

Sema4D silencing increase the sensitivity of nivolumab to B16-F10 resistant melanoma via inhibiting the PI3K/AKT signaling pathway

Zhuo Zhang ^{Equal first author, 1, 2}, **Duoli Zhang** ^{Equal first author, 2}, **Fang Wang** ^{1, 2}, **Jiao Liu** ³, **Yuhong Sun** ², **Songyot Anuchapreeda** ¹, **Singkome Tima** ¹, **Zhangang Xiao** ^{Corresp., 2}, **Suwit Duangmano** ^{Corresp. 1}

¹ Chiang Mai University, Chiang Mai, Thailand

² Southwest Medical University, Luzhou, China

³ Affiliated Hospital of Southwest Medical University, Luzhou, China

Corresponding Authors: Zhangang Xiao, Suwit Duangmano

Email address: zhangangxiao@swmu.edu.cn, suwit.du@cmu.ac.th

Melanoma is a common skin tumor in high mortality, especially in Europe, North America and Oceania. Immunosuppressants such as PD-1 have been proved to be effective in the treatment of malignant melanoma. However, 60% of patients have no immune response. Sema4D, also called CD100, is distributed to T cells and other types of cancer. Sema4D and its receptor, Plexin-B1, plays crucial roles in the immune regulation, angiogenesis, and tumor progression. However, Sema4D in melanoma with anti-PD-1 resistance are still poorly understood. we use a combination of molecular biology techniques and bioinformatics to study the roles of Sema4D in anti-PD-1 resistance melanoma. The results showed that the expression of Sema4D, Plexin-B1 was significantly overexpressed in B16-F10R cell. Sema4D knockdown synergizes with anti-PD-1 treatment, cell viability, cell invasion and migration are significantly decreased, while the apoptosis was increased, the growth of tumors on the body surface of mice was also inhibited. Mechanistically, bioinformatics analysis revealed that Sema4D is involved in the PI3K/AKT signaling pathway. Downregulation of p-PI3K/PI3K and p-AKT/AKT expression were observed in Sema4D knockdown, therefore, nivolumab resistance is related to Sema4D and Sema4D deficiency can improve sensitivity to nivolumab via inhibition PI3K/AKT signaling pathway.

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¹ Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand.

² Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou 646000, China.

³ Department of Pharmacy, Affiliated Hospital of Southwest Medical University, Luzhou 646000, China.

[^]These authors contributed equally to this work.

[#]Corresponding authors:

Zhangang Xiao, Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou, 646000, Sichuan, PR China; E-mail: zhangangxiao@swmu.edu.cn.

Suwit Duangmano, Cancer Research Unit of Associated Medical Sciences (AMS CRU), Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, 50200, Thailand; E-mail: suwit.du@cmu.ac.th.

Abstract

Melanoma is a common skin tumor in high mortality, especially in Europe, North America and Oceania. Immunosuppressants such as PD-1 have been proved to be effective in the treatment of malignant melanoma. However, 60% of patients have no immune response. Sema4D, also called CD100, is distributed to T cells and other types of cancer. Sema4D and its receptor, Plexin-B1, plays crucial roles in the immune regulation, angiogenesis, and tumor progression. However, Sema4D in melanoma with anti-PD-1 resistance are still poorly understood. we use a combination of molecular biology techniques and bioinformatics to study the roles of Sema4D in anti-PD-1 resistance melanoma. The results showed that the expression of Sema4D, Plexin-B1 was significantly overexpressed in B16-F10R cell. Sema4D knockdown synergizes with anti-PD-1 treatment, cell viability, cell invasion and migration are significantly decreased, while the apoptosis was increased, the growth of tumors on the body surface of mice was also inhibited. Mechanistically, bioinformatics analysis revealed that Sema4D is involved in the PI3K/AKT signaling pathway. Downregulation of p-PI3K/PI3K and p-AKT/AKT expression were observed in Sema4D knockdown, therefore, nivolumab resistance is related to Sema4D and Sema4D deficiency can improve sensitivity to nivolumab via inhibition PI3K/AKT signaling pathway.

Keywords: Sema4D, Melanoma, Nivolumab, PI3K/AKT, resistance.

INTRODUCTION

Melanoma, which is prevalent in Europe and North America, has been on the rise in recent years (Rodriguez-Cerdeira et al., 2017). Melanoma is the most aggressive and fatal skin cancer, which can easily metastasize to the whole body through lymph and blood vessels. Melanoma has high tolerance to treatment and poor prognosis. If the tumor metastasizes to distant and visceral organs, the 5-year survival rate is only 15-20% (Huang and Zappasodi, 2022, Eggermont et al., 2014). The current treatment for melanoma includes surgery, chemoradiotherapy, targeted therapy and immunotherapy (Miller and Mihm, 2006). Because cutaneous melanoma is often associated with UV exposure and elevated tumor mutational load, it is one of the most immunogenic of all cancer types and one of the most effective for immunotherapy (Melixetian et al., 2022).

Immune checkpoints include programmed cell death protein-1(PD-1) and programmed death-ligand 1(PD-L1) play important role in immunotherapy, PD-L1 is distributed in tumor cell and PD-1 is generally distributed in T-cells, when PD-L1 bind to its receptor PD-1, triggers inhibitory signaling to attenuate T cell activity (Sun et al., 2018). PD-1 or PD-L1 inhibitors such as nivolumab and pembrolizumab can prevent the binding of PD-1 to PD-L1, thus restoring T cell activity and killing tumors (Larkin et al., 2015, Ribas et al., 2015, Weber et al., 2015). Clinical evidence indicated that there are only 30-40% of patients receiving PD-1 or PD-L1 inhibitors could achieve a complete response (Hugo et al., 2016). Furthermore, there are a growing subset of responders acquired resistance within two years after treatment induction in melanoma (Robert et al., 2015b, Robert et al., 2015a). Therefore, it is urgent to develop new biomarkers with predictive value in the treatment of PD-1 inhibitor.

Semaphorins has become a key factor in regulating tumor growth and metastasis. Especially in the tumor microenvironment, the disturbance between different cell types can control the development and progression of cancer(Valentini et al., 2021, Lontos et al., 2018). Semaphorin 4D (Sema4D) is a member of the Semaphorins, which has immunoregulatory activity, platelet inactivation, angiogenesis stimulation and bone formation regulation (Lontos et al., 2018). Sema4D increased in most tumors, such as prostate, colon, breast, melanoma, head, and neck carcinomas (Liu et al., 2015). As a ligand of Plexin-B1, Sema4D plays important roles in tumor cell proliferation, survival, and migration(Ch'ng and Kumanogoh, 2010).

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway is involved in the occurrence and development of melanoma, lncRNA OR3A4, GRB2 and Hey1 can promote melanoma migration and invasion via this signaling pathway(Shayan et al., 2017, Wu et al., 2020). When PI3K combines with GRB2, it is phosphorylated, recruited to the cell membrane and activated, then AKT phosphorylated and acts on its target genes to regulate a variety of cellular processes (Yu et al., 2006).

However, in melanoma, roles of Sema4D in the anti-PD-1 tolerance is unclear. In this study, we use a combination of molecular biology techniques and bioinformatics to study the roles of Sema4D in anti-PD-1 resistance melanoma. We provide the evidence for the first time that Sema4D is involved in anti-PD-1 resistance of melanoma. Moreover, Sema4D deficiency can overcome resistance to anti-PD-1 therapy, the mechanism is related to PI3K/AKT signaling

pathway.

MATERIALS AND METHODS

Cell culture and B16-F10 resistance cell line establishment

293T cells, B16-F10 were purchased by Nanjing Cobioer Biotechnology Co., LTD (Nanjing, China), cultured at 37°C in 5% CO₂ in RPMI-1640 (Life Technologies, Invitrogen, Canada) with 10% FBS (Gibco, USA). The combination of high dose shock and gradually increasing dose was applied to establish the cell lines insensitive to nivolumab (anti-PD-1). The B16-F10 cell line in the logarithmic growth phase were cultured with nivolumab (AbMole Bioscience Inc., shanghai, China) 10 ng/mL and TIL at a 1:5 ratio. After 24 hours, the solution was changed, and dead cells were discarded. The remaining cells were then cultured with RPMI-1640 without nivolumab, when the remaining cells have stabilized, then cultured with nivolumab 25 ng/ml and repeat these steps until the cells could grow continuously and stably in the medium with nivolumab 50 ng/mL, and then cultured drug-free for 1 month, B16-F10 resistance cell line (B16-F10R) were obtained. B16-F10R also needs with nivolumab 5 ng/mL to maintain the resistance (Maeda et al., 1991).

Tumor Infiltrating Lymphocyte (TIL)

The tumors were removed from the Balb/c mice, about 2-3 cubic millimeters in size, and cultured with 2 mL complete medium in 24 well plates. The complete medium included 6000 IU/mL IL-2 which purchased by Novartis, RPMI-1640 and 2.05 mM L-glutamine were the major component and get from Thermo Fisher Scientific, there is also a need to add 10 % heat-inactivated human AB serum (Omega Scientific, USA), 55 µM 2-mercaptoethanol and 10 mM of HEPES Buffer (Mediatech, USA), gentamicin(50 µg/mL) , penicillin(100 I.U./mL), streptomycin(100 µg/mL) were purchased from Invitrogen and prevent infection (Arneth, 2019).

Western blotting

We investigated the expression of PD-L1, Sema4D, Plexin-B1, PI3K, p-PI3K, AKT, p-AKT, these protein were purchased from Abcam Shanghai Trading Co., LTD and cell signaling technology, Inc. (Shanghai, China). Total protein was extracted and BCA protein detection kit (Beyotime, China) was used to evaluate the protein concentration. after electrophoresis, transfer membrane, antibody incubation process, an ECL chromogenic substrate was applied for detecting the signals (Beyotime, China).

RT-qPCR

Total RNA was obtained by processing the samples with TRIzol reagent and Dnase-free DNase Set (Beyotime, China). The synthesis of complementary DNA was performed using a cDNA synthesis kit (Thermo Fisher Scientific, USA). Finally, RT-PCR analysis was performed using an ABI PRISM 7900-HT sequence detection system (Applied Biosystems, USA). For qRT-PCR, the following primers were used.

PD-L1:5'-AGAACCTGGCTGCACCTAAC-3'(F),
5'-GAGAAGGTCAAACCGCCTCA-3'(R);

Plexin-B1:5'-AAGCCCAGCCTACTAACAACC-3'(F),
5'-CAGCCCCACTGTACTGACTG-3'(R);
Sema4D:5'-TCAAAGCAGACGGAATGCCTA-3'(F),
5'-CCCCAACCATGACTGATGTGTA-3'(R);
GAPDH:5'-TTGTCATGGGAGTGAACGAGA-3'(F),
5'-CAGGCAGTTGGTGGTACAGG-3'(R).

shRNA and expression plasmid transfection

Short hairpin RNA (shRNA) targeting Sema4D and the lentivirus containing this shRNA were designed and synthesized by Genepharma Co. (Shanghai, China), sh-sema4D (5'-GGATGGGACTG TCTATGATGT-3') and the control NC-sema4D (5'-TTCTCCGAACGTGTCACGT-3'). The shuttle plasmid and three other packaging plasmids (pGag/Pol, pRev, pVSV-G) were co-transfected into 293T cells to generate a lentivirus expressing Sema4D shRNA or a control lentivirus expressing shRNA. Cells were inoculated into 6-well plates at 1×10^5 and infected with lentivirus while 5 μ g/mL of Polybrene was added. The culture media was changed after 24 hours of infection, and the infected cells were screened with 1 μ g/mL of puromycin for 7 days after the culture media change to obtain stable lentiviral shRNA expressing cell lines for subsequent experimental analysis (Moore et al., 2010).

Sema4D overexpression

B16-F10 cells were treated with RPMI 1640 containing 10% fetal bovine serum culture medium was placed in 5% CO₂ incubator at 37°C, and logarithmic growth was taken long-term cells were used in the experiment. Construct Sema4D lentivirus overexpression vector LV-Sema4D and negative control LV-NC, LV-Sema4D and LV-NC commissioned by Gemma Pharmaceutical Technology Co., LTD. (Shanghai) to synthesize. B16-F10 cell was inoculated in 24-well plates and cultured for 24 h multiplicity of infection, complete culture medium was replaced for further culture 48 h, the fluorescence intensity was observed under an inverted fluorescence microscope.

MTT

Cell viability was measured by MTT assay. MTT was dissolved in DMSO and 0.5 mg/mL MTT diluted in 1×PBS for 4 hours, Turn on Benchmark Plus microplate reader (Bio-Rad, American) and detect absorbance at 570 nm (Algazi et al., 2020).

Cell apoptosis analysis

Cell apoptosis were detected by AnnexinV-AlexaFluor647/PI Kit Apoptosis Assay Kit(Biotech, China). 5×10^5 B16-F10R cells were seeded in 10-cm culture dishes (Corning, USA) and incubated overnight. 5×10^5 treated cells were collected and resuspended with 500 μ L binding buffer. 5 μ L Annexin V/AlexaFluor647 were incubate for 5 minutes at room temperature, then add 10 μ L propidium iodide (PI), fluorescent dyes should be protected from light.

Wound healing and invasion analysis

B16-F10R cells invasion and migration was analyzed by Matrigel-coated invasion chambers and scratch assay. The initial number of cells cultured was 5×10^5 , a sterile 10 μ L pipette tip was used to mark a line in the monolayer of each well, the wounds were observed at 0, 24 and 48 hours under a microscope (Nikon, Japan). ImageJ software was used to measure the wound areas. Cell free migration area=(cell free area 0h - cell free area 24hs)/cell free area 0h (Ji et al., 2018). Matrigel-coated invasion chambers (Corning, USA) was used to incubation with 48 hours. The top of Matrigel and invading cells were fixed and stained with 0.1% crystal violet. The microscope (Nikon, Japan) was used to photograph invading cells, and 3 fields were randomly selected and counted each time.

Animal and tumorigenesis

Balb/c mice with 12-week-old were purchased from Chendu dossy experimental animals CO., LTD (SCXK (Chuan) 2020-030, Chengdu, China). The mice were fed unrestricted diet and water intake, and were kept under specific pathogen-free conditions in a laboratory where a 12-hour light/dark cycle was strictly enforced(SYXK(Chuan) 2018-065, Luzhou, China). For each group, at least 5 mice were used (Sanmamed et al., 2015). Tumor volumes were measured on 1, 3, 6, 9, 12, 15 and 18 days with caliper, at days 18, mice were sacrificed, tumor weight was measured, tumor volumes were calculated with the formula: tumor volume = $0.52 \times \text{length} \times \text{width}^2$.

Data collection

Melanoma gene expression profiling data were extracted from the Cancer Genome Consortium (TCGA) database using the "TCGAbiolinks" software package. Expression profiles were converted to transcripts per million mapped reads per kilobase exon model (TPM) and normalized by log2.

Weighted co-expression network analysis

To explore the signaling pathways involved in Sema4D, gene co-expression network analysis (WGCNA) was first conducted to determine genes with significant change between high- and low- expression of Sema4D through "WGCNA" package on R project. Topological overlapping measurement was utilized to identify modules most associated with Sema4D expressions. Correlation between modules and traits is calculated by Spearman correlation analysis.

Protein-Protein interaction

To explore the potential functions played by Sema4D in various biological processes, we constructed Sema4D-related protein-protein interaction network by STRING (<https://cn.string-db.org/>).

Gene set variation analysis

Gene set variation analysis was conducted to reveal the molecular mechanisms under the different Sema4D expression populations in melanoma. The h.all.v7.5.1.symbols.gmt gene set

was used to enrichment analysis and was downloaded from MSigDB (<https://www.gsea-msigdb.org/gsea/index.jsp>).

Functional enrichment analysis of Sema4D

To explore the mechanism of action of Sema4D involved in PD-1 inhibitor resistance, enrichment analysis on hub genes was performed. "ClusterProfiler" package in R project was utilized to identify the signaling pathways involved in Sema4D within TCGA cohort (which contains 471 melanoma samples).

Statistical analysis

GraphPad Prism V.7.00 software was utilized for statistical analysis. Different statistical methods were selected according to different experimental purposes, compare the differences between two groups, we chose Student's t test, one-way ANOVA was used to compare the differences between the overall differences. $P < 0.05$ is considered to be statistically significant.

RESULTS

Sema4D and Plexin-B1 overexpression in nivolumab resistance B16-F10 cells

To investigate the mRNA and protein expression of Sema4D and Plexin-B1 in B16-F10R and B16-F10 groups, Sema4D and Plexin-B1 were detected. Compared with B16-F10 group, mRNA and protein expression of Sema4D and Plexin-B1 were significantly overexpressed in B16-F10R group (*Figure 1A-E*).

PD-L1 overexpression in nivolumab resistance B16-F10 cells

mRNA and protein expression of PD-L1 in B16-F10R and B16-F10 cells were detected. Compared with B16-F10 group, mRNA and protein expression of PD-L1 were significantly overexpressed in B16-F10R group (*Figure 1A, F-G*).

Sema4D deficiency inhibit PD-L1 expression upon anti-PD-1 therapy

We analyzed the correlation of PD-L1 and Sema4D with bioinformatics, it showed that PD-L1 and Sema4D are positively correlated (*Figure 2A*). Compared with Sema4D-NC group, protein expression of Sema4D in Sema4D-shRNA group was significantly downregulated (*Figure 2B, C*), it indicated that Sema4D knockdown is success. Compared with Sema4D-NC group, mRNA and protein expression of PD-L1 in Sema4D-shRNA group was significantly downregulated after nivolumab treatment (*Figure 2B, D, E*), which was consistent with the results of bioinformatics. Therefore, inhibition of SEMA4D significantly repressed the mRNA and protein expression of PD-L1.

Sema4D deficiency potentiates anti-PD-1 treatment efficacy

To explore the relationship between Sema4D deficiency and nivolumab treatment efficacy in B16-F10 and B16-F10R cells, we investigated the cell viability and cell apoptosis. First, we observed

the effects of nivolumab on the cell viability of B16-F10 and B16-F10 resistant cell lines, the results showed that nivolumab had inhibitory effect on B16-F10 cell lines, but no effect on B16-F10R cell lines, which was statistically significant(**Figure 3A**). Next, we explored the effects of nivolumab on the cell viability of Sema4D overexpressed in B16-F10 cell lines. The results showed that nivolumab showed little inhibitory effect on B16-F10 cell lines with overexpressed Sema4D. This suggesting that Sema4D was related to nivolumab sensitivity to B16-F10 cell lines(**Figure 3B**). We further explored the effect of nivolumab on the cell viability of Sema4D knockdown B16-F10R cell lines, the results showed Sema4D deficiency potentiates anti-PD-1 treatment efficacy in B16-F10R cells. Compared with Sema4D-NC group, the cell viability of Sema4D-shRNA group in combination with nivolumab is decreased significantly (**Figure 3C**) and rate of apoptosis cell in Sema4D-shRNA group after treatment with anti-PD-1 is notably increased (**Figure 3D**). Finally, we tested the effect of nivolumab on Sema4D-knockdown melanoma in mice. In vivo experiment, we transplanted B16-F10R cells in the mice and observed the size and weight of tumors treated with nivolumab, tumor volume and weight were significantly delayed in Sema4D-shRNA group mice as compared to Sema4D-NC group mice (**Figure 3E, F, G**).

Sema4D deficiency potentiates the inhibitory effect of anti-PD-1 on cell invasion and migration

The affection of Sema4D deficiency with anti-PD-1 treatment on B16-F10R cell invasion and migration was analyzed by Matrigel-coated invasion chambers and scratch assay. The results showed that when knockdown of Sema4D, cell migration of Sema4D-shRNA group was decreased ($P < 0.0001$), cell migration of Sema4D-shRNA group was wider than those of Sema4D-NC group cells (**Figure 4A, B**). In addition, the number of invaded cells was significantly less in Sema4D-shRNA group in contrast to Sema4D-NC group (**Figure 4C, D**), these data suggesting that lowering Sema4D could enhance the inhibitory effect of anti-PD-1 on cell migration and invasion.

Downregulation of Sema4D inhibit PI3K/AKT expression

The brown module was determined to be most significantly associated with Sema4D expression ($r = +0.67$, $p\text{-value} = 1e-62$), which contained a total of 778 genes (**Figure 5A-B**). Gene set variation analysis (GSVA) revealed several significantly upregulated malignancy-associated events (IL-6, JAK, STAT3, MTORC1, and KRAS signaling pathways) in the Sema4D high expression group as well as downregulation of p53 signaling (**Figure 5C**). Sema4D was significantly associated with oncogenes RHOA, RRAS and immunomodulatory-related genes PTPRC and PLXNC1(**Figure 5D**). Pathway enrichment analysis revealed that Sema4D is involved in multiple immune-related pathways as well as the PI3K/AKT pathway (**Figure 5E**). Numerous studies have demonstrated that excessive activation of PI3K/AKT signaling pathway is associated with excessive proliferation of tumor cells, inhibition of apoptosis and attenuation of the efficacy of immune checkpoint inhibitors (Zhang et al., 2022). The expression of PI3K/AKT protein was explored by Western blot. p-PI3K/PI3K, p-AKT/AKT of Sema4D-shRNA group were decreased than those of Sema4D-NC group, which suggesting aberrant activation of the PI3K/AKT signaling

pathway in the Sema4D-NC group (*Figure 5F*).

DISCUSSION

Immunotherapy is one important method for melanoma treatment, when alone or in combination with anti-PD-1 and anti-PD-L1 may improve survival rates (Simeone et al., 2015). Nivolumab is a PD-1 inhibitor with high affinity to PD-1 (Hirano et al., 2005). However, the ineffectiveness or resistance of anti-PD-1 to melanoma is a serious problem. In general, anti-PD-1 resistance are mainly derived into acquired resistance and innate resistance (Bridgeman et al., 2014, Marincola et al., 2000, Bronte et al., 2005, O'Donnell et al., 2017). The causes of anti-PD-1 or anti-PD-L1 resistance to melanoma are very complex and not fully understood. This study intends to elucidate that Sema4D may be involved in the sensitivity of melanoma to nivolumab therapy via PI3K/AKT signaling pathway.

Nivolumab is a PD-1 inhibitor, PD-1 exists in T cells while PD-L1 exists in tumor cells, so the effect of nivolumab on tumor cells requires the presence of T cells. In this experiment, Tumor Infiltrating Lymphocyte(TIL) was obtained from mice and added to cell culture to simulate tumor microenvironment. B16-F10 is a melanoma cell line derived from C57BL/6J mice, which can be inoculated subcutaneously in C57BL/6J mice, tumor formation can also be inoculated subcutaneously in Balb/c mice(Lucas L Colombo et al., 2019). Balb/c mice is the most common strains of mice in the laboratory, widely used in immunological animal experiments and melanoma grows well and is easy to observe, therefore, in this experiment, mouse B16-F10 cells were selected for vivo experiment and Balb/c mic was selected for vitro experiment.

Sema4D expressed on many cancer cells, when it combined with Plexin-B1, it correlates with tumor immune infiltration, angiogenesis, and tumor progression in melanoma (Lu et al., 2021). To understand the expression of Sema4D in melanoma resistance to anti-PD-1, B16-F10 resistant cell lines was established, after administered 50 mg nivolumab, the killing ability of nivolumab was significantly weaker than that of B16-F10 cell lines, this means that resistant cell lines are established successfully. Then we explored mRNA and protein expression of Sema4D and Plexin-B1 in B16-F10 cell lines and B16-F10R cell lines, our results revealed that the mRNA and protein expression of Sema4D and Plexin-B1 in drug-resistant cell lines were overexpressed. Therefore, we provide the evidence for the first time that Sema4D and Plexin-B1 is related to anti-PD-1 resistance of melanoma.

To further analyzed the relationship between Sema4D and nivolumab, Sema4D were overexpression in B16-F10 cells and Sema4D expression was inhibited by shRNA in B16-F10R cells. It was observed that overexpression of Sema4D can attenuates the therapeutic effect of nivolumab in B16-F10 cells and downregulation of Sema4D will increase the sensitivity of B16-F10R cell lines to nivolumab. In addition, we also investigated the effects of Sema4D knockdown on B16-F10R cells in response to nivolumab, downregulation of Sema4D also increased the inhibiting the growth of tumor cells and reducing cell invasion and migration. As reported by

Rezaeepoor that silencing of Sema4D serve as a therapeutics target for the suppression of invasion, migration(Rezaeepoor et al., 2021).A study conducted by Rashidi illustrated silencing of Sema4D can elevated apoptosis rate of SW48 cells in response to 5-FU treatment (Rashidi et al., 2020). Moreover, we verified the enhancement of anti-PD-1 sensitivity by Sema4D knockdown in vivo xenograft models, mice treated with nivolumab after Sema4D knockdown showed significantly slower tumor growth. Taken together, these finding demonstrated that Sema4D deficiency can potentiates nivolumab treatment efficacy.

We further analyzed the correlation between Sema4D and PD-L1 expression in anti-PD-1 resistance. It has been demonstrated that the presence of PD-L1 determines the response to anti-PD-1/PD-L1 therapy (Sunshine et al., 2017), regorafenib can promotes antitumor immunity via inhibiting PD-L1 expression in melanoma (Wu et al., 2019). JAK1/2 mutation leads to the lack of reactive PD-L1 expression, which is involved in the resistance of PD-1/PD-L1 inhibitors (Shin et al., 2017). In this study, we evaluated PD-L1 expression in B16-F10, B16-F10R cell and B16-F10R cell knockdown with Sema4D-shRNA, the results showed PD-L1 expression in B16-F10R was increased and Sema4D knockdown group is decrease, these suggest that the effects of Sema4D knockdown on Nivolumab in B16-F10R cell lines may be related to the decrease of PD-L1 expression. However, which way contributes to the PD-L1 downregulation after knockdown Sema4D remains unclear and needs further investigation.

PI3K is phosphorylated by binding to GRB2, and AKT phosphorylates target genes to regulate cell function (Pu et al., 2021). Sema4D and plexin-B1 is associated with osteosarcoma, in which PYK2-PI3K-Akt pathway is activated to promote tumor progression(Li et al., 2021). To explore the relationship between Sema4D and PI3K/AKT signaling pathways in anti-PD-1 resistance, we detected the expression of PI3K/AKT protein, in B16-F10R cell, p-PI3K/PI3K, p-AKT/AKT expression were significantly increased, when inhibited the Sema4D, p-PI3K/PI3K, p-AKT/AKT expression of Sema4D-shRNA group were decreased, these shown that Sema4D can regulate PI3K/AKT expression and activity. Bioinformatics analysis revealed that Sema4D is involved in the PI3K/AKT signaling pathway which is activated and plays important roles such as being involved in the development and occurrence in melanoma and it also activate receptor tyrosine kinases (Davies, 2012). However, whether the relationship between Sema4D and nivolumab sensitivity is related to JAK-STAT signal pathway and other signal pathways needs further study.

In conclusion, we explored the role of Sema4D in the process of PD-1 inhibitor resistance. In anti-PD-1 resistant cell line, upregulation of Sema4D and Plexin-B1 was observed. When Sema4D was downregulated, it prevented the phosphorylation of PI3K and AKT, thus inhibiting the PI3K/AKT pathway activity. Therefore, these results may have therapeutic implication and may be exploited for the development of novel treatment for anti-PD-1 resistance melanoma in the future.

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Not applicable.

Authors' contributions

Conceptualization: Zhuo Zhang; Formal analysis and investigation: Duoli Zhang and Fang Wang; Cell and animal experiments: Jiao Liu; Writing original draft preparation: Zhuo Zhang, Songyot Anuchapreeda and Singkome Tima; Writing review and editing: Zhangang Xiao , Yuhong Sun and Suwit Duangmano.

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Availability of data and materials

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the ethic committee (Southwest medical university, China) and in compliance with the international regulations on care and protection of laboratory animals (SWMU20210429).

Consent for publication

Not applicable.

Competing interests

The authors declare that they do not have any conflicts of interest.

References

- ALGAZI, A. P., TWITTY, C. G., TSAI, K. K., LE, M., PIERCE, R., BROWNING, E., HERMIZ, R., CANTON, D. A., BANNAVONG, D., OGLESBY, A., FRANCISCO, M., FONG, L., PITTET, M. J., ARLAUCKAS, S. P., GARRIS, C., LEVINE, L. P., BIFULCO, C., BALLESTEROS-MERINO, C., BHATIA, S., GARGOSKY, S., ANDTBACKA, R. H. I., FOX, B. A., ROSENBLUM, M. D. & DAUD, A. I. 2020. Phase II Trial of IL-12 Plasmid Transfection and PD-1 Blockade in Immunologically Quiescent Melanoma. *Clin Cancer Res*, 26, 2827-2837.
- ARNETH, B. 2019. Tumor Microenvironment. *Medicina (Kaunas)*, 56.
- BRIDGEMAN, J. S., LADELL, K., SHEARD, V. E., MINERS, K., HAWKINS, R. E., PRICE, D. A. & GILHAM, D. E. 2014. CD3zeta-based chimeric antigen receptors mediate T cell activation via cis- and trans-signalling mechanisms: implications for optimization of receptor structure for adoptive cell therapy. *Clin Exp Immunol*, 175, 258-67.

- BRONTE, V., KASIC, T., GRI, G., GALLANA, K., BORSELLINO, G., MARIGO, I., BATTISTINI, L., IAFRATE, M., PRAYER-
GALETTI, T., PAGANO, F. & VIOLA, A. 2005. Boosting antitumor responses of T lymphocytes infiltrating
human prostate cancers. *J Exp Med*, 201, 1257-68.
- CH'NG, E. S. & KUMANOGOH, A. 2010. Roles of Sema4D and Plexin-B1 in tumor progression. *Mol Cancer*, 9, 251.
- DAVIES, M. A. 2012. The role of the PI3K-AKT pathway in melanoma. *Cancer J*, 18, 142-7.
- EGGERMONT, A. M., SPATZ, A. & ROBERT, C. 2014. Cutaneous melanoma. *Lancet*, 383, 816-27.
- HIRANO, F., KANEKO, K., TAMURA, H., DONG, H., WANG, S., ICHIKAWA, M., RIETZ, C., FLIES, D. B., LAU, J. S., ZHU, G.,
TAMADA, K. & CHEN, L. 2005. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer
therapeutic immunity. *Cancer Res*, 65, 1089-96.
- HUANG, A. C. & ZAPPASODI, R. 2022. A decade of checkpoint blockade immunotherapy in melanoma: understanding
the molecular basis for immune sensitivity and resistance. *Nat Immunol*, 23, 660-670.
- HUGO, W., ZARETSKY, J. M., SUN, L., SONG, C., MORENO, B. H., HU-LIESKOVAN, S., BERENT-MAOZ, B., PANG, J.,
CHMIELEWSKI, B., CHERRY, G., SEJA, E., LOMELI, S., KONG, X., KELLEY, M. C., SOSMAN, J. A., JOHNSON, D.
B., RIBAS, A. & LO, R. S. 2016. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in
Metastatic Melanoma. *Cell*, 165, 35-44.
- JI, Y., XIAO, Y., XU, L., HE, J., QIAN, C., LI, W., WU, L., CHEN, R., WANG, J., HU, R., ZHANG, X., GU, Z. & CHEN, Z. 2018.
Drug-Bearing Supramolecular MMP Inhibitor Nanofibers for Inhibition of Metastasis and Growth of Liver
Cancer. *Adv Sci (Weinh)*, 5, 1700867.
- LARKIN, J., CHIARION-SILENI, V., GONZALEZ, R., GROB, J. J., COWEY, C. L., LAO, C. D., SCHADENDORF, D., DUMMER,
R., SMYLLIE, M., RUTKOWSKI, P., FERRUCCI, P. F., HILL, A., WAGSTAFF, J., CARLINO, M. S., HAANEN, J. B.,
MAIO, M., MARQUEZ-RODAS, I., MCARTHUR, G. A., ASCIERTO, P. A., LONG, G. V., CALLAHAN, M. K.,
POSTOW, M. A., GROSSMANN, K., SZNOL, M., DRENO, B., BASTHOLT, L., YANG, A., ROLLIN, L. M., HORAK,
C., HODI, F. S. & WOLCHOK, J. D. 2015. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated
Melanoma. *N Engl J Med*, 373, 23-34.
- LI, C., WAN, L., WANG, P., GUAN, X., LI, C. & WANG, X. 2021. Sema4D and Plexin-B1 promotes the progression of
osteosarcoma cells by activating Pyk2-PI3K-AKT pathway. *J Musculoskelet Neuronal Interact*, 21, 577-583.
- LIU, X. L., SONG, J., LIU, K. J., WANG, W. P., XU, C., ZHANG, Y. Z. & LIU, Y. 2015. Role of inhibition of osteogenesis
function by Sema4D and Plexin-B1 signaling pathway in skeletal fluorosis in vitro. *J Huazhong Univ Sci
Technolog Med Sci*, 35, 712-715.
- LONTOS, K., ADAMIK, J., TSAGIANNI, A., GALSON, D. L., CHIRGWIN, J. M. & SUVANNASANKHA, A. 2018. The Role of
Semaphorin 4D in Bone Remodeling and Cancer Metastasis. *Front Endocrinol (Lausanne)*, 9, 322.
- LU, Q., CAI, P., YU, Y., LIU, Z., CHEN, G. & ZENG, Z. 2021. Sema4D correlates with tumour immune infiltration and is
a prognostic biomarker in bladder cancer, renal clear cell carcinoma, melanoma and thymoma.
Autoimmunity, 54, 294-302.
- Lucas L Colombo, Silvia I Vanzulli, Alfonso Blázquez-Castro, Clara Sanchez Terrero, Juan C
Stockert.2019.Photothermal effect by 808-nm laser irradiation of melanin: a proof-of-concept study of
photothermal therapy using B16-F10 melanotic melanoma growing in BALB/c mice[J].BiomeAEDA, K.,
LAFRENIERE, R. & JERRY, L. M. 1991. Production and characterization of tumor infiltrating lymphocyte
clones derived from B16-F10 murine melanoma. *J Invest Dermatol*, 97, 183-9.
- MARINCOLA, F. M., JAFFEE, E. M., HICKLIN, D. J. & FERRONE, S. 2000. Escape of human solid tumors from T-cell
recognition: molecular mechanisms and functional significance. *Adv Immunol*, 74, 181-273.

- MELIXETIAN, M., PELICCI, P. G. & LANFRANCONE, L. 2022. Regulation of LncRNAs in Melanoma and Their Functional Roles in the Metastatic Process. *Cells*, 11.
- MILLER, A. J. & MIHM, M. C., JR. 2006. Melanoma. *N Engl J Med*, 355, 51-65.
- MOORE, C. B., GUTHRIE, E. H., HUANG, M. T. & TAXMAN, D. J. 2010. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol*, 629, 141-58.
- O'DONNELL, J. S., LONG, G. V., SCOLYER, R. A., TENG, M. W. & SMYTH, M. J. 2017. Resistance to PD1/PDL1 checkpoint inhibition. *Cancer Treat Rev*, 52, 71-81.
- PU, Y., LEI, M., CHEN, Y., HUANG, Y., ZHANG, L., CHEN, J., ZHANG, Y., SHAO, X., LIU, L. & CHEN, J. 2021. Hey1 promotes migration and invasion of melanoma cells via GRB2/PI3K/AKT signaling cascade. *J Cancer*, 12, 6979-6988.
- RASHIDI, G., REZAEIPOOR, M., MOHAMMADI, C., SOLGI, G. & NAJAFI, R. 2020. Inhibition of semaphorin 4D enhances chemosensitivity by increasing 5-fluorouracil-induced apoptosis in colorectal cancer cells. *Mol Biol Rep*, 47, 7017-7027.
- REZAEIPOOR, M., RASHIDI, G., POURJAFAR, M., MOHAMMADI, C., SOLGI, G. & NAJAFI, R. 2021. SEMA4D Knockdown Attenuates beta-Catenin-Dependent Tumor Progression in Colorectal Cancer. *Biomed Res Int*, 2021, 8507373.
- RIBAS, A., PUZANOV, I., DUMMER, R., SCHADENDORF, D., HAMID, O., ROBERT, C., HODI, F. S., SCHACHTER, J., PAVLICK, A. C., LEWIS, K. D., CRANMER, L. D., BLANK, C. U., O'DAY, S. J., ASCIERTO, P. A., SALAMA, A. K., MARGOLIN, K. A., LOQUAI, C., EIGENTLER, T. K., GANGADHAR, T. C., CARLINO, M. S., AGARWALA, S. S., MOSCHOS, S. J., SOSMAN, J. A., GOLDINGER, S. M., SHAPIRA-FROMMER, R., GONZALEZ, R., KIRKWOOD, J. M., WOLCHOK, J. D., EGGERMONT, A., LI, X. N., ZHOU, W., ZERNHELT, A. M., LIS, J., EBBINGHAUS, S., KANG, S. P. & DAUD, A. 2015. Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised, controlled, phase 2 trial. *Lancet Oncol*, 16, 908-18.
- ROBERT, C., LONG, G. V., BRADY, B., DUTRIAUX, C., MAIO, M., MORTIER, L., HASSEL, J. C., RUTKOWSKI, P., MCNEIL, C., KALINKA-WARZOGA, E., SAVAGE, K. J., HERNBERG, M. M., LEBBE, C., CHARLES, J., MIHALCIOIU, C., CHIARION-SILENI, V., MAUCH, C., COGNETTI, F., ARANCE, A., SCHMIDT, H., SCHADENDORF, D., GOGAS, H., LUNDGREN-ERIKSSON, L., HORAK, C., SHARKEY, B., WAXMAN, I. M., ATKINSON, V. & ASCIERTO, P. A. 2015a. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med*, 372, 320-30.
- ROBERT, C., SCHACHTER, J., LONG, G. V., ARANCE, A., GROB, J. J., MORTIER, L., DAUD, A., CARLINO, M. S., MCNEIL, C., LOTEM, M., LARKIN, J., LORIGAN, P., NEYNS, B., BLANK, C. U., HAMID, O., MATEUS, C., SHAPIRA-FROMMER, R., KOSH, M., ZHOU, H., IBRAHIM, N., EBBINGHAUS, S., RIBAS, A. & INVESTIGATORS, K.-. 2015b. Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med*, 372, 2521-32.
- RODRIGUEZ-CERDEIRA, C., CARNERO GREGORIO, M., LOPEZ-BARCENAS, A., SANCHEZ-BLANCO, E., SANCHEZ-BLANCO, B., FABBROCINI, G., BARDHI, B., SINANI, A. & GUZMAN, R. A. 2017. Advances in Immunotherapy for Melanoma: A Comprehensive Review. *Mediators Inflamm*, 2017, 3264217.
- SANMAMED, M. F., RODRIGUEZ, I., SCHALPER, K. A., ONATE, C., AZPILIKUETA, A., RODRIGUEZ-RUIZ, M. E., MORALES-KASTRESANA, A., LABIANO, S., PEREZ-GRACIA, J. L., MARTIN-ALGARRA, S., ALFARO, C., MAZZOLINI, G., SARNO, F., HIDALGO, M., KORMAN, A. J., JURE-KUNKEL, M. & MELERO, I. 2015. Nivolumab and Urelumab Enhance Antitumor Activity of Human T Lymphocytes Engrafted in Rag2^{-/-}IL2Rgamma^{null} Immunodeficient Mice. *Cancer Res*, 75, 3466-78.
- SHAYAN, G., SRIVASTAVA, R., LI, J., SCHMITT, N., KANE, L. P. & FERRIS, R. L. 2017. Adaptive resistance to anti-PD1 therapy by Tim-3 upregulation is mediated by the PI3K-Akt pathway in head and neck cancer.

Oncoimmunology, 6, e1261779.

SHIN, D. S., ZARETSKY, J. M., ESCUIN-ORDINAS, H., GARCIA-DIAZ, A., HU-LIESKOVAN, S., KALBASI, A., GRASSO, C. S., HUGO, W., SANDOVAL, S., TORREJON, D. Y., PALASKAS, N., RODRIGUEZ, G. A., PARISI, G., AZHDAM, A., CHMIELOWSKI, B., CHERRY, G., SEJA, E., BERENT-MAOZ, B., SHINTAKU, I. P., LE, D. T., PARDOLL, D. M., DIAZ, L. A., JR., TUMEH, P. C., GRAEBER, T. G., LO, R. S., COMIN-ANDUIX, B. & RIBAS, A. 2017. Primary Resistance to PD-1 Blockade Mediated by JAK1/2 Mutations. *Cancer Discov*, 7, 188-201.

SIMEONE, E., GRIMALDI, A. M. & ASCIERTO, P. A. 2015. Anti-PD1 and anti-PD-L1 in the treatment of metastatic melanoma. *Melanoma Manag*, 2, 41-50.

SUN, C., MEZZADRA, R. & SCHUMACHER, T. N. 2018. Regulation and Function of the PD-L1 Checkpoint. *Immunity*, 48, 434-452.

SUNSHINE, J. C., NGUYEN, P. L., KAUNITZ, G. J., COTTRELL, T. R., BERRY, S., ESANDRIO, J., XU, H., OGURTSOVA, A., BLEICH, K. B., CORNISH, T. C., LIPSON, E. J., ANDERS, R. A. & TAUBE, J. M. 2017. PD-L1 Expression in Melanoma: A Quantitative Immunohistochemical Antibody Comparison. *Clin Cancer Res*, 23, 4938-4944.

VALENTINI, E., DI MARTILE, M., DEL BUFALO, D. & D'AGUANNO, S. 2021. SEMAPHORINS and their receptors: focus on the crosstalk between melanoma and hypoxia. *J Exp Clin Cancer Res*, 40, 131.

VAN DEN BERG, J. H., HEEMSKERK, B., VAN ROOIJ, N., GOMEZ-EERLAND, R., MICHELS, S., VAN ZON, M., DE BOER, R., BAKKER, N. A. M., JORRITSMA-SMIT, A., VAN BUUREN, M. M., KVISTBORG, P., SPITS, H., SCHOTTE, R., MALLO, H., KARGER, M., VAN DER HAGE, J. A., WOUTERS, M., PRONK, L. M., GEUKES FOPPEN, M. H., BLANK, C. U., BEIJNEN, J. H., NUIJEN, B., SCHUMACHER, T. N. & HAANEN, J. 2020. Tumor infiltrating lymphocytes (TIL) therapy in metastatic melanoma: boosting of neoantigen-specific T cell reactivity and long-term follow-up. *J Immunother Cancer*, 8.

WEBER, J. S., D'ANGELO, S. P., MINOR, D., HODI, F. S., GUTZMER, R., NEYNS, B., HOELLER, C., KHUSHALANI, N. I., MILLER, W. H., JR., LAO, C. D., LINETTE, G. P., THOMAS, L., LORIGAN, P., GROSSMANN, K. F., HASSEL, J. C., MAIO, M., SZNOL, M., ASCIERTO, P. A., MOHR, P., CHMIELOWSKI, B., BRYCE, A., SVANE, I. M., GROB, J. J., KRACKHARDT, A. M., HORAK, C., LAMBERT, A., YANG, A. S. & LARKIN, J. 2015. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*, 16, 375-84.

WU, J., ZHOU, M. Y., YU, X. P., WU, Y. & XIE, P. L. 2020. Long noncoding RNA OR3A4 promotes the migration and invasion of melanoma through the PI3K/AKT signaling pathway. *Eur Rev Med Pharmacol Sci*, 24, 10917.

WU, R. Y., KONG, P. F., XIA, L. P., HUANG, Y., LI, Z. L., TANG, Y. Y., CHEN, Y. H., LI, X., SENTHILKUMAR, R., ZHANG, H. L., SUN, T., XU, X. L., YU, Y., MAI, J., PENG, X. D., YANG, D., ZHOU, L. H., FENG, G. K., DENG, R. & ZHU, X. F. 2019. Regorafenib Promotes Antitumor Immunity via Inhibiting PD-L1 and IDO1 Expression in Melanoma. *Clin Cancer Res*, 25, 4530-4541.

YU, M., LOWELL, C. A., NEEL, B. G. & GU, H. 2006. Scaffolding adapter Grb2-associated binder 2 requires Syk to transmit signals from FcepsilonRI. *J Immunol*, 176, 2421-9.

ZHANG, Z., RICHMOND, A. & YAN, C. 2022. Immunomodulatory Properties of PI3K/AKT/mTOR and MAPK/MEK/ERK Inhibition Augment Response to Immune Checkpoint Blockade in Melanoma and Triple-Negative Breast Cancer. *Int J Mol Sci*, 23.

Figure Legends

Figure 1. | Sema4D, Plexin-B1 and PD-L1 expression in B16-F10 and B16-F10R cells. Cells were derived into B16-F10R group(B16-F10R cells) and B16-F10 (B16-F10 cells), 24 hours after cell culture, Sema4D, Plexin-B1 and PD-L1 expression were detected. (A) Protein bands of Sema4D, Plexin-B1 and PD-L1. (B) Ratio to GAPDH of Sema4D. (C) Ratio to GAPDH of Plexin-B1. (D) Ratio to GAPDH of PD-L1. (E) mRNA expression of Sema4D. (F) mRNA expression of Plexin-B1. (G) mRNA expression of PD-L1. Compared with B16-F10 group, Sema4D and Plexin-B1 were significantly upregulated in B16-F10R group. * is $p<0.05$, ** is $P<0.01$, *** is $P<0.001$.

Figure 2. | Sema4D deficiency inhibit PD-L1 expression. B16-F10R Cell were derived into Sema4D-shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50 ng/ml 24 hours, collect the cell to detect the expression of PD-L1. (A) Correlation between PD-L1 and Sema4D (B) Protein bands of PD-L1and Sema4D. (C) Ratio to GAPDH of Sema4D. (D)Ratio to GAPDH of PD-L1. (E) mRNA expression of PD-L1. Compared with Sema4D-NC group, ** is $p<0.01$, *** is $P<0.001$.

Figure 3. | Sema4D deficiency renders B16-F10R cells sensitive to nivolumab treatment. (A) Effects of nivolumab on cell viability of B16-F10 and B16-F10R. B16-F10 cells were derived into B16-F10 and B16-F10+nivolumab group, B16-F10R cells were derived into B16-F10R and B16-F10R+nivolumab groups, B16-F10 and B16-F10R groups were as control group without treatment, B16-F10+nivolumab and B16-F10R+nivolumab groups were treatment with 50ng/mL nivolumab and co-cultured with TIL at a 1:5 ratio (van den Berg et al., 2020). Compared with B16-F10R+nivolumab group, ** is $p<0.01$.(B) Effects of nivolumab on B16-F10 cell viability after Sema4D overexpression. B16-F10 cells were derived into B16-F10, B16-F10+nivolumab, B16-F10+LV-Sema4D and B16-F10+LV-NC group, B16-F10 is control group, another 3 groups were treatment with 50ng/mL nivolumab and co-cultured with TIL at a 1:5 ratio, B16-F10+LV-Sema4D group was also treated with overexpression Sema4D, B16-F10+LV-NC group was also treated with Sema4D negative control. Compared with B16-F10 group, **** is $p<0.0001$; Compared with B16-F10+LV-NC group, ##### is $p<0.0001$.(C) Effects of nivolumab on B16-F10R cell after Sema4D knockdown. B16-F10R cells were derived into B16-F10R, B16-F10R+nivolumab, Sema4D-shRNA and Sema4D-NC group, B16-F10R is control group, another 3 groups were treatment with 50ng/mL nivolumab and co-cultured with TIL at a 1:5 ratio, Sema4D-shRNA group was knockdown Sema4D, and Sema4D-NC group was Sema4D-shRNA negative control group. Compared with Sema4D-shRNA group, * is $p<0.05$, ** is $p<0.01$; (D) B16-F10R cell apoptosis rate after treatment with nivolumab. B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-NC group, the treatment is the same as C. (E) Bar graph of apoptosis rate after treatment with anti-PD1. The grouping and processing methods are the same as D. (F) Tumor volume of knockdown Sema4D with nivolumab treatment. Balb/C mice were derived into Sema4D-shRNA group and Sema4D-NC group, B16-F10R-Sema4D-shRNA cells and B16-F10R-Sema4D-NC cells were intradermal injected into the right side of mice, then intraperitoneally injected nivolumab 10 mg/kg once at day 1. (G) Tumor weight of knockdown Sema4D with nivolumab

treatment. the treatment is the same as F. (H) Tumor of mice. the treatment is the same as F. Compared with Sema4D-NC group, ** is $p<0.01$, *** is $p<0.001$, **** is $p<0.0001$.

Figure 4. | Sema4D deficiency potentiates nivolumab inhibitory effects on B16-F10R cell invasion and migration. B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50 ng/ml, cell invasion and migration were detected. (A) cell migration. Representative image of cell migration in the absence and presence of shRNA Sema4D at 0 h, 24 h, and 48 h. The scratched areas were measured in three random fields in each group. Wound healing analysis showed a significant difference in the cell-free area of Sema4D-shRNA group was significantly wider than that of Sema4D-NC group at 24 and 48 hours ($P < 0.0001$). (B) Ratio of cell free area. (C) chamber invasion of cells. Chamber invasion analysis showed a significant difference lower number of invasive cells in Sema4D-shRNA group than Sema4D-NC group ($\times 100$) at 48h. (D) cell invasion number per field. Compared with Sema4D-NC group, **** is $p<0.0001$.

Figure 5. | Sema4D knockdown is significantly associated with inhibition of PI3K-AKT signaling pathway. (A) Association of clustered modules with Sema4D expression. The brown module was identified as the most relevant module for Sema4D expression. (B) Module-trait associations. (C) Gene set variation analysis revealed several significantly dysregulation malignancies events between different Sema4D expression population. (D) Protein-protein interactions of Sema4D. (E) Pathway enrichment analysis showed that Sema4D is involved in the PI3K-AKT signaling pathway. (F) PI3K-AKT signaling pathway proteins expression after Sema4D knockdown. B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50 ng/ml, PI3K, AKT protein expression were detected. Western blotting showed that Sema4D was significantly associated with inhibition of PI3K-AKT signaling pathway. Compared with Sema4D-NC group, ** is $p<0.01$.

Figure 1

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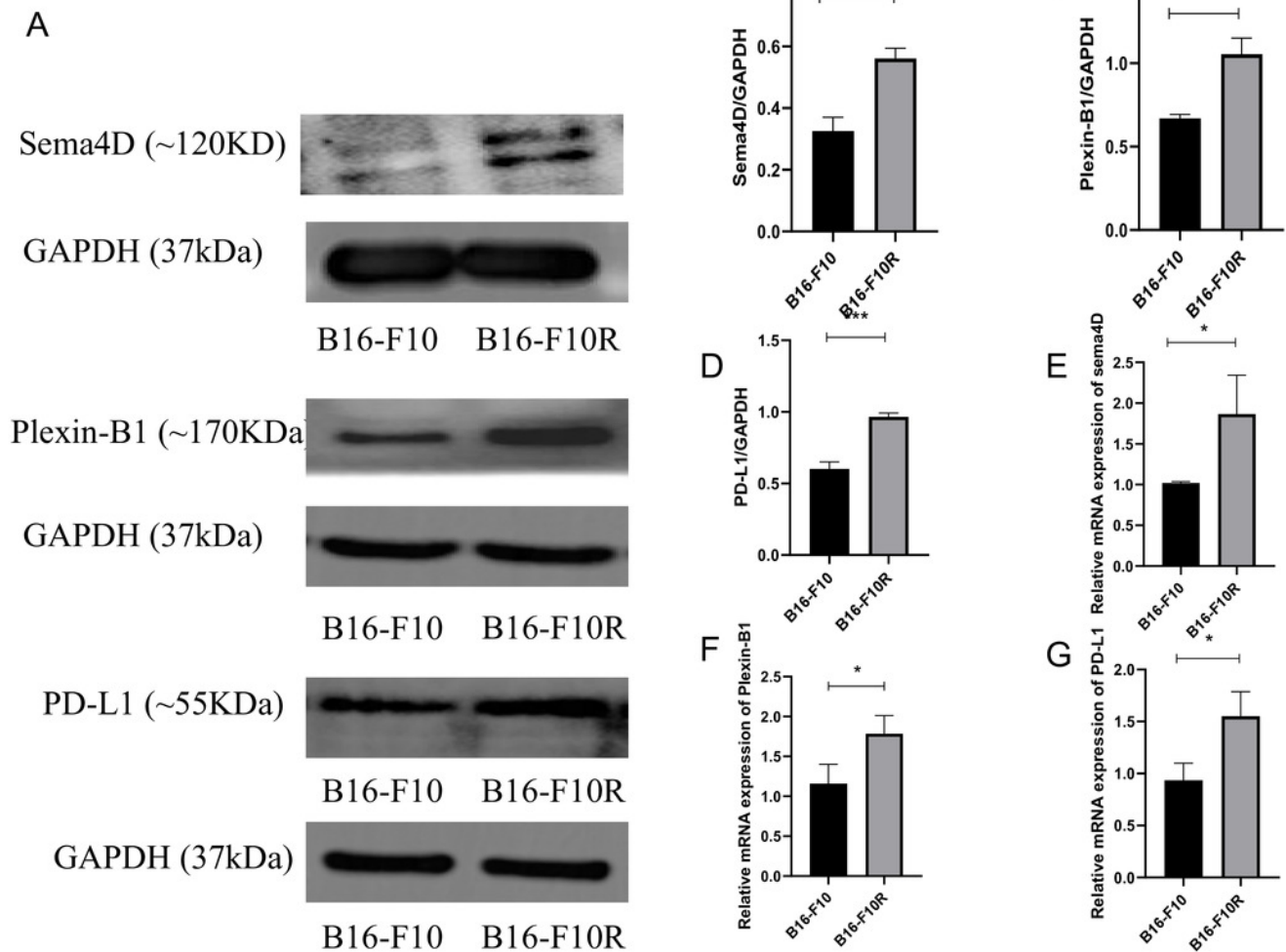


Figure 2

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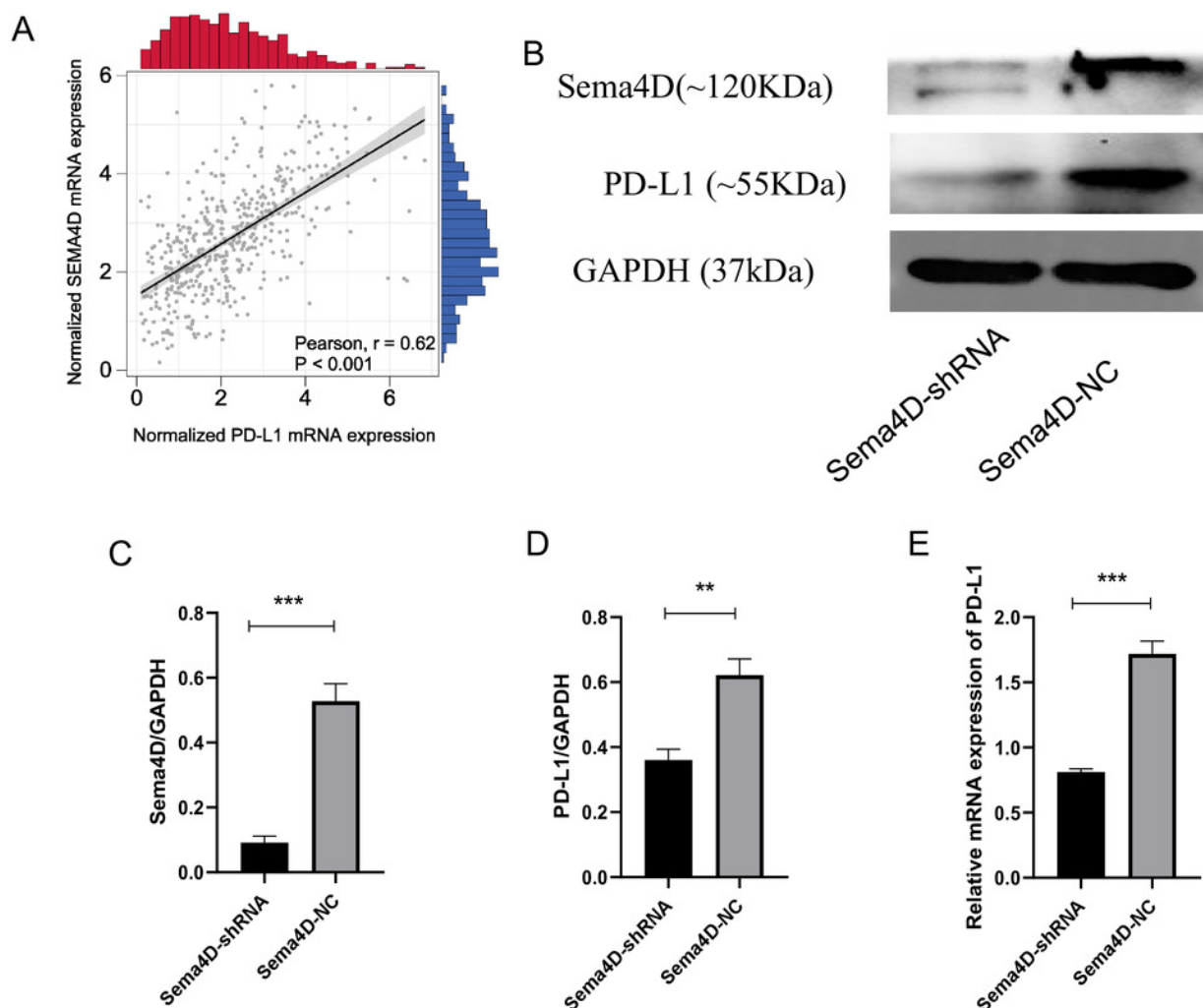


Figure 3

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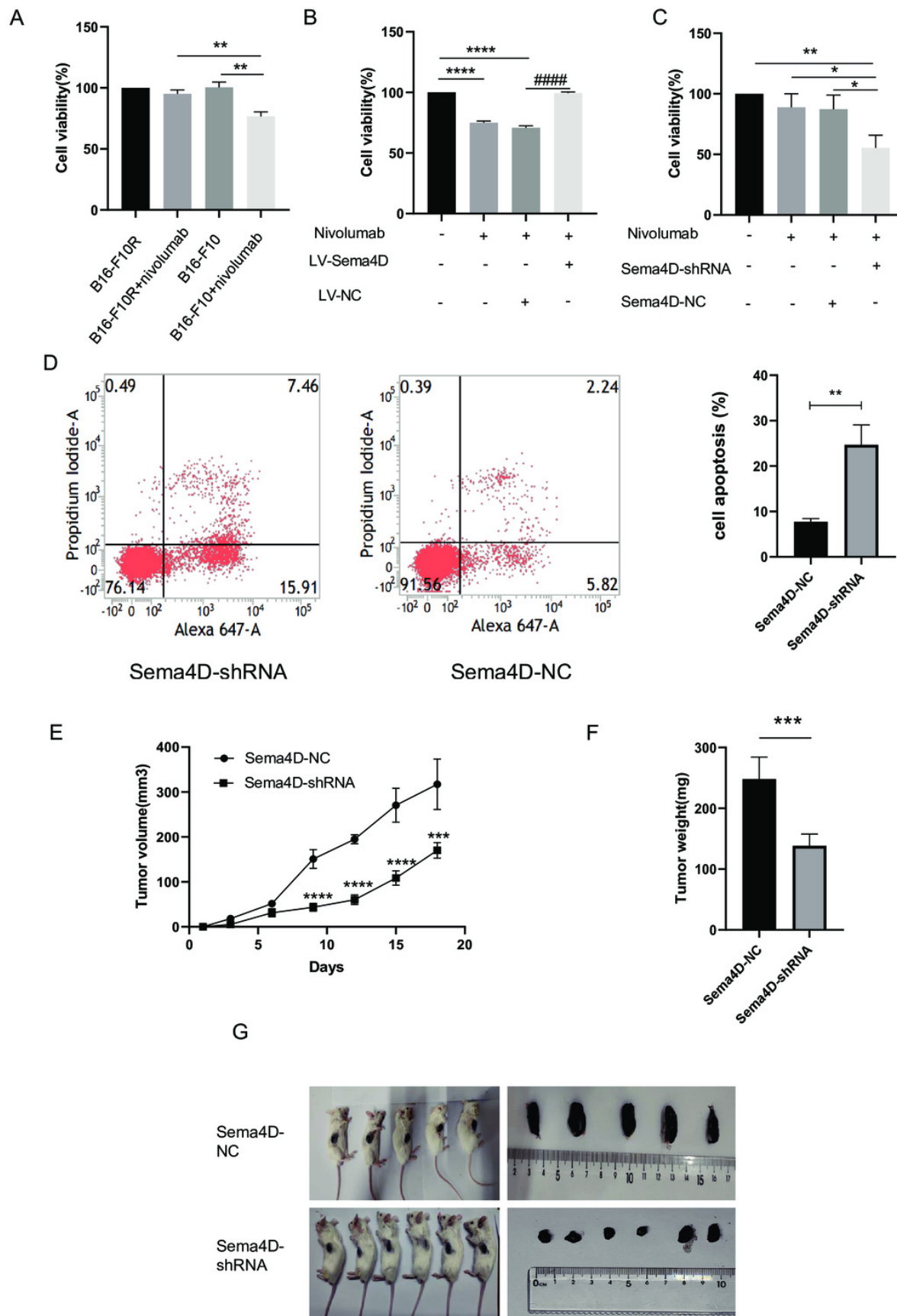


Figure 4

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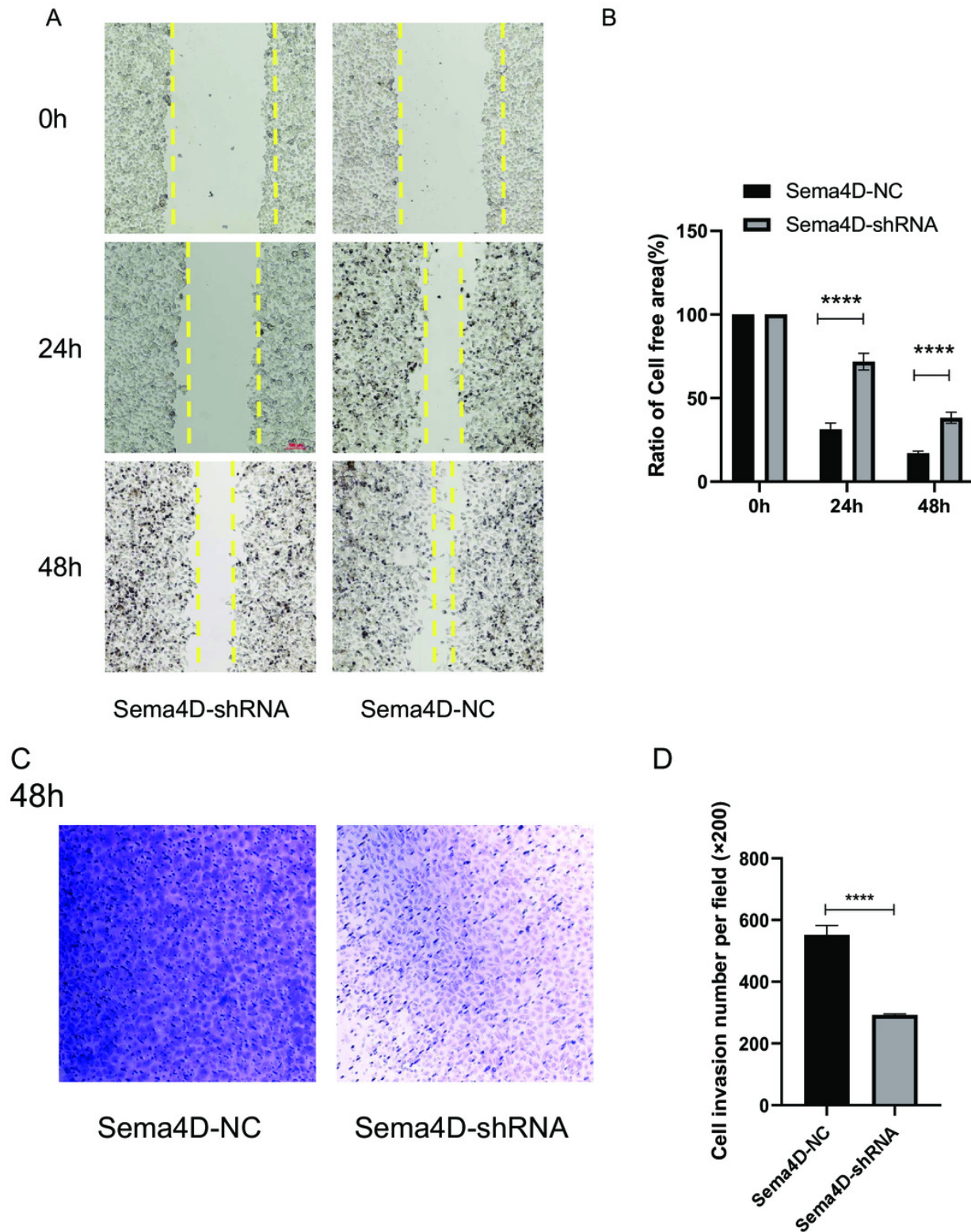


Figure 5

Figure 5

Figure 5. | Sema4D knockdown is significantly associated with inhibition of PI3K-AKT signaling pathway. (A) Association of clustered modules with Sema4D expression. The brown module was identified as the most relevant module for Sema4D expression. (B) Module-trait associations. (C) Gene set variation analysis revealed several significantly dysregulation malignancies events between different Sema4D expression population. (D) Protein-protein interactions of Sema4D. (E) Pathway enrichment analysis showed that Sema4D is involved in the PI3K-AKT signaling pathway. (F) PI3K-AKT signaling pathway proteins expression after Sema4D knockdown. B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50 ng/ml, PI3K, AKT protein expression were detected. Western blotting showed that Sema4D was significantly associated with inhibition of PI3K-AKT signaling pathway . Compared with Sema4D-NC group, ** is $p<0.01$.

