Title: Serglycin is a theranostic target in nasopharyngeal carcinoma that promotes metastasis

Authors:
Xin-Jian Li, Choon Kiat Ong, Yun Cao, Yan-Qun Xiang, Jian-Yong Shao, Aikseng Ooi, Li-Xia Peng, Wen-Hua Lu, Zhongfa Zhang, David Petillo, Li Qin, Ying-Na Bao, Fang-Jing Zheng, Claramee Shulyn Chia, N. Gopalkrishna Iyer, Tie-Bang Kang, Yi-Xin Zeng, Khee Chee Soo, Jeffrey M. Trent, Bin Tean Teh, Chao-Nan Qian

Author affiliations:
1. State Key Laboratory on Oncology in South China, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou, Guangdong 510060, P. R. China.

2. NCCS-VARI Translational Research Laboratory, National Cancer Center Singapore, 11 Hospital Drive, Singapore 169610

3. Department of Pathology, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou, Guangdong 510060, P. R. China

4. Department of Nasopharyngeal Carcinoma, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou, Guangdong 510060, P. R. China

5. Laboratory of Cancer Genetics, Van Andel Research Institute, 333 Bostwick Ave NE, Grand Rapids, MI 49503

7. Division of Pharmacoproteomics, Institute of Pharmacy and Pharmacology, University of South China, Hengyang, Hunan 42100, P. R. China

8. Department of General Surgery, Singapore General Hospital, Outram Road, Singapore 169608, Singapore

9. Department of Surgical Oncology, National Cancer Centre Singapore, 11 Hospital Drive, Singapore 169610, Singapore

10. Division of Medical Sciences, National Cancer Centre Singapore, 11 Hospital Drive, Singapore 169610, Singapore

11. Laboratory of Genome Biology, Van Andel Research Institute, 333 Bostwick Ave NE, Grand Rapids, MI 49503

Financial supports:
This work was supported by grants from the State Key Program of National Natural Science Foundation of China (Grant No. 81030043, authors Chao-Nan Qian, Xin-Jian Li, Yun Cao, Yan-Qun Xiang, Li-Xia Peng, Wen-Hua Lu, Ying-Na Bao, Fang-Jing Zheng), the National High Technology Research and Development Program of China (863 Program) (Grant No. 20060102A4002, authors Xin-Jian Li, Li-Xia Peng, Wen-Hua Lu, Chao-Nan Qian, Jian-Yong Shao, Yi-Xin Zeng), the Major State Basic Research Program (973 Project) of China (Grant No. 2006CB910104, author Yi-Xin Zeng), the Youth Science Fund of the National Natural Science Foundation of China (Grant No. 81000946, author Li Qin), the Van Andel Foundation (authors Aikseng Ooi, David Petillo), the National Cancer Center Research Found of Singapore
(Choon Kiat Ong, Aikseng Ooi, Claramae Shulyn Chia, N. Gopalakrishna Iyer, Bin Tean Teh), and the Singapore Millennium Foundation (authors Choon Kiat Ong, Bin Tean Teh).

**Corresponding author:**
Chao-Nan Qian, MD, PhD
Department of Nasopharyngeal Carcinoma
Sun Yat-sen University Cancer Center
651 Dongfeng East Road, Guangzhou, Guangdong 510060, P. R. China.
Phone: +86-20-87343457
Fax: +86-20-87343624
Email: qianchn@sysucc.org.cn

**Potential conflicts of interest:**
All authors declare no competing financial interests.

**Notes about this manuscript:**
The total word number is 5505, with 7 figures, 2 supplementary figures, 3 supplementary tables, and 1 supplementary materials and methods.
**Running title:**
Serglycin regulates metastasis of nasopharyngeal carcinoma

**Key words:**
Serglycin, metastasis, nasopharyngeal carcinoma, prognosis, autocrine, paracrine.

**Abstract:**
Nasopharyngeal carcinoma (NPC) is known for its high metastatic potential. Here we report the identification of the proteoglycan serglycin (SRGN) as a functionally significant marker of metastasis in this setting. Comparative genomic expression profiling of NPC cell line clones with high and low metastatic potential revealed SRGN as one of the most upregulated genes in highly metastatic cells. RNAi-mediated inhibition of SRGN expression blocked serglycin secretion and the invasive motility of highly metastatic cells, reducing metastatic capacity in vivo. Conversely, SRGN overexpression in poorly metastatic cells increased their motile behavior and metastatic capacity in vivo. Growth rate was not influenced by serglycin in either highly or poorly metastatic cells. Secreted but not bacterial recombinant serglycin promoted motile behavior, suggesting a critical role for glycosylation in serglycin activity. SRGN inhibition was associated with reduced expression of vimentin but not other epithelial-mesenchymal transition proteins. In clinical specimens, serglycin expression was elevated significantly in liver metastases from NPC relative to primary NPC tumors. We evaluated the prognostic value of serglycin by immunohistochemical staining of tissue microarrays from 263 NPC patients followed by multivariate analyses. High serglycin expression in primary NPC was found to be an unfavorable independent indicator of distant...
metastasis-free and disease-free survival. Our findings establish that glycosylated serglycin regulates NPC metastasis via autocrine and paracrine routes, and that it serves as a prognostic indicator of metastasis-free survival and disease-free survival in NPC patients.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common human
malignant tumors in southern China and Southeast Asia (1, 2). NPC has the highest metastasis rate among head and neck cancers (3-5), with the majority of the patients having metastases to regional lymph nodes or even distant organs at the time of diagnosis (6). However, the molecular mechanisms underlying NPC metastasis are poorly understood, and understanding the molecular basis is important for developing novel strategies for prevention and treatment of metastatic NPC. In the present study, high throughput screening identified serglycin correlated with NPC metastasis.

Serglycin is a proteoglycan consisting of a core protein to which negatively charged glycoaminoglycan (GAG) chains of either chondroitin sulfate or heparin are attached (7, 8). The core protein containing 158 amino acid residues can be divided into 3 domains: a signal peptide domain spanning amino acid residues 1–27 and responsible for secretion; an N-terminal domain consisting of residues 28–76, its function unknown; and a C-terminal domain consisting of residues 77–158 containing a long region of Ser-Gly repeats for GAG attachment (9). The functions of SG in various cells depend on the type and size of the GAG chains decorating the core protein (8, 10-19).

Serglycin mRNA or protein has been detected in many kinds of cells, e.g., embryonic stem cells, hematopoietic cells, and endothelial cells (11, 14, 20-23). Serglycin is thought to be important for homeostasis of positively charged components (e.g., proteases) in storage granules due to its negatively charged GAG chains (24-28). In cytotoxic lymphocytes (CTLs) or natural killer T (NT) cells, a macromolecular complex of granzyme B and perforin complexed with serglycin induces the apoptosis of target cells (29-34). Serglycin has been associated with tumorigenesis in acute myeloid leukemia (AML) and myeloma, and it was found to be a selective marker for distinguishing AML from Philadelphia chromosome-negative chronic myeloproliferative disorders. It is constitutively secreted at high levels by multiple myeloma cell lines (35, 36). Although the involvement of serglycin in tumor metastasis has been
speculated (37), the exact role of serglycin in NPC remains unknown.

The epithelial-mesenchymal transition (EMT), a fundamental process in embryonic development, is involved in the metastasis and progression of tumors (38, 39). Activation of the EMT program, accompanied by an increase in the mesenchymal marker vimentin and loss of the epithelial marker E-cadherin, endows carcinoma cells with enhanced migratory and invasive properties that facilitate dissemination to permissive niches (39).

Materials and Methods

Cell culture and cellular growth rate

The human NPC cell line CNE-2 and its clones (S18, S22, and S26, cultured in less than 50 passages), and SUNE-1 and its clone 5-8F were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C. Cellular growth curves were plotted using the cellular viability values assessed by the MTS method (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay solution, Promega, Madison, WI).

In vitro migration and invasion assays

Migration and invasion assays were performed according to the methods described previously with minor modifications (40). More details were described in the Supplementary Materials and Methods.

Detection of serglycin in conditioned medium

A total of $2 \times 10^6$ cells were plated in 100-mm culture plates and incubated for 48 h in a regular medium. The medium was then replaced with a serum-free medium (10 ml) and cells were incubated for an additional 24 h. Ten milliliters of conditioned medium was collected and concentrated to a volume of 200 μl using Amicon Ultra centrifuge filters (10 kDa molecular weight
cutoff pore size; Millipore, Bedford, MA). Twenty microliters of the concentrated conditioned medium was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted with anti-human serglycin (Cat No.: H00005552-M03, Abnova, Taiwan) antibodies.

**Human tissues and tissue microarray**

All of the human tissue samples were obtained from the Department of Pathology, Sun Yat-sen University Cancer Center, with prior patient consents and the approval of the Institutional Clinical Ethics Review Board at Sun Yat-sen University Cancer Center. The TMAs contained qualified primary NPC samples from 263 pathologically diagnosed patients at Sun Yat-sen University Cancer Center between December 30, 1997, and September 6, 2002. Of these patients, 199 were male and 64 female, with a median age of 46 years old (ranging from 17-77 years old). All patients received radiotherapy, with doses of 70-74 Gy to the primary tumor, 60-64 Gy to the involved areas of the neck, and 50 Gy to the uninvolved areas of the neck. For the patients with late-stage disease (stages III and IV), 2-3 cycles of induction or concurrent platinum-based chemotherapy was given. The patients were followed up regularly with a median follow-up time of 83 months. TMAs were constructed as described previously (41) and the methodology is described in the Supplementary Materials and Methods.

**Histologic evaluation and immunohistochemical and immunofluorescent staining**

Mouse lymph nodes were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 µm throughout the lymph nodes. To evaluate the micrometastases in mouse lymph nodes, one section in every 20 sequential sections was selected for H&E staining.
For IHC staining of serglycin and vimentin, paraffin-embedded tissues were sectioned at 5 µm and immunohistochemical staining was performed as described previously (42). Briefly, the sections were incubated with a rabbit anti-human SG polyclonal antibody (Cat No.: HPA000759, Sigma-Aldrich, St. Louis, MO, working dilution 1:50) or mouse anti-human E-cadherin monoclonal antibody (Abcam, Cambridge, MA) overnight at 4 °C, or mouse anti-human Vimentin monoclonal antibody (Neomarkers, Fremont, CA, working dilution 1:100) for 30 min at room temperature. An EnVision kit (DAKO, Denmark) was used to detect the primary antibodies followed by 3,3-diaminobenzidine substrate visualization and counterstaining with hematoxylin. The intensity of IHC staining in the tumor cells was scored independently by two pathologists using the semiquantitative IRS (immuno-reactive score) scale according to Remmele and Stegner (43), which takes into account both the intensity of the color reaction (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and the percentage of stained cells (0% = 0, 1–10% = 1, 11–50% = 2, 51–80% = 3, 81–100% = 4). The average value from the two referees was used as the final score.

Quantitative PCR

The methodology of quantitative PCR is described in the Supplementary Materials and Methods. The sequences of PCR primers used for amplification of SG and GAPDH were as follows: GAPDH forward: 5'-AAGGTCATCCCTGAGCTGAA-3'; GAPDH reverse: 5'-TGACAAAGTGGTCGTTGAGG-3'; SG forward: 5'-TATCCTACGCGGAGAGCCAGGTAC-3'; SG reverse: 5'-TTCCGTTAGGAAGCCACTCCC AGATC-3'. The experiments were performed in triplicate.

Lentiviral transduction studies
Cell lines stably expressing either SG shRNA or a scrambled non-target shRNA were established using a BLOCK-iT™ Lentiviral Pol II miR RNAi system (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The targets of human serglycin shRNAs are 5’-CTGGTTCTGGAATCCTCAGTT-3’ and 5’-CGCTGCAATCCAGACAGTAAT-3’. See the Supplementary Materials and Methods for more details.

**Immunoblotting**

The primary antibodies, including mouse anti-human serglycin monoclonal antibody (Abnova, Taiwan), mouse anti-human E-cadherin monoclonal antibody (Abcam, Cambridge, MA), rabbit anti-human vimentin polyclonal antibody (Cell Signalling Technology, Beverly, MA), and mouse anti-human β-actin monoclonal antibody (Sigma Aldrich, St. Louis, MO) were used at a dilution of 1:1,000. See the Supplementary Materials and Methods for more details.

**Wound healing assay**

Wound healing assays were performed by plating cells in 6-well plates (3 × 10^5 cells/well). After cells were allowed to attach and reach confluence, a scratch was made using a sterile yellow 100 µl pipette tip and detached cells were removed by washing with PBS. Phase contrast images were taken in the same field at the indicated time points. The experiments were performed in triplicate.

**Deglycosylation assay**

Concentrated conditioned medium containing secreted serglycin protein was treated with an enzymatic Glycoprotein Deglycosylation Kit (EMD Chemicals, Gibbstown, NJ) according to the manufacturer’s instructions.
Briefly, 20 µl of concentrated conditioned medium, mixed with denaturation buffer (2% SDS, 1 M β-mercaptoethanol) and reaction buffer (250 mM sodium phosphate buffer, pH 7.0), was incubated at 100 °C for 5 min. After adding 1 µl each of N-glycosidase F, α2-3,6,8,9-neuraminidase, endo-α-N-acetylgalactosaminidase, β1,4-galactosidase, and β-N-acetylglucosaminidase, plus 2.5 µl of TRITON® X-100 (15% solution), the conditioned medium was incubated at 37 °C for 48 h. Immunoblots were carried out to determine the efficiency of SG deglycosylation.

Animals and spontaneous lymph node metastasis assay

Female athymic BALB/c nu/nu mice between 5 to 6 weeks of age were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China) and maintained in the vivarium of Sun Yat-sen University Cancer Center (Guangzhou, China). All of the animal studies were conducted in accordance with the principles and procedures outlined in the guidelines of Institutional Animal Care and Use Committee at Sun Yat-sen University Cancer Center. For the generation of xenograft tumors for genomic expression profiling, 1 × 10^5 cells were subcutaneously injected into the left flank of a mouse, and the tumors were collected when their volume was 150-250 mm^3. The spontaneous lymph node metastasis model has been published previously (40). Briefly, a total of 1 × 10^5 cells were injected into the left hind footpad of each mouse to generate a primary tumor. After 7-8 weeks, the popliteal lymph node of the left hind foot was collected on the terminal day for routine tissue processing.

Genomic expression profiling

The methodology of gene expression profiling is described in the Supplementary Materials and Methods. To identify genes that were differentially expressed between high- and low-metastasis cells/xenografts, the
log2 transformed expression value of each gene in S18 cells/xenograft was subtracted by the mean of log2 transformed expression value of the corresponding gene in the 3 low-metastasis cells/xenografts (i.e., CNE2, S22, S26). The results from this calculation were then sorted; the 25 most up-regulated and 25 most down-regulated genes are presented in Figure 1A and 1B as heatmaps. The gene expression data for this study have been uploaded to the Gene Expression Omnibus, with the accession number GSE24154.

**Statistical methods**

Single comparisons were performed using Student's t test, Mann-Whitney test, or Chi-square test (two-tailed; \( P < 0.05 \) was considered significant). The Spearman correlation test (2-tailed) was used to calculate the correlation coefficient (\( r \)) and P value between the serglycin and vimentin staining scores, or between the serglycin and E-cadherin staining scores. The median of the IHC score value was used as the cutoff value to divide the patients into high- and low-SG-expression groups. The censoring time distribution was estimated by the Kaplan-Meier method and P values were calculated by Log Rank analysis. Cox's regression model was used to assess the contribution of SG in multivariate statistical models of disease-free, distant-metastasis-free, and overall survival. Predictors were judged to be significant at \( P \leq 0.05 \).

**Results**

**Serglycin expression is elevated in NPC cells with higher metastasis potential**

We have previously isolated and established cellular clones having different metastatic abilities from the parental NPC cell line, CNE-2 (40). Among these clones, clone 18 (S18) had the highest metastatic ability, while clone 22 (S22) and clone 26 (S26), as well as the parental line CNE-2, had low
metastatic ability. To explore the molecular mechanism(s) underlying this important biological behavior, we performed genomic expression profiling of these four cell lines from in vitro cultured cells. In the high-metastasis clone S18, the serglycin gene (SRGN) was the second most highly up-regulated (Figure 1A). Considering the potential differences between in vivo and in vitro conditions, xenograft tumors from these four cell lines were generated in nude mice and were collected for gene expression profiling using the same technology. Again, serglycin was the second most up-regulated gene in S18 xenograft (Figure 1B).

To confirm the finding from genomic expression profiling, real-time quantitative PCR was performed to quantify the mRNA levels of serglycin among the four lines, with consistent results (Figure 1C). Moreover, a high level of serglycin expression was also detected in the high-metastasis clone 5-8F isolated from another parental NPC cell line SUNE-1 with low-metastasis potential (44) (Figure 1C). The protein levels of serglycin were also evaluated. The results showed that S18 and 5-8F cells could secrete serglycin into culture medium, while the other low-metastasis cell lines could not (Figure 1D). The specificity of the anti-human serglycin antibody was confirmed and shown in Supplementary Figure 1. After partial deglycosylation of secreted serglycin, a protein with a lower molecular weight (about 80 kDa) could be detected (Figure 1E), confirming the glycosylation of the serglycin protein secreted by high-metastasis cells.

**Serglycin is up-regulated in liver metastases from NPC and in highly migratory/invasive NPC cells**

To evaluate the clinical relevance of our hypothesis that serglycin regulates NPC metastasis, we collected archival tissues of liver metastases from NPC (n = 16) for comparison with primary NPC tissues (n = 48). Immunohistochemical (IHC) staining showed that the protein levels of
serglycin were significantly higher in the liver metastases (Figures 2A and 2B), implying that serglycin could be important in clinical scenarios.

To confirm that up-regulation of serglycin in highly migratory cells could be repeatedly found, a two-chamber (Boyden chamber) assay was used to isolate highly migratory cells from the parental CNE-2 line. After three sequential passages of screening, highly migratory/invasive cells (CNE-1 M3) were isolated (Figure 2C), and serglycin was found to be highly expressed in these cells (Figure 2D).

Lower serglycin secretion suppresses the migration and invasion of NPC cells without influencing growth rate

To examine the causal role of serglycin in motility of NPC cells, we engineered cell lines from S18 that stably expressed either shRNAs targeting serglycin expression (SG KD1 and SG KD2) or a scrambled non-target shRNA. The suppression of serglycin expression at the mRNA level was confirmed by quantitative real-time PCR (Figure 3A). A decrease in secreted serglycin protein was confirmed via immunoblotting (Figure 3B). Yet, knocking down serglycin in S18 cells did not alter the growth rate of the cells measured by an MTS assay (Figure 3C), implying that serglycin did not have a role in controlling cellular growth. The migration ability of S18 cells was significantly suppressed after the loss of secreted serglycin, as evaluated by a scratch-wound healing assay and a two-chamber assay (Figures 3D and 3E). The invasion ability of S18 cells was also significantly reduced by knocking down serglycin (Figure 3E). These results proved that secreted serglycin could promote NPC cell migration and invasion in an autocrine mode.

Secreted SG promotes the motility of low-metastasis NPC cells

To validate the motility-enhancing activity of serglycin in NPC cells, we used lentiviral transduction to generate an S26 cell line stably overexpressing
serglycin. Overexpression was confirmed by immunoblotting, and secreted serglycin protein was detected in the conditioned medium of overexpressing S26 cells, but not in the conditioned medium of control cells (Figure 4A). Overexpression of serglycin did not alter the growth rate of the cells (Figure 4B).

The conditioned medium of S26 cells overexpressing serglycin or the control vector was subjected to 50X concentration (from 10 ml to 200 µl) using 10 K ultrafilters, and concentrated serglycin protein was then confirmed using immunoblotting (Figure 4C). The concentrated conditioned medium was used to stimulate migration and invasion by wild-type S26 cells. The numbers of migrated and invaded cells were significantly increased after the stimulation (Figures 4D and 4E). These results suggest that serglycin could also promote NPC motility via a paracrine mode, by which secreted serglycin from a rare clone could stimulate the motility of cells unable to secrete serglycin. Interestingly, prokaryotic recombinant serglycin without glycosylation modification could not promote migration of CNE-2 and S26 cells (Supplementary Figure 2), suggesting that the glycosylation of serglycin is critical for promoting cellular motility.

**Expression of SG mediates the metastasis rate of NPC in vivo**

To determine the effect of serglycin in vivo, we used a spontaneous lymph node metastasis model (40). As expected, serglycin-knockdown cells showed a significant reduction in metastasis rate, and serglycin-overexpressing cells showed an increased metastasis rate (Figure 5). These results confirmed that serglycin was involved in the control of NPC metastasis in vivo.

**Serglycin expression is associated with the epithelial-mesenchymal transition**

To explore the relationship of serglycin and EMT, IHC staining of serglycin,
vimentin, and E-cadherin was performed in 48 primary human NPC tissue samples. The results indicated that serglycin expression level positively correlated to the expression level of vimentin (Figures 6A and 6B), and inversely correlated to the expression level of E-cadherin (Figure 6A and 6C). To confirm this finding, the protein levels of several EMT markers in four cellular populations were evaluated. A high level of secreted serglycin accompanied elevated levels of mesenchymal markers vimentin, N-cadherin, and fibronectin, and a reduced level of epithelial protein E-cadherin in the high-metastasis S18 cells (Figure 6D). The suppression of serglycin resulted in a lower vimentin level, but did not influence the levels of other EMT proteins (Figure 6E). All of these data suggest that serglycin is correlated with EMT by mediating the vimentin level.

High-level serglycin expression is an independent, unfavorable prognostic indicator for NPC

Finally, to evaluate the prognostic value of serglycin expression in primary NPC, we performed IHC staining for serglycin in a set of tissue microarrays containing 263 NPC samples with sufficient clinical follow-up information about the patients (Supplementary Table 1, Figures 7A and 7B). The patients were divided into low- and high- serglycin expression groups using the median of serglycin staining scores as the cut-off value. The correlations between serglycin expression and clinicopathological characteristics are presented in Supplementary Table 2. High serglycin expression was significantly correlated with the occurrence of disease progression or distant metastasis. Multivariate analyses using Cox’s regression model revealed that a high level of serglycin expression was an independent, unfavorable prognostic indicator for disease-free survival and distant-metastasis-free survival (Supplementary Table 3). The survival curves of disease-free survival and distant-metastasis-free survival are presented in Figures 7C and 7D. Taken
together, these analyses revealed that high serglycin expression level in NPC significantly correlated with adverse patient outcomes.

**Discussion**

In the present study, we have identified a metastasis-enhancing glycoprotein, serglycin, by using high-throughput gene expression profiling analyses of CNE-2 clones having low- or high-metastasis potential. There are two advantages of using cellular clones derived from a typical cell line for hunting the gene(s) regulating tumor metastasis. The first is to minimize the influences from confounding factors. When different cell lines (or tumor tissues) derived from different patients are used for comparisons, many confounding factors might appear, including gender, race, age, and other disease backgrounds. All of these factors will be perfectly balanced when we compare cellular clones derived from a single patient. The second advantage is to amplify the impact of critical cellular population on metastasis. As reported previously (40), among 29 clones isolated from the CNE-2 line, only 2 clones possessed high-metastasis ability, with S18 being the most aggressive. Obviously, if we extract the mRNA from the parental line, the signal from this aggressive clone will be overwhelmed by the majority of low-metastasis populations. However, tumor metastases usually arise from rare clones in the primary tumor (45). Using the identified culprit clone for comparison can therefore easily reveal the key molecules regulating metastasis.

Among the most up-regulated genes in S18, serglycin is second on the list under both *in vitro* and *in vivo* conditions, implying the heavily involvement of this glycoprotein in regulating NPC metastasis. To our knowledge, no published data has directly linked serglycin with tumor metastasis. Our present study has discovered that serglycin is involved in regulating NPC metastasis by promoting the migratory and invasive abilities of NPC cells via autocrine and paracrine modes, with autocrine mechanism seems to be more
important. Serglycin expression can also mediate the level of vimentin, which not only is a marker of EMT but also has an important role in regulating cellular migration (46). Moreover, serglycin is an independent, unfavorable prognostic indicator for distant-metastasis-free survival and disease-free survival in NPC patients.

As shown in Figure 1A and 1B, other genes are also highly expressed in S18 cells, implying that multiple factors could be involved in regulating NPC metastasis. When serglycin expression was knocked down in high-metastasis S18 cells, a 15% reduction in the metastasis rate was achieved, suggesting that targeting multiple factors is a hope for complete suppression of NPC metastasis.

In summary, our study demonstrates that serglycin plays a pivotal role in regulating NPC metastasis by way of enhancing cellular migration, cellular invasiveness, vimentin expression level, and in vivo spread of cancer cells. Moreover, a high level of serglycin expression can potentially be used to predict shorter disease-free survival and shorter metastasis-free survival of NPC patients. Targeting serglycin could be a novel option for prevention of NPC metastasis.

Acknowledgements

We thank David Nadziejka, Grand Rapids, Michigan, for critical reading of the manuscript.

References

3. Ahmad A, Stefani S. Distant metastases of nasopharyngeal carcinoma: a
19. Zernichow L, Dalen KT, Prydz K, Winberg JO, Kolset SO. Secretion of

Figure legends

Figure 1. Serglycin expression and glycosylation in high-metastasis clones.

Gene expression profiling using Affymetrix microarray analysis was performed on cultured cells of the four lines (A), as well as on the
corresponding xenograft tumors grown subcutaneously in the nude mice (B). Red and blue respectively indicate up-regulation and down-regulation of a gene relative to the average of the three low-metastasis lines (CNE-2, S22, and S26). (C) The mRNA levels of serglycin (normalized to GAPDH) in the four lines were confirmed by quantitative real-time PCR, showing that serglycin expression was significantly higher in S18 cells than in the other lines. Moreover, high level of serglycin mRNA was also found in another high-metastasis clone 5-8F in contrast to its parental low-metastasis NPC cell line SUNE-1. Column, mean; error bar, ± standard deviation from triplicates. (D) The serglycin protein levels in these lines as determined by western blotting. (E) The concentrated conditioned medium of S18 cells was deglycosylated at 37 °C for 48 h using an enzymatic deglycosylation kit. After partial deglycosylation, a protein band of 80 kDa was detected by immunoblotting of serglycin, in contrast to the control sample with no enzyme.

Figure 2. Serglycin expression is up-regulated in liver metastases and in migrated CNE-2 cells.

(A) We used immunohistochemical staining to evaluate the protein expression levels of serglycin in primary NPC tissues and in liver metastases from NPC. The ranges of serglycin expression (brown) were shown, with prominent staining in metastatic lesions. Scale bars, 50 μm. (B) Statistical analysis (Mann-Whitney test) revealed a significant increase of serglycin expression in liver metastases from NPC relative to expression in primary NPC (*, p < 0.001). (C) A two-chamber assay (modified Boyden chamber assay) with and without Matrigel was used to evaluate migration and invasion abilities, respectively. Parental CNE-2 cells and CNE-2 M3 cells were studied. Sequential screening successfully selected the CNE-2 M3 cells that had increased migration and invasion abilities. Column, mean; bars, ± standard deviation (from triplicates). Note: * indicates p < 0.001 relative to parental
CNE-2 cells (Student’s t test). (D) Serglycin mRNA levels in CNE-2 and CNE-2 M3 cells were determined by quantitative real-time PCR normalized to GAPDH. Serglycin expression was dramatically up-regulated in the CNE-2 M3 cells, consistent with the previous findings.

**Figure 3. Suppression of serglycin inhibits the migration and invasion abilities of S18 cells.**

S18 cells were transduced with lentiviruses expressing scrambled shRNA or shRNAs targeting serglycin at different loci (KD 1 and KD 2). (A) Serglycin mRNA levels (normalized to GAPDH) determined by quantitative real-time PCR in the serglycin knock-down cells were significantly reduced. (B) Serglycin protein levels in both conditioned medium (CM) and whole cell lysate (WCL) were determined by immunoblotting. Suppression of serglycin in SG KD 1 and SG KD 2 cells completely eliminated the secretion of serglycin protein into the medium. (C) Suppression of serglycin did not alter the proliferative rates of the three cell populations as determined by MTS assay. (D) Suppression of serglycin dramatically reduced the migration ability of the cells as determined by a wound-healing assay. Yellow dashed lines denote the margins of the wound. (E) A two-chamber assay was used for further evaluation of the invasion/migration ability of the S18 cells. Suppression of serglycin significantly reduced the cells’ migration and invasion abilities. Column, mean; bars, standard errors from triplicates. Student’s t test was used for the statistical analyses. Note: *, p < 0.001 relative to that of scrambled shRNA; #, p = 0.0027 for SG KD 1 and 0.0061 for SG KD 2 when compared with that of scrambled shRNA.

**Figure 4. Secreted serglycin enhances migration and invasion abilities of low-metastasis S26 cells.**

(A) Low-metastasis S26 cells were transduced with lentiviruses
expressing serglycin cDNA or an empty vector as control. The serglycin expression levels were determined by western blotting. Protein levels of serglycin in conditioned medium (CM) and whole cell lysate (WCL) were increased by stable ectopic expression of serglycin cDNA in S26 cells. (B) S26 cells stably overexpressing serglycin cDNA or the control vector were subjected to MTS assays. There was no statistical difference between these two cellular populations. (C) A high level of secreted serglycin was detected by immunoblotting in the concentrated conditioned medium of the S26 cells carrying serglycin plasmid. Twenty-four hours of stimulation of wild-type S26 cells with the two conditioned media resulted in an increase of migration ability (D) and an increase of invasion ability (E) of the S26 cells. Column, mean; bars ± standard error (from triplicates). Note: * p < 0.01 with Student’s t test.

Figure 5. In vivo metastasis rates of serglycin-knockdown cells and serglycin-overexpressing cells.

Subcutaneous injection of S18 cells stably expressing serglycin shRNAs (SG KD 1 and KD 2) or scrambled shRNA into the left hind footpad of nude mice led to metastasis to the left popliteal lymph node in some animals. (A) Image of a popliteal lymph node 38 days after injection of S18 cells expressing a scrambled shRNA. (B) Enlarged image of the framed area in A, showing metastatic S18 cells invading the normal lymphoid tissue. (C) The proportion of popliteal lymph node metastasis after inoculation. Knocking down serglycin in S18 cells significantly reduced the metastasis rate from 88.1% (52/59) to 72.4% (42/58). (D) S26 cells stably overexpressing serglycin or a control vector were inoculated into the of the left hind footpad. The image shows a metastatic popliteal lymph node 38 days after injection. (E) Enlarged image of the framed area in D, showing metastatic serglycin-expressing S26 cells in the sub-capsular area of the lymph node invading the normal lymphoid tissue. (F) The proportion of popliteal lymph node metastasis after inoculation of S26
cells. Overexpression of serglycin in S26 cells significantly increased the metastasis rate from 67.7% (42/62) to 85.2% (52/61).

**Figure 6. Serglycin expression modulates vimentin expression in NPC cells.**

(A) Continuous sections of human NPC tissue were subjected to IHC staining with antibodies against serglycin, vimentin, and E-cadherin. The high expression of serglycin in the tumor tissue in Case 1 was accompanied by an elevated level of vimentin and absence of E-cadherin. Conversely, the low expression of serglycin in the tumor tissue of Case 2 was accompanied by absence of vimentin and an elevated level of E-cadherin. Scale bars, 50 μm. (B) Using the same methods of continuous sectioning and IHC staining, 47 primary NPC tissues satisfactorily stained with antibodies against vimentin and SG were scored and plotted. A significant positive correlation between serglycin and vimentin was shown. (C) For E-cadherin staining, 46 NPC tissues were qualified for analysis. A significant negative correlation between SG and E-cadherin was found. (D) Immunoblotting of serglycin, and EMT markers in CNE-2, S26, and S18 cells showed a high level of secreted serglycin in the conditioned medium of S18 cells, which was accompanied by a high level of vimentin, N-cadherin, and fibronectin, and a low level of E-cadherin. Conversely, low expression of secreted serglycin in the conditioned medium of CNE-2 and S26 cells was accompanied by a low level of vimentin, N-cadherin, and fibronectin, and a high level of E-cadherin. (E) Knocking down serglycin expression using shRNAs (SG KD1 and SG KD2) dramatically suppressed the vimentin level in a whole cell lysate of S18 cells, but the levels of other EMT markers were not influenced.

**Figure 7. Elevated serglycin level correlates with shorter disease-free survival and distant-metastasis-free survival in NPC patients.**
Tissue microarray (TMA) analyses of a cohort of 263 NPC patients diagnosed at M0 were conducted to explore the correlation between patient survival and serglycin expression, as evaluated by IHC staining. (A) High and low levels of serglycin protein expression in TMA are shown under both low and high magnifications of a light microscope. The scale bars represent 100 μm. (B) The median follow-up time for this cohort of patients was 83 months. (C) The median score for serglycin expression was used as a cut-off to divide the patients into high- and low-expression groups. The disease-free survival (DFS) rate was significantly higher in the low- serglycin group. (D) The distant-metastasis-free survival (DMFS) rate was also significantly higher in the low- serglycin group. P values were calculated by Log Rank test.
Fig 2

A

Primary NPC

Liver metastases

B

Serglycin staining scores

Primary NPCs (n = 48)

Liver metastases from NPC (n = 16)

C

Number of cells / field

CNE-2

CNE-2 M3

Migration

Invasion

D

Relative serglycin mRNA level

CNE-2

CNE-2 M3

*
Fig 3

A

Relative serglycin mRNA level

<table>
<thead>
<tr>
<th></th>
<th>Scrambled</th>
<th>SG KD 1</th>
<th>SG KD 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70000</td>
<td>60000</td>
<td>50000</td>
</tr>
<tr>
<td>1</td>
<td>60000</td>
<td>50000</td>
<td>40000</td>
</tr>
<tr>
<td>2</td>
<td>50000</td>
<td>40000</td>
<td>30000</td>
</tr>
</tbody>
</table>

B

CM

WCL

β-actin

C

MTS activity (A_{490} nm)

<table>
<thead>
<tr>
<th></th>
<th>Scrambled</th>
<th>SG KD 1</th>
<th>SG KD 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>1.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

D

Scrambled

SG KD 1

SG KD 2

0.5 mm

0 h

24 h

36 h

E

Number of S18 cells / field

<table>
<thead>
<tr>
<th></th>
<th>Scrambled</th>
<th>SG KD 1</th>
<th>SG KD 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migration</td>
<td>900</td>
<td>800</td>
<td>700</td>
</tr>
<tr>
<td>Invasion</td>
<td>800</td>
<td>700</td>
<td>600</td>
</tr>
</tbody>
</table>

* * # #
Fig 5

(A) Non-metastasis

(B) Metastasis

(C) Number of mice

(D) Number of mice

(E) Number of mice

(F) Number of mice

- Vector: 42 non-metastasis, 20 metastasis
- Serglycin overexpression: 52 non-metastasis, 9 metastasis
- Scrambled: 52 non-metastasis, 7 metastasis
- SG KD1: 42 non-metastasis, 16 metastasis
- SG KD2: 43 non-metastasis, 16 metastasis

P-values:
- P = 0.036
- P = 0.032
- P = 0.022
- P = 0.036
**Fig 6**

A. Case 1 and Case 2 images showing Serglycin, Vimentin, and E-cadherin.

B. Scatter plot showing the correlation between Serglycin score and Vimentin score (n = 47, r = 0.305, P = 0.037).

C. Scatter plot showing the correlation between Serglycin score and E-cadherin score (n = 46, r = -0.388, P = 0.008).

D. Western blots of CM and WCL from CNE-2, S26, and S18 cells showing Serglycin, Vimentin, N-cadherin, Fibronectin, and β-actin.

E. Western blots of S18 cells with CM and WCL showing Scrambled, SG KO 1, and SG KO 2 conditions for Serglycin, Vimentin, E-cadherin, Fibronectin, N-cadherin, Snail, Twist, and β-actin.
Serglycin is a theranostic target in nasopharyngeal carcinoma that promotes metastasis

Xin-Jian Li, Choon Kiat Ong, Yun Cao, et al.

Cancer Res  Published OnlineFirst February 2, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-3557

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/02/02/0008-5472.CAN-10-3557.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.