig. 2F). These data indicated that LASP-1 was critical for the migration and invasion of PDAC cells.

To determine whether LASP-1 overexpression is sufficient to promote PDAC cell migration, LASP-1 was ectopically expressed in two PDAC cell lines with low endogenous LASP-1 levels (BxPC-3 and Panc-1; Supplementary Fig. S1). Cell migration and invasion analysis by Transwell assay suggested that LASP-1 upregulation in BxPC-3 and Panc-1 cells evidently increased cell migration and invasion (Fig. 2G). Wound-healing assays showed that the migratory activity of the BxPC-3 and Panc-1 cells was enhanced by LASP-1 overexpression when compared with the control cells (Fig. 2H). Importantly, BxPC-3/pcDNA3.1-LASP1 and Panc-1/pcDNA3.1-LASP1 displayed some pseudopods extending from cell bodies (Fig. 2I). These results showed that ectopic overexpression of LASP-1 was sufficient to promote PDAC cell migration and invasion.

Figure 1.

LASP-1 is overexpressed in the specimens of PDAC. A. Western blot analysis (left) of LASP-1 levels in ten total paired human PDAC tumorous and matched adjacent nontumorous tissues. LASP-1 protein expression levels were normalized by those of β-actin. The chart shows LASP-1 protein expression levels in PDAC specimens versus paired adjacent normal pancreatic tissues. N, normal; T, tumor; **, P < 0.01 (right). B. RT-PCR analysis of LASP-1 levels in seven total paired human PDAC tumorous and matched adjacent nontumorous tissues (left). LASP-1 mRNA expression levels were normalized by β-actin. The chart shows LASP-1 mRNA expression levels in PDAC specimens versus paired adjacent normal pancreatic tissues. N, normal; T, tumor; *, P < 0.05 (right). C. expression analysis of LASP-1 protein in PDAC, serous cystadenoma, and neuroendocrine tumor tissues by immunohistochemistry (magnification, ×200 or ×400).
HIF1α directly regulated the expression of LASP-1 through binding to the HRE in the LASP-1 gene promoter

Our previous study indicated HIF1α as a critical transcriptional factor in pancreatic cancer cell migration (18, 23). To determine whether HIF1α regulates transcription of LASP-1 in pancreatic cancer cells, we used specific siRNAs targeting HIF1α to effectively reduce HIF1α expression (Supplementary Fig. S2). Knockdown of HIF1α expression decreased LASP-1 mRNA (Fig. 3A, left) and protein (Fig. 3A, right) expression (P < 0.05). Moreover, when HIF1α was overexpressed in Panc-1 cells, LASP-1 mRNA (Fig. 3B, left) and protein (Fig. 3B, right) expression markedly increased (P < 0.05). Taken together,
these results suggest that HIF1α plays a critical role in LASP-1 expression.

To determine whether hypoxia may induce LASP-1 overexpression in PDAC cells, four PDAC cell lines were cultured under normoxia (21% O2) and hypoxia (1.5% O2) for 12 hours and the LASP-1 expression levels were determined by Western blot analysis. As shown in Fig. 3C, LASP-1 expression increased about 3.85-fold after hypoxic treatment when compared with normoxia-cultured cells, suggesting that hypoxic PDAC microenvironment might be responsible for LASP-1 overexpression.

To understand the molecular mechanism underlying LASP-1 overexpression in PDAC, we surveyed the promoter region of human LASP-1 gene and identified four HRE (Fig. 3D).

To investigate whether HIF1α directly binds to LASP-1 promoter, chromatin immunoprecipitation assay was performed in Panc-1 cells at 1.5% O2 or 21% O2. In chromatin fractions pulled down by an anti-HIF1α antibody, only the HRE of LASP-1 promoter located at −1,005 to −1,001 was detected (Fig. 3E, top). The fragment immunoprecipitated by anti-HIF1α antibody significantly increased (Fig. 3E, bottom; * P < 0.01) under hypoxia, suggesting that hypoxia promoted the binding of HIF1α to LASP-1 promoter.

To determine whether the binding of HIF1α activates LASP-1 promoter, we constructed a full-length LASP-1 luciferase promoter vector (containing HREs, −1,005 to 1,001) and cotransfected this reporter construct with or without HIF1α cDNA into Panc-1 cells. Luciferase analysis showed that HIF1α overexpression (pcDNA-HIF1α) significantly increased LASP-1 promoter activity in Panc-1 cells (−2.9-fold, * P < 0.05; Fig. 3F). To determine whether the HRE1 site is required for HIF1α to transactivate LASP-1 promoter, this HIF1α binding site was mutated from ACGTG to ACATG. As shown in Fig. 3F, the mutation of HRE1 almost abolished the transactivation of LASP-1 promoter by HIF1α. (VEGF promoter was used as the positive control).
HIF1α upregulates LASP-1 expression in xenograft mouse model

To determine whether HIF1α regulates LASP-1 expression in vivo, we injected Panc-1 cells subcutaneously into the right flank of nude nu/nu mice. When the tumors reached 100 mm3, the mice were intraperitoneally injected with saline or digoxin (2 mg/kg) on a daily basis to inhibit HIF activity (21, 24, 25). The tumor was palpable at 5 days after inoculation and all of the mice had developed tumors by the end of the experiment. Compared with the saline control group, the average tumor volume in the digoxin group was reduced obviously (P < 0.01; Fig. 4A–C). Next, we evaluated the association between HIF1α and LASP-1 by Western blot analysis (Fig. 4D) and immunohistochemistry (Fig. 4E) and the results suggest that expression of LASP-1 was decreased as a result of the HIF1α level reduction by digoxin.

HIF1 correlates with LASP-1 expression in specimens of human PDAC

To determine whether HIF1 indeed regulates the expression of LASP-1 in patients with PDAC, we performed immunohistochemical staining to determine HIF1α and LASP-1 levels in PDAC specimens. As shown in Fig. 4F, LASP-1 expression colocalized with HIF1α in consecutive sections of PDAC tissues at different grades. Importantly, HIF1α expression in PDAC specimens significantly correlated with the levels of LASP-1 (Fig. 4G and H), implicating that HIF1α is a critical regulator for LASP-1 overexpression in patients with PDAC.

LASP-1 overexpression is able to rescue the inhibition of PDAC metastasis by HIF1 knockdown

To understand the role of LASP-1 in HIF1α-mediated migration, we ectopically expressed LASP-1 in Panc-1 cells. As shown in Fig. 5A (left, Panc-1; right, CFPA-1), LASP-1 overexpression at least partially rescued the inhibitory effect of HIF1α knockdown on PDAC cell migration (P < 0.05), suggesting that LASP-1 was involved in HIF1α-mediated migration.

To confirm the role for LASP-1 in pancreatic cancer cell invasion and metastasis in vivo, we developed an orthotopic pancreatic cancer mouse model using Panc-1/pLV Vector and Panc-1/pLV LASP-1 cells. When compared with Panc-1/pLV Vector cells, the morphology of Panc-1/pLV LASP-1 cells became irregular and had thin and long pseudopods (Supplementary Fig. S3A). The upregulation of LASP-1 protein in Panc-1/pLV LASP-1 cells was confirmed by Western blot analysis (Supplementary Fig. S3B). When injected orthotopically, Panc-1 cells developed primary tumor in the pancreas and distant metastases in the liver, gut, and mesentry over the course of 6 weeks. The size of primary pancreatic tumor and metastases of liver, gut, and mesentry in Panc-1/pLV Vector group was suppressed by digoxin treatment (mean ± SD: 9.17 ± 1.47 vs. 3.33 ± 1.03, P < 0.01; Fig. 5B and C). However, the Panc-1/pLV LASP-1 + digoxin group developed a significantly larger primary pancreatic tumor and higher number of metastatic lesions in liver, gut, and mesentry when compared with Panc-1/pLV Vector + digoxin tumors (mean ± SD: 3.33 ± 1.03 vs. 22.83 ± 2.79, P < 0.01; Fig. 5B and C). The tumors from pancreas, liver, gut, and spleen were further confirmed by hematoxylin and eosin staining (Fig. 5D). Taken together, these results suggested that overexpression of LASP-1 is critical for HIF1α-mediated PDAC metastasis.
There was no obvious correlation between expression of LASP-1 and age, gender, and histologic grade of patients with PDAC. However, LASP-1 expression was correlated with the pathologic tumor node metastasis stage ($\chi^2 = 21.806, P < 0.05$) and lymph node metastasis ($\chi^2 = 17.481, P < 0.01$) of PDAC samples (Table 1). Importantly, patients with PDAC with high or medium (++) or +++ LASP-1 protein expression had significantly worse overall survival than those with negative or low (- or +) LASP-1 expression ($P = 0.008$; median time, 8 and 16 months; Fig. 6). Taken together, these data indicated that LASP-1 correlates with lymph node metastasis and influences the prognosis of patients with PDAC.

### Discussion

In this study, we investigated the role of LASP-1 in PDAC progression and metastasis. Our data showed that LASP-1 expression levels were higher in PDAC than in adjacent non-tumorous tissues. Intriguingly, LASP-1 overexpression was also observed in metastatic breast (13), ovarian (14), colorectal (15), and hepatocellular cancer tissues (26) and cell lines. Our mechanistic studies revealed that LASP-1 overexpression in PDAC was mainly mediated by HIF1α, which directly binds to a HRE on LASP-1 promoter. Importantly, HIF1α inhibition

### Table 1. Correlation of LASP-1 expression to clinicopathologic features in PDAC

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**NOTE:** Statistical data of LASP-1 expression in relation to clinicopathologic features in PDAC surgical samples. Values of $P$ were calculated by the $\chi^2$ test. N0 and N1 refer to the absence and presence of regional lymph node (LN) metastasis, respectively. pTNM stage refers to the pathologic tumor node metastasis (pT/NM) stage.

*Statistically significant ($P < 0.05$).
PDAC metastasis. Understanding the interaction among these pathways may provide new clues for inhibiting metastasis of PDAC.

Our data indicated that LASP-1 might play a causal role in PDAC metastasis. Indeed, LASP-1 expression in patients with PDAC strongly associated with lymph node metastasis and poor clinical prognosis. Furthermore, we showed that LASP-1 indeed promoted the liver, gut, and mesentery metastases by using the orthotopic xenograft mouse model of pancreatic cancer. In summary, results from in vitro and in vivo experiments indicated that LASP-1 overexpression was a critical driver for PDAC cell migration and metastasis.

Thus, we demonstrated that LASP-1 was upregulated in PDAC particularly in those with metastasis, thereby indicating its relationship with poor clinical prognosis. LASP-1 stimulated cancer cell metastasis and aggressive phenotypes in vitro and in vivo. Most importantly, HIF1α regulated LASP-1 expression by binding to the HRE. Therefore, inhibiting LASP-1 expression may be more effective for treating metastatic PDAC.

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

Authors’ Contributions
Conception and design: T. Zhao, H. Ren, S. Yang, J. Hao
Development of methodology: T. Zhao, H. Ren, J. Chen, Y. Sun, J. Sun, H. Zhang, J. Hao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Zhao, H. Ren, J. Li, J. Chen, W. Xin, Y. Sun, L. Sun, J. Sun, X. Wang, J. Hao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Zhao, H. Ren, J. Sun, C. Huang, H. Zhang, S. Yang, J. Hao
Writing, review, and/or revision of the manuscript: T. Zhao, H. Ren, J. Chen, J. Sun, S. Gao, S. Yang, J. Hao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Zhao, H. Ren, J. Chen, H. Zhang, Y. Sun, S. Gao, J. Hao
Study supervision: H. Ren, Y. Sun, J. Hao
Other (performed the immunohistochemistry experiments and prepared the regents and buffer): Y. Yang

Grant Support
This work was supported by the National Natural Science Foundation of China (grants 81302082, 81272685, 31301151, 81172355, 31471340, 31470937, 81472264, and 81401957), the Key Program of Natural Science Foundation of Tianjin (grants 11ZCDJJC18400 and 13CYBJYC37400), and the Major Anticancer Technologies R&D Program of Tianjin (grant 12ZCDJSY16700). S. Yang is supported by the NIH (grant R01CA175741).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 10, 2014; revised September 11, 2014; accepted October 17, 2014; published OnlineFirst November 10, 2014.

References
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Cancer Res  Published OnlineFirst November 10, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-2040

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/11/07/0008-5472.CAN-14-2040.DC1

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