Sema4D/Plexin-B1 is involved in anti-PD-1 resistance in melanoma via PI3K/AKT signaling pathway

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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 is called CD100 and 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/Plexin-B1 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 anti-PD-1 resistance cell. Cell viability, cell invasion and migration are significantly decreased, while the apoptosis was increased by Sema4D knockdown synergizes with anti-PD-1 treatment. Moreover, 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, PD-1 inhibitor resistance is related to Sema4D/Plexin-B1 and Sema4D deficiency can overcome resistance to anti-PD-1 therapy via PI3K/AKT signaling pathway.

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2 **signaling pathway**

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23 **Abstract**

24 Melanoma is a common skin tumor in high mortality, especially in Europe, North America and

25 Oceania. Immunosuppressants such as PD-1 have been proved to be effective in the treatment of

26 malignant melanoma. However, 60% of patients have no immune response. Sema4D is called

27 CD100 and is distributed to T cells and other types of cancer. Sema4D and its receptor, Plexin-

28 B1, plays crucial roles in the immune regulation, angiogenesis, and tumor progression, while

29 Sema4D/Plexin-B1 in melanoma with anti-PD-1 resistance are still poorly understood. To verify

30 the roles of Sema4D/Plexin-B1 in melanoma with anti-PD-1 resistance and the mechanism of

31 Sema4D involvement in nivolumab resistance was related to PI3K/AKT pathway, we first

32 detected the expression differences of Sema4D and Plexin-B1 in drug-resistant cell lines(B16-

33 F10R) and sensitive cell lines(B16-F10), then B16-F10R cells were divided into Sema4D-

34 shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50

35 ng/mL for 24 hours, cell viability, cell apopotosis, cell invasion,cell migration and PI3K/AKT

36 expressionwere detected. In vitro, 12 Balb/c mice with 12-week-old and half male and female

37 were divided into Sema4D-shRNA group and Sema4D-NC group which intradermal injected

38 with B16-F10R-Sema4D-shRNA cells and B16-F10R-Sema4D-NC cells respectively, then

39   intraperitoneally injected nivolumab 10 mg/kg once at day 1, tumor volumes were measured on
40   1, 3, 6, 9, 12, 15, 18 days and tumor weight was measured at days 18. The results showed that
41   the expression of Sema4D, Plexin-B1 was significantly overexpressed in B16-F10R anti-PD-1
42   resistance cell. Cell viability, cell invasion and migration are significantly decreased, while the
43   apoptosis was increased by Sema4D knockdown synergizes with anti-PD-1 treatment. Moreover,
44   the growth of tumors on the body surface of mice was also inhibited. Mechanistically,
45   bioinformatics analysis revealed that Sema4D is involved in the PI3K/AKT signaling pathway.
46   Downregulation of p-PI3K/PI3K and p-AKT/AKT expression were observed in Sema4D
47   knockdown. Therefore, PD-1 inhibitor resistance is related to Sema4D/Plexin-B1 and Sema4D
48   deficiency can overcome resistance to anti-PD-1 therapy via PI3K/AKT signaling pathway.
49
50   **Keywords:** Sema4D, Melanoma, Nivolumab, PI3K/AKT, PD-1 inhibitor resistance.
51

52   **INTRODUCTION**
53   Melanoma, which is prevalent in Europe and North America, has been on the rise in recent
54   years (Rodriguez-Cerdeira et al., 2017). Melanoma is the most aggressive and fatal skin cancer,
55   which can easily metastasize to the whole body through lymph and blood vessels.Melanoma has
56   high tolerance to treatment and poor prognosis. If the tumor metastasizes to distant and visceral
57   organs, the 5-year survival rate is only 15-20% (Huang and Zappasodi, 2022, Eggermont et al.,
58   2014). The current treatment for melanoma includes surgery, chemoradiotherapy, targeted therapy
59   and immunotherapy (Miller and Mihm, 2006). Because cutaneous melanoma is often associated
60   with UV exposure and elevated tumor mutational load, melanoma is one of the most immunogenic
61   of all cancer types and one of the most effective for immunotherapy (Melixetian et al., 2022).
62   Immune checkpoints include programmed cell death protein-1(PD-1) and programmed death-
63   ligand 1(PD-L1) play important role in immunotherapy , PD-L1 is distributed in tumor cell and
64   PD-1 is generally distributed in T-cells, when PD-L1 bind to its receptor PD-1, triggers inhibitory
65   signaling to attenuate T cell activity (Sun et al., 2018). PD-1 or PD-L1 inhibitors such as nivolumab
66   and pembrolizumab can prevent the binding of PD-1 to PD-L1, thus restoring T cell activity and
67   killing tumors (Larkin et al., 2015, Ribas et al., 2015, Weber et al., 2015). Clinical evidence
68   indicated that there are only 30-40% of patients receiving PD-1 or PD-L1 inhibitors could achieve
69   a complete response (Hugo et al., 2016). Furthermore, there are a growing subset of responders
70   acquired resistance within two years after treatment induction in melanoma (Robert et al., 2015b,
71   Robert et al., 2015a). Therefore, it is urgent to develop new biomarkers with predictive value in
72   the treatment of PD-1 inhibitor.
73   Semaphorins has become a key factor in regulating tumor growth and metastasis. Especially
74   in the tumor microenvironment, the disturbance between different cell types can control the
75   development and progression of cancer(Valentini et al., 2021, Lontos et al., 2018). Semaphorin
76   4D (Sema4D) is a member of the Semaphorins, which has immunoregulatory activity, platelet
77   inactivation, angiogenesis stimulation and bone formation regulation   (Lontos et al., 2018).
78   Sema4D increased in most tumors, such as prostate, colon, breast, melanoma, head, and neck
79   carcinomas (Liu et al., 2015). As a ligand of Sema4D, Plexin-B1 plays important roles in tumor

80 cell proliferation, survival, and migration via Sema4D/Plexin-B1 signal pathway (Ch'ng and
81 Kumanogoh, 2010).
82   The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway is involved in the
83 occurrence and development of melanoma, Long noncoding RNA OR3A4, GRB2 and Hey1 can
84 promote melanoma migration and invasion via this signaling pathway(Shayan et al., 2017, Wu et
85 al., 2020). When PI3K combines with GRB2, it is phosphorylated, recruited to the cell
86 membrane and activated, then AKT phosphorylated and acts on its target genes to regulate a
87 variety of cellular processes (Yu et al., 2006).
88   However, in melanoma, roles of Sema4D in the anti-PD-1 tolerance is unclear. In this study,
89 we use a combination of molecular biology techniques and bioinformatics to study the roles of
90 Sema4D in anti-PD-1 resistance melanoma. We provide the evidence for the first time that
91 Sema4D/Plexin-B1 is involved in anti-PD-1 resistance of melanoma. Moreover, Sema4D
92 deficiency can overcome resistance to anti-PD-1 therapy, the mechanism is related to PI3K/AKT
93 signaling pathway.
94

95 **MATERIALS AND METHODS**
96 **Cell culture and B16-F10 resistance cell line establishment**
97   293T cells, B16-F10 were purchased by Nanjing Cobioer Biotechnology Co., LTD (Nanjing,
98 China), cultured at 37℃ in 5% CO_2 in RPMI-1640 (Life Technologies, Invitrogen, Canada) with
99 10% FBS (Gibco, USA). The combination of high dose shock and gradually increasing dose was
100 applied to establish the cell lines insensitive to nivolumab (anti-PD-1). The B16-F10 cell line in
101 the logarithmic growth phase were cultured with nivolumab (AbMole Bioscience Inc., shanghai,
102 China) 10 ng/mL and TIL at a 1:5 ratio. After 24 hours, the solution was changed, and dead cells
103 were discarded. The remaining cells were then cultured with RPMI-1640 without nivolumab, when
104 the remaining cells have stabilized, then cultured with nivolumab 25 ng/ml and repeat these steps
105 until the cells could grow continuously and stably in the medium with nivolumab 50 ng/mL, and
106 then cultured drug-free for 1 month, B16-F10 resistance cell line (B16-F10R) were obtained. B16-
107 F10R also needs with nivolumab 5 ng/mL to maintain the resistance (Maeda et al., 1991).
108

109 **Tumor Infiltrating Lymphocyte (TIL)**
110   The tumors were removed from the Balb/c mice, about 2-3 cubic millimeters in size, and
111 cultured with 2 mL complete medium in 24 well plates. The complete medium included 6000
112 IU/mL IL-2 which purchased by Novartis , RPMI-1640 and 2.05 mM L-glutamine were the major
113 component and get from Thermo Fisher Scientific, there is also a need to add 10 % heat-inactivated
114 human AB serum (Omega Scientific, USA), 55 µM 2-mercaptoethanol and 10 mM of HEPES
115 Buffer (Mediatech, USA), gentamicin(50 µg/mL) , penicillin(100 I.U./mL), streptomycin(100
116 µg/mL) were purchased from Invitogen and prevent infection (Arneth, 2019).
117

118 **Western blotting**
119   We investigated the expression of PD-L1, Sema4D, Plexin-B1, PI3K, p-PI3K, AKT, p-AKT ,
120 these protein were purchased from Abcam Shanghai Trading Co., LTD and cell signaling

121 technology, Inc. (Shanghai, China).Total protein was extracted and BCA protein detection kit
122 (Beyotime, China) was used to evaluate the protein concentration. after electrophoresis, transfer
123 membrane, antibody incubation process, an ECL chromogenic substrate was applied for detecting
124 the signals (Beyotime, China).
125
126 **RT-qPCR**
127 Total RNA was obtained by processing the samples with TRIzol reagent and Dnase-free DNase
128 Set (Beyotime, China) . The synthesis of complementary DNA was performed using a cDNA
129 synthesis kit (Thermo Fisher Scientific, USA). Finally, RT-PCR analysis was performed using an
130 ABI PRISM 7900-HT sequence detection system (Applied Biosystems, USA).For qRT-PCR, the
131 following primers were used.
132 PD-L1:5'-AGAACCTGGCTGCACCTAAC-3'(F),
133 5'-GAGAAGGTCAAACCGCCTCA-3'(R);
134 Plexin-B1:5'-AAGCCCAGCCTACTAACAACC-3'(F),
135 5'-CAGCCCCACTGTACTGACTG-3'(R);
136 Sema4D:5'-TCAAAGCAGACGGAATGCCTA-3'(F),
137 5'-CCCCAACCATGACTGATGTGTA-3'(R);
138 GAPDH:5'-TTGTCATGGGAGTGAACGAGA-3'(F),
139 5'-CAGGCAGTTGGTGGTACAGG-3'(R).
140
141 **shRNA and expression plasmid transfection**
142   Short hairpin RNA (shRNA) targeting Sema4D and the lentivirus containing this shRNA were
143 designed and synthesized by Genepharm Co. (Shanghai, China), sh-sema4D (5'-
144 GGATGGGACTG   TCTATGATGT-3')   and   the   control   NC-sema4D   (5'-
145 TTCTCCGAACGTGTCACGT-3'). The shuttle plasmid and three other packaging plasmids
146 (pGag/Pol, pRev, pVSV- G) were co-transfected into 293T cells to generate a lentivirus expressing
147 Sema4D shRNA or a control lentivirus expressing shRNA. Cells were inoculated into 6-well plates
148 at 1×10^5 and infected with lentivirus while 5 μg/mL of Polybrene was added. The culture media
149 was changed after 24 hours of infection, and the infected cells were screened with 1 μg/mL of
150 puromycin for 7 days after the culture media change to obtain stable lentiviral shRNA expressing
151 cell lines for subsequent experimental analysis (Moore et al., 2010).
152
153 **MTT**
154   Cell viability was measured by MTT assay. 1×10^4 B16-F10R cells were cultured and transfected
155 with Sema4D-shRNA and Sema4D-NC after 24hours, so cell were divided into Sema4D-shRNA
156 and Sema4D-NC group, and these two groups were co-cultured with TIL at a 1:5 ratio (van den
157 Berg et al., 2020) and nivolumab 50 ng/mL for 24 hours. MTT was dissolved in DMSO and 0.5
158 mg/mL MTT diluted in 1×PBS for 4 hours, Turn on Benchmark Plus microplate reader (Bio-Rad,
159 American) and detect absorbance at 570 nm (Algazi et al., 2020).
160
161 **Cell apoptosis analysis**

162 Cell apoptosis were detected by AnnexinV-AlexaFluor647/PI Kit Apoptosis Assay
163 Kit(Biotech, China). 5×10^5 B16-F10R cells were seeded in 10-cm culture dishes (Corning, USA)
164 and incubated overnight. Cells were divided into Sema4D-shRNA and Sema4D-NC group which
165 transfected with Sema4D-shRNA and Sema4D-NC respectively, co-cultured with TIL at a 1:5
166 ratio and nivolumab 50 ng/mL for 24 hours. 5×10^5 cells were collected and resuspended with
167 500 µL binding buffer . 5 µL Annexin V/AlexaFluor647 were incubate for 5 minutes at room
168 temperature, then add 10 µL propidium iodide (PI) , fluorescent dyes should be protected from
169 light.
170

Wound healing and invasion analysis

172 B16-F10R cells were divided into Sema4D-shRNA group and Sema4D-NC group. cell invasion
173 and migration was analyzed by Matrigel-coated invasion chambers and scratch assay. The initial
174 number of cells cultured was 5×10^5 , a sterile 10 µL pipette tip was used to mark a line in the
175 monolayer of each well, the wounds were observed at 0, 24 and 48 hours under a microscope
176 (Nikon, Japan). ImageJ software was used to measure the wound areas.Cell free migration
177 area=(cell free area 0h - cell free area 24hs)/cell free area 0h (Ji et al., 2018). Matrigel-coated
178 invasion chambers (Corning, USA) was used to incubation with 48 hours. The top of Matrigel and
179 invading cells were fixed and stained with 0.1% crystal violet.The microscope (Nikon, Japan) was
180 used to photograph invading cells, and 3 fields were randomly selected and counted each time.
181

Animal and tumorigenesis

183 Balb/c mice with 12-week-old were purchased from Chendu dossy experimental animals CO.,
184 LTD (SCXK (Chuan) 2020-030, Chengdu, China). The mice were fed unrestricted diet and water
185 intake, and were kept under specific pathogen-free conditions in a laboratory where a 12-hour
186 light/dark cycle was strictly enforced(SYXK(Chuan) 2018-065, Luzhou, China). According to the
187 minimum requirements of congeners, there were six mice in each group of the same strain, making
188 a total of 12 mice (Sanmamed et al., 2015), according to the random count table, mice were divided
189 into Sema4D-shRNA group and Sema4D-NC group randomly, six mice in each group were
190 labeled with picric acid as head, neck, back, left back, right back, and left forelimb. B16-F10R-
191 Sema4D-shRNA cells and B16-F10R-Sema4D-NC cells were intradermal injected into the right
192 side of two group mice respectively , so Sema4D-NC group is the control group, then
193 intraperitoneally injected nivolumab 10 mg/kg once at day 1 , 1 mice of Sema4D-NC group was
194 died during the experiment. Tumor volumes were measured on 1, 3, 6, 9, 12, 15 and 18 days with
195 caliper, at days 18, the mice were anesthetized with 10% chloral hydrate and sacrificed by cervical
196 dislocation, tumor weight was measured, tumor volumes were calculated with the formula: tumor
197 volume = $0.52 \times$ length $\times$ width2. The people who make the models are different from the people
198 who measure the tumors, and the tumor testers don't know the grouping and the measured data are
199 handed over to the statisticians for processing. All experimental protocols were approved by the
200 ethic committee (Southwest medical university, China) and in compliance with the international
201 regulations on care and protection of laboratory animals (SWMU20210429).
202

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

Tumor size, apoptosis rate and other measurement data were expressed as mean plus or minus standard deviation. 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 anti-PD-1 resistance melanoma cells

To investigate the mRNA and protein expression of Sema4D and Plexin-B1 in B16-F10R and

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

247

248 **Sema4D deficiency inhibit PD-L1 expression upon anti-PD-1 therapy**
249 We analyzed the correlation of PD-L1 and Sema4D with bioinformatics, it showed that PD-L1
250 and Sema4D are positively correlated *(Figure 2A)*. Compared with Sema4D-NC group, mRNA
251 and protein expression of PD-L1 in Sema4D-shRNA group was significantly downregulated after
252 nivolumab treatment *(Figure 2B, C, D)*, which was consistent with the results of bioinformatics.
253 Therefore, inhibition of SEMA4D significantly repressed the mRNA and protein expression of
254 PD-L1.

255

256 **Sema4D deficiency potentiates anti-PD-1 treatment efficacy**
257 To explore the relationship between Sema4D deficiency and anti-PD-1 treatment efficacy in
258 B16-F10R cells, we investigated the cell viability and cell apoptosis. The results showed Sema4D
259 deficiency potentiates anti-PD-1 treatment efficacy in B16-F10R cells. Compared with Sema4D-
260 NC group, the cell viability of Sema4D-shRNA group in combination with anti-PD-1 is decreased
261 significantly *(Figure 3A)* and rate of apoptosis cell in Sema4D-shRNA group after treatment with
262 anti-PD-1 is notably increased *(Figure 3B, C)*. In vivo experiment, we transplanted B16-F10R
263 cells in the mice and observed the size and weight of tumors treated with nivolumab, tumor volume
264 and weight were significantly delayed in Sema4D-shRNA group mice as compared to Sema4D-
265 NC group mice *(Figure 3D, E, F)*.

266

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

277

278 **Downregulation of Sema4D inhibit PI3K/AKT expression**
279 The brown module was determined to be most significantly associated with Sema4D expression
280 (r = +-0.67, p-value = 1e-62), which contained a total of 778 genes *(Figure 5A-B)*. Gene set
281 variation analysis (GSVA) revealed several significantly upregulated malignancy-associated
282 events (IL-6, JAK, STAT3, MTORC1, and KRAS signaling pathways) in the Sema4D high
283 expression group as well as downregulation of p53 signaling *(Figure 5C)*. Sema4D was
284 significantly associated with oncogenes RHOA, RRAS and immunomodulatory-related genes

285   PTPRC and PLXNC1*(Figure 5D)*. Pathway enrichment analysis revealed that Sema4D is involved
286   in multiple immune-related pathways as well as the PI3K/AKT pathway *(Figure 5E)*. Numerous
287   studies have demonstrated that excessive activation of PI3K/AKT signaling pathway is associated
288   with excessive proliferation of tumor cells, inhibition of apoptosis and attenuation of the efficacy
289   of immune checkpoint inhibitors (Zhang et al., 2022). The expression of PI3K/AKT protein was
290   explored by Western blot. p-PI3K/PI3K, p-AKT/AKT of Sema4D-shRNA group were decreased
291   than those of Sema4D-NC group, which suggesting aberrant activation of the PI3K/AKT signaling
292   pathway in the Sema4D-NC group *(Figure 5F)*.
293
294   **DISCUSSION**
295   Immunotherapy is one important method for melanoma, PD-1 and PD-L1 are two of the
296   most attractive new immune checkpoints, for melanoma patients, when alone or incombination
297   with anti-PD-1 and anti-PD-L1 may improve survival rates (Simeone et al., 2015). Nivolumab is
298   a PD1 inhibitor that has anti-melanoma effects with high affinity to PD-1 (Hirano et al., 2005).
299   However, the ineffectiveness or resistance of anti-PD-1 to melanoma is a serious problem. In
300   general, anti-PD-1 resistance are mainly divided into acquired resistance and innate resistance
301   (Bridgeman et al., 2014, Marincola et al., 2000 , Bronte et al., 2005, O'Donnell et al., 2017). The
302   causes of anti-PD-1 or anti-PD-L1 resistance to melanoma are very complex and the genes
303   involved are not fully understood. This study elucidated the mechanism of anti-PD-1resistance is
304   associated with PI3K/AKT signaling pathway via Sema4D expression.
305   Sema4D expressed on T cells and many cancers, when it combined with Plexin-B1, it
306   correlates with tumor immune infiltration, angiogenesis, and tumor progression in melanoma (Lu
307   et al., 2021). In this study, we explored mRNA and protein expression of Sema4D and Plexin-B1
308   in mouse melanoma tumor cell lines and drug-resistant cell lines, our results revealed that the
309   mRNA and protein expression of Sema4D and Plexin-B1 in drug-resistant cell lines were
310   overexpressed. Therefore, we provide the evidence for the first time that Sema4D/Plexin-B1 is
311   related to anti-PD-1 resistance of melanoma.
312   To understand the expression of Sema4D in melanoma resistance to anti-PD-1, we inhibited
313   Sema4D expression by shRNA in combination with anti-PD-1 (Nivolumab). It was observed that
314   downregulation of Sema4D sensitized B16-F10R cells to nivolumab by inhibiting the growth of
315   tumor cells and reducing cell invasion and migration. As reported by Rezaeepoor that silencing of
316   Sema4D serve as a therapeutics target for the suppression of invasion, migration(Rezaeepoor et
317   al., 2021).
318   In addition, we also investigated the effects of Sema4D knockdown on B16-F10R cells
319   apoptosis in response to nivolumab. We found that rate of apoptosis cell in Sema4D-shRNA
320   group after treatment with anti-PD-1 is markedly increased. A study conducted by Rashidi
321   illustrated silencing of Sema4D can elevated apoptosis rate of SW48 cells in response to 5-FU
322   treatment (Rashidi et al., 2020). Moreover, we verified the enhancement of anti-PD-1 sensitivity
323   by Sema4D knockdown in vivo xenograft models, mice treated with nivolumab after Sema4D
324   knockdown showed significantly slower tumor growth. Taken together, these finding
325   demonstrated that Sema4D deficiency can potentiates anti-PD-1 treatment efficacy.

326   We further analyzed the correlation between Sema4D and PD-L1 expression in anti-PD-1
327   resistance. It has been demonstrated that the presence of PD-L1 determines the response to anti-
328   PD-1 /PD-L1 therapy (Sunshine et al., 2017), regorafenib can promotes antitumor immunity via
329   inhibiting PD-L1 expression in melanoma (Wu et al., 2019). JAK1/2 mutation leads to the lack
330   of reactive PD-L1 expression, which is involved in the resistance of PD-1/PD-L1 inhibitors (Shin
331   et al., 2017). In this study, we evaluated PD-L1 expression in B16-F10R cell when knockdown
332   with Sema4D-shRNA, the results showed PD-L1 expression in Sema4D knockdown group is
333   decrease, it suggest that   the effects of Sema4D knockdown on Nivolumab in B16-F10R cell
334   lines may be related to the decrease of PD-L1 expression. However, which way contributes to
335   the PD-L1 downregulation after knockdown Sema4D remains unclear and needs further
336   investigation.
337   Bioinformatics analysis revealed that Sema4D is involved in the PI3K/AKT signaling
338   pathway which is activated and plays important roles such as being involved in the development
339   and occurrence in melanoma and it also activate receptor tyrosine kinases (Davies, 2012). PI3K
340   is phosphorylated by binding to GRB2, and AKT phosphorylates target genes to regulate cell
341   function (Pu et al., 2021). Sema4D/ plexin-B1 is associated with osteosarcoma, in which PYK2-
342   PI3K-Akt pathway is activated to promote tumor progression(Li et al., 2021). To explore the
343   relationship between Sema4D and PI3K/AKT signaling pathways in anti-PD-1 resistance, we
344   detected the expression of PI3K/AKT protein, in B16-F10R cell, p-PI3K/PI3K, p-AKT/AKT
345   expression were significantly increased, when inhibited the Sema4D, p-PI3K/PI3K, p-AKT/AKT
346   expression of Sema4D-shRNA group were decreased, these shown that Sema4D can regulate
347   PI3K/AKT expression and activity.
348   However, this study only examined the differences in Sema4D expression between sensitive
349   cells and resistant cells , and the effects of nivolumab on cells and animals after Sema4D
350   blockade were also evaluated. In this study , the number of cell lines involved and the methods
351   of blocking Sema4D in animals were limited, so in future studies, we will further expand the cell
352   types and try to study the mechanism of Sema4D in vivo.
353   In conclusion, we explored the role of Sema4D/Plexin-B1 in the process of PD-1 inhibitor
354   resistance. In anti-PD-1 resistant cell line, upregulation of Sema4D/Plexin-B1 was observed.
355   When Sema4D was downregulated, it prevented the phosphorylation of PI3K and AKT, thus
356   inhibiting the PI3K/AKT pathway activity. Therefore, these results may have therapeutic
357   implication and may be exploited for the development of novel treatment for anti-PD-1
358   resistance melanoma in the future.
359

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361   Not applicable.

362

363   **Authors' contributions**
364   Conceptualization: Zhuo Zhang; Formal analysis and investigation: Duoli Zhang   and
365   Fang Wang; Cell and animal experiments: Jiao Liu; Writing original draft
366   preparation: Zhuo Zhang, Songyot Anuchapreeda and Singkome Tima; Writing

367 review and editing: Zhangang Xiao and Suwit Duangmano.
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373

Availability of data and materials

374
375 The raw data supporting the conclusions of this article will be made available by the authors,
376 without undue reservation.
377

Declarations

378
Ethics approval and consent to participate

379
380 All experimental protocols were approved by the ethic committee (Southwest medical
381 university, China) and in compliance with the international regulations on care and protection of
382 laboratory animals (SWMU20210429).
383

Consent for publication

384
385 Not applicable.
386

Competing interests

387
388 The authors declare that they do not have any conflicts of interest.
389

References

390
391 ALGAZI, A. P., TWITTY, C. G., TSAI, K. K., LE, M., PIERCE, R., BROWNING, E., HERMIZ,
392 R., CANTON, D. A., BANNAVONG, D., OGLESBY, A., FRANCISCO, M., FONG, L.,
393 PITTET, M. J., ARLAUCKAS, S. P., GARRIS, C., LEVINE, L. P., BIFULCO, C.,
394 BALLESTEROS-MERINO, C., BHATIA, S., GARGOSKY, S., ANDTBACKA, R. H. I.,
395 FOX, B. A., ROSENBLUM, M. D. & DAUD, A. I. 2020. Phase II Trial of IL-12 Plasmid
396 Transfection and PD-1 Blockade in Immunologically Quiescent Melanoma. *Clin Cancer*
397 *Res,* 26, 2827-2837.
398 ARNETH, B. 2019. Tumor Microenvironment. *Medicina (Kaunas),* 56.
399 BRIDGEMAN, J. S., LADELL, K., SHEARD, V. E., MINERS, K., HAWKINS, R. E., PRICE,
400 D. A. & GILHAM, D. E. 2014. CD3zeta-based chimeric antigen receptors mediate T cell
401 activation via cis- and trans-signalling mechanisms: implications for optimization of
402 receptor structure for adoptive cell therapy. *Clin Exp Immunol,* 175, 258-67.
403 BRONTE, V., KASIC, T., GRI, G., GALLANA, K., BORSELLINO, G., MARIGO, I.,
404 BATTISTINI, L., IAFRATE, M., PRAYER-GALETTI, T., PAGANO, F. & VIOLA, A.
405 2005. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers.
406 *J Exp Med,* 201, 1257-68.
407 CH'NG, E. S. & KUMANOGOH, A. 2010. Roles of Sema4D and Plexin-B1 in tumor progression.

408   *Mol Cancer,* 9**,** 251.

409   DAVIES, M. A. 2012. The role of the PI3K-AKT pathway in melanoma. *Cancer J,* 18**,** 142-7.

410   EGGERMONT, A. M., SPATZ, A. & ROBERT, C. 2014. Cutaneous melanoma. *Lancet,* 383**,**
411   816-27.

412   HIRANO, F., KANEKO, K., TAMURA, H., DONG, H., WANG, S., ICHIKAWA, M., RIETZ,
413   C., FLIES, D. B., LAU, J. S., ZHU, G., TAMADA, K. & CHEN, L. 2005. Blockade of B7-
414   H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer*
415   *Res,* 65**,** 1089-96.

416   HUANG, A. C. & ZAPPASODI, R. 2022. A decade of checkpoint blockade immunotherapy in
417   melanoma: understanding the molecular basis for immune sensitivity and resistance. *Nat*
418   *Immunol,* 23**,** 660-670.

419   HUGO, W., ZARETSKY, J. M., SUN, L., SONG, C., MORENO, B. H., HU-LIESKOVAN, S.,
420   BERENT-MAOZ, B., PANG, J., CHMIELOWSKI, B., CHERRY, G., SEJA, E.,
421   LOMELI, S., KONG, X., KELLEY, M. C., SOSMAN, J. A., JOHNSON, D. B., RIBAS,
422   A. & LO, R. S. 2016. Genomic and Transcriptomic Features of Response to Anti-PD-1
423   Therapy in Metastatic Melanoma. *Cell,* 165**,** 35-44.

424   JI, Y., XIAO, Y., XU, L., HE, J., QIAN, C., LI, W., WU, L., CHEN, R., WANG, J., HU, R.,
425   ZHANG, X., GU, Z. & CHEN, Z. 2018. Drug-Bearing Supramolecular MMP Inhibitor
426   Nanofibers for Inhibition of Metastasis and Growth of Liver Cancer. *Adv Sci (Weinh),* 5**,**
427   1700867.

428   LARKIN, J., CHIARION-SILENI, V., GONZALEZ, R., GROB, J. J., COWEY, C. L., LAO, C.
429   D., SCHADENDORF, D., DUMMER, R., SMYLIE, M., RUTKOWSKI, P., FERRUCCI,
430   P. F., HILL, A., WAGSTAFF, J., CARLINO, M. S., HAANEN, J. B., MAIO, M.,
431   MARQUEZ-RODAS, I., MCARTHUR, G. A., ASCIERTO, P. A., LONG, G. V.,
432   CALLAHAN, M. K., POSTOW, M. A., GROSSMANN, K., SZNOL, M., DRENO, B.,
433   BASTHOLT, L., YANG, A., ROLLIN, L. M., HORAK, C., HODI, F. S. & WOLCHOK,
434   J. D. 2015. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated
435   Melanoma. *N Engl J Med,* 373**,** 23-34.

436   LI, C., WAN, L., WANG, P., GUAN, X., LI, C. & WANG, X. 2021. Sema4D/Plexin-B1 promotes
437   the progression of osteosarcoma cells by activating Pyk2-PI3K-AKT pathway. *J*
438   *Musculoskelet Neuronal Interact,* 21**,** 577-583.

439   LIU, X. L., SONG, J., LIU, K. J., WANG, W. P., XU, C., ZHANG, Y. Z. & LIU, Y. 2015. Role
440   of inhibition of osteogenesis function by Sema4D/Plexin-B1 signaling pathway in skeletal
441   fluorosis in vitro. *J Huazhong Univ Sci Technolog Med Sci,* 35**,** 712-715.

442   LONTOS, K., ADAMIK, J., TSAGIANNI, A., GALSON, D. L., CHIRGWIN, J. M. &
443   SUVANNASANKHA, A. 2018. The Role of Semaphorin 4D in Bone Remodeling and
444   Cancer Metastasis. *Front Endocrinol (Lausanne),* 9**,** 322.

445   LU, Q., CAI, P., YU, Y., LIU, Z., CHEN, G. & ZENG, Z. 2021. Sema4D correlates with tumour
446   immune infiltration and is a prognostic biomarker in bladder cancer, renal clear cell
447   carcinoma, melanoma and thymoma. *Autoimmunity,* 54**,** 294-302.

448   MAEDA, K., LAFRENIERE, R. & JERRY, L. M. 1991. Production and characterization of tumor

449 infiltrating lymphocyte clones derived from B16-F10 murine melanoma. *J Invest*
450 *Dermatol,* 97**,** 183-9.
451 MARINCOLA, F. M., JAFFEE, E. M., HICKLIN, D. J. & FERRONE, S. 2000. Escape of human
452 solid tumors from T-cell recognition: molecular mechanisms and functional significance.
453 *Adv Immunol,* 74**,** 181-273.
454 MELIXETIAN, M., PELICCI, P. G. & LANFRANCONE, L. 2022. Regulation of LncRNAs in
455 Melanoma and Their Functional Roles in the Metastatic Process. *Cells,* 11.
456 MILLER, A. J. & MIHM, M. C., JR. 2006. Melanoma. *N Engl J Med,* 355**,** 51-65.
457 MOORE, C. B., GUTHRIE, E. H., HUANG, M. T. & TAXMAN, D. J. 2010. Short hairpin RNA
458 (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol,* 629**,**
459 141-58.
460 O'DONNELL, J. S., LONG, G. V., SCOLYER, R. A., TENG, M. W. & SMYTH, M. J. 2017.
461 Resistance to PD1/PDL1 checkpoint inhibition. *Cancer Treat Rev,* 52**,** 71-81.
462 PU, Y., LEI, M., CHEN, Y., HUANG, Y., ZHANG, L., CHEN, J., ZHANG, Y., SHAO, X., LIU,
463 L. & CHEN, J. 2021. Hey1 promotes migration and invasion of melanoma cells via
464 GRB2/PI3K/AKT signaling cascade. *J Cancer,* 12**,** 6979-6988.
465 RASHIDI, G., REZAEEPOOR, M., MOHAMMADI, C., SOLGI, G. & NAJAFI, R. 2020.
466 Inhibition of semaphorin 4D enhances chemosensitivity by increasing 5-fluorouracile-
467 induced apoptosis in colorectal cancer cells. *Mol Biol Rep,* 47**,** 7017-7027.
468 REZAEEPOOR, M., RASHIDI, G., POURJAFAR, M., MOHAMMADI, C., SOLGI, G. &
469 NAJAFI, R. 2021. SEMA4D Knockdown Attenuates beta-Catenin-Dependent Tumor
470 Progression in Colorectal Cancer. *Biomed Res Int,* 2021**,** 8507373.
471 RIBAS, A., PUZANOV, I., DUMMER, R., SCHADENDORF, D., HAMID, O., ROBERT, C.,
472 HODI, F. S., SCHACHTER, J., PAVLICK, A. C., LEWIS, K. D., CRANMER, L. D.,
473 BLANK, C. U., O'DAY, S. J., ASCIERTO, P. A., SALAMA, A. K., MARGOLIN, K. A.,
474 LOQUAI, C., EIGENTLER, T. K., GANGADHAR, T. C., CARLINO, M. S.,
475 AGARWALA, S. S., MOSCHOS, S. J., SOSMAN, J. A., GOLDINGER, S. M.,
476 SHAPIRA-FROMMER, R., GONZALEZ, R., KIRKWOOD, J. M., WOLCHOK, J. D.,
477 EGGERMONT, A., LI, X. N., ZHOU, W., ZERNHELT, A. M., LIS, J., EBBINGHAUS,
478 S., KANG, S. P. & DAUD, A. 2015. Pembrolizumab versus investigator-choice
479 chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised,
480 controlled, phase 2 trial. *Lancet Oncol,* 16**,** 908-18.
481 ROBERT, C., LONG, G. V., BRADY, B., DUTRIAUX, C., MAIO, M., MORTIER, L., HASSEL,
482 J. C., RUTKOWSKI, P., MCNEIL, C., KALINKA-WARZOCHA, E., SAVAGE, K. J.,
483 HERNBERG, M. M., LEBBE, C., CHARLES, J., MIHALCIOIU, C., CHIARION-
484 SILENI, V., MAUCH, C., COGNETTI, F., ARANCE, A., SCHMIDT, H.,
485 SCHADENDORF, D., GOGAS, H., LUNDGREN-ERIKSSON, L., HORAK, C.,
486 SHARKEY, B., WAXMAN, I. M., ATKINSON, V. & ASCIERTO, P. A. 2015a.
487 Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med,* 372**,**
488 320-30.
489 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-/-IL2Rgammanull 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

531   FOPPEN, M. H., BLANK, C. U., BEIJNEN, J. H., NUIJEN, B., SCHUMACHER, T. N.
532   & HAANEN, J. 2020. Tumor infiltrating lymphocytes (TIL) therapy in metastatic
533   melanoma: boosting of neoantigen-specific T cell reactivity and long-term follow-up. *J*
534   *Immunother Cancer*, 8.
535   WEBER, J. S., D'ANGELO, S. P., MINOR, D., HODI, F. S., GUTZMER, R., NEYNS, B.,
536   HOELLER, C., KHUSHALANI, N. I., MILLER, W. H., JR., LAO, C. D., LINETTE, G.
537   P., THOMAS, L., LORIGAN, P., GROSSMANN, K. F., HASSEL, J. C., MAIO, M.,
538   SZNOL, M., ASCIERTO, P. A., MOHR, P., CHMIELOWSKI, B., BRYCE, A., SVANE,
539   I. M., GROB, J. J., KRACKHARDT, A. M., HORAK, C., LAMBERT, A., YANG, A. S.
540   & LARKIN, J. 2015. Nivolumab versus chemotherapy in patients with advanced
541   melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised,
542   controlled, open-label, phase 3 trial. *Lancet Oncol*, 16, 375-84.
543   WU, J., ZHOU, M. Y., YU, X. P., WU, Y. & XIE, P. L. 2020. Long noncoding RNA OR3A4
544   promotes the migration and invasion of melanoma through the PI3K/AKT signaling
545   pathway. *Eur Rev Med Pharmacol Sci*, 24, 10917.
546   WU, R. Y., KONG, P. F., XIA, L. P., HUANG, Y., LI, Z. L., TANG, Y. Y., CHEN, Y. H., LI, X.,
547   SENTHILKUMAR, R., ZHANG, H. L., SUN, T., XU, X. L., YU, Y., MAI, J., PENG, X.
548   D., YANG, D., ZHOU, L. H., FENG, G. K., DENG, R. & ZHU, X. F. 2019. Regorafenib
549   Promotes Antitumor Immunity via Inhibiting PD-L1 and IDO1 Expression in Melanoma.
550   *Clin Cancer Res*, 25, 4530-4541.
551   YU, M., LOWELL, C. A., NEEL, B. G. & GU, H. 2006. Scaffolding adapter Grb2-associated
552   binder 2 requires Syk to transmit signals from FcepsilonRI. *J Immunol*, 176, 2421-9.
553   ZHANG, Z., RICHMOND, A. & YAN, C. 2022. Immunomodulatory Properties of
554   PI3K/AKT/mTOR and MAPK/MEK/ERK Inhibition Augment Response to Immune
555   Checkpoint Blockade in Melanoma and Triple-Negative Breast Cancer. *Int J Mol Sci*, 23.
556

557   **Figure Legends**
558   Figure 1. | Sema4D/Plexin-B1 expression in cells. Cells were divided into B16-F10R group(B16-
559   F10R cells) and B16-F10 (B16-F10 cells), 24 hours after cell culture, Sema4D/Plexin-B1
560   expression were detected. (A) Protein bands of Sema4D and Plexin-B1. (B) Ratio to GAPDH of
561   Sema4D. (C) Ratio to GAPDH of Plexin-B1. (D) mRNA expression of Sema4D. (E) mRNA
562   expression of Plexin-B1. Compared with B16-F10 group, Sema4D and Plexin-B1 were
563   significantly upregulated in B16-F10R group. [*] is $p<0.05$, [**] is $P<0.01$.
564
565   Figure 2. | Sema4D deficiency inhibit PD-L1 expression. B16-F10R Cell were divided into
566   Sema4D-shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab
567   50 ng/ml 24 hours, collect the cell to detect the expression of PD-L1. (A) Correlation between PD-
568   L1 and Sema4D (B) Protein bands of PD-L1. (C) Ratio to GAPDH of PD-L1. (D) mRNA
569   expression of PD-L1. Compared with Sema4D-NC group, [**] is $p<0.01$, [***] is $P<0.001$.
570
571   Figure 3. | Sema4D deficiency potentiates anti-PD-1 treatment efficacy. (A) cell viability after

572 treatment with anti-PD-1. (B) cell apoptosis rate after treatment with anti-PD1. (C) Bar graph of
573 apoptosis rate after treatment with anti-PD1. (D) Tumor volume of knockdown Sema4D with anti-
574 PD-1 treatment. (E) Tumor weight of knockdown Sema4D with anti-PD-1 treatment. (F) Tumor
575 of Sema4D-shRNA(n=6) and Sema4D-NC mice(n=5). Compared with Sema4D-NC group, ** is
576 p<0.01, *** is p<0.001,**** is p<0.0001.

577

578 Figure 4. | Sema4D deficiency potentiates the inhibitory effect of anti-PD-1 on cell invasion and
579 migration. Cells were divided into Sema4D-shRNA group and Sema4D-NC group, co-cultured
580 with TIL at a 1:5 ratio and nivolumab 50 ng/ml 24hours, cell invasion and migration were
581 detected, wound healing analysis showed a significant difference in the cell-free area of
582 Sema4D-shRNA group was significantly wider than that of Sema4D-NC group at 24 and 48
583 hours (P < 0.0001). Chamber invasion analysis showed a significant difference lower number of
584 invasive cells in Sema4D-shRNA group than Sema4D-NC group (×100). (A) Representative
585 image of cell migration in the absence and presence of shRNA Sema4D at 0 h, 24 h, and 48 h.
586 The scratched areas were measured in three random fields in each group. (B) Ratio of cell free
587 area (C) chamber invasion of cell (D) cell invasion number per field. Compared with Sema4D-
588 NC group, **** is p<0.0001.

589

590 Figure 5. | Sema4D knockdown is significantly associated with inhibition of PI3K-AKT
591 signaling pathway. (A) Association of clustered modules with Sema4D expression. The brown
592 module was identified as the most relevant module for Sema4D expression. (B) Module-trait
593 associations. (C) Gene set variation analysis revealed several significantly dysregulation
594 malignancies events between different Sema4D expression population. (D) Protein-protein
595 interactions of Sema4D. (E) Pathway enrichment analysis showed that Sema4D is involved in
596 the PI3K-AKT signaling pathway. (F) Western blotting showed that knockdown of Sema4D was
597 significantly associated with inhibition of PI3K-AKT signaling pathway. Compared with
598 Sema4D-NC group, ** is p<0.01.

599

Figure 1

Sema4D/Plexin-B1 expression in cells. Cells were divided into B16-F10R group(B16-F10R cells) and B16-F10 (B16-F10 cells), 24 hours after cell culture, Sema4D/Plexin-B1 expression were detected.

(A) Protein bands of Sema4D and Plexin-B1. (B) Ratio to GAPDH of Sema4D. (C) Ratio to GAPDH of Plexin-B1. (D) mRNA expression of Sema4D. (E) mRNA expression of Plexin-B1. Compared with B16-F10 group, Sema4D and Plexin-B1 were significantly upregulated in B16-F10R group. * is p<0.05, ** is P<0.01.

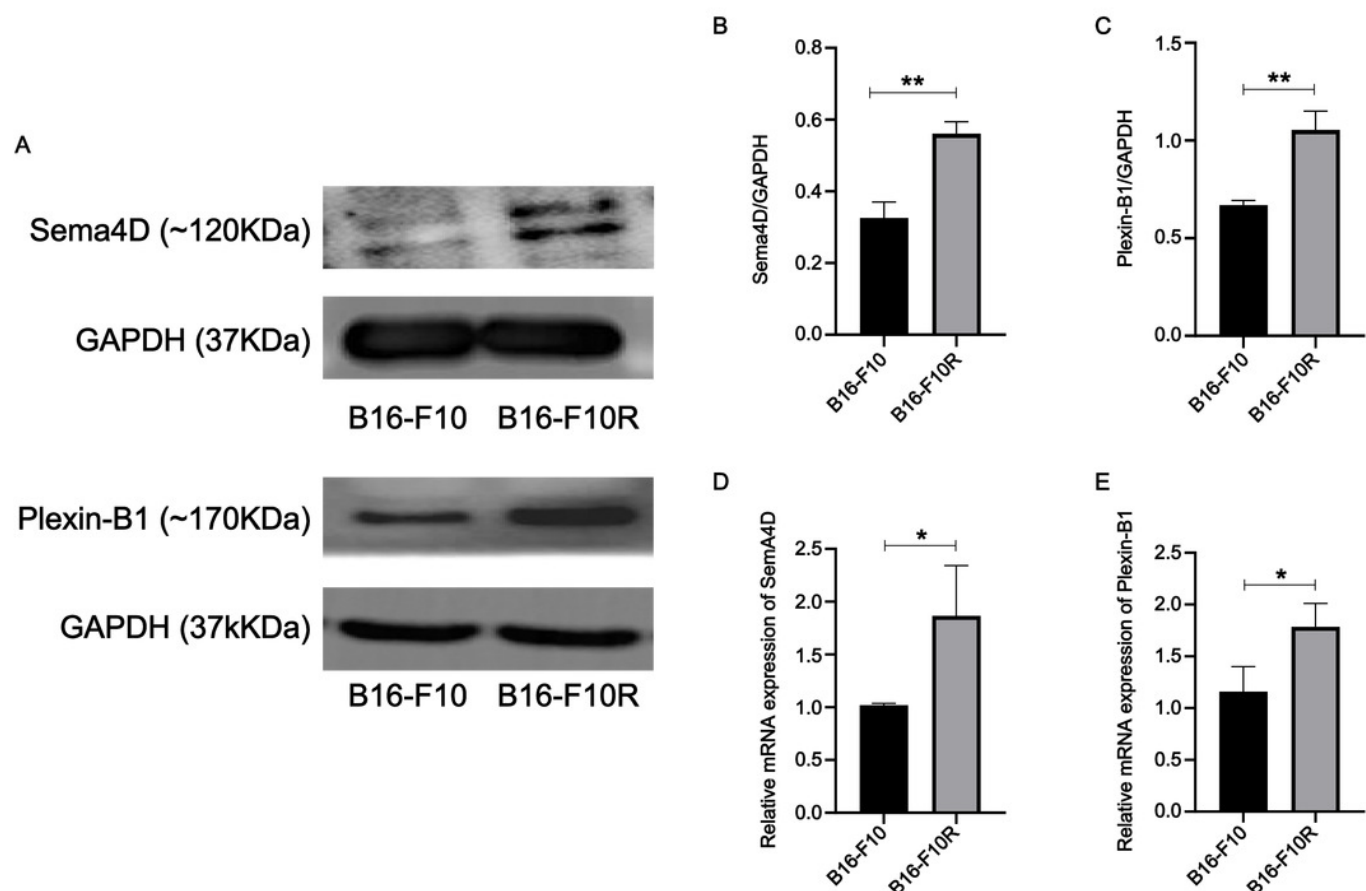



Figure 2

Sema4D deficiency inhibit PD-L1 expression. B16-F10R Cell were divided 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-L1. (C) Ratio to GAPDH of PD-L1. (D) mRNA expression of PD-L1. Compared with Sema4D-NC group, [**] is p<0.01, [***] is P<0.001.

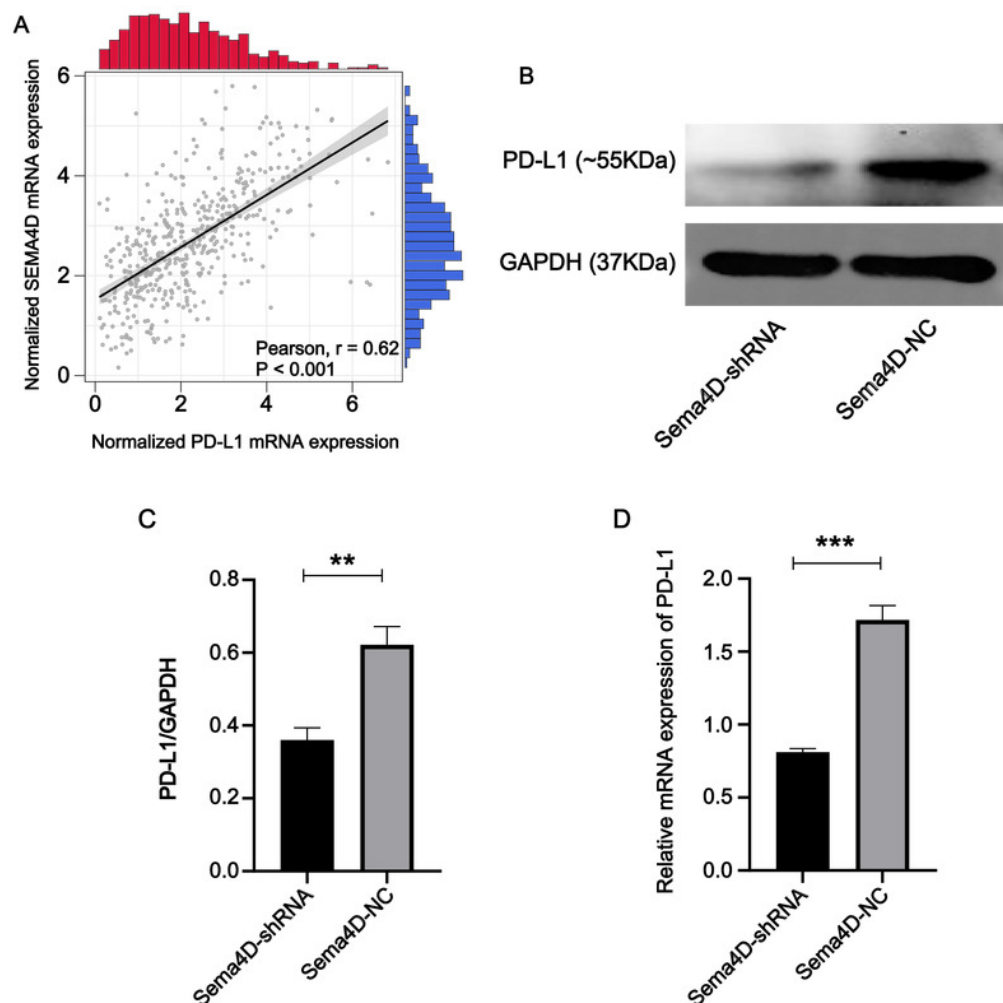
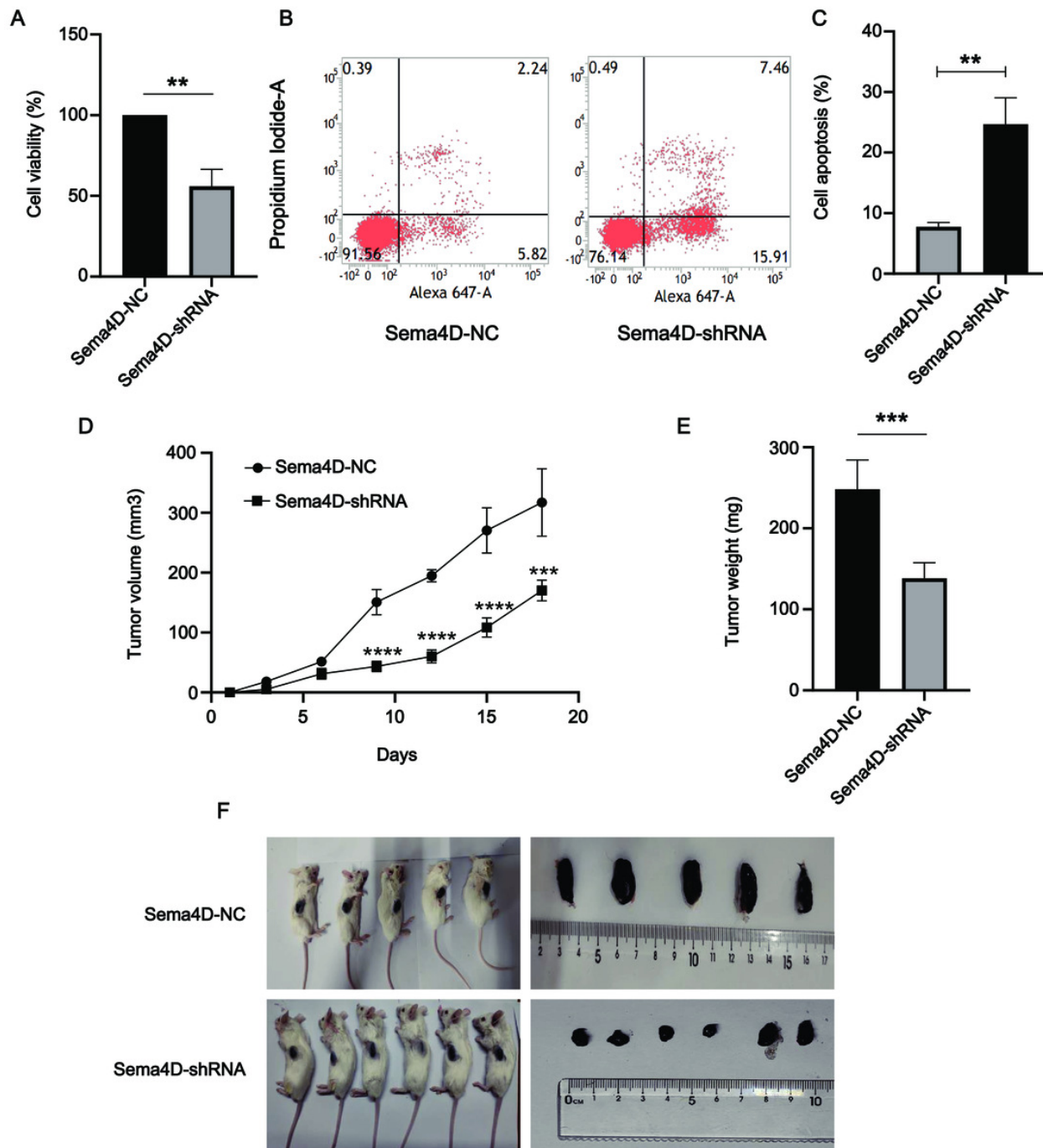



Figure 3

Sema4D deficiency potentiates anti-PD-1 treatment efficacy.

(A) cell viability after treatment with anti-PD-1. (B) cell apoptosis rate after treatment with anti-PD1. (C) Bar graph of apoptosis rate after treatment with anti-PD1. (D) Tumor volume of knockdown Sema4D with anti-PD-1 treatment. (E) Tumor weight of knockdown Sema4D with anti-PD-1 treatment. (F) Tumor of Sema4D-shRNA and Sema4D-NC mice. Compared with Sema4D-NC group, ** is p<0.01, *** is p<0.001,**** is p<0.0001.

A

Cell viability (%)

**

Sema4D-NC   Sema4D-shRNA

B

Propidium Iodide-A

0.39   2.24
91.56   5.82

Alexa 647-A

Sema4D-NC

0.49   7.46
76.14   15.91

Alexa 647-A

Sema4D-shRNA

C

Cell apoptosis (%)

**

Sema4D-NC   Sema4D-shRNA

D

Tumor volume (mm3)

- Sema4D-NC
- Sema4D-shRNA

****   ****   ****   ***

Days

E

Tumor weight (mg)

***

Sema4D-NC   Sema4D-shRNA

F

Sema4D-NC

Sema4D-shRNA

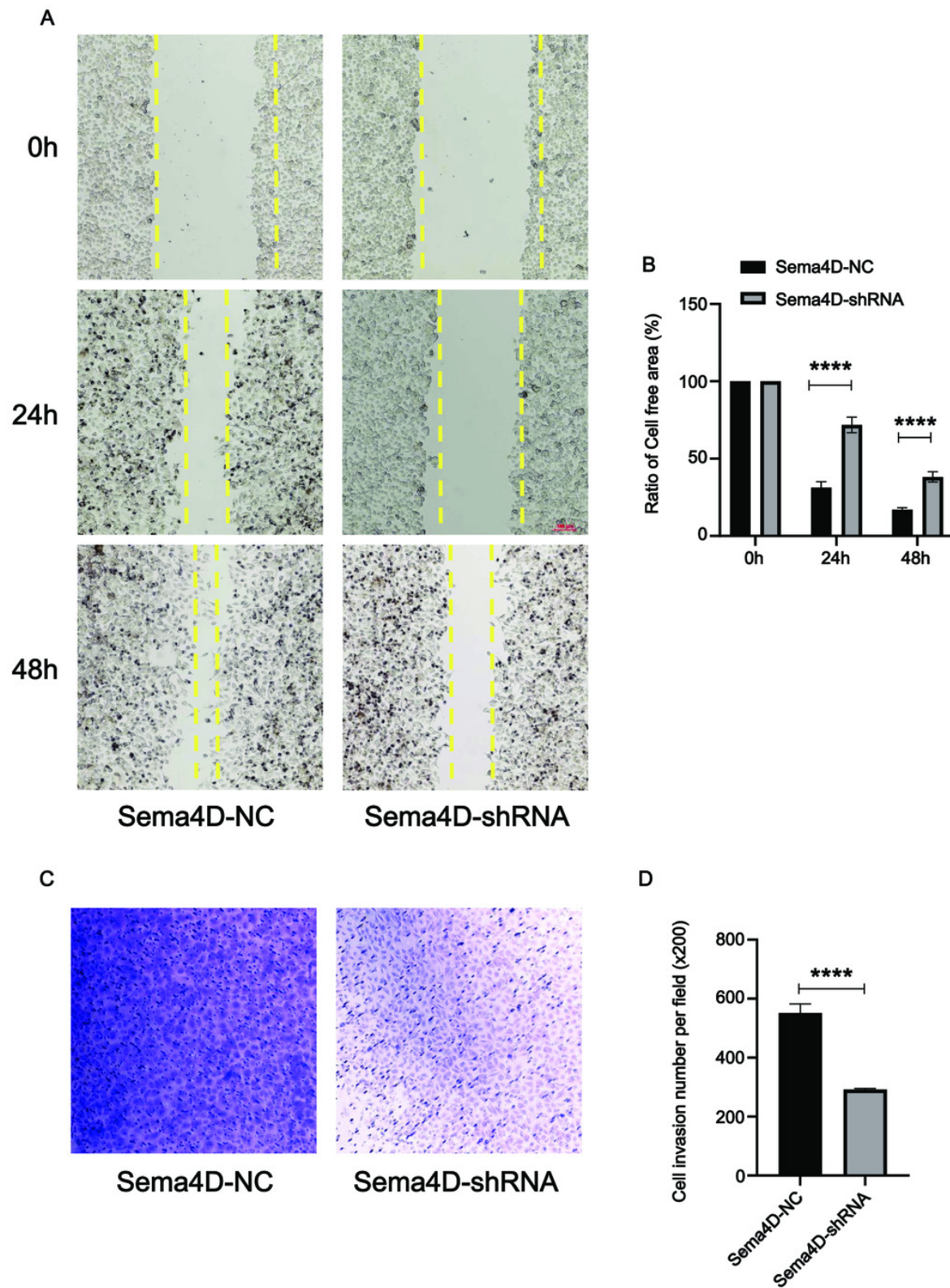
Figure 4

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

(A) 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. (B) Ratio of cell free area (C) chamber invasion of cell (D) cell invasion number per

field. Compared with Sema4D-NC group, **** is p<0.0001.

A

0h

24h

48h

Sema4D-NC   Sema4D-shRNA

B

Ratio of Cell free area (%)

■ Sema4D-NC
▨ Sema4D-shRNA

****   ****

0h   24h   48h

C

Sema4D-NC   Sema4D-shRNA

D

Cell invasion number per field (x200)

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Sema4D-NC   Sema4D-shRNA

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) Western blotting showed that knockdown of Sema4D was significantly associated with inhibition of PI3K-AKT signaling pathway. Compared with Sema4D-NC group, [**] is $p<0.01$.