Serglycin Is a Theranostic Target in Nasopharyngeal Carcinoma that Promotes Metastasis

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Abstract

Nasopharyngeal carcinoma (NPC) is known for its high-metastatic potential. Here we report the identification of the proteoglycan serglycin as a functionally significant regulator of metastasis in this setting. Comparative genomic expression profiling of NPC cell line clones with high- and low-metastatic potential revealed the serglycin gene (SRGN) as one of the most upregulated genes in highly metastatic cells. RNAi-mediated inhibition of serglycin expression blocked serglycin secretion and the invasive motility of highly metastatic cells, reducing metastatic capacity in vivo. Conversely, serglycin 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. Serglycin 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 an independent prognostic indicator of metastasis-free survival and disease-free survival in NPC patients. Cancer Res; 71(8); 3162–72. ©2011 AACR.

Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy 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 (LN) or even distant organs at the time of diagnosis (6). However, the molecular mechanisms underlying NPC metastasis are poorly understood.

Serglycin is a proteoglycan consisting of a core protein to which negatively charged glycosaminoglycan (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, an N-terminal domain which negatively charged GAG chains of

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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

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 Dulbecco’s modified Eagle’s medium supplemented with 10% FBS at 37°C. Cellular growth curves were plotted by using the cellular viability values assessed by the MTS method (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay solution; Promega).

In vitro migration and invasion assays

Wound healing assays and transwell assays were used to evaluate the migration and invasion abilities of the cells (40). See the Supplementary Methods for more details.

Detection of serglycin in conditioned medium

A total of 2 × 10⁶ cells were plated in 100-mm culture plates and incubated for 48 hours in a regular medium, then replaced with a serum-free medium and incubated for an additional 24 hours. Ten milliliters of conditioned medium was collected and concentrated to a volume of 200 μL by using Amicon Ultra centrifuge filters (10 kDa molecular weight cutoff pore size; Millipore). Twenty microliters of the concentrated conditioned medium was subjected to SDS-PAGE and blotted with anti-human serglycin (Cat no. H00005552-M03, Abnova) antibodies.

Human tissues and tissue microarray

All the human tissue samples were obtained from the Department of Pathology, Sun Yat-sen University Cancer Center (SYSUCC), with prior patient consents and the approval of the Institutional Clinical Ethics Review Board at SYSUCC. The tissue microarrays (TMA) contained qualified primary NPC samples from 263 pathologically diagnosed patients at SYSUCC between December 30, 1997, and September 6, 2002. Of these patients, 199 were men and 64 women, with a median age of 46 years (ranging from 17 to 77 years). All patients received radiotherapy, with doses of 70 to 74 Gy to the primary tumor, 60 to 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 to 3 cycles of induction or concurrent platinum-based chemotherapy was given. The patients were followed up regularly. TMAs were constructed as described previously (41) and the methodology is described in the Supplementary Methods.

Histologic evaluation and immunohistochemical staining

Mouse lymph nodes were routinely fixed 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 hematoxylin and eosin staining.

For immunohistochemical (IHC) staining of serglycin and vimentin, paraffin-embedded tissues were sectioned at 5 μm and IHC staining was performed as described previously (42). Briefly, the sections were incubated with a rabbit anti-human serglycin polyclonal antibody (Cat No.: HPA000759, Sigma-Aldrich; working dilution 1:50) or mouse anti-human E-cadherin monoclonal antibody (Abcam) overnight at 4°C, or mouse anti-human Vimentin monoclonal antibody (Neomarkers; working dilution 1:100) for 30 minutes at room temperature. An EnVision kit (DAKO) 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 2 pathologists by using the semiquantitative IRS (immunoreactive 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 2 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 serglycin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: GAPDH forward, 5′-AAGGTCAATCCCCGAGCTGA-3′; GAPDH reverse, 5′-TGACCAAGTGGTCTGTTGAGG-3′; serglycin forward, 5′-TATCCTAGCCGGGAGCCAGGTAC-3′; serglycin reverse, 5′-TTCGTTAGGAAGGCCTACCCAGATC-3′. The experiments were performed in triplicate.

Lentiviral transduction studies

Cell lines stably expressing either serglycin short hairpin RNA (shRNA) or a scrambled nontarget shRNA were established by a BLOCK-it Lentiviral Pol II miR RNAi system (Invitrogen) according to the manufacturer’s instructions. The targets of human serglycin shRNAs are 5′-CTCTTCTGGAATCCTCAGTT-3′ and 5′-CGCTGCAATCAGACAGTAAT-3′. See the Supplementary Methods for more details.

Immunoblotting

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

Concentrated conditioned medium containing secreted serglycin protein was treated with an enzymatic Glyceropyranoside Deglycosylation Kit (EMD Chemicals) according to the manufacturer’s instructions. Briefly, 20 μL of concentrated conditioned medium, mixed with denaturation buffer (2% SDS, 1 mol/L β-mercaptoethanol) and reaction buffer (250 mmol/L sodium phosphate buffer, pH 7.0), was incubated at 100°C for 5 minutes. After adding 1 μL each of N-glycosidase F, α2-3,6,8,9-neuraminidase, endo-α-2-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 hours. Immobots were carried out to determine the efficiency of serglycin deglycosylation.

Animals and spontaneous lymph node metastasis assay

Female athymic mice between 5 and 6 weeks of age were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). All the animal studies were conducted in accordance with the guidelines of Institutional Animal Care and Use Committee at SYSUCC. Xenograft tumors for genomic expression profiling were generated by subcutaneous injection of the cancer cells in the mice. The spontaneous LN 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 to 8 weeks, the popliteal LN 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 Methods. To identify genes that were differentially expressed between high- and low-metastasis cells/xenografts, the log_2 transformed expression value of each gene in S18 cells/xenograft was subtracted by the mean of log_2 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 upregulated and 25 most downregulated genes are presented in Figure 1A and B as heat maps. The gene expression data for this study have been uploaded to the

Figure 1. Serglycin expression and glycosylation in high-metastasis clones. Gene expression profiling was performed on cultured cells of the 4 lines (A), and on the corresponding xenograft tumors grown subcutaneously in the nude mice (B). Red and blue, respectively, indicate upregulation and downregulation of a gene relative to the average of the 3 low-metastasis lines (CNE-2, S22, and S26). C, the mRNA levels of serglycin (normalized to GAPDH) in the 4 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, ±SD (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 hours by 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. CM, conditioned medium; WCL, whole cell lysate.
Gene Expression Omnibus, with the accession number GSE24154.

**Statistical methods**

Single comparisons were performed by Student’s t test, Mann–Whitney test, or $\chi^2$ test (2-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-serglycin 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 for multivariate survival analysis. Predictors were judged to be significant at $P < 0.05$. 

Figure 2. Serglycin expression is upregulated in liver metastases and in migrated CNE-2 cells. A, IHC staining was used 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 $\mu$m. 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 2-chamber assay (transwell 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, ±SD (from triplicates). *, $P < 0.001$ 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 upregulated in the CNE-2 M3 cells, consistent with the previous findings.

Serglycin Regulates Metastasis of Nasopharyngeal Carcinoma
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, whereas clone 22 (S22) and clone 26 (S26), and the parental line CNE-2, had low-metastatic abilities. To explore the underlying molecular mechanism(s), we performed genomic expression profiling of these 4 cell lines from in vitro cultured cells. In the high-metastasis clone S18, the serglycin gene (SRGN) was the second most highly upregulated (Fig. 1A). Considering the potential differences between in vivo and in vitro conditions, xenograft tumors from these 4 cell lines were also collected for gene expression profiling. Again, serglycin was the second most upregulated gene in S18 xenograft (Fig. 1B).

To confirm the findings described above, quantitative real-time PCR was performed to quantify the mRNA levels of serglycin among the 4 lines, with consistent results (Fig. 1C). Moreover, a high level of serglycin expression was also detected in the high-metastasis clone 5-8F isolated from another low-metastasis parental NPC cell line SUNE-1 (ref. 44; Fig. 1C). Moreover, S18 and 5-8F cells could secrete serglycin into culture medium, whereas the other low-metastasis cell lines could not (Fig. 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 (approximately 80 kDa) could be detected (Fig. 1E), confirming the glycosylation of the serglycin protein secreted by high-metastasis cells.

Results

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Serglycin is upregulated 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). IHC staining showed that the protein levels of serglycin were significantly higher in the liver metastases (Fig. 2A and B), implying that serglycin could be important in clinical scenarios.

To confirm that upregulation of serglycin in highly migratory cells could be repeatedly found, a 2-chamber (Boyden chamber) assay was used to isolate highly migratory cells from the parental CNE-2 line. After 3 sequential passages of screening, highly migratory/invasive cells (CNE-1 M3) were isolated (Fig. 2C), and serglycin was found to be highly expressed in these cells (Fig. 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 nontarget shRNA. The suppression of serglycin expression at the mRNA level was confirmed by quantitative real-time PCR (Fig. 3A). A decrease in secreted serglycin protein was confirmed via immunoblotting (Fig. 3B). Yet, knocking down serglycin in S18 cells did not alter the growth rate of the cells (Fig. 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 (Fig. 3D and E). The invasion ability of S18 cells was also significantly reduced by knocking down serglycin (Fig. 3E). These results proved that secreted serglycin could promote NPC cell migration and invasion in an autocrine mode.

Secreted serglycin promotes the motility of low-metastasis NPC cells

To validate the motility-enhancing activity of serglycin, we generated an S26 cell line stably overexpressing serglycin in the conditioned medium (Fig. 4A), without altering the cellular growth (Fig. 4B).

The conditioned medium of S26 cells overexpressing serglycin was subjected to 50× concentration (from 10 mL to 200 μL), and concentrated serglycin protein was then confirmed by immunoblotting (Fig. 4C). The concentrated conditioned medium was used to stimulate migration and invasion by wild-type S26 cells. The number of migrated and invaded cells were significantly increased after the stimulation (Fig. 4D and E). 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 the cells (Supplementary Fig. 2), suggesting that the glycosylation of serglycin is critical for promoting cellular motility.

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

The effect of serglycin in vivo was determined by using an LN metastasis model (40). As expected, serglycin knockdown cells showed a significant reduction in metastasis rate, and serglycin-overexpressing cells showed an increased metastasis rate (Fig. 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 (Fig. 6A and B), and inversely correlated to the expression level of E-cadherin (Fig. 6A and C). To confirm this finding, the protein levels of several EMT markers in 4 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 cells (Fig. 6D). The suppression of serglycin resulted in a lower vimentin level, but did not influence the levels of other EMT proteins (Fig. 6E). All 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 values of serglycin expression, we performed IHC staining for serglycin in a set of tissue microarrays containing 263 NPC samples (Supplementary Table 1; Fig. 7A and B). The correlations between serglycin expression and clinicopathologic characteristics are presented in Supplementary Table 2. High serglycin expression was significantly correlated with the occurrence of disease progression or distant metastasis. Multivariate analyses 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 Figure 7C and D. Taken together, these analyses revealed that high serglycin expression level in NPC significantly correlated with adverse patient outcomes.

**Discussion**

In this 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. The first advantage of our approach 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 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 tumor (45). Using the identified culprit clone for comparison can therefore easily reveal the key molecules regulating metastasis.

Among the most upregulated genes in S18, serglycin is second on the list under both in vitro and in vivo conditions, implying the heavy involvement of this glycoprotein in regulating NPC metastasis. To our knowledge, no published data have directly linked serglycin with tumor metastasis. This

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, 47 primary NPC tissues satisfactorily stained with antibodies against vimentin and serglycin 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 serglycin and E-cadherin was found. D, Immunoblotting of serglycin and EMT markers showed a high level of secreted serglycin in the conditioned medium of high-metastasis S18 and 5-8F cells, which was accompanied by high levels of vimentin, N-cadherin, and fibronectin, and a low level of E-cadherin. Conversely, low expression of secreted serglycin in low-metastasis cells (CNE-2, S26, and SUNE-1) was accompanied by low levels of vimentin, N-cadherin, and fibronectin, and a high level of E-cadherin. E, knocking down serglycin expression by 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.
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 seeming 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 B, 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 shows 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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