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Original Article

SIRT1 is highly expressed in brain metastasis tissues of non-small cell lung cancer (NSCLC) and in positive regulation of NSCLC cell migration

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Abstract: Brain metastases are a frequent and ongoing major complication of non-small cell lung cancer (NSCLC). To deepen our understanding to the underlying mechanisms by which NSCLC cells metastasize to brain and hence to improve the therapy, a high throughput RNAi screening with shRNA library of 153 epigenetic genes was subjected to A549, a NSCLC cell line with high migration ability, to examine the effects of these genes on cell migration by wound-healing assay. The screening results showed that knockdown of 2 genes (KDM5B and SIRT1) dramatically and specifically inhibits A549 migration but not affects the proliferation, which was subsequently confirmed through transwell migration assay. Furthermore, SIRT1 is found to be highly expressed in brain metastasis tissues of NSCLC, compared to the NSCLC tissues, suggesting that SIRT1 may play roles in brain metastasis of NSCLC. The relationship between SIRT1 expression and cell migration ability was further investigated in three NSCLC cell lines and the result indicated that SIRT1 expression is tightly correlated with cell migration ability. Collectively, our work provides potential biomarker and therapeutic target for brain metastasis of NSCLC.

Keywords: Non-small cell lung cancer, brain metastasis, RNAi screening, epigenetics, SIRT1

Introduction

Brain metastases are a frequent and ongoing major complication of non-small cell lung cancer (NSCLC). Among NSCLC patients, approximately 20% to 40% will develop brain metastases at some point [1, 2]. The prognosis is generally poor and available standard therapeutic options provide a limited improvement in local control, overall survival and symptom relief. The median survival for untreated patients is 1-2 months, which may be extended to 6 months with surgery, radiotherapy, and chemotherapy [3-5]. The emerging targeted therapies, such as EGFR tyrosine kinase inhibitor (TKI), erlotinib and gefitinib, and anti-VEGF Bevacizumab treatment, have been proved to be promising therapy for these patients in some clinical trials [6, 7]. However, hemorrhage of brain metastasis from non-small cell lung cancer post targeted therapies [8, 9] suggests that these therapies need further safety evaluation in more patients. Therefore, it is urgent to deepen our understanding of the underlying mechanism by which NSCLC cells metastasize to brain and hence to improve the therapy.

Cancer metastasis is a multistep process during which tumor cells, responding to different intrinsic and extrinsic stimuli, detach from the primary tumor mass, invade the contiguous stroma, migrate over a long distance, and colonize distant organs. In this complex process, reciprocal regulation of tumor cells and the microenvironment leads to the consequence of metastasis. Except that EGFR mutations [10], αv-Integrin isoform [11], CXCR4/CXCL12 [12], E-cadherin [13], S100B [14] and MMP2 expression [15] in NSCLC cells and junctional adhesion molecules and microRNAs [16] in brain [17] are involved into brain metastases of NSCLC, growing evidences suggest that epigenetic regulation plays vital roles in cancer metastasis.

Epigenetic regulation, including genomic DNA methylation, histone modifications, chromatin

remodeling and microRNAs regulation, implicates in tumorigenesis and cancer metastasis. It is proposed that epigenetic control of epithelial-to-mesenchymal transition (EMT) is also critical for the conversion of early stage tumors into invasive malignancies and therefore contributes to cancer metastasis [18]. Li et al reported that silencing of Wnt5a during colon cancer metastasis involves histone modifications, including lower levels of acetylated histone H3, H4 and H3K4me2 and higher levels of H3K27me3 in the promoter region [19]. Carmona et al found that DNA methylation-associated transcriptional silencing of CDH11, an inhibitor of tumor growth, motility and dissemination, occurred in the corresponding lymph node metastases of melanoma and head and neck cancer cells but not in the primary tumors [20]. Wu et al found epigenetic inactivation of the canonical Wnt antagonist secreted frizzled-related protein 1 by promoter hypermethylation in hepatocellular carcinoma cells contributes to metastasis in HCC progression [21]. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells [22] and the ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells [23]. All these suggest that epigenetic regulation may dynamically control the expression of key genes involved in tumor progress and contribute to cancer metastasis.

Yet, there is not systemic evaluation of epigenetic enzymes on NSCLC migration until now. To this end, an RNAi screening with epigenetics genes was applied to A549, a NSCLC cell line with high migration ability, to systematically investigate the roles of these genes in NSCLC cells migration. Wound-healing experiments showed that the knockdown of 2 genes dramatically inhibits A549 migration, which was further validated through transwell migration experiments. And SIRT1 is also found to highly express in tissues of brain metastasis of NSCLC, compared to the NSCLC tissues, suggesting that SIRT1 may play roles in brain metastasis of NSCLC. Our results might propose a potential therapeutic target of brain metastases in NSCLC.

Materials and methods

Cell culture

NSCLC cell lines (A549, NCI-H460, 95C, 95D and U1752) were maintained in Dulbecco's modified Eagle's medium (DMEM) or RMPI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) at 37°C and 5% CO_2.

Construction of shRNA lentiviral library and lentivirus infection

A customized lentiviral shRNA library targeting 153 epigenetic genes (see Supplementary Table 1A) was constructed into lentivirus vector (Invitrogen, BLOCK-iT™ Lentiviral RNAi Expression System, K4944-00), shRNA lentivirus were packaged and titered in HEK293T cells according to the manufacturer's protocol. Each gene in this library contained two pools of four distinct shRNA species targeting different positions of the target transcript. A549 cells were infected by shRNA lentivirus at MOI of 5 in the presence of polybrene (8 μg/ml).

RNAi screening

A549 cells (2500 per well) were plated in 96 well plates and infected 24 h later with shRNA library, screened as 306 pools of shRNAs per pool in independent triplicates in the primary screening. 96 h after infection, wound-healing experiments were performed to investigate the migration ability following shRNA treatments. Those genes that inhibited cell migration by higher than 20% were designated as positive hits. And then most positive hits were validated by secondary RNAi screening.

In vitro wound-healing assay

Cells in 96-well plates were transfected with shRNA lentiviral libraries. 96 h later, a 100-μL pipette tip was used to scribe a line across the cell monolayer, medium was then changed to DMEM containing 0~1% FBS. The relative migrated distances were measured using a fluorescence microspore 24 h later, compared to the NC group.

Cell migration assay

Migration assays were performed in Transwells (Corning, Inc.) (8.0-μm pore size). For migration assay, 96 h post transduction of lentiviral vectors, 20,000~10,000 cells (according to the cell types) in serum-free medium were added to the upper wells. Media containing 10% FBS were added to the lower wells. Cells that migrat-

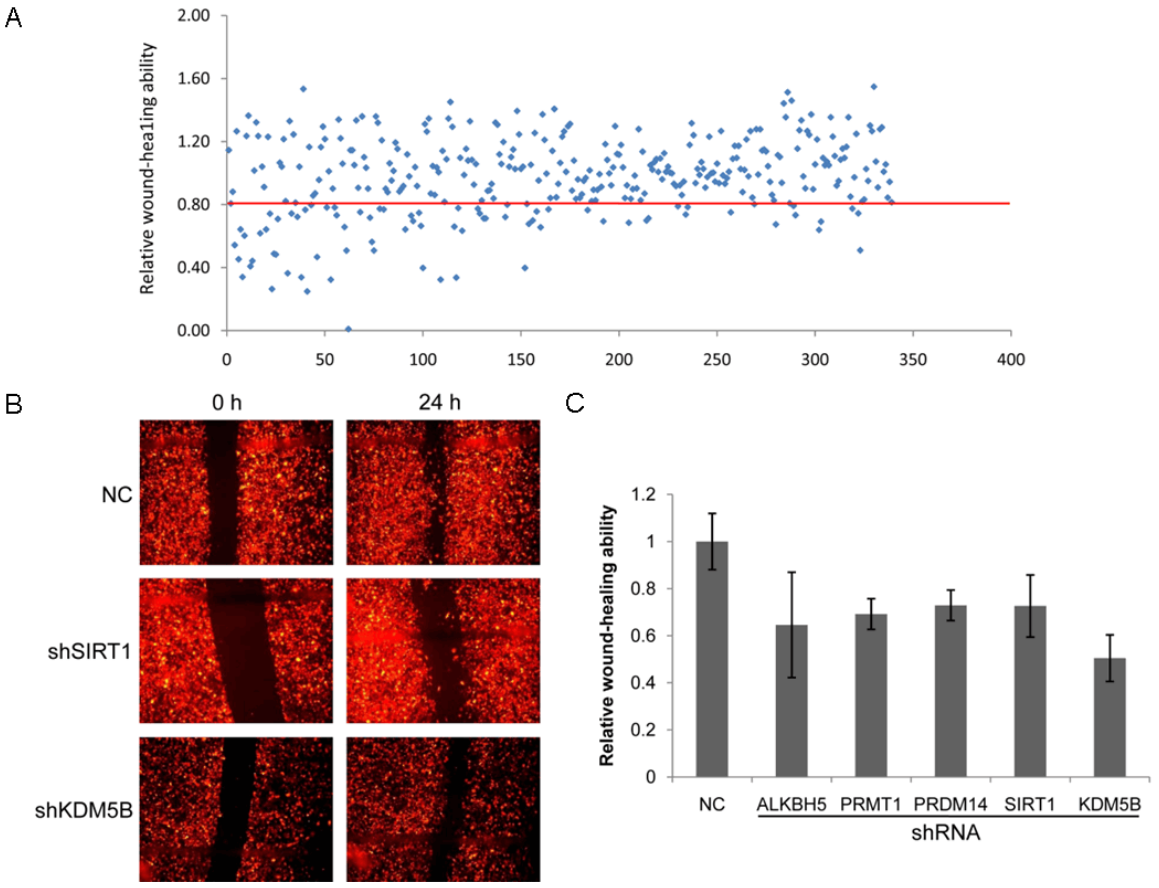

Figure 1. A high throughput screening with shRNAs library of 153 epigenetic genes. A: Primary screening. A549 cells were treated with shRNA lentiviral library for 96 h, and then wound-healing assay was performed to examine the effects of these genes on cell migration ability. There are two pools of shRNAs for each gene; every pool of some gene is comprised of four distinct shRNA species targeted to this gene. Those genes that cell migration was decreased by higher than 20% following knockdown of this gene (the dots below the red line) and $p<0.05$ were designed as positive hits. B: Wound-healing pictures of two positive hits (KDM5B and SIRT1) in the secondary screening. C: The histogram of positive hits in the secondary screening. Every treatment was triplicate in the same experiment. Bars represent the standard errors.

ed through the filter after 24 h were stained and counted by phase microscopy.

Tissue samples

Twelve brain metastasis tissues of NSCLC were obtained from the Department of Neurosurgery, Huashan Hospital (Shanghai, China) for quantitative real-time PCR (qPCR) analysis. All tissue samples were obtained from the surgical removal and immediately snap frozen in liquid nitrogen and stored in liquid nitrogen until use. In addition, two primary NSCLC tissues and three normal lung tissues were collected for qPCR analysis to investigate the expression profile of SIRT1. The study was approved by the ethics committee of Huashan Hospital. 100 mg or so tissues were homogenized in 1 ml Trizol (Life technologies), the total RNA was extracted according to the manufacturer's protocol.

Quantitative real-time PCR (qPCR)

Total RNA was synthesized to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). The primers used for qPCR validation were list in Supplementary Table 2. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in negative control cells (for gene silencing effi-

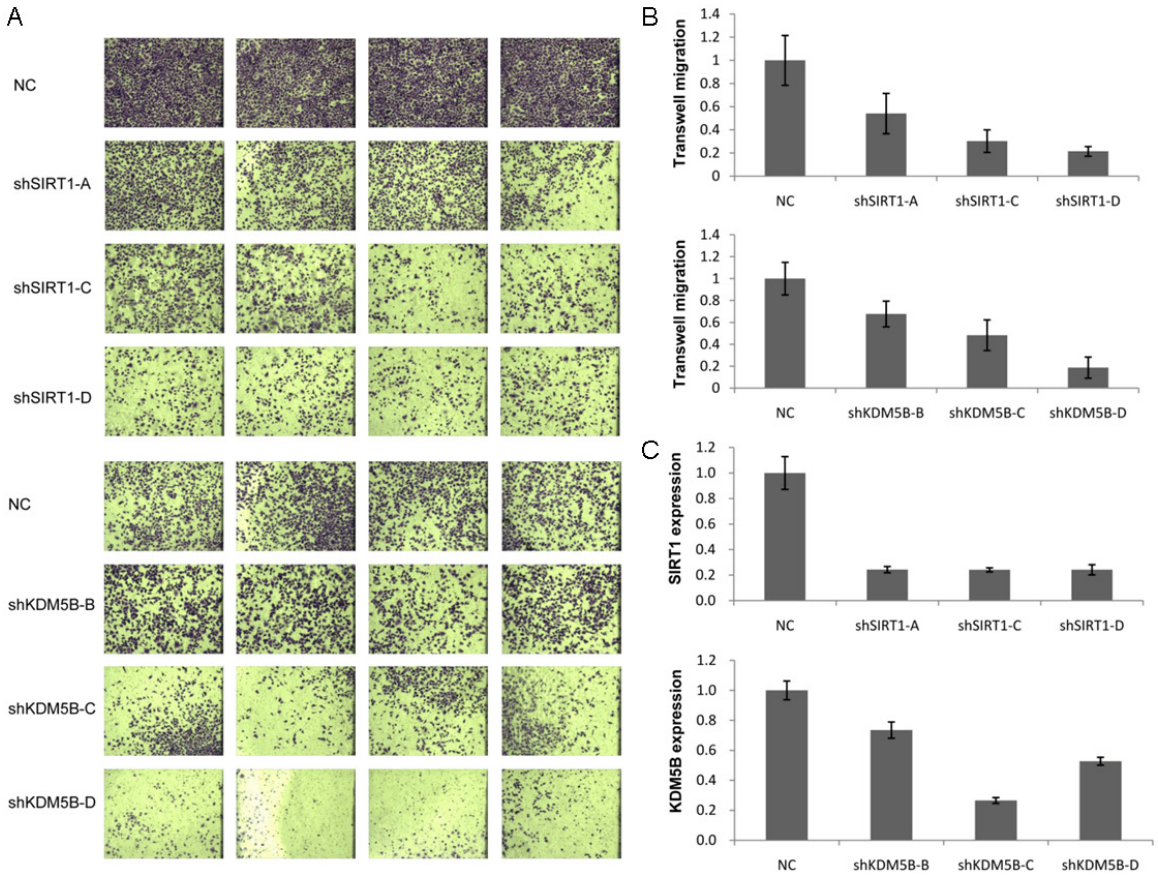

Figure 2. SIRT1 and KDM5B silencing by shRNAs specifically inhibits the migration ability of NSCLC A549 cells. A: Transwell migration assay for SIRT1 or KDM5B knockdown. SIRT1 or KDM5B knockdown (by three distinct shRNA species of each gene, respectively, for 96 h) cells were seeded into the upper well, 24 h later the migrated cells were stained and photographed. For eah shRNA specie, two repeats were performed. Two fields of each repeat were list in the figure. B: The histogram of migration ability for KDM5B or SIRT1 knockdown. C: Gene silencing by shRNA was assayed by qPCR. Bars represent the standard errors.

cacy detection) or in normal tissue 1 (for SIRT1 expression in tissues) or in A549 (for SIRT1 expression in NSCLC cell lines).

MTS cell proliferation assay

The tetrazolium compound (MTS) cell proliferation assay is a quantitative colorimetric assay for mammalian cell survival and proliferation. A549 cells (4×10^3) were grown in 100 µl of culture medium containing serum per well in a 96-well plate. After 24 h, the cells were treated by shSIRT1-AD or shSIRT1-EH lentivirus for 96 h (shSIRT1-AD is a shRNAs pool composed of shSIRT1-A, shSIRT1-B, shSIRT1-C and shSIRT1-D; shSIRT1-EH is a shRNAs pool composed of shSIRT1-E, shSIRT1-F, shSIRT1-G and shSIRT1-H). Every treatment for each cell line was triplicate in the same experiment. Then 20 µl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 2 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol.

Results

RNAi screening in A549 cells for migration-related epigenetic genes

Primary screening with shRNA library of 153 epigenetic genes through wound-healing assay showed that 25 epigenetic genes positively regulate the NSCLC cell migration ability. Knockdown of these genes significantly ($p<0.05$) reduced migration ability of A549 cells (**Figure 1A**). Interestingly, the other 26 epigenetic genes were involved in the negative

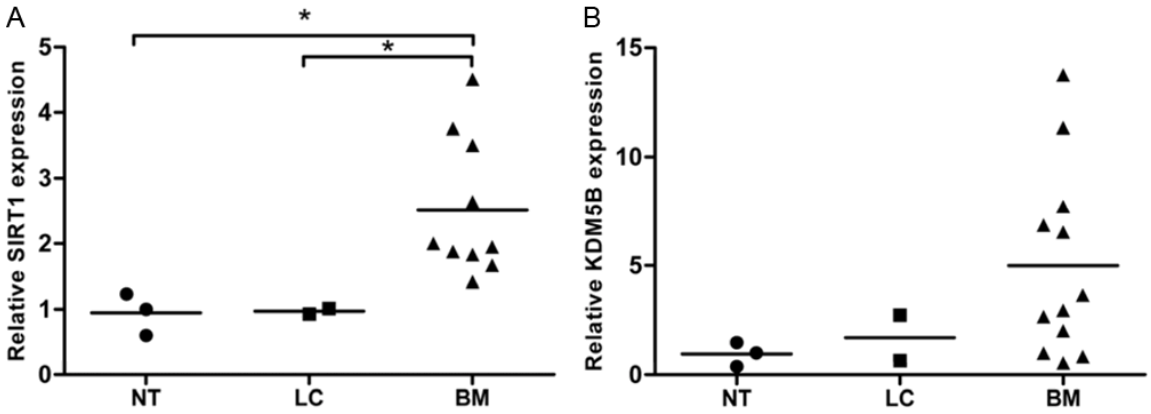

Figure 3. SIRT1 and KDM5B expression in brain metastasis tissues of NSCLC (n=12), NSCLC tissues (n=2) and normal tissues (n=3) detected by qPCR. NT, normal tissues of lung; LC, NSCLC tissues; BM, brain metastasis tissues of NSCLC.

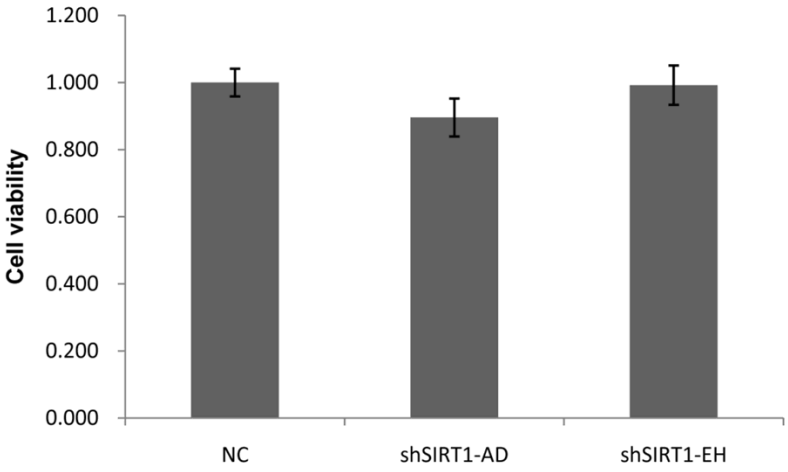

Figure 4. A549 cell proliferation following SIRT1 silencing detected by MTS assay. A549 cells were treated with two pools for SIRT1, 96 h later MTS assay was performed.

regulation of A549 cells migration (cell migration ability was increased by higher than 20% following shRNA treatments, $p<0.05$, see Supplementary Table 1B), such as HDAC6. Silence of HDAC6 increased A549 migration ability by 24% or 40% (two pools of HDAC6 shRNAs induced A549 migration by different extents). In total, 1/3 of epigenetic genes in our library were associated with the regulation of A549 cells migration. A secondary screening was performed for those 25 positive hits to validate their roles in cell migration and 5 genes were found to be positive in these two cycles of screening (**Figure 1C**). Among these 5 genes, ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism [24], KDM5B is a histone demethylase, PRDM14 may possess histone methyltransferase activity, PRMT1 encodes a member of the protein arginine N-methyltransferase (PRMT) family, while the functions of human SIRT1 have not yet been determined; however, yeast sirtuin proteins are known to regulate epigenetic gene silencing.

Validation of positive genes by transwell migration assay and selection of effective shRNA species by qPCR

To further validate the roles of positive genes in A549 cells migration, transwell migration assay was performed. The 4 distinct shRNA species comprised of each positive pool for KDM5B and SIRT1 were constructed, respectively, and infected A549 cells. The transwell migration results showed that knockdown of KDM5B and SIRT1 by more than 2 single shRNA species really suppressed the migration ability of A549 cells (**Figure 2A**, **2B**), suggesting these inhibition is not caused by off-target effect [25].

KDM5B and SIRT1 silencing by shRNAs was confirmed by quantitative PCR (**Figure 2C**). The two KDM5B/SIRT1-targeting shRNAs that caused the most significant effects on tran-

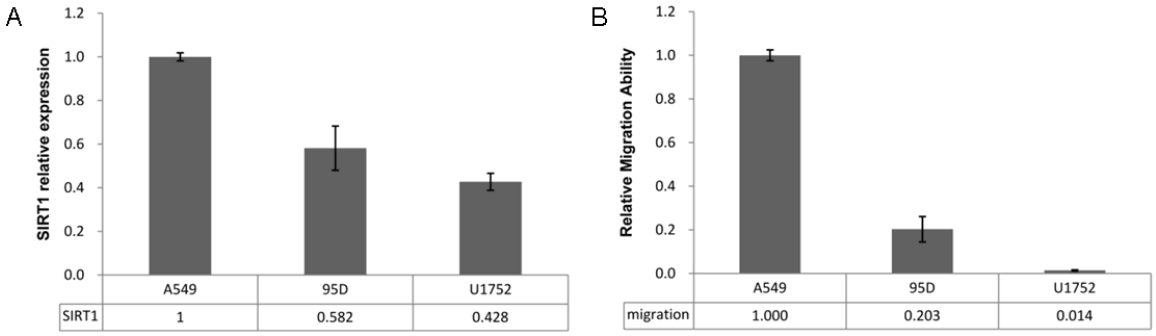

Figure 5. The relationship between SIRT1 expression and cell migration ability in five NSCLC cell lines. A: SIRT1 expression in five NSCLC cell lines examined by qPCR. B: Migration ability of five NSCLC cell lines detected by transwell migration assay.

swell migration ability were also shown to cause the most significant silencing (**Figure 2B**, **2C**).

Collectively, KDM5B and SIRT1 silencing by shRNAs specifically inhibits the migration ability of NSCLC A549 cells.

SIRT1 is highly expressed in brain metastasis tissues of NSCLC patients

To evaluate the clinical importance of KDM5B and SIRT1 for NSCLC metastasis, we examined the mRNA expression of these two genes in NSCLC. 2 NSCLC tissues and corresponding normal tissues and 12 brain metastasis tissues of NSCLC patients were collected. qPCR results showed that SIRT1 expression in brain metastasis tissues of NSCLC patients was significantly increased, compared to that in NSCLC tissues and corresponding normal tissues (**Figure 3A**). However, there was not marked difference in KDM5B expression between these 3 types of tissues (**Figure 3B**). This suggests that high expression of SIRT1 is highly correlated to the brain metastasis of NSCLC in clinic.

SIRT1 knockdown does not affect A549 cell proliferation

SIRT1 is proposed to be involved in proliferation of cancer cells [26, 27], so we investigated cell viability following SIRT1 knockdown (**Figure 4**). The results showed that A549 cell proliferation was not significantly changed after SIRT1 silencing, suggesting that the effect of SIRT1 on cell migration above mentioned is not caused by anti-proliferation of SIRT1 knockdown.

SIRT1 expression level is correlated to migration ability of NSCLC cell lines

And then the relationship between SIRT1 expression and cell migration ability was investigated in 3 NSCLC cell lines (A549, 95D and U1752). The results showed that Sirt1 expression was decreased gradually according to the following order: A549, 95D and U1752 (**Figure 5A**), and the cell migration ability was decreased stepwise according to the same order (**Figure 5B**). There are positive correspondences between these two data sets. Yet, due to limited cell line number, this correlation is still need to be further examined in more NSCLC cell lines and with functional confirmation by establishing over-expression stable cell lines. This indicates that SIRT1 expression is positively correlated to the migration ability of NSCLC cell lines.

Discussion

Brain metastasis of NSCLC is a great challenge for approximately 1/3 NSCLC patients, it is urgent to broaden and deepen our understandings on the mechanisms by which NSCLC cells metastasize to brain. As the first step, it is of great interest to find genes related to the migration of NSCLC cells *in vitro.*

Growing evidence suggsets that epigenetic regulation implicates in tumorigenesis and metastasis. But the systemic investigation of whole epigenetic genes in metastasis regulation has not been reported. In present work, a high throughput RNAi screening with shRNA library of 153 epigenetic genes was applied to A549, a NSCLC cell line with high migration ability.

Primary screening found that 1/3 or so epigenetic genes are involved in positive or negative regulation of A549 migration. This suggests an all-around regulation was carried out by epigenetic genes, some genes promote migration, and others inhibit migration, cell migration ability is controlled in a balance manner. Genetic variations in tumor cells or stimuli from microenvironment induce or inactivate some genes of this system and hence break this balance and contribute to the alteration on migration ability. So, it is the genetic variations in tumor cells or stimuli from microenvironment that is worthy of further studies to find the underlying reasons for alterations of migration ability in future.

Secondary screening found that 5 epigenetic genes were in positive regulation on A549 migration. 2 out of these 5 genes, KDM5B and SIRT1, were further validated through transwell migration assay and the gene silencing of shRNA was verified by qPCR. Knockdown of KDM5B and SIRT1 was found to specifically inhibit A549 migration. Subsequent experiments showed that SIRT1 was highly expressed in brain metastasis tissues of NSCLC patients, compared to that in NSCLC tissues and the corresponding normal tissues, suggesting the clinical importance of SIRT1 in brain metastasis of NSCLC.

The class III histone deacetylase SIRT1 is a nicotinamide adenine dinucleotide (NAD(+))-dependent deacetylase, SIRT1 is reported to be overexpressed and catalytically activated in a number of human cancers and implicated in tumorigenesis and metastasis of several types of cancer, including gastric carcinoma, pancreatic cancer, breast cancer, prostate cancer, colorectal adenocarcinoma and NSCLC. Cha et al reported in 2009 that SIRT1 expression is significantly correlated with lymph node metastasis and tumor invasion and is associated with poor prognosis of gastric carcinoma [28]. Noh et al reported SIRT1 expression is associated with lymph node metastasis and advanced tumor invasion [29]. Several studies suggest that SIRT1 can induce epithelial-mesenchymal transition (EMT) and invasion and hence promote cancer metastasis, silencing of SIRT1 inhibit cancer cell migration or invasion [30-32], which is consistent with our data. However, there have been conflicting data supporting whether SIRT1 act as a tumour promoter or as a tumour suppressor. It is proposed that SIRT1 suppresses EMT and hence inhibit cancer cell migration or invasion, the downregulation of SIRT1 expression plays roles in metastasis of ovarian cancer, lung cancer and colorectal adenocarcinoma [33-35]. The reason for this inconsistency may be in that cancer cell migration/invasion is probably controlled by a complex network comprised of many epigenetic genes, while SIRT1 is just one member of this network. Furthermore, SIRT1 is under control of some microRNAs, such as miR-204 [36], miR-520c and miR-373 [37], and miR-22 [38]. Therefore, it is necessary to reveal the upstream regulators and the downstream effectors of SIRT1 and to find out the other migration-regulated genes in further study.

Taken together, our high throughput RNAi screening showed that many epigenetic genes are involved in migration regulation, SIRT1 is associated with positive regulation of A549 cells migration and SIRT1 is highly expressed in brain metastasis tissues of NSCLC. SIRT1 is a drugable target, which might do benefit to patients in clinical therapy combination with SIRT1 specific inhibitor.

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References

[1] Mujoomdar A, Austin JH, Malhotra R, Powell CA, Pearson GD, Shiau MC, Raftopoulos H. Clinical predictors of metastatic disease to the brain from non-small cell lung carcinoma: primary tumor size, cell type, and lymph node metastases. Radiology 2007; 242: 882-888.

[2] Barnholtz-Sloan JS, Sloan AE, Davis FG, Vigneau FD, Lai P, Sawaya RE. Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the Metropolitan Detroit Cancer Surveillance System. J Clin Oncol 2004; 22: 2865-2872.

[3] Knisely JP, Berkey B, Chakravarti A, Yung AW, Curran WJ Jr, Robins HI, Movsas B, Brachman DG, Henderson RH, Mehta MP. A phase III study of conventional radiation therapy plus

thalidomide versus conventional radiation therapy for multiple brain metastases (RTOG 0118). Int J Radiat Oncol Biol Phys 2008; 71: 79-86.
[4] McHaffie DR, Chabot P, Dagnault A, Suh JH, Fortin MA, Chang E, Timmerman R, Souhami L, Grecula J, Nabid A, Schultz C, Werner-Wasik M, Gaspar LE, Brachman D, Mody T, Mehta MP. Safety and feasibility of motexafin gadolinium administration with whole brain radiation therapy and stereotactic radiosurgery boost in the treatment of </= 6 brain metastases: a multi-institutional phase II trial. J Neurooncol 2011; 105: 301-308.
[5] Fidler IJ. The role of the organ microenvironment in brain metastasis. Semin Cancer Biol 2011; 21: 107-112.
[6] Welsh JW, Komaki R, Amini A, Munsell MF, Unger W, Allen PK, Chang JY, Wefel JS, McGovern SL, Garland LL, Chen SS, Holt J, Liao Z, Brown P, Sulman E, Heymach JV, Kim ES, Stea B. Phase II trial of erlotinib plus concurrent whole-brain radiation therapy for patients with brain metastases from non-small-cell lung cancer. J Clin Oncol 2013; 31: 895-902.
[7] Crino L, Dansin E, Garrido P, Griesinger F, Laskin J, Pavlakis N, Stroiakovski D, Thatcher N, Tsai CM, Wu YL, Zhou C. Safety and efficacy of first-line bevacizumab-based therapy in advanced non-squamous non-small-cell lung cancer (SAiL, MO19390): a phase 4 study. Lancet Oncol 2010; 11: 733-740.
[8] Yan DF, Yan SX, Yang JS, Wang YX, Sun XL, Liao XB, Liu JQ. Hemorrhage of brain metastasis from non-small cell lung cancer post gefitinib therapy: two case reports and review of the literature. BMC Cancer 2010; 10: 49.
[9] Socinski MA, Langer CJ, Huang JE, Kolb MM, Compton P, Wang L, Akerley W. Safety of bevacizumab in patients with non-small-cell lung cancer and brain metastases. J Clin Oncol 2009; 27: 5255-5261.
[10] Breindel JL, Haskins JW, Cowell EP, Zhao M, Nguyen DX, Stern DF. EGF Receptor Activates MET through MAPK to Enhance Non-Small Cell Lung Carcinoma Invasion and Brain Metastasis. Cancer Res 2013; 73: 5053-65.
[11] Vogetseder A, Thies S, Ingold B, Roth P, Weller M, Schraml P, Goodman SL, Moch H. alphav-Integrin isoform expression in primary human tumors and brain metastases. Int J Cancer 2013; 133: 2362-71.
[12] Wald O, Shapira OM, Izhar U. CXCR4/CXCL12 axis in non small cell lung cancer (NSCLC) pathologic roles and therapeutic potential. Theranostics 2013; 3: 26-33.
[13] Yoo JY, Yang SH, Lee JE, Cho DG, Kim HK, Kim SH, Kim IS, Hong JT, Sung JH, Son BC, Lee SW. E-cadherin as a predictive marker of brain metastasis in non-small-cell lung cancer, and its regulation by pioglitazone in a preclinical model. J Neurooncol 2012; 109: 219-227.
[14] Pang X, Min J, Liu L, Liu Y, Ma N, Zhang H. S100B protein as a possible participant in the brain metastasis of NSCLC. Med Oncol 2012; 29: 2626-2632.
[15] Rojiani MV, Alidina J, Esposito N, Rojiani AM. Expression of MMP-2 correlates with increased angiogenesis in CNS metastasis of lung carcinoma. Int J Clin Exp Pathol 2010; 3: 775-781.
[16] Teplyuk NM, Mollenhauer B, Gabriely G, Giese A, Kim E, Smolsky M, Kim RY, Saria MG, Pastorino S, Kesari S, Krichevsky AM. MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity. Neuro Oncol 2012; 14: 689-700.
[17] Jia W, Martin TA, Zhang G, Jiang WG. Junctional adhesion molecules in cerebral endothelial tight junction and brain metastasis. Anticancer Res 2013; 33: 2353-2359.
[18] Wang Y, Shang Y. Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. Exp Cell Res 2013; 319: 160-169.
[19] Li Q, Chen H. Silencing of Wnt5a during colon cancer metastasis involves histone modifications. Epigenetics 2012; 7: 551-558.
[20] Carmona FJ, Villanueva A, Vidal A, Munoz C, Puertas S, Penin RM, Goma M, Lujambio A, Piulats JM, Mesia R, Sanchez-Cespedes M, Manos M, Condom E, Eccles SA, Esteller M. Epigenetic disruption of cadherin-11 in human cancer metastasis. J Pathol 2012; 228: 230-240.
[21] Wu Y, Li J, Sun CY, Zhou Y, Zhao YF, Zhang SJ. Epigenetic inactivation of the canonical Wnt antagonist secreted frizzled-related protein 1 in hepatocellular carcinoma cells. Neoplasma 2012; 59: 326-332.
[22] Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 2008; 9: 582-589.
[23] Brabletz S, Bajdak K, Meidhof S, Burk U, Niedermann G, Firat E, Wellner U, Dimmler A, Faller G, Schubert J, Brabletz T. The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells. EMBO J 2011; 30: 770-782.
[24] Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, Vagbo CB, Shi Y, Wang WL, Song SH, Lu Z, Bosmans RP, Dai Q, Hao YJ, Yang X, Zhao WM, Tong WM, Wang XJ, Bogdan F, Furu K, Fu Y, Jia G, Zhao X, Liu J, Krokan HE, Klungland A, Yang YG, He C. ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. Mol Cell 2013; 49: 18-29.
[25] Echeverri CJ, Beachy PA, Baum B, Boutros M, Buchholz F, Chanda SK, Downward J, Ellenberg

J, Fraser AG, Hacohen N, Hahn WC, Jackson AL, Kiger A, Linsley PS, Lum L, Ma Y, Mathey-Prevot B, Root DE, Sabatini DM, Taipale J, Perrimon N, Bernards R. Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat Methods 2006; 3: 777-779.

[26] Bae HJ, Noh JH, Kim JK, Eun JW, Jung KH, Kim MG, Chang YG, Shen Q, Kim SJ, Park WS, Lee JY, Nam SW. MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. Oncogene 2013; [Epub ahead of print].

[27] Moore RL, Faller DV. SIRT1 represses estrogen-signaling, ligand-independent ERalpha-mediated transcription, and cell proliferation in estrogen-responsive breast cells. J Endocrinol 2013; 216: 273-285.

[28] Cha EJ, Noh SJ, Kwon KS, Kim CY, Park BH, Park HS, Lee H, Chung MJ, Kang MJ, Lee DG, Moon WS, Jang KY. Expression of DBC1 and SIRT1 is associated with poor prognosis of gastric carcinoma. Clin Cancer Res 2009; 15: 4453-4459.

[29] Noh SJ, Baek HA, Park HS, Jang KY, Moon WS, Kang MJ, Lee DG, Kim MH, Lee JH, Chung MJ. Expression of SIRT1 and cortactin is associated with progression of non-small cell lung cancer. Pathol Res Pract 2013; 209: 365-370.

[30] Zhao G, Cui J, Zhang JG, Qin Q, Chen Q, Yin T, Deng SC, Liu Y, Liu L, Wang B, Tian K, Wang GB, Wang CY. SIRT1 RNAi knockdown induces apoptosis and senescence, inhibits invasion and enhances chemosensitivity in pancreatic cancer cells. Gene Ther 2011; 18: 920-928.

[31] Nakane K, Fujita Y, Terazawa R, Atsumi Y, Kato T, Nozawa Y, Deguchi T, Ito M. Inhibition of cortactin and SIRT1 expression attenuates migration and invasion of prostate cancer DU145 cells. Int J Urol 2012; 19: 71-79.

[32] Byles V, Zhu L, Lovaas JD, Chmilewski LK, Wang J, Faller DV, Dai Y. SIRT1 induces EMT by cooperating with EMT transcription factors and enhances prostate cancer cell migration and metastasis. Oncogene 2012; 31: 4619-4629.

[33] Sun L, Li H, Chen J, Iwasaki Y, Kubota T, Matsuoka M, Shen A, Chen Q, Xu Y. PIASy mediates hypoxia-induced SIRT1 transcriptional repression and epithelial-to-mesenchymal transition in ovarian cancer cells. J Cell Sci 2013; 126: 3939-47.

[34] Sun L, Li H, Chen J, Dehennaut V, Zhao Y, Yang Y, Iwasaki Y, Kahn-Perles B, Leprince D, Chen Q, Shen A, Xu Y. A SUMOylation-dependent pathway regulates SIRT1 transcription and lung cancer metastasis. J Natl Cancer Inst 2013; 105: 887-898.

[35] Jang SH, Min KW, Paik SS, Jang KS. Loss of SIRT1 histone deacetylase expression associates with tumour progression in colorectal adenocarcinoma. J Clin Pathol 2012; 65: 735-739.

[36] Zhang L, Wang X, Chen P. MiR-204 down regulates SIRT1 and reverts SIRT1-induced epithelial-mesenchymal transition, anoikis resistance and invasion in gastric cancer cells. BMC Cancer 2013; 13: 290.

[37] Liu P, Wilson MJ. miR-520c and miR-373 upregulate MMP9 expression by targeting mTOR and SIRT1, and activate the Ras/Raf/MEK/Erk signaling pathway and NF-kappaB factor in human fibrosarcoma cells. J Cell Physiol 2012; 227: 867-876.

[38] Xu D, Takeshita F, Hino Y, Fukunaga S, Kudo Y, Tamaki A, Matsunaga J, Takahashi RU, Takata T, Shimamoto A, Ochiya T, Tahara H. miR-22 represses cancer progression by inducing cellular senescence. J Cell Biol 2011; 193: 409-424.

Table S1A. 153 epigenetic genes

Gene	Gene	Gene	Gene	Gene	Gene
ACTL6A	C17ORF49	JMJD1C	MECOM	PRMT6	SUV39H1
ALKBH4	CARM1	JMJD4	MEN1	PRMT7	SUV39H2
ALKBH5	CBX2	JMJD5	MLL	PRMT8	SUV420H1
ALKBH6	CBX7	JMJD6	MLL2	RAD54L	SUV420H2
ALKBH7	CHD4	JMJD7	MLL3	RAD54L2	UTY
ALKBH8	CIITA	JMJD8	MLL5	REST	WHSC1
ARID1A	CREBBP	KAT2A	MPG	RNF2	WHSC1L1
ARID2	CSRP2BP	KAT2B	MTA3	RNF20	ZMYND8
ASF1A	DOT1L	KAT5	NSD1	RPS6KA3	
ASF1B	DZIP3	KAT6A	PHF6	RPS6KA5	
ASH1L	EGLN2	KAT6B	PRDM1	SETD1A	
ASH2L	EHMT1	KAT7	PRDM10	SETD1B	
ASXL1	EHMT2	KAT8	PRDM11	SETD2	
ATAD2	EPC1	KDM1A	PRDM12	SETD3	
ATF2	ESCO1	KDM1B	PRDM13	SETD4	
ATF7IP	ESCO2	KDM2A	PRDM14	SETD5	
ATRX	EZH1	KDM2B	PRDM15	SETD6	
BAZ1A	EZH2	KDM3A	PRDM16	SETD7	
BAZ1B	FBXO11	KDM3B	PRDM2	SETD8	
BAZ2A	HAT1	KDM4A	PRDM4	SETDB1	
BAZ2B	HDAC1	KDM4B	PRDM5	SETDB2	
BMI1	HDAC2	KDM4C	PRDM6	SETMAR	
BRCA2	HDAC3	KDM4D	PRDM7	SIRT1	
BRD1	HDAC5	KDM5A	PRDM8	SIRT2	
BRD2	HDAC6	KDM5B	PRDM9	SMYD1	
BRD8	HDAC7	KDM5C	PRMT1	SMYD2	
BRDT	HDAC8	KDM5D	PRMT2	SMYD3	
BRWD3	JARID2	KDM6A	PRMT3	SMYD4	
C14ORF169	JHDM1D	KDM6B	PRMT5	SMYD5	

Table S1B. 25 genes in positive regulation and 26 genes in negative regulation of A540 cell migration

Gene	relative migration	*P* value	Gene	relative migration	*P* value
ESCO2	0.010	0.00002	KDM4B	1.278	0.039
ASF1B	0.264	0.002	SETD1B	1.278	0.034
SIRT2	0.323	0.001	KDM4A	1.278	0.034
KAT2A	0.324	0.005	PRMT5	1.279	0.016
CBX2	0.336	0.001	WHSC1L1	1.290	0.047
ATRX	0.338	0.006	MEN1	1.298	0.038
HDAC7	0.340	0.006	PRDM4	1.298	0.029
ATAD2	0.364	0.009	BRDT	1.311	0.022
BRDT	0.398	0.004	SUV420H1	1.317	0.046
BRWD3	0.398	0.004	ALKBH7	1.320	0.020
ALKBH4	0.408	0.007	ALKBH6	1.320	0.032
ALKBH5	0.443	0.010	REST	1.329	0.015
HDAC5	0.453	0.033	KDM2B	1.335	0.010
BRD2	0.467	0.033	BMI1	1.346	0.008
ASH1L	0.483	0.049	MPG	1.346	0.013
ZMYND8	0.508	0.018	MECOM	1.354	0.012
PRDM6	0.510	0.007	KDM5D	1.354	0.038
BRCA2	0.634	0.036	SETD3	1.372	0.021
ARID1A	0.642	0.041	C17ORF49	1.372	0.015
KDM5B	0.674	0.014	HDAC6	1.394	0.004
PRMT1	0.684	0.008	EHMT1	1.407	0.004
PRDM14	0.694	0.016	SETD6	1.451	0.001
SIRT1	0.721	0.032	UTY	1.460	0.002
SUV39H2	0.737	0.035	KDM6A	1.513	0.0004
PRDM1	0.765	0.037	ATRX	1.534	0.019
			SETD2	1.548	0.001

Table S2. Primers for qPCR

gene	forward	reverse
Actb	caccatgtaccctggcatt	gtacttgcgctcaggaggag
KDM5B	atcgagtgggctcaggact	tgccaggaggttgagatacc
SIRT1	agagtggcaaaggagcagattagt	cctcagcgccatggaaaat