Research Paper

Role of Semaphorin 3D in Inhibition of Colorectal Cancer Cell Migration by Regulating Actin Filament Associated Protein 1 Like 1

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The abnormal expression of semaphorin 3D is associated with various tumors. Nevertheless, the function and mechanism in colorectal cancer have not been fully understood. This study aims at exploring the migration pattern and related mechanisms of semaphorin 3D in colorectal cancer cells *in vitro*. Human SW480 colorectal cancer cells lines were used in this study. Semaphorin 3D lentiviral overexpressed vector and control lentiviral vector were used to transfect SW480 cells. Quantification of semaphorin 3D, actin filament associated protein 1 like 1 messenger ribonucleic acid and protein levels were done by real-time quantitative polymerase chain reaction and western blotting. Ribonucleic acid sequencing is used for analysis of differential gene expression and transwell assay for determination of migration capacity. The research results showed that semaphorin 3D gene expression was dramatically overexpressed in SW480 cells that were transfected by semaphorin 3D lentiviral overexpressed vector. Compared to control group, cell migration capacity of semaphorin 3D overexpression SW480 cells were strikingly reduced. Ribonucleic acid sequencing, quantitative polymerase chain reaction and western blotting assays hinted actin filament associated protein 1 like 1 was significantly downregulated in semaphorin 3D overexpression cells. Based on these findings, overexpression of semaphorin 3D might prevent migration of colorectal cancer cells *via* down regulating actin filament associated protein 1 like 1. Therefore, semaphorin 3D may inhibit colorectal cancer cell migration and suppress colorectal cancer carcinogenesis and progression.

Key words: Semaphorin 3D, colorectal cancer, actin filament associated protein 1 like 1, lentiviral vector

As a common gastrointestinal carcinoma, Colorectal Cancer (CRC) is associated with high malignancy, rapid progression and poor therapeutic efficacy[1]. In 2023, CA: A Cancer Journal for Clinicians reported that the number of expected new cases and deaths of CRC has ranked third in the United States for both men and women[2]. According to the latest study of Sung *et al.* the incidence of CRC in China has surged to the second place in cancer in 2020 and simultaneously its mortality rate has soared to the fifth[3]. CRC is less sensitive to chemotherapy[4] and metastasis is a vital cause of death in CRC patients. However, the molecular mechanism underlying CRC metastasis is yet to be elucidated[5]. Therefore, exploring the possible mechanisms of metastatic CRC and looking for new therapeutic targets would be the promising treatment approach for CRC.

Semaphorin 3D (SEMA3D) is a protein belonging to the semaphorins family. SEMA3D plays an important physiological role in heart[6], blood vessels[7], embryonic development[8], regeneration[9] and neural development[10]. However, abnormal expression of SEMA3D can cause corresponding diseases. Some studies have demonstrated that abnormal expression of SEMA3D can regulate the occurrence and development of Hirschsprung disease by affecting the development of enteric nervous system[11-13]. Nevertheless, whether SEMA3D is correlated with Ovine Johnes Disease (OJD) is still controversial[14,15]. With further study, the role played by SEMA3D in tumor carcinogenesis and progression is increasingly concerned. Recently, SEMA3D has been suggested to suppress the occurrence and progression of glioblastoma, breast and thyroid carcinomas[16-18]. On

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the contrary, Foley *et al.* suggested that SEMA3D can promote the metastasis of Pancreatic Ductal Adenocarcinoma (PDA)[19].

These studies indicated that SEMA3D may play a role in promoting or inhibiting cancer carcinogenesis and progression in different tumors. Yet, its mechanism of action in CRC has not been fully elucidated.

In our earlier research, SEMA3D expression was found to be markedly reduced in CRC tissues versus adjacent counterparts, with an inverse connection with lymph node metastasis, manifesting that SEMA3D is a favorable prognostic factor for CRC patients[20]. Therefore, SEMA3D may inhibit CRC development and metastases.

Herein, we further investigate whether SEMA3D suppresses CRC cells migration and preliminarily explores its inhibition mechanisms.

MATERIALS AND METHODS

Source and cultivation of cells:

We Human CRC cell lines like RKO, SW620, HCT116, HT29, LOVO, CW2, LS513 and SW480 ordered from the American Type Culture Collection (ATCC, Manassas, Virginia, United States), were placed in an Roswell Park Memorial Institute (RPMI) 1640 medium, routinely supplemented with 10 % Fetal Bovine Serum (FBS) (HyClone, Tauranga, New Zealand) for cultivation at 37° with 95 % Oxygen (O_2) and 5 % Carbon dioxide (CO_2).

Real-time quantitative Polymerase Chain Reaction (qPCR):

We used an RNeasy Mini Kit (Qiagen, Catalog no. 74106) to isolate cellular total Ribonucleic Acid (RNA) and a PrimeScript Reverse Transcriptase (RT) reagent kit (Takara Biotechnology, Dalian, China, Catalog no. RR037A) to synthesize complementary Deoxyribonucleic Acid (cDNA). This was followed by qPCR using a SYBR Premix Ex (Taq Takara, Catalog no. RR420A) and the calculation of relative gene expression levels with 2-delta delta threshold cycle ($2^{-\Delta\Delta Ct}$) (Internal control: Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)). Primer sequences are GAPDH Forward (F): 5'-ACCACAGTCCATGCCATCAC-3' and GAPDH Reverse (R): 5'-TCCACCACCCTGTTGCTGTA-3', SEMA3D F: 5'-TGGGACATCGAAGACAGCAT-3' and SEMA3D R: 5'-AAAGTGTGCTCCTGGGCTTT-3', Actin Filament Associated Protein 1 Like 1 (AFAP1L1) F: 5'-GTGACCTGAGTGACCTTCGG-3' and AFAP1L1 R: 5'-AGAGTCATTATTGTGGGAGCTGA-3'.

Western Blotting (WB) analysis:

The WB program was performed as previously described[21] and the information of antibodies was followed. SEMA3D (Abcam, Catalog no. AB18074, 1:1000), AFAP1L1 (Santa Cruz Biotech, Inc., Catalog no. SC-514788, 1:1000), beta (β)-actin (Affinity, Catalog no. T0022, 1:5000) and β-actin was used as a control.

Lentiviral vector constructs and cell transfection:

Human SEMA3D lentiviral overexpressed vector and control lentiviral vector were constructed and packaged by Wuhan GeneCreate Biological Engineering Co., Ltd (Wuhan, China). Full-length SEMA3D cDNA was amplified and then inserted into lentiviral-protocadherin (pCDH)-Cytomegalovirus (CMV)-Multiple Cloning Sites (MCS)-human Elongation Factor 1 alpha (EF1α)-Green Fluorescent Protein (GFP)-P2A-Puro vector to obtain pCDH-SEMA3D overexpressed plasmid. Packaging plasmids (pMDLg-pRRE, pMD2.G and pRSV-Rev) were co-transfected into Human Embryonic Kidney 293 T antigen (HEK293T) to obtain pCDH-SEMA3D lentiviral particles. The SW480 cell strain was then infected with concentrated pCDH-SEMA3D lentiviral particles and screened by puromycin to obtain stable SEMA3D-overexpressed SW480 cell line. Lentiviral-pCDH-CMV-MCS-EF1α-GFP-P2A-Puro vector was used as negative control and quantification of SEMA3D messenger RNA (mRNA) was made by real-time qPCR.

RNA-Sequencing (RNA-Seq) and Differentially Expressed Gene (DEG) analyses:

We separated total RNA from SW480 SEMA3D overexpressed cells and negative control cells and then enriched mRNA with Oligo deoxythymidine (dT) beads and reverse transcribed it into cDNA. The cDNA fragments were then treated for purification and ligation with Illumina sequencing adapters. After the selection of ligated products of a proper size using Agarose gel electrophoresis, PCR amplification and sequencing using Illumina HiSeqTM 4000 were carried out.

DEGs were analyzed by edgeR package (http://www.r-project.org/) across overexpressed cells and

negative controls. We identified genes with a False Discovery Rate (FDR)<0.05 and a fold change≥2 as significant DEGs and validated them by qPCR.

Cell migration assays:

Migratory capacities of cells with transfected or untransfected SEMA3D gene are evaluated by transwell chambers (8 μm pore, Corning, Catalog no. 3422). 600 μl 10 % FBS added RPMI 1640 medium was placed into the lower chambers as a chemoattractant and then 100 μl cells of 1×10^5 density with RPMI 1640 (serum-free) were suspended in upper chambers. After 24 h of incubation, cells located on the upper chamber surface were erased, while those on the lower surface were fixed with 4 % neutral formaldehyde and subsequently stained with 0.1 % crystal violet. After photographing, the lower surface cells were treated with 33 % acetic acid. Then the absorbance was determined at 570 nm in a 96-well plate by a microplate reader (Bio-TEK, ELx 800, Saxony).

Statistical analysis:

All experiments were run in triplicates and the obtained data was statistically described as mean±Standard Deviation (SD). GraphPad Prism software was used for statistical analysis and statistical significance was indicated by p<0.05.

RESULTS AND DISCUSSION

SEMA3D gene expression in CRC cell lines was shown in fig. 1. Real-time PCR quantified SEMA3D expression. SEMA3D mRNA levels were highly expressed in RKO and SW620 CRC cell lines, while lower in HCT116, HT29, LOVO, CW2, LS513 and SW480 CRC cell lines. Thus, SW480 cells with low SEMA3D expression were randomly selected as overexpression transfection cell lines for subsequent functional and mechanism studies.

Overexpression of SEMA3D using lentiviral vector was shown in fig. 2. To demonstrate the role of SEMA3D in CRC cell line SW480, SEMA3D overexpressed lentiviral vector and control lentiviral vector were performed. Results showed that SEMA3D successfully overexpressed at mRNA levels in overexpressed SW480 cells compared to control vector (fig. 2A). Concurrently, WB analysis also demonstrated dramatical increase of SEMA3D protein in SEMA3D overexpressed SW480 cells, compared with control cells (fig. 2B). These results suggested that construction of stably transfected cell lines overexpressing SEMA3D is successful.

SEMA3D inhibits *in vitro* migration of CRC cells as shown in fig. 3. To manifest the migration function of SEMA3D in CRC, transwell assay was used. Compared to the control cells, the migration ability of SEMA3D overexpressed SW480 cells were notably decreased.

To probe into the regulatory mechanisms of SEMA3D in CRC cell migration, changes in downstream genes expression profiles in SW480 with overexpression of SEMA3D were analyzed. RNA-Seq analysis found that 38 genes were significantly changed after overexpression of SEMA3D (fig. 4). Consistently, qPCR further validated dramatically down-regulated AFAP1L1 mRNA in SEMA3D overexpressing SW480 cells (fig. 5A). According to WB analysis, AFAP1L1 protein was also remarkably down-regulated in SEMA3D overexpressing SW480 cells versus control group (fig. 5B).

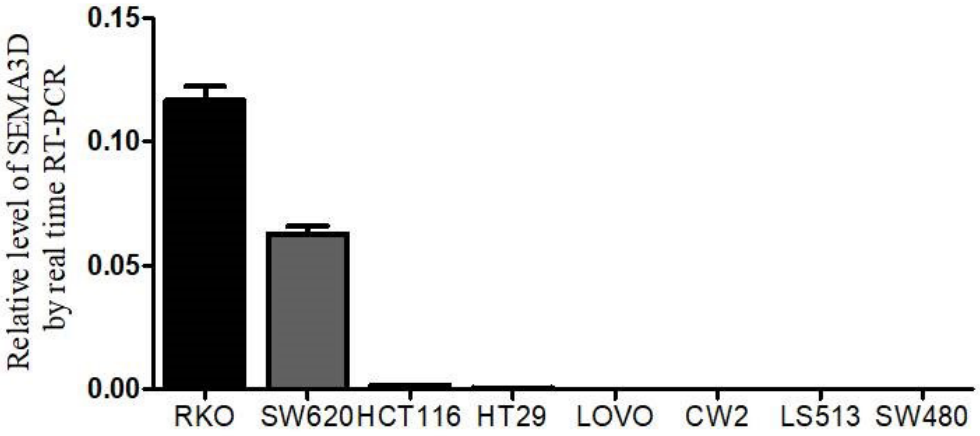

Fig. 1: SEMA3D mRNA expression in different CRC cells and the relative level of SEMA3D by real time RT-PCR was calculated by $2^{-\Delta\Delta Ct}$, ΔΔCt=Ct (GAPDH)-Ct (SEMA3D)

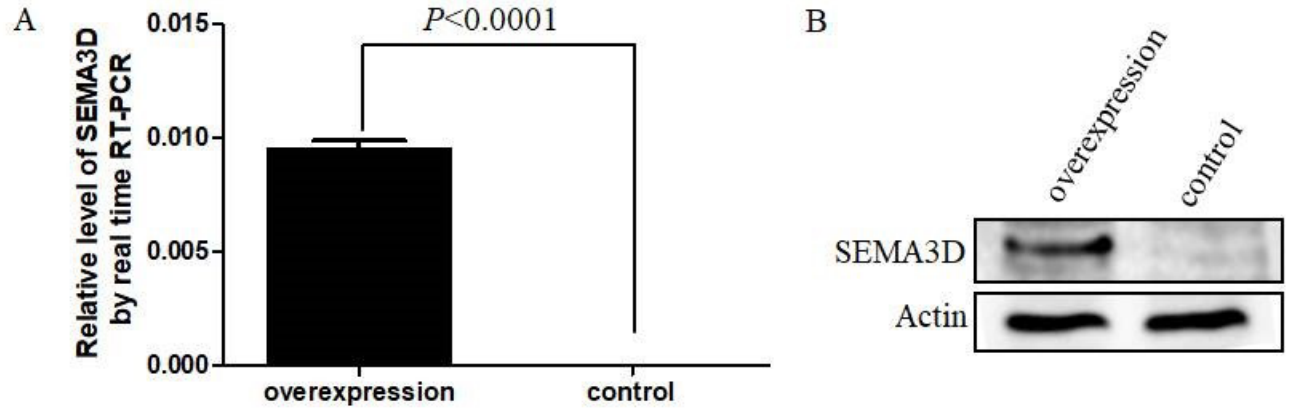

Fig. 2: Overexpression of SEMA3D using SEMA3D lentiviral vector

Note: (A) SEMA3D mRNA levels in SEMA3D or control lentiviral vector-transfected CRC SW480 cells and (B) SEMA3D protein levels in SEMA3D- or control lentiviral vector-transfected CRC SW480, overexpression: Cells transfected with SEMA3D lentiviral vector and control: Cells transfected with control lentiviral vector

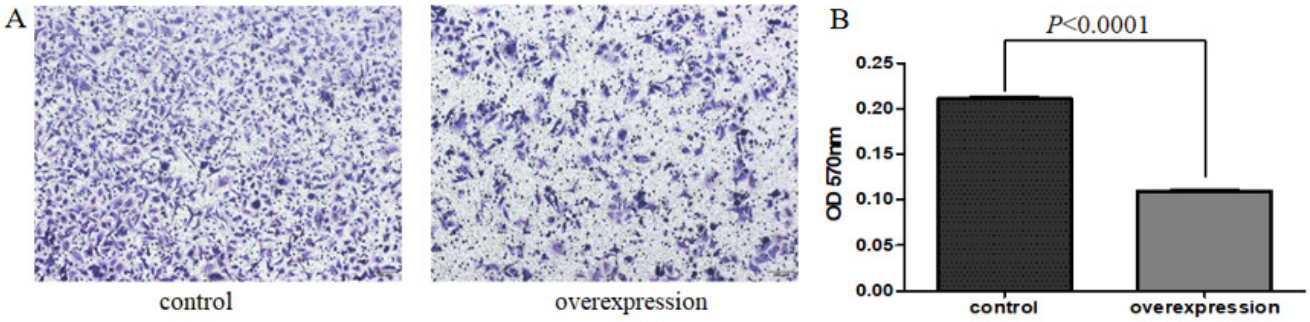

Fig. 3: Effects of SEMA3D overexpression on SW480 cell migration

Note: (A) Migration ability of transfected SW480 cells was detected with a transwell chamber (original magnification, 100×) and (B) Quantitatively determined the migration of SW480 cells in lower surface by 33 % acetic acid, overexpression: Cells transfected with SEMA3D lentiviral vector and control: Cells transfected with control lentiviral vector

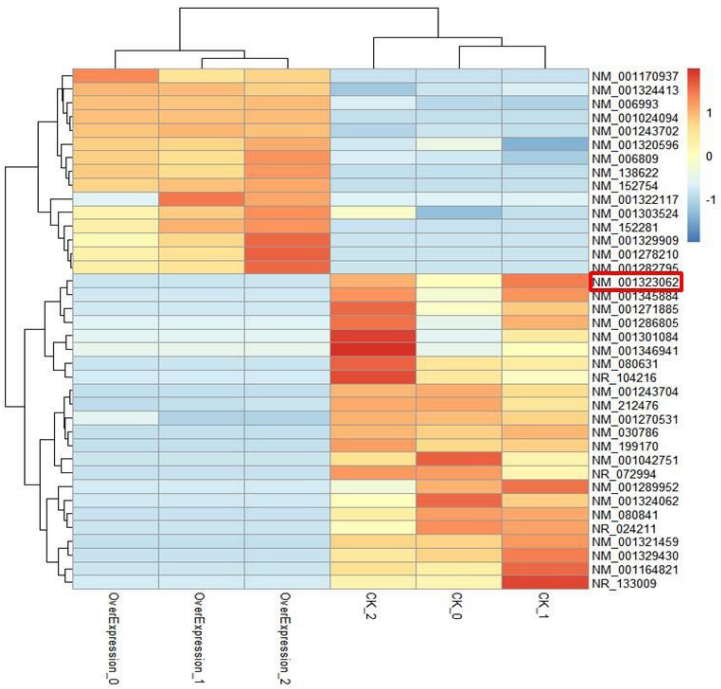

Fig. 4: Heat map of hierarchical clustering of DEG in SW480 cells

Note: AFAP1L1 is lower expressed in SEMA3D overexpressed cells than control cells. Three independent experiments were performed and determined NM_001323062 as AFAP1L1, overexpression: Cells transfected with SEMA3D lentiviral vector and CK: Cells transfected with control lentiviral vector

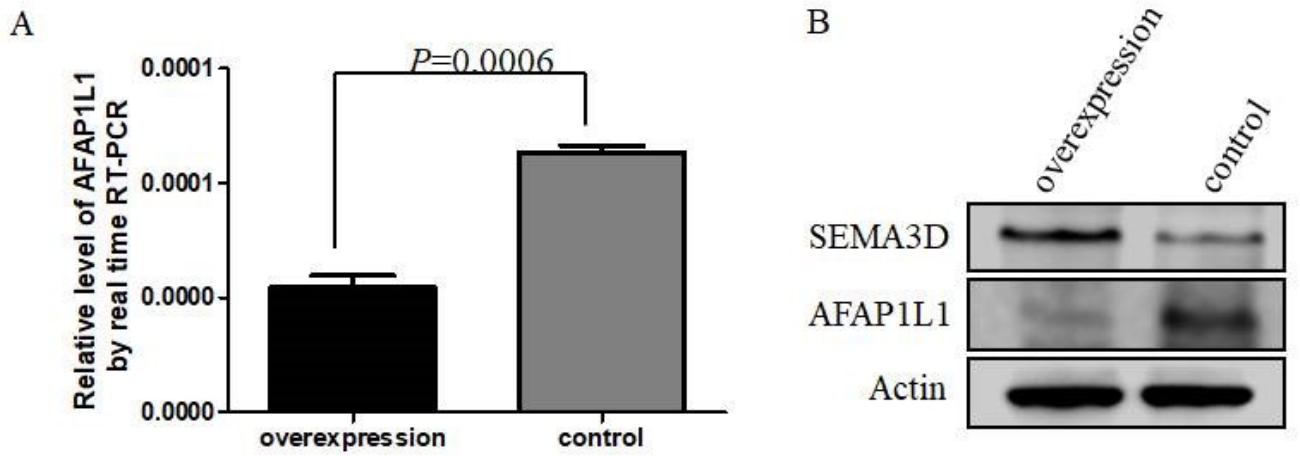

Fig. 5: Mechanism of SEMA3D in CRC cells
Note: (A) AFAP1L1 mRNA levels in SEMA3D- or control lentiviral vector-transfected CRC SW480 cells and (B) SEMA3D and AFAP1L1 protein levels in SEMA3D- or control lentiviral vector-transfected SW480, overexpression: Cells transfected with SEMA3D lentiviral vector and control: Cells transfected with control lentiviral vector

The semaphorins protein family was originally thought to be an inducer of axonal growth cones that plays a vital part in nervous system development[22,23]. SEMA3D is a secreted protein of the semaphorins protein family that plays a crucial role in heart[6], blood vessels[7,24], embryonic development[8], neural regeneration[9] and development[10]. For example, Aghajanian *et al.* found that SEMA3D inhibits human umbilical vein epithelial cell migration by regulating the Phosphoinositide 3-Kinase/ Protein kinase B (PI3K/AKT) axis[24]. However, abnormal expression of SEMA3D can cause various diseases. In a comprehensive analysis of SEMA3D variation in Asian ancestry, Gunadi *et al.* found abnormal expression of SEMA3D in Indonesian Hirschsprung patients[25]. Furthermore, other research studies showed that SEMA3D is essential in carcinogenesis. The occurrence and development of glioma[16,26], breast[17], thyroid[18,27], pancreatic[19,28] and Non-Small Cell Lung Cancer (NSCLC)[29], as well as meningioma[30], cervical[31], bladder[32] and endometrial cancers[33] are all correlated with abnormal expression of SEMA3D. Sabag *et al.* showed that SEMA3D inhibited glioblastoma angiogenesis in the cerebral cortex of mice, thereby inhibiting tumor genesis[16]. In human meningioma, the mutation of Nuclear receptor Coactivator 3 (NCOA3) gene can up-regulate the expression of SEMA3D, which can be used as a biomarker for the occurrence and development of meningioma[30]. Sun *et al.* confirmed the inverse connection between SEMA3D expression and the prognosis of bladder cancer, which shows the potential role of SEMA3D in promoting bladder carcinogenesis[32]. However, another study shown that SEMA3D may play an inhibition role in clear-cell renal-cell carcinoma[34]. SEMA3D can also inhibit the onset and development of human breast carcinoma[17]. Gomez-Rueda *et al.* reported that SEMA3D was down-regulated in thyroid cancer, which may be a new diagnostic marker for thyroid cancer with cytological uncertainty cases[18]. Hai *et al.* reached similar conclusions by finding that SEMA3D prevented papillary thyroid carcinoma from proliferating and migrating through the Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) axis[27]. These studies suggested that SEMA3D may have a tumor suppressive effect.

Foley *et al.* indicated that SEMA3D was upregulated by Annexin A2 (AnxA2) in PDA and the patients with high expression of SEMA3D were more prone to metastasis than those with low expression[19]. Another research manifested that knockdown of SEMA3D could reduce invasion and metastasis in mice. Increased expression of SEMA3D could promote perineural invasion in human PDA and SEMA3D might be a positive factor in PDA[28]. Wang *et al.* demonstrated the important role of SEMA3D in the malignant transformation of Peptic Ulcer Disease (PUD) and Gastrointestinal Cancer (GC)[35]. According to the study of Li *et al.* SEMA3D may inhibit liver carcinogenesis and its progression by blocking PI3K/AKT axis *via* Filamin A (FLNA) [36]. Through whole-genome profiles assay of Malay CRC patients with intact Mismatch Repair (MMR) proteins, Juhari *et al.* illustrated that the mutation of SEMA3D can result in the development of CRC[37]. In addition, SEMA3D has been shown to be

associated with multidrug resistance in leukemia and NSCLC[38,39].

All these studies suggested that SEMA3D may play promotion or inhibition roles in different types of tumors and may also be essential in drug resistance in tumors. Our previous work has shown markedly reduced SEMA3D expression in CRC tissues compared to paired paracancerous tissues and an inverse association of SEMA3D with lymph node metastasis, suggesting that SEMA3D may be an inhibitor of CRC metastasis and a relatively independent prognostic indicator[20]. Therefore, SEMA3D is a promising prognostic factor for CRC patients.

In the current work, overexpressing SEMA3D in SW480 cells evidently reduced cancer cell migration ability compared with control cells, suggesting that SEMA3D may inhibit CRC cell migration. To probe into the mechanism underlying SEMA3D inhibition of CRC migration, RNA-Seq and DEG assays were used in overexpressed SEMA3D cells and control cells. Thirty-eight DEGs were identified. Subsequently, the validated results by qPCR showed visibly down-regulated AFAP1L1 mRNA in SEMA3D overexpressed SW480 cells compared to control cells, which is consistent with the sequencing results. AFAP1L1 protein was also remarkably down-regulated in SEMA3D overexpressed CRC cells, as indicated by the WB analysis.

AFAP1L1 is an adaptor protein belonging to Actin Filament Associated Protein (AFAP) family. AFAP1L1 can interact with cortactin by binding to the Src Homology 3 (SH3) domain and promoting formation of invadosome[40]. Furu *et al.* have shown that AFAP1L1 is a marker for predicting metastasis in spindle cell sarcomas. Knocking out AFAP1L1 gene in sarcomas cells can inhibit cell invasion, while in overexpressed AFAP1L1 sarcoma cell lines, the growth rate and invasion ability were significantly enhanced[41]. Further studies by the same team suggested that the transcription factor Specificity proteins 3 (Sp3) directly regulates the expression of AFAP1L1, thereby promoting the metastasis of sarcoma cells[42].

Another recent research came to similar conclusion that AFAP1L1 can control migration and invasion in sarcoma cells *via* a novel pathway that AFAP1L1 is phosphorylated by Vav guanine nucleotide exchange factor 2 (Vav2) and Non-catalytic region of tyrosine kinase adaptor protein 2 (Nck2), and then activate Lyn/Src family tyrosine kinase signaling pathway[43]. In the research of NSCLC, Wang *et al.* found that AFAP1L1 can accelerate the cell cycle process, promote cell proliferation and inhibit apoptosis[44]. Zimmermann *et al.* identified that AFAP1L1 can fuse with Platelet-Derived Growth Factor Receptor beta (PDGFRβ) gene in myeloid neoplasm through next-generation sequencing which can provide opportunities for the development of therapeutic drugs[45].

In the study of CRC, Takahashi *et al.* discovered that AFAP1L1 expression was distinctly increased in tumor tissues compared to adjacent normal counterparts. AFAP1L1 can interact with vinculin protein to regulate cell morphology, movement and promote the development of CRC, indicating that AFAP1L1 plays a crucial part in CRC metastases *via* modulating cell morphology and promoting cell movement[46]. Nevertheless, in future studies, we should discuss the multiple biological functions of SEMA3D in CRC cells such as epithelial-mesenchymal transition, proliferation and apoptosis. In addition, the role of SEMA3D in CRC *in vivo* still needs further investigation.

Taken together, combined with our research results and literature review, we speculated that SEMA3D might inhibit the migration of CRC cells and ultimately inhibit CRC progression by inhibiting AFAP1L1 expression.

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Conflict of interests:

The authors declared no conflict of interest.

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