Visualized Cancer Medicine 2023, **4**, 6

https://doi.org/10.1051/vcm/2023002

Available online at:
https://vcm.edpsciences.org

PERSPECTIVE ARTICLE OPEN ACCESS

Semaphorin 4C accelerates disease progression and enables disease detection in breast cancer

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Received 14 February 2023, Accepted 6 May 2023, Published online 31 May 2023

Abstract – Semaphorins constitute a diverse family of widely expressed transmembrane, diffusible, and GPI-linked proteins with versatile physiologic functions in orchestrating nerve system development, immune homeostasis, angiogenesis, and cell metabolism. Accumulating evidence highlights semaphorins as essential regulators of tumorigenesis by coordinating the cell-cell communications in the tumor microenvironment. Semaphorin 4C (SEMA4C) is a member of the fourth class of semaphorins with high affinity to Plexin-B2 and its interplay with cancer has long been a significant knowledge gap. Here, this perspective summarizes the recent progress in the understanding of SEMA4C in cancer and comprehensively delineates the discovery of SEMA4C in lymphatic vessels of breast cancer, the mechanisms by which SEMA4C promotes the invasiveness, proliferation, metastasis, and drug resistance of breast cancer, and the explorations of leveraging serum SEMA4C in breast cancer detection, highlighting SEMA4C as a critical driver of breast cancer progression, an effective biomarker for breast cancer diagnosis, and potential therapeutic target for breast cancer treatment.

Key words: Breast cancer, Semaphorin 4C, Biomarker, Diagnosis, Metastasis, Tumor microenvironment.

Introduction

Semaphorins constitute a large and diverse family of widely expressed transmembrane, diffusible, and GPI-linked proteins with versatile physiologic functions in orchestrating nerve system development, immune homeostasis, angiogenesis, and cell metabolism [1]. Accumulating evidence indicates that semaphorins act as essential regulators of tumor development by coordinating the complex cell-cell communications in the tumor microenvironment [2]. In humans, 20 semaphorins are identified and divided into eight classes according to the terminal carboxyl domain, and most semaphorins exert effects via plexins receptors [3]. SEMA4C is a pivotal member of the fourth class of semaphorins with high affinity to Plexin-B2 and SEMA4C-Plexin B2 signaling substantially contributes to the polarization of B cells, ureteric branching, and cerebellar granule cell precursor migration [4–6]. The association between SEMA4C and cancer progression and the underlying mechanisms have long been a significant knowledge gap until recently our studies and others unveiled overexpressed SEMA4C in multiple human cancers, the critical roles of SEMA4C in abetting breast cancer progression, the signaling pathways that mediate the SEMA4C-cancer interplay, and the promising potential of serum SEMA4C as a diagnostic biomarker for breast cancer [7–14].

Breast cancer is the most frequent malignancy worldwide with 2,261,419 new cases and 684,996 cancer-related deaths annually [15]. Breast cancer harbors intricate heterogeneity and is clinically divided into four subtypes including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-positive, and triple-negative breast cancer by the expression of hormonal receptors (estrogen receptor and progesterone receptor) and HER2. The heterogeneity is further perplexed by genomic aberrations featured by *BRCA1/2* mutations and immunomodulation such as PD-L1 expression and lymphocyte infiltration [16]. Owing to the tailored treatment strategies to molecular subtypes, breast cancer without distant organ metastases has an encouraging five-year relative survival rate of over 90%, in contrast to below 30% for metastatic disease [17]. Dissemination of breast cancer cells occurs mainly through

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the lymphatic vessels and lymph node metastasis predicts impaired prognosis of breast cancer. Lymphatic endothelial cells control leukocyte transport and interact with immune cells to regulate lymph node metastasis [18].

Semaphorin 4C accelerates disease progression in breast cancer

Leveraging a technique that combines laser capture microdissection and gene microarray analysis we developed [19], we molecularly portraited tumor-associated lymphatic endothelial cells in breast cancer, identified *Semophorin 4C* (*SEMA4C*) as the most upregulated gene compared with normal lymphatic endothelial cells, and deciphered its roles in accelerating lymphatic metastasis [7]. Tumor-associated lymphatic endothelial cells not only enhanced the expression of membrane-bound SEMA4C but produced a plethora of soluble SEMA4C with the assistance of matrix metalloproteinase. Intriguingly, soluble SEMA4C was capable of modulating the behaviors of both tumor cells and lymphatic endothelial cells via disparate pathways. Specifically, soluble SEMA4C fueled lymphangiogenesis via the activation of PlexinB2-ERBB2 signaling cascades in lymphatic endothelial cells and boosted the proliferation and migration of tumor cells mediated by PlexinB2-MET signaling, which orchestrated lymphatic metastasis in an RHOA-dependent manner. Moreover, RHOA signaling blockade could suppress the promoting effects of soluble SEMA4C on lymph node metastasis.

Besides the intimate association between SEMA4C and lymphatic dissemination, we also investigated the biological effects of SEMA4C within tumor cells and their impacts on tumor microenvironment architecture [10]. We screened SEMA4C expression in frequently used human tumor cell lines of eight cancer types (melanoma and breast, prostate, lung, ovarian, cervical, pancreatic, and colorectal cancers). Interestingly, high levels of SEMA4C protein were found in cell lines of all eight cancer types, which was consistent with the literature that reported elevated SEMA4C expression in multiple human cancers (osteosarcoma and breast, colorectal, ovarian, esophageal, gastric, and cervical cancers) and indicated the intriguing association between SEMA4C and tumorigenesis [8, 9, 11, 20–23]. Besides, enhanced SEMA4C expression predicted worsened prognosis of breast, ovarian, lung, and gastric cancers, and significantly correlated with advanced stages and lymphatic invasion in breast cancer. Knockdown of SEMA4C/PlexinB2 signals *in vitro* induced cell growth arrest and cellular senescence in breast cancer cell line mediated by the p53 signaling pathway. Consistently, the interference of SEMA4C in subcutaneous breast tumors curbed tumor growth and metastatic dissemination. Importantly, SEMA4C knockdown reshaped the tumor microenvironment by decreasing angiogenesis and macrophage recruitment. Mechanistically, SEMA4C in tumor cells promoted angiogenesis and macrophage recruitment by regulating the release of diffusible factors characterized by angiogenin (ANG) and colony-stimulating factor (CSF-1) via the classical NF-kB signaling pathway.

Contemporarily, the importance of SEMA4C in breast cancer progression is independently demonstrated by the other research team based in Italy [11, 12]. They found that SEMA4C was widely expressed in human breast cancers and its elevation was associated with curtailed survival of patients with breast cancer [11]. SEMA4C-PlexinB2 signaling blockade by genetic knockdown-induced growth arrest associated with cell senescence, cell cycle inhibition, and cytokinesis defects. The SEMA4C-PlexinB2 signaling sustained breast cancer cell proliferation by maintaining elevated RhoA signaling through a SEMA4C/PlexinB2/LARG-dependent RhoA signaling pathway. Furthermore, enhanced SEMA4C signaling disrupts cell polarity and renders luminal breast cancer mesenchymal features in RhoA- and ERBB2-dependent manner. Overexpressed SEMA4C in indolent luminal estrogen receptor-positive breast tumor cells *in vivo* resulted in a phenotypic polarization towards strengthened invasiveness characterized by resistance to tamoxifen, estrogen independence, and increased metastases through upregulated protumor transcription factors such as Snail, Slug, and SOX-2.

These studies categorize SEMA4C as a ligand for PlexinB2 to form a forward signaling cascade that bolsters breast cancer cell viability and growth. Interestingly, a reverse signaling mode also exists for SEMA4C to function as a signaling receptor to determine tumor progression [12]. SEMA4C reverse signaling partially triggered mesenchymal-epithelial transition (MET) in tumor cells and facilitated metastatic colonization *in vivo*. The MET induced by SEMA4C reverse signaling could be attributable to escalated ID1/3 transcriptional factors, suppressing the attributes of epithelial-mesenchymal transition. Specifically, SEMA4C interacted on the cell surface with TGFβ/BMP receptors and induced the phosphorylation of SMAD1/5, thereby enhancing the activity of downstream *ID* genes.

Semaphorin 4C enables disease detection in breast cancer

Increasingly advanced understandings of SEMA4C in breast cancer provide opportunities to leverage SEMA4C for improved breast cancer management. One approach to maximize breast cancer survival is to implement effective screening in the target population and detect it early when curative treatments are available. For instance, breast cancer screening using mammography significantly reduces breast cancer mortality in women 50–74 years of age [24]. Currently investigated screening methods are mainly comprised of imaging techniques (mammography, ultrasonography, and magnetic resonance imaging) and clinical examinations. Though widely used tumor biomarkers such as carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), and cancer antigen (CA153) are expressed at variable levels in the tumor microenvironment of breast cancer subtypes (Figure 1, Video 1), their serum concentrations lack sensitivity in diagnosing breast cancer [25, 26]. An effective blood-derived biomarker will greatly benefit breast cancer diagnosis because it is easy to operate, radiation-free, subject to analysis, less restricted to complications, and could complement mammography in women with dense breasts or low tumor burden. Unfortunately, no serum biomarker has been proven effective in the early detection of breast cancer to date [27]. Recent studies have started to concentrate on discovering

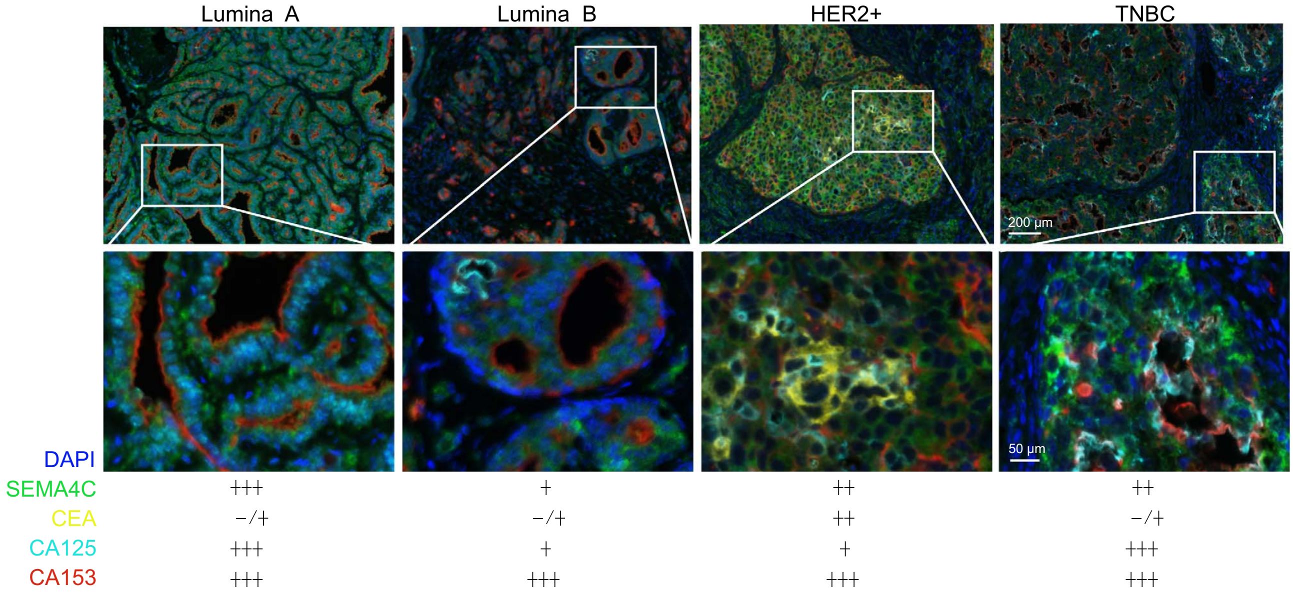
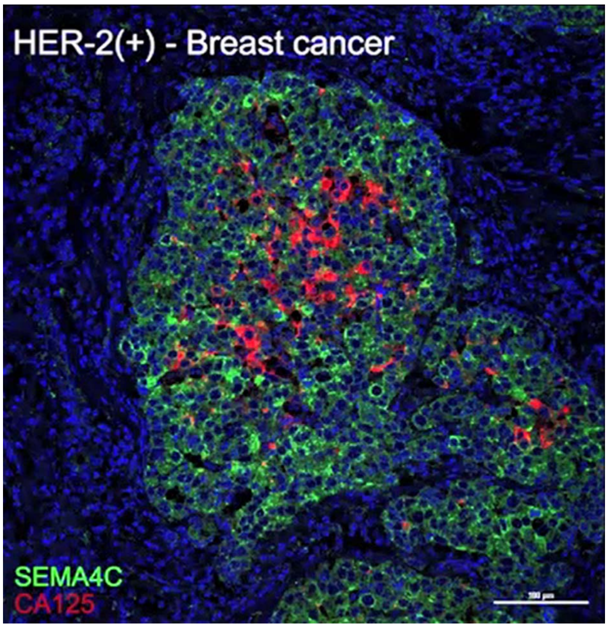


Figure 1. Multiplex Immunofluorescence of tumor biomarkers in breast cancer tumor microenvironment. Multiplex immunofluorescence staining of formaldehyde fixed paraffin embedded tumor slide specimens of four breast cancer subtypes were performed using Opal IHC Multiplex Assay kit (NEL821001KT, Akoya Biosciences) according to the manufacturer's protocol. Sections were sequentially stained as follows: MUC16/CA125 (clone EPR1020(2), dilution 1:1000, Abcam), MUC1/CA153 (clone EPR1023, dilution 1:500, Abcam), CEA (clone EPCEAR7, dilution 1:2000, Abcam), and SEMA4C (28402-1-AP, dilution 1:500, Proteintech). Briefly, slides were microwave heat-treated in Epitope retrieval solution 1 or 2 buffer, blocked in Antibody Diluent, and incubated with the primary antibody, HRP-conjugated secondary polymer, OPAL fluorescent reagents, and DAPI. Whole slide scans were acquired using the Vectra3 automated quantitative pathology imaging system (Akoya Biosciences).

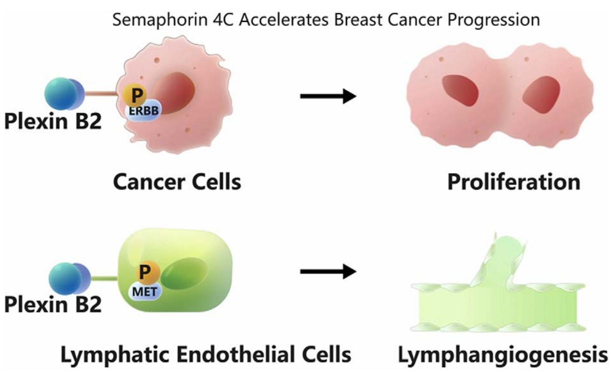
novel diagnostic biomarkers in breast cancer including circulating tumor cells and their products, proteins, autoantibodies, miRNAs, and circRNAs [28, 29].

Motivated by the findings that SEMA4C was evidently expressed in the tumor microenvironment of four breast cancer subtypes (Figure 1) and soluble SEMA4C was significantly elevated in the serum of patients with breast cancer [7], we evaluated the utility and robustness of serum SEMA4C as a diagnostic biomarker for breast cancer [13, 14]. Taking advantage of an enzyme-linked immunosorbent assay kit we optimized, we measured serum SEMA4C concentrations of 6,213 consecutive inpatients comprising 1,233 patients with breast cancer, 600 patients with benign breast tumors, 1,168 healthy controls, and 3,212 patients with other 14 types of solid tumors (cervical, pancreatic, gastric, liver, kidney, ovarian, lung, prostate, thyroid, colorectal, brain, esophageal, bladder, and endometrial cancers). To evaluate the values of serum SEMA4C levels in breast cancer diagnosis, we established a training cohort to determine the optimal threshold for SEMA4C tests, two validation cohorts for performance assessment, and a pan-cancer cohort to prove the specificity of serum SEMA4C for breast cancer diagnosis. These cohorts were geographically representative. Specifically, the training cohort was comprised of 661 patients with breast cancer, 253 patients with benign breast tumors, and 301 healthy women from Tongji Hospital of Huazhong University of Science and Technology and Qilu Hospital of Shandong University between January 2013 and June 2016. Validation cohort 1 included 332 patients with breast cancer, 183 patients with benign breast tumors, and 170 healthy women enrolled in Tongji Hospital between July 2016 and September 2017, while validation cohort 2 was composed of 240 patients with breast cancer, 164 patients with benign breast tumors, and 132 healthy women derived from Hubei Cancer Hospital from July 2016 to June 2017. The pan-cancer cohort was composed of 3,212 patients with cancer and 565 healthy women from Tongji Hospital and Qilu Hospital from July 2017 to June 2018. After determining 5 ng per mL as the best discriminative threshold in the training cohort, serum SEMA4C exhibited high area under the curve, sensitivity, and specificity to detect breast cancer in two validation cohorts (Area under the curve, 0.920 and 0.938; Sensitivity, 82.8% and 86.7%; Specificity, 87.5% and 87.8%). More importantly, for early-stage breast cancer and ductal carcinoma in situ (DCIS) that lack symptoms and effective biomarkers, serum SEMA4C displayed impressive area under the curve and accuracy to separate them from noncancerous controls. The pan-cancer cohort revealed that high serum SEMA4C levels were specifically observed among patients with breast cancer. Surgical removal of malignant breast lesions significantly decreased serum SEMA4C concentrations, indicating the potential of serum SEMA4C in predicting tumor burden and treatment response monitoring.

Next, we compared the diagnostic capabilities of serum SEMA4C in breast cancer with those of mammography and ultrasonography and investigated the combinatorial diagnosis of serum SEMA4C and imaging [14]. Serum SEMA4C levels were quantified in 1,833 consecutive women patients with pathologically confirmed breast lesions, among which mammography results were available in 802 patients and ultrasonography in 1,424 patients. Interestingly, serum SEMA4C yielded a higher area under the curve (0.927 vs. 0.788),



Video 1. Semaphorin 4C, CA125, and CA153 expression in breast cancer tumor microenvironment. This video shows the expression of semaphorin 4C, CA125, and CA153 in four molecular subtypes of breast cancer including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-positive, and triple-negative breast cancer. https://vcm.edpsciences.org/10.1051/vcm/2023002#V1.



Video 2. Semaphorin 4C accelerates disease progression and enables disease detection in breast cancer. This video presents the biological processes by which semaphorin 4C accelerates breast cancer progression and enables breast cancer detection based on two of our previous reports [7, 13]. The icons in the video were created with Biorender.com. https://vcm.edpsciences.org/10.1051/vcm/2023002#V2.

enhanced specificity (84.8% vs. 61.3%), and compromised sensitivity (83.9% vs. 96.4%) than mammography to diagnose breast cancer. Compared with ultrasonography, serum SEMA4C demonstrated an improved area under the curve (0.907 vs. 0.804), ameliorated specificity (83.1% vs. 73.0%), and impaired sensitivity (81.8% vs. 87.8%). In diagnosing Breast Imaging Reporting and Data System (BI-RADS) category 3 and 4 breast lesions, the relative weakness for mammography and ultrasonography interpretations [30], joint diagnostic efficacy of serum SEMA4C and imaging displayed significantly increased area under the curve to diagnose breast cancer than imaging alone for both mammography and ultrasonography.

Conclusion

In conclusion, this perspective comprehensively delineates the roadmap from the discovery of SEMA4C in lymphatic vessels of breast cancer and the ensuing elucidation of SEMA4C in breast cancer biology to the exploratory applications of serum SEMA4C in breast cancer diagnosis (Video 2). These studies spanned over ten years and showed a paradigm of bedside-bench side-bedside science, highlighting SEMA4C as a critical driver of breast cancer progression, an effective serum biomarker for breast cancer diagnosis, and a potential therapeutic target for breast cancer treatment. Studies are ongoing to decode the complicated interactions between SEMA4C and the tumor microenvironment of breast cancer and will hopefully advance the understanding of SEMA4C and facilitate better management of breast cancer.

Conflict of interest

The authors have nothing to disclose.

Acknowledgements. This study was supported by the National Key Technology Research and Development Programme of China (2022YFC2704200 and 2022YFC2704205), National Science and Technology Major Sub-Project (2018ZX10301402-002), National Natural Science Foundation of China (81772787 and 82072889), Technical Innovation Special Project of Hubei Province (2018ACA138), and Fundamental Research Funds for the Central Universities (2019kfyXMBZ024).

Author contributions

Conceptualization, Qinglei Gao and Huayi Li; Data curation, formal analysis, methodology, resources, and visualization: Xin Li, Sen Xu, Yu Xia, and Hongfeng Zhang; Funding acquisition: Qinglei Gao; Writing-original draft: Huayi Li; Writing-reviewing and editing: all authors.

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Cite this article as: Li H, Li X, Xu S, Xia Y, Zhang H & Gao Q. Semaphorin 4C accelerates disease progression and enables disease detection in breast cancer. Visualized Cancer Medicine. 2023; **4**, 6.