SLC39A4 expression is associated with enhanced cell migration, cisplatin resistance, and poor survival in non-small cell lung cancer

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The zinc transporter SLC39A4 influences epithelial cell morphology and migration in various cancers; however, its role in regulating cell invasion and chemotherapeutic resistance in human lung cancer is not yet clear. Here, integrated analysis of gene expression in non-small cell lung cancer revealed that SLC39A4 expression is significantly correlated with increased tumour size and regional lymph node spread, as well as shorter overall survival (OS) and disease-free survival (DFS). SLC39A4 silencing by lentivirus-mediated shRNA blocked human lung cancer cell epithelial-mesenchymal transition and metastasis in vitro and in vivo, respectively. Moreover, SLC39A4 knockdown enhanced cancer cell sensitivity to cisplatin-induced death by inhibiting stemness in lung cancer cells. Collectively, these data suggest that SLC39A4 may be a novel therapeutic target and predictive marker of tumour metastasis in non-small cell lung cancer.

Recent evidence indicates that the zinc transporter SLC39A4 promotes cell invasion and apoptotic resistance in pancreatic cancer1, 2, glioma3, 4, and prostate carcinoma5 through multiple mechanisms such as IL6/STAT3 pathway activation6 or regulation of protein expression7. However, although these findings indicate that SLC39A4 plays an important role in cancer cell migration and survival, the molecular mechanisms underlying these processes require further elucidation.

The epithelial-mesenchymal transition (EMT) drives polarized, immotile epithelial cells to acquire apolar, highly migratory fibroblastoid-like features. This process is indispensable for organogenesis, tissue remodelling, and wound healing8, but it is also a means for cancer metastasis. The functional significance of EMT in cancer metastasis is based on the observation that the acquisition of mesenchymal markers such as vimentin or fibroblast-specific protein 1 (FSP1) and loss of cell adhesion molecules such as E-cadherin in epithelial carcinoma cells is associated with increased metastatic potential9, 10. Moreover, the EMT also plays a dominant role in the initial stage of cancer cell migration, where epithelial-derived tumour cells obtain a propensity for migration and deformation that enables their invasion into the basement membrane11. Concurrently, the EMT can induce stem cell-like characteristics in cancer cells, enhancing their resistance to chemotherapy12. Nevertheless, the participation of SLC39A4 in EMT, stemness, and the chemotherapeutic resistance of cancer cells requires further research.

Lung cancer is one of the most common malignancies worldwide and the leading cause of cancer-related death in men and women13, 14. Tumours are often classified into two main subtypes, i.e., small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), based on the size and appearance of cancer cells under the microscope. NSCLC accounts for the majority of lung cancers and is generally characterized by regional lymph node metastasis and hematogenous spread15. Lung cancer development and progression are complex processes that involve multiple causes, including environmental factors, long-term exposure to radioactive materials and their derivatives, and genetic mutations16. Metastatic lung cancer cells are usually resistant to radiation and chemotherapy, greatly limiting the prognosis of these patients17; thus, the identification of metastasis-related biomarkers in lung cancer is particularly relevant for the development of precision therapies18.

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The growing prevalence of high-throughput genomics has generated large amounts of data pertinent to cancer research, leading to the formation of multiple cancer-specific gene expression databases, such as the Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA). To date, little is known about the clinical significance and function of SLC39A4 in NSCLC pathogenesis; therefore, this study aimed to investigate its relevance in meta-analyses of data obtained from GEO and TCGA. We subsequently confirmed our results by tissue arrays, SLC39A4 functional studies in cultured cells and animal models.

Here, we showed that SLC39A4 upregulation is a prognostic indicator of NSCLC. Cell-based assays also demonstrated an important role for SLC39A4 in the diminished migratory potential, stemness, and chemoresistance of A549 lung cancer cells both in vitro and in vivo. Collectively, this research suggests that SLC39A4 may be a novel therapeutic target in NSCLC.

### Results

**SLC39A4 is an independent predictor of NSCLC.** To study the role and function of SLC39A4 in NSCLC pathogenesis, we first assessed the levels of SLC39A4 expression in NSCLC samples and normal tissues. Mean differences were estimated using an inverse variance (IV)-weighted random-effects model (Mean difference, 1.48; 95% CI, 0.88–2.08). Metabolic analysis of SLC39A4 specificity and sensitivity as a biomarker of NSCLC (c) SLC39A4 expression association with tumour stage using a Mantel-Haenszel (M-H)-weighted random-effects model (Odds Ratio [OR], 0.52; 95% CI, 0.34–0.80).
SLC39A4 expression is negatively correlated with overall survival (OS) and disease-free survival (DFS) in NSCLC. We queried the GEO and TCGA databases and found seven datasets that included data on various survival outcomes. A meta-analysis of these studies revealed that increased tumour SLC39A4 expression was associated with shortened OS ($Z = 2.05, P < 0.04$) and DFS ($Z = 2.46, P < 0.01$) (Supplementary Figure 1a,b). To confirm this finding, we examined the association between SLC39A4 expression and survival outcomes in lung tumour arrays by immunohistochemistry. For this, the 90 tumour samples were dichotomized into groups with high or low SLC39A4 expression based on the median staining intensity observed in the array ($\mu = 6.516$) (Fig. 2a), and then evaluated for OS and DFS by Kaplan-Meier analysis (Fig. 2b,c). Notably, we found that high SLC39A4 expression was a significant indicator of poor OS and DFS in NSCLC (OS: $HR = 2.918, 95\% CI: 2.353–3.482, P = 0.0333$; DFS: $HR = 2.498, 95\% CI: 1.924–3.072, P = 0.0180$), consistent with the results of our meta-analysis. Moreover, we also determined that SLC39A4 expression was associated with increased tumour volume ($P = 0.0105$), regional lymph node spread ($P = 0.0030$), and clinical stage ($P = 0.0412$) (Table 1). More importantly, regional lymph nodes ($HR = 2.562, P = 0.013$), clinical stage ($HR = 2.118, P = 0.022$) and SLC39A4 overexpression ($HR = 3.067, P = 0.019$) were also independent predictors for OS in the multivariate analysis (Table 2). As shown in Fig. 2d, we also observed an increase in SLC39A4 expression corresponding with the progression of in situ tumours to metastatic lesions.

SLC39A4 silencing inhibits lung cancer cell metastasis in vitro. EMT is a key step in the metastatic initiation of cancer cells$^{22}$. Because tumour metastasis is significantly detrimental to the survival of patients with lung cancer, we further investigated the effects of SLC39A4 expression on lung cancer cell migration. A549
Cells were infected with lentivirus expressing SLC39A4 shRNA or control lentivirus and potent knockdown was confirmed by qPCR and western blot analysis for mRNA and protein expression, respectively (P < 0.01; Fig. 3a,b). We found that SLC39A4 knockdown inhibits the Zn$^{2+}$ concentration of cells in vivo (Fig. 3c). Wound-healing, transwell, and matrigel invasion assays revealed that SLC39A4 knockdown significantly inhibited A549 cell migration (Fig. 3d–i). Consistently, immunofluorescence and western blot analysis showed a marked upregulation of the epithelial marker E-cadherin accompanied by a concomitant downregulation of the mesenchymal markers FSP-1 and N-cadherin in response to SLC39A4 silencing (Fig. 3j,k). The same experimental results were obtained from SPC-A-1 cells (Supplementary Figure 2). In addition, the function of SLC39A4 in EMT was investigated in normal lung epithelial cell line BEAS-2B; we found that SLC39A4 knockdown reduced the Zn$^{2+}$ concentration and inhibited the expression of EMT markers (Supplementary Figure 3). This pattern was also observed in the GEO meta-analysis, where SLC39A4 levels were negatively correlated with E-cadherin expression (Z = 7.60, P < 0.00001; Supplementary Figure 4a), but positively associated with FSP-1 (Z = 6.56, P < 0.00001) and N-cadherin (Z = 6.59, P < 0.0001; Supplementary Figure 4b,c) expression.

SLC39A4 silencing attenuates metastatic spread in a mouse model of lung cancer. We further investigated the metastatic capability of shSLC39A4 cells in mouse model of lung cancer. Notably, mice injected with shSLC39A4 knockdown cells exhibited a smaller loss in body weight (Fig. 4a) that corresponded with diminished pulmonary metastasis when compared to control mice (P < 0.01; Fig. 4b–d). Moreover, immunofluorescence of A549 subcutaneous tumours confirmed the increased E-cadherin and decreased FSP-1 and N-cadherin expression in shSLC39A4 A549 cells.

SLC39A4 silencing promotes sensitivity to cisplatin in human NSCLC cells. Our previous results suggested that shSLC39A4 depressed the mesenchymal phenotype of A549 cells. Because many studies have

| Factors | No. of Cases | Average Staining Score ± SD | P-value |
|---------|--------------|-----------------------------|---------|
| Sex     |              |                             |         |
| Male    | 49           | 6.441 ± 0.2136              | 0.7848  |
| Female  | 41           | 6.607 ± 0.5352              |         |
| Age     |              |                             |         |
| <60     | 41           | 6.955 ± 0.3254              | 0.0196  |
| ≥60     | 49           | 6.149 ± 0.3868              |         |
| Primary tumour (T) | | | |
| T1-T2  | 67           | 5.950 ± 0.3147              | 0.0105  |
| T3-T4  | 23           | 8.166 ± 0.5445              |         |
| Regional lymph nodes (N) | | | |
| N0-N1  | 56           | 5.412 ± 0.2272              | 0.0030  |
| N2-N3  | 19           | 9.771 ± 0.6109              |         |
| Clinical stage | | | |
| I–II   | 57           | 5.852 ± 0.2542              | 0.0412  |
| III–IV | 29           | 7.793 ± 0.6199              |         |

Table 1. Association of SLC39A4 expression and clinicopathological findings in NSCLC. Clinical and TNM staging were scored according to the American Joint Committee on Cancer: AJCC Cancer Staging Manual, 7th edition.

| Parameters | Univariate analysis | Multivariate analysis |
|------------|---------------------|-----------------------|
|            | HR                  | 95% CI                | P-value   | HR                  | 95% CI                | P-value   |
| Sex        |                     |                       |           |                     |                       |           |
| Male vs. Female | 1.314              | 0.752–1.972           | 0.453     |                     |                       |           |
| Age        |                     |                       |           |                     |                       |           |
| <60 vs. ≥60 | 1.118              | 0.683–1.593           | 0.166     |                     |                       |           |
| Primary tumour (T) | | | |
| T1-T2 vs. T3-T4 | 1.910          | 0.962–2.872           | 0.041     |                     |                       |           |
| Regional lymph nodes (N) | | | |
| N0-N1 vs. N2-N3 | 2.238            | 1.929–3.682           | 0.006     | 2.562              | 1.992–3.232           | 0.013     |
| Clinical stage | | | |
| I–II vs. III–IV | 2.109            | 1.848–3.563           | 0.031     | 2.118              | 1.890–2.441           | 0.022     |
| SLC39A4 expression | | | |
| Low vs. High | 2.918            | 2.353–3.482           | 0.033     | 3.067              | 2.190–3.566           | 0.019     |

Table 2. Summary of univariate and multivariate analysis of overall survival in NSCLC.
Figure 3. SLC39A4 silencing inhibits A549 cells metastasis in vitro. (a,b) SLC39A4 expression in A549 knockdown and empty vector control (Ctl) cells by western blot analysis (a) and qPCR (b,c). Zn2+ concentration in A549 cells after knockdown of SLC39A4 in vivo. (d,e) Analysis of SLC39A4 knockdown and Ctl cell migration in wound-healing assays (scale bar, 500 μm). Representative images (d) and quantitation (e) are shown. (f,g) Cell migration was monitored in transwell assays with SLC39A4 knockdown and Ctl A549 cells (scale bar, 100 μm). Representative images (f) and quantitation (g) are shown. (h,i) Cell invasion was monitored in matrigel transwell assays with SLC39A4 knockdown and Ctl A549 cells (scale bar, 100 μm). Representative images (h) and quantitation (i) are shown. (j,k) Analysis of E-cadherin (epithelial marker) and FSP-1 and N-cadherin (mesenchymal markers) expression in knockdown and control cells by immunofluorescence staining (j) and western blotting (k) (scale bar, 50 μm).
shown that the EMT conveys resistance to radiotherapy or chemotherapy, we speculated that SLC39A4 silencing could affect the chemotherapeutic response by regulating stemness. SLC39A4 expression was significantly correlated with the expression of the lung cancer stem cell biomarkers CD44 and CD133 (Z = 7.11, P < 0.00001; Fig. 5a,b). This finding was further confirmed by immunofluorescence and western blot analysis of CD44 and CD133 expression in shSLC39A4 or Ctl A549 subcutaneous tumours (scale bar, 100 μm).

Figure 4. SLC39A4 silencing limits metastatic spread in a mouse model of lung cancer. (a) Body weight measurements of tumour-bearing mice. (b) Representative images of mouse lungs 30 d after shSLC39A4 or Ctl A549 cell engraftment. (c) Representative images of H&E staining in lung tissue sections from each group (scale bar, 100 μm). (d) The average number of metastatic tumour nodules in the lungs xenograft mice. E. Immunofluorescence for E-cadherin, FSP-1, and N-cadherin expression in A549 shSLC39A4 or Ctl subcutaneous tumours (scale bar, 100 μm).
formation assays revealed that shSLC39A4 sensitized A549 cells to cisplatin-induced cell death (Fig. 5e–j), suggesting that SLC39A4 enhances cellular resistance to cisplatin therapy. Similar findings were also observed in a subcutaneous tumour model with cisplatin treatment, where shSLC39A4 A549 tumours caused less body weight loss (Fig. 5h) and were less proliferative and resistant to cisplatin treatment (Fig. 5i,j).

Figure 5. Inhibition of SLC39A4 enhances cisplatin resistance in human NSCLC cells. (a) Correlation of SLC39A4 and CD44 expression, Fisher’s Z = 0.26 (95% CI, 0.19–0.33). (b) Correlation of SLC39A4 and CD133 expression, Fisher’s Z = 0.21 (95% CI, 0.14–0.28). (c,d) Analysis of CD44 and CD133 expression by immunofluorescence (c) and western blotting (d) in shSLC39A4 or Ctl A549 cells (scale bar, 50 μm). (e,f) Cisplatin-induced cell death was monitored in CCK-8 arrays (e) and colony formation assays (f,g). Quantitation of colony formation. (h–j) Body weight (h) and tumour size (i,j) were used to examine cisplatin sensitivity in shSLC39A4 and Ctl A549 tumour-bearing mice.
Discussion
Lung cancer is the leading cause of cancer-related death in men and women\textsuperscript{25}, with more than one million new cases diagnosed each year. Despite recent advances in treatment modalities, including surgery, chemotherapy, and radiotherapy, cancer metastasis remains the major determinant of poor outcome, with an overall 5-year survival rate of 15%\textsuperscript{36}. Thus, it is important to discover novel lung cancer metastasis–related genes and their functional mechanisms to encourage the development of additional cancer therapeutics.

The combined use of tumour-related gene expression data and clinical specimens has proven to be a valuable method for identification of potential drug targets\textsuperscript{27–29}. Studies have indicated that zinc transport and homeostasis play important roles in cancer progression\textsuperscript{19–32}; however, their functional significance in lung cancer is not clear. In this study, a meta-analysis of data extracted from the GEO and TCGA databases revealed the clinical significance of SLC39A4 in lung cancer. Specifically, SLC39A4 expression was markedly higher in NSCLC tissues than in normal controls and was a prognostic indicator of increased metastatic stage, as well as poor OS and DFS. Collectively, these data suggest that SLC39A4 may play an important role in lung cancer pathophysiology.

The EMT plays a significant role during tumour invasion and metastasis\textsuperscript{33}, and is characterized by the acquisition of mesenchymal markers such as vimentin or FSP-1, and loss of epithelial cell adhesion molecules, such as E-cadherin\textsuperscript{22,34,35}. To further study the effect of SLC39A4 on the aggressive cellular characteristics of NSCLC, we infected A549 cells with lentivirus expressing shSLC39A4 or empty vector control. Notably, knockdown of SLC39A4 induced a more epithelial-like phenotype with increased E-cadherin and decreased FSP-1 expression, suggesting that SLC39A4 promotes EMT in A549 cells. The clinical relevance of these results was confirmed with \textit{in vitro} migration assays and a mouse model of tumour metastasis, indicating that SLC39A4 plays an important role in NSCLC cell migration.

Many studies have demonstrated that the EMT facilitates resistance to radiotherapy and chemotherapy\textsuperscript{36,37}. Cisplatin (cis-diamminedichloro-platinum II) is the primary chemotherapeutic agent used in lung cancer therapy, particularly NSCLC\textsuperscript{38}. Because acquired resistance is a common occurrence in NSCLC patients\textsuperscript{39}, insights into the molecular mechanisms underlying cisplatin resistance is necessary for the development of novel therapeutic strategies. Consistently, SLC39A4 knockdown cells displayed heightened sensitivity to cisplatin-induced cell death when compared to control cells.

In conclusion, the present study demonstrated that SLC39A4 is overexpressed in NSCLC and correlates with increased staging and diminished patient survival. Moreover, silencing of SLC39A4 induced an epithelial-like phenotype, decreased cancer stem cell marker expression, and increased cisplatin sensitivity. Thus, these findings suggest that SLC39A4 may serve as a prognostic biomarker and putative therapeutic target to enhance chemosensitivity in NSCLC.

Materials and Methods
Public data analysis. Gene expression data were obtained from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. The meta-analysis included eight datasets encompassing 942 lung cancer patients (Supplementary Table S1), whereas the prognostic analysis included seven datasets with 1623 total patients (Supplementary Table S2). Raw CEL files were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), and background correction and data extraction was performed in R software (R version 3.3.0). The meta-analysis was conducted in Review Manager (RevMan Version 5.3, Copenhagen, Denmark), using a random-effects model because the expression data were acquired by different means. Results are presented in forest plots. Cochran $\chi^2$ and $F$ analyses were performed to assess heterogeneity among the studies involved.

Reagents. The anti-SLC39A4 antibody was obtained from Abcam (Cambridge, UK). All other antibodies were obtained from Proteintech Group, Inc. (Wuhan, China). Secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other kits and reagents were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Tissue arrays were from Outdo Biotech Co., Ltd. (Shanghai, China).

SLC39A4 immunohistochemistry. Tissue arrays were dewaxed and antigens retrieved using high pressure. Endogenous peroxidases were blocked with 3% hydrogen peroxide for 10 min. After immersion in normal goat serum for 30 min, tissues were incubated with the primary antibody at 4°C overnight, washed with phosphate-buffered saline (PBS), and then incubated with a biotin-conjugated secondary antibody for 30 min at 37°C. After washing, the sections were incubated with horseradish peroxidase (HRP) complex for 30 min at 37°C and visualized using diaminobenzidine (DAB). All immunohistochemical images were obtained under an Olympus BX51 microscope equipped with a 40× objective lens (Olympus, Tokyo, Japan) and a DP 50 camera (Olympus). Images were processed using DPC controller software (Olympus).

Immunohistochemical staining was evaluated by a semiquantitative scoring method. The SLC39A4 staining was scored as follows: no staining (0), light positive staining (1), medium positive staining (2), and strong positive staining (3). The area of positive staining was scored as: $<5\%$ (0), 5–25\% (1), 26–50\% (2), 51–75\% (3), and $>75\%$ (4). An overall score was calculated by multiplying the intensity and expression scores for each sample. SLC39A4 expression was dichotomized using median staining intensity as the cutoff to define “high” or “low” as above or below the median, respectively.

Cell culture. The A549 and SPC-A-1 lung cancer cells and the BEAS-2B normal lung epithelial cell line were maintained in RPMI-1640 medium supplemented with 10% (vol/vol) foetal bovine serum (FBS), 10 mM L-glutamine, and 5 mg/mL penicillin/streptomycin at 37°C with 5% CO\textsubscript{2}. All media and supplements were purchased from Invitrogen (Carlsbad, CA, USA).

SLC39A4 lentiviral silencing. A549 cells were infected with a lentivirus encoding SLC39A4 shRNA (5′-ACGTAGCAGCTCCTGGACATGGTGCAAGATG-3′) following the manufacturer’s instructions. Briefly,
lentiviral particles were purchased from GeneCopoeia (Guangzhou, China) and used to infect A549 cells. Stable cell lines expressing shSLC39A4 or psi-LVRH1MP empty vector (C1) were then selected in medium containing 0.5 μg/mL puromycin.

**Detection of Zn\(^{2+}\) concentration.** Zn\(^{2+}\) concentration was measured by atomic absorption spectrometry.

**Immunofluorescence.** Cultured cells were fixed with 4% paraformaldehyde, washed twice with PBS, and then blocked with PBS containing 10% normal goat serum. Cells were then stained with anti-E-cadherin, anti-vimentin, or anti-FSP1 polyclonal antibody for 30 min at 37 °C, washed twice with PBS, stained with Cy3 (red) or Alexa Flour 488 (green)-conjugated secondary antibody for 30 min at 37 °C, and then washed twice again before imaging. All immunofluorescence images were obtained with an Olympus BX51 microscope equipped with a 20 × or 40 × objective lens (Olympus) and a DP 50 camera (Olympus). Images were processed using DPC controller software (Olympus).

**Transwell assays.** Cells were cultured in 10-cm plates, and fresh medium was added 18 h before each assay. Cells were trypsinized, washed twice, resuspended in serum-free medium, and then counted using a haemocytometer. RPMI 1640 with 10% serum was added to the lower wells of the transwell chamber apparatus, and 10,000 cells in 200 μL of serum-free media were added to each upper well. The loaded chamber was incubated for 24 h at 37 °C, at which time the cells on the upper membrane surface were removed by scraping to leave only those that had migrated through the membrane. The transwell membranes were then fixed in methanol, stained with 0.1% crystal violet, and air-dried. The number of cells in each field was quantified and presented as an average from five fields of triplicate wells for each test condition.

**Western blotting.** Protein samples were resolved by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes, which were then blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% fat-free milk. Primary antibody incubations were performed for 18 h at 4 °C. HRP-conjugated secondary antibody incubations were performed at room temperature for 1 h and visualized with enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL) or ECL Plus (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) substrates according to the manufacturers’ instructions.

**Cell viability assays.** Cell viability was assessed by colony formation assays and cell counting kit (CCK)-8 assay. Briefly, cells were plated at 500 cells per well in a 6-well plate (Corning, Corning, NY, USA) after being treated with different concentrations of cisplatin (0, 1.25, 2.5, or 5 μg/mL). Cells were cultured for 10 days with medium changes every 3 days. Colonies were washed with PBS, fixed in methanol, and then stained with crystal violet. The CCK-8 assay was completed according to the manufacturers’ instructions.

**Mouse xenograft model.** All xenograft experiments were performed in accordance with the guidelines of the Laboratory Animal Ethical Committee at Chengdu Medical College. All experimental protocols were approved by the Laboratory Animal Ethical Committee at Chengdu Medical College (Approval date: 2017.07.20; Approval number: CYYFYEC2016003). An A549 lung metastasis model was established to investigate the effect of SLC39A4 on cell migration and invasion in vivo.

After centrifugation, cells were treated with 2% HCL for 1 hour, detection by atomic absorption spectrometer (Beijing Bohui Innovation Technology Co., LTD, BH2100S-5500S).

**Statistical analysis.** Each experiment was performed at least three times independently. The association of SLC39A4 expression with clinicopathological parameters was analysed by paired t-test or one-way ANOVA in GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Statistical significance was defined as P < 0.05.

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Acknowledgements

This work was funded by the Foundation of Sichuan Province Education Office (15ZB0245 and17TD0012), Foundation of Sichuan Medicine Institute (16PJ112) and Foundation of Chengdu Medical College (CYTD15-03).

Author Contributions

D.W. and Y.X. conceived and designed the study. D.W. contributed to the design and editing of this manuscript. D.W. and T.L. performed experiments. D.W., S.D., and R.H. analysed the data. D.W. and T.L. wrote the manuscript. All authors approved the final version.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-07830-4

Competing Interests: The authors declare that they have no competing interests.

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