Sema4D silencing increase the sensitivity of nivolumab to B16-F10 resistant melanoma via inhibiting the 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, 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.

1 **Sema4D silencing increases the sensitivity of nivolumab to B16-F10 resistant melanoma via**
2 **inhibiting the PI3K/AKT signaling pathway**

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23 **Abstract**
24 Melanoma is a common skin tumor that causes a high rate of mortality, especially in Europe,
25 North America and Oceania. Immunosuppressants such as anti-PD-1 have been used in the
26 treatment of malignant melanoma, however, nearly 60% of patients do not respond to these
27 treatments. Sema4D, also called CD100, is expressed in T cells and tumor tissues. Sema4D and
28 its receptor, Plexin-B1, play crucial roles in the process of immune regulation, angiogenesis, and
29 tumor progression. Role of Sema4D in melanoma with anti-PD-1 resistance is poorly
30 understood. Through a combination of molecular biology techniques and in silico analysis, the
31 role of Sema4D in improving anti-PD-L1 sensitivity in melanoma was explored. The results
32 showed that the expression of Sema4D, Plexin-B1 and PD-L1 was significantly increased in
33 B16-F10R cells. Sema4D knockdown synergizes with anti-PD-1 treatment, cell viability, cell
34 invasion and migration are significantly decreased, while the apoptosis was increased, the growth
35 of tumors on the mice was also inhibited. Mechanistically, bioinformatics analysis revealed that
36 Sema4D is involved in the PI3K/AKT signaling pathway, downregulation of p-PI3K/PI3K and
37 p-AKT/AKT expression were observed in Sema4D knockdown, therefore, nivolumab resistance
38 is related to Sema4D and Sema4D silencing can improve sensitivity to nivolumab via inhibition
39 PI3K/AKT signaling pathway.

40
41 **Keywords:** Sema4D, Melanoma, Nivolumab, PI3K/AKT, resistance.

42

INTRODUCTION

43

44 Melanoma, which is prevalent in Europe and North America, has been on the rise in recent years
45 (Rodriguez-Cerdeira et al. 2017). Melanoma is the most aggressive and fatal skin cancer, which
46 can easily metastasize to the whole body through lymph and blood vessels. If the tumor
47 metastasizes to distant and visceral organs, the 5-year survival rate is only 15-20% (Eggermont et
48 al. 2014; Huang & Zappasodi 2022). Surgery is an important treatment for melanoma,
49 chemotherapy such as paclitaxel and cisplatin are also effective, immunotherapy such as anti-PD-
50 1/PD-L1 also plays an important role in changing the survival rate of melanoma(Miller & Mihm
51 2006). Because cutaneous melanoma is often associated with UV exposure and elevated tumor
52 mutational load, it is one of the most immunogenic of all cancer types and one of the most effective
53 for immunotherapy(Melixetian et al. 2022).

54 Immune checkpoints such as programmed cell death protein-1(PD-1) and programmed death-
55 ligand 1(PD-L1) play an important role in immunotherapy. PD-L1 is distributed in tumor cell and
56 PD-1 is generally distributed in T-cells, when PD-L1 bind to its receptor PD-1, triggers inhibitory
57 signaling to attenuate T cell activity (Sun et al. 2018). PD-1 or PD-L1 inhibitors such as nivolumab
58 and pembrolizumab can prevent the binding of PD-1 to PD-L1, thus restoring T cell activity and
59 killing melanoma, non-small lung cancer (NSCLC) and other tumors (Larkin et al. 2015; Ribas et
60 al. 2015; Weber et al. 2015). However, clinical evidence indicated that there are only 30-40% of
61 patients receiving PD-1 or PD-L1 inhibitors could achieve a complete response (Hugo et al. 2016).
62 Furthermore, there are a growing subset of responders acquired resistance within two years after
63 treatment in melanoma (Robert et al. 2015a; Robert et al. 2015b). Therefore, it is an urgent to
64 find new biomarkers with predictive value for melanoma or other solid tumors that are
65 ineffective or resistance in the treatment of PD-1 or PD-L1 inhibitors, and ultimately improve
66 the efficacy of PD-1 or PD-L1 inhibitors.

67 Semaphorins is involved in regulating tumor growth and metastasis. Especially in the tumor
68 microenvironment, the disturbance between different cell types can control the development and
69 progression of cancer(Lontos et al. 2018; Valentini et al. 2021). Semaphorin 4D (Sema4D) is a
70 member of the Semaphorins, which plays a important role in the progress of immunoregulatory,
71 platelet inactivation, angiogenesis stimulation and bone formation regulation (Lontos et al. 2018).
72 Sema4D is upregulated in most tumor tissues, such as prostate, colon, breast, melanoma, head, and
73 neck carcinomas (Liu et al. 2015). As a ligand of Plexin-B1, Sema4D plays important roles in
74 tumor cell proliferation, survival, and migration(Ch'ng & Kumanogoh 2010).

75 The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway is involved in the
76 occurrence and development of melanoma. lncRNA OR3A4, GRB2 and Hey1 can promote
77 melanoma migration and invasion via this signaling pathway(Shayan et al. 2017; Wu et al.
78 2020). When PI3K combines with GRB2, it will be phosphorylated and recruited to the cell
79 membrane, then AKT phosphorylated and acts on its target genes to regulate a variety of cellular
80 processes (Yu et al. 2006).

81 In this study, we use a combination of molecular biology techniques and bioinformatics to
82 explore the roles of Sema4D in anti-PD-1 resistance melanoma. We demonstrated for the first time

83 that Sema4D is involved in anti-PD-1 resistance in melanoma. Moreover, Sema4D deficiency can
84 overcome resistance to anti-PD-1 therapy and the mechanism is related to PI3K/AKT signaling
85 pathway.
86
87 **MATERIALS AND METHODS**
88 **Cell culture and B16-F10 resistance cell line establishment**
89   293T cells, B16-F10 were purchased by Nanjing Cobioer Biotechnology Co., LTD (Nanjing,
90 China), cultured at 37℃ in 5% CO_2 in RPMI-1640 (Life Technologies, Invitrogen, Canada) with
91 10% FBS (Gibco, USA). The combination of high dose shock and gradually increasing dose was
92 applied to establish the cell lines insensitive to nivolumab (anti-PD-1). The B16-F10 cell line in
93 the logarithmic growth phase were cultured with nivolumab (AbMole Bioscience Inc., shanghai,
94 China) 10 ng/mL and TIL at a 1:5 ratio. After 24 hours, the solution was changed, and dead cells
95 were discarded. The remaining cells were then cultured with RPMI-1640 without nivolumab, when
96 the remaining cells have stabilized, then cultured with nivolumab 25 ng/ml and repeat these steps
97 until the cells could grow continuously and stably in the medium with nivolumab 50 ng/mL, and
98 then cultured drug-free for 1 month, B16-F10 resistance cell line (B16-F10R) were obtained. B16-
99 F10R also needs with nivolumab 5 ng/mL to maintain the resistance (Maeda et al. 1991).
100
101 **Tumor Infiltrating Lymphocyte (TIL)**
102   The tumors were removed from the Balb/c mice, about 2-3 cubic millimeters in size, and
103 cultured with 2 mL complete medium in 24 well plates. The complete medium included 6000
104 IU/mL IL-2 which purchased by Novartis, RPMI-1640 and 2.05 mM L-glutamine were the major
105 component and get from Thermo Fisher Scientific, there is also a need to add 10 % heat-inactivated
106 human AB serum (Omega Scientific, USA), 55 μM 2-mercaptoethanol and 10 mM of HEPES
107 Buffer (Mediatech, USA), gentamicin(50 μg/mL) , penicillin(100 I.U./mL), streptomycin(100
108 μg/mL) were purchased from Invitogen and prevent infection (Arneth, 2019).
109
110 **Western blotting**
111 We investigated the expression of PD-L1, Sema4D, Plexin-B1, PI3K, p-PI3K, AKT and p-AKT.
112 Antibodies against these proteins were purchased from Abcam Shanghai Trading Co., LTD and
113 cell signaling technology, Inc. (Shanghai, China). Total protein was extracted and BCA protein
114 detection kit (Beyotime, China) was used to evaluate the protein concentration. after
115 electrophoresis, transfer membrane, antibody incubation process, the dilute concentration of
116 primary antibody is PD-L1(1:500), Sema4D(1:1000), Plexin-B1(1:1000), PI3K(1:100), p-
117 PI3K(1:1000), AKT(1:1000), p-AKT(1:500), an ECL chromogenic substrate was applied for
118 detecting the signals (Beyotime, China).
119
120 **RT-qPCR**
121 Total RNA was obtained by processing the samples with TRIzol reagent and Rnase-free DNase
122 Set (Beyotime, China). The synthesis of complementary DNA was performed using a cDNA
123 synthesis kit (Thermo Fisher Scientific, USA). Finally, RT-qPCR analysis was performed using

124   an ABI PRISM 7900-HT sequence detection system (Applied Biosystems, USA). For RT-qPCR,

125   the following primers were used.

126   PD-L1:5'-AGAACCTGGCTGCACCTAAC-3'(F),

127   5'-GAGAAGGTCAAACCGCCTCA-3'(R);

128   Plexin-B1:5'-AAGCCCAGCCTACTAACAACC-3'(F),

129   5'-CAGCCCCACTGTACTGACTG-3'(R);

130   Sema4D:5'-TCAAAGCAGACGGAATGCCTA-3'(F),

131   5'-CCCCAACCATGACTGATGTGTA-3'(R);

132   GAPDH:5'-TTGTCATGGGAGTGAACGAGA-3'(F),

133   5'-CAGGCAGTTGGTGGTACAGG-3'(R).

134

135   **shRNA and expression plasmid transfection**

136   Short hairpin RNA (shRNA) targeting Sema4D and the lentivirus containing this shRNA were

137   designed and synthesized by Genepharm Co. (Shanghai, China), sh-sema4D (5′-

138   GGATGGGACTG TCTATGATGT-3′) and the control NC-sema4D (5′-

139   TTCTCCGAACGTGTCACGT-3′). The shuttle plasmid and three other packaging plasmids

140   (pGag/Pol, pRev, pVSV- G) were co-transfected into 293T cells to generate a lentivirus expressing

141   Sema4D shRNA or a control lentivirus expressing shRNA. Cells were inoculated into 6-well plates

142   at 1×10^5 and infected with lentivirus while 5 μg/mL of Polybrene was added. The culture media

143   was changed after 24 hours of infection, and the infected cells were screened with 1 μg/mL of

144   puromycin for 7 days after the culture media change to obtain stable lentiviral shRNA expressing

145   cell lines for subsequent experimental analysis (Moore et al. 2010).

146

147   **Sema4D overexpression**

148   B16-F10 cells were treated with RPMI 1640 containing 10% fetal bovine serum

149   culture medium was placed in 5% CO_2 incubator at 37℃, and logarithmic growth was taken long-

150   term cells were used in the experiment. Construct Sema4D lentivirus overexpression vector LV-

151   Sema4D and negative control LV-NC, these vectors were commissioned by Gemma

152   Pharmaceutical Technology Co., LTD. (Shanghai) to synthesize. B16-F10 cell was inoculated in

153   24-well plates and cultured for 24 h multiplicity of infection, complete culture medium was

154   replaced for further culture 48 h, the expression of Sema4D was detected by RT-qPCR.

155

156   **MTT**

157   Cell viability was measured by Methyl Thiazolyl Tetrazolium(MTT)assay. MTT was

158   dissolved in DMSO and 0.5 mg/mL MTT diluted in 1×PBS for 4 hours, Turn on Benchmark Plus

159   microplate reader (Bio-Rad, 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 Kit(Biotech,

163   China). 5×10^5 B16-F10R cells were seeded in 10-cm culture dishes (Corning, USA) and incubated

164   overnight. 5×10^5 treated cells were collected and resuspended with 500 μL binding buffer. 5 μL

165 Annexin V/AlexaFluor647 were incubate for 5 minutes at room temperature, then add 10 μL
166 propidium iodide (PI), fluorescent dyes should be protected from light. All operations are
167 performed as per manufacturer instructions.
168

169 **Wound healing and invasion analysis**
170 B16-F10R cells invasion and migration was analyzed by Matrigel-coated invasion chambers
171 and scratch assays respectively. The initial number of cells cultured was 5×10^5, a sterile 10 μL
172 pipette tip was used to mark a line in the monolayer of each well, the wounds were observed at 0,
173 24 and 48 hours under a microscope (Nikon, Japan). ImageJ software was used to measure the
174 wound areas. Cell free migration area=(cell free area 0h - cell free area 24h)/cell free area 0h (Ji
175 et al. 2018). 5×10^5 cells were placed into the Matrigel-coated invasion chambers (Corning, USA)
176 to incubation, the upper and lower cultures were separated by polycarbonate membranes, and the
177 cells under study were planted in the chamber, after 48h, the top of Matrigel and invading cells
178 were fixed and stained with 0.1% crystal violet. The microscope (Nikon, Japan) was used to
179 photograph invading cells, and 3 fields were randomly selected and counted each time.
180

181 **Animal and tumorigenesis**
182 Balb/c mice with 12-week-old were purchased from Chendu dossy experimental animals CO.,
183 LTD (SCXK (Chuan) 2020-030, Chengdu, China). The mice were fed unrestricted diet and water
184 intake, and were kept under specific pathogen-free conditions in a laboratory where a 12-hour
185 light/dark cycle was strictly enforced(SYXK(Chuan) 2018-065, Luzhou, China). For each group,
186 at least 5 mice were used (Sanmamed et al. 2015). Tumor volumes were measured on 1, 3, 6, 9,
187 12, 15 and 18 days with caliper, at days 18, mice were sacrificed, tumor weight was measured,
188 tumor volumes were calculated with the formula: tumor volume = $0.52 \times$ length $\times$ width^2 (mm^3).
189

190 **Data collection**
191 Melanoma gene expression profiling data were extracted from the Cancer Genome
192 Consortium (TCGA) database using the "TCGAbiolinks" software package. Expression profiles
193 were converted to transcripts per million mapped reads per kilobase exon model (TPM) and
194 normalized by log2.
195

196 **Weighted co-expression network analysis**
197 To explore the signaling pathways involved in Sema4D, gene co-expression network analysis
198 (WGCNA) was first conducted to determine genes with significant change between high- and
199 low- expression of Sema4D through "WGCNA" package on R project. Topological overlapping
200 measurement was utilized to identify modules most associated with Sema4D expressions.
201 Correlation between modules and traits is calculated by Spearman correlation analysis.
202

203 **Protein-Protein interaction**
204 To explore the potential functions played by Sema4D in various biological processes, we
205 constructed Sema4D-related protein-protein interaction network by STRING (https://cn.string-

206   db.org/).
207

Gene set variation analysis

209   Gene set variation analysis was conducted to reveal the molecular mechanisms under the
210   different Sema4D expression populations in melanoma. The h.all.v7.5.1.symbols.gmt gene set
211   was used to enrichment analysis and was downloaded from MSigDB (https://www.gsea-
212   msigdb.org/gsea/index.jsp).
213

Functional enrichment analysis of Sema4D

215   To explore the mechanism of action of Sema4D involved in PD-1 inhibitor resistance,
216   enrichment analysis on hub genes was performed. "ClusterProfiler" package in R project was
217   utilized to identify the signaling pathways involved in Sema4D within TCGA cohort (which
218   contains 471 melanoma samples).
219

Statistical analysis

221   GraphPad Prism V.7.00 software was utilized for statistical analysis. Different statistical
222   methods were selected according to different experimental purposes, compare the differences
223   between two groups, we chose Student's t test, one-way ANOVA was used to compare the
224   differences between the overall differences. P<0.05 is considered to be statistically significant.
225

RESULTS

Sema4D, Plexin-B1 and PD-L1 upregulation in nivolumab resistance B16-F10 cells

228   To investigate the mRNA and protein expression of Sema4D, Plexin-B1and PD-L1 in
229   sensitive cell line and insensitive cell line of melanoma, mRNA and protein expression of
230   Sema4D, Plexin-B1 and PD-L1 of B16-10 and B16-F10R were detected. Compared with B16-10
231   group, mRNA and protein expression of Sema4D, Plexin-B1and PD-L1 were significantly
232   upregulation in B16-F10R group (*Figure 1A-G)*.
233

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

235   We analyzed the correlation of PD-L1 and Sema4D with bioinformatics, it showed that PD-L1
236   and Sema4D are positively correlated *(Figure 2A)* in TCGA-cohort. Compared with Sema4D-NC
237   group, protein expression of Sema4D in Sema4D-shRNA group was significantly downregulated*,*
238   it indicated that Sema4D silencing is success *(Figure 2B, C)*. Compared with Sema4D-NC group,
239   mRNA and protein expression of PD-L1 in Sema4D-shRNA group was significantly
240   downregulated after nivolumab treatment, which was consistent with the results of bioinformatics
241   *(Figure 2B, D, E)*. Therefore, Sema4D silencing significantly repressed the mRNA and protein
242   expression of PD-L1.
243

Sema4D repression renders B16-F10R cells sensitive to PD-1 treatment

245   To explore the relationship between Sema4D downregulation and nivolumab treatment efficacy

246 in B16-F10 and B16-F10R cells, we investigated the cell viability and cell apoptosis. First, we
247 observe the effects of nivolumab on the cell viability of B16-F10 and B16-F10 resistant cell
248 lines. Compared with the B16-F10 cell lines, nivolumab had inhibitory effect on B16-F10 cell
249 lines(p=0.0018), it is suggested that nivolumab has inhibitory effect on sensitive cell lines.
250 Compared with the B16-F10+nivolumab group, the inhibitory effect of nivolumab on drug-
251 resistant cell lines was decreased significantly(p=0.0025), it is suggested that nivolumab has
252 weak inhibitory effect on resistance cell lines *(Figure 3A)*. Next, we explored the effects of
253 nivolumab on the cell viability of Sema4D overexpressed in B16-F10 cells. First, the mRNA
254 expression of Sema4D was detected, and the results showed that Sema4D expression of LV-
255 Sema4D group was increased significantly compared with the LV-NC group(p=0.0083), it
256 indicates that Sema4D transfection is successful (*Figure 3B*). Through MTT, compared with LV-
257 NC group, nivolumab have little inhibitory effect on B16-F10 cells with Sema4D
258 overexpression(p<0.0001), this suggest that Sema4D overexpression could weaken
259 nivolumab effect, so Sema4D was related to nivolumab sensitivity to B16-F10 cells. (*Figure
260 3C*). We further explored the effect of nivolumab on the cell viability of Sema4D knockdown
261 B16-F10R cell lines, the results showed Sema4D downregulation potentiates anti-PD-1 treatment
262 efficacy in B16-F10R cells. Compared with Sema4D-NC group, the cell viability of Sema4D-
263 shRNA group in combination with nivolumab was decreased significantly(p=0.0186) *(Figure 3D)*
264 and rate of apoptosis cell in Sema4D-shRNA group after treatment with anti-PD-1 was notably
265 increased(p=0.0027) *(Figure 3E)*. Finally, we tested the effect of nivolumab on Sema4D-
266 knockdown melanoma in mice. In vivo experiment, we transplanted B16-F10R cells in the mice
267 and observed the size and weight of tumors treated with nivolumab, tumor volume and weight
268 were significantly delayed in Sema4D-shRNA group mice as compared to Sema4D-NC group
269 mice *(Figure 3F, G, H)*.
270
271
272 **Sema4D repression potentiates the effect of anti-PD-1 on cell invasion and migration**
273 The effect of Sema4D repression with anti-PD-1 treatment on B16-F10R cell invasion and
274 migration was analyzed by Matrigel-coated invasion chambers and scratch assay. The results
275 showed that when knockdown of Sema4D, cell migration of Sema4D-shRNA group was
276 decreased (p<0.0001), the cell-free area between migrating cells from Sema4D-shRNA group
277 was wider than the gap between cells of the Sema4D-NC group *(Figure 4A, B)*. In addition, the
278 number of invaded cells was significantly less in Sema4D-shRNA group in contrast to Sema4D-
279 NC group *(Figure 4C, D)*, these data suggesting that lowering Sema4D could enhance the
280 inhibitory effect of anti-PD-1 on cell migration and invasion.
281
282 **Downregulation of Sema4D inhibit PI3K/AKT expression**
283 The brown module was determined to be most significantly associated with Sema4D expression
284 (r = +-0.67, p-value = 1e-62), which contained a total of 778 genes *(Figure5A-B)*. Gene set
285 variation analysis (GSVA) revealed several significantly upregulated malignancy-associated
286 events (IL-6, JAK, STAT3, MTORC1, and KRAS signaling pathways) in the Sema4D high

287 expression group as well as downregulation of p53 signaling *(Figure 5C)*. Sema4D was
288 significantly associated with oncogenes RHOA, RRAS and immunomodulatory-related genes
289 PTPRC and PLXNC1*(Figure 5D)*. Pathway enrichment analysis revealed that Sema4D is involved
290 in multiple immune-related pathways as well as the PI3K/AKT pathway *(Figure 5E)*. Numerous
291 studies have demonstrated that excessive activation of PI3K/AKT signaling pathway is associated
292 with excessive proliferation of tumor cells, inhibition of apoptosis and attenuation of the efficacy
293 of immune checkpoint inhibitors (Zhang et al. 2022). The expression of PI3K/AKT protein was
294 explored by Western blot. p-PI3K/PI3K, p-AKT/AKT of Sema4D-shRNA group were decreased
295 than those of Sema4D-NC group, which suggesting aberrant activation of the PI3K/AKT signaling
296 pathway in the Sema4D-NC group *(Figure 5F)*.
297
298 **DISCUSSION**
299   Immunotherapy is one important method for melanoma treatment, when alone or in combination
300 with anti-PD-1 and anti-PD-L1 may improve survival rates (Simeone et al. 2015). Nivolumab is a
301 PD-1 inhibitor with high affinity to PD-1 (Hirano et al. 2005). However, the ineffectiveness or
302 resistance of anti-PD-1 to melanoma is a serious problem. In general, anti-PD-1 resistance are
303 mainly derived into acquired resistance and innate resistance (Bridgeman et al. 2014; Marincola
304 et al. 2000), (Bronte et al. 2005; O'Donnell et al. 2017). The causes of anti-PD-1 or anti-PD-L1
305 resistance to melanoma are very complex and not fully understood. This study intends to elucidate
306 that Sema4D may be involved in the sensitivity of melanoma to nivolumab therapy via PI3K/AKT
307 signaling pathway.
308   Nivolumab is a PD-1 inhibitor, PD-1 exists in T cells while PD-L1 exists in tumor cells, so the
309 effect of nivolumab on tumor cells requires the presence of T cells. B16-F10 is a melanoma cell
310 line derived from C57BL/6J mice, but tumor formation can also be inoculated subcutaneously in
311 Balb/c mice(Lucas L Colombo et al., 2019). In this experiment, Tumor Infiltrating Lymphocyte
312 (TIL) was obtained from Balb/c mice and added to B16-F10 or B16-F10R cell to simulate tumor
313 microenvironment. B16-F10 resistance cells were implanted subcutaneously in Balb/c mice by
314 subcutaneous injection to observe tumor volume and weight.
315 Sema4D expressed on many cancer cells, Plexin-B1 also expression in cancer such as ovarian,
316 melanoma, when Sema4D combined with Plexin-B1, it correlates with tumor immune infiltration,
317 angiogenesis, and tumor progression in melanoma (Ch'ng & Kumanogoh 2010; Lu et al. 2021).
318 To understand the expression of Sema4D in melanoma resistance to anti-PD-1, B16-F10 resistant
319 cell lines was established, after administered 50 ng/ml nivolumab, the killing ability of nivolumab
320 was significantly weaker than that of B16-F10 cell lines, this means that resistant cell lines are
321 established successfully. Then we explored mRNA and protein expression of Sema4D and Plexin-
322 B1 in B16-F10 cell lines and B16-F10R cell lines, our results revealed that the mRNA and protein
323 expression of Sema4D and Plexin-B1 in drug-resistant cell lines were overexpressed. Therefore,
324 we provide the evidence for the first time that Sema4D and Plexin-B1 is related to anti-PD-1
325 resistance of melanoma.
326   To further analyzed the relationship between Sema4D and nivolumab, Sema4D were
327 overexpression in B16-F10 cells and Sema4D expression was silencing by shRNA in B16-F10R

328   cells. It was observed that overexpression of Sema4D can attenuates the therapeutic effect of
329   nivolumab in B16-F10 cells and downregulation of Sema4D will increase the sensitivity of B16-
330   F10R cell lines to nivolumab. In addition, we also investigated the effects of Sema4D knockdown
331   on B16-F10R cells in response to nivolumab, downregulation of Sema4D also increased the
332   inhibiting the growth of tumor cells and reducing cell invasion and migration. As reported by
333   Rezaeepoor that silencing of Sema4D serve as a therapeutics target for the suppression of invasion,
334   migration(Rezaeepoor et al. 2021).A study conducted by Rashidi illustrated silencing of Sema4D
335   can elevated apoptosis rate of SW48 cells in response to 5-FU treatment (Rashidi et al. 2020).
336   Moreover, we verified the enhancement of anti-PD-1 sensitivity by Sema4D knockdown in vivo
337   xenograft models, mice treated with nivolumab after Sema4D knockdown showed significantly
338   slower tumor growth. Taken together, these finding demonstrated that Sema4D deficiency can
339   potentiates nivolumab treatment efficacy.
340   We further analyzed the correlation between Sema4D and PD-L1 expression in anti-PD-1
341   resistance. It has been demonstrated that the presence of PD-L1 determines the response to anti-
342   PD-1/PD-L1 therapy (Sunshine et al. 2017), regorafenib can promotes antitumor immunity via
343   inhibiting PD-L1 expression in melanoma (Wu et al. 2019). JAK1/2 mutation leads to the lack of
344   reactive PD-L1 expression, which is involved in the resistance of PD-1/PD-L1 inhibitors (Shin et
345   al. 2017). In this study, we evaluated PD-L1 expression in B16-F10, B16-F10R cell and B16-F10R
346   cell knockdown with Sema4D-shRNA, the results showed PD-L1 expression in B16-F10R was
347   increased and Sema4D knockdown group is decrease, these suggest that the effects of Sema4D
348   knockdown on Nivolumab in B16-F10R cell lines may be related to the decrease of PD-L1
349   expression. However, which way contributes to the PD-L1 downregulation after knockdown
350   Sema4D remains unclear and needs further investigation.
351   PI3K is phosphorylated by binding to GRB2, and AKT phosphorylates target genes to regulate
352   cell function (Pu et al. 2021). Sema4D and plexin-B1 is associated with osteosarcoma, in which
353   PYK2-PI3K-Akt pathway is activated to promote tumor progression(Li et al. 2021). To explore
354   the relationship between Sema4D and PI3K/AKT signaling pathways in anti-PD-1 resistance, we
355   detected the expression of PI3K/AKT protein, in B16-F10R cell, p-PI3K/PI3K, p-AKT/AKT
356   expression were significantly increased, when inhibited the Sema4D, p-PI3K/PI3K, p-AKT/AKT
357   expression of Sema4D-shRNA group were decreased, these shown that Sema4D can regulate
358   PI3K/AKT expression and activity. Bioinformatics analysis revealed that Sema4D is involved in
359   the PI3K/AKT signaling pathway which is activated and plays important roles such as being
360   involved in the development and occurrence in melanoma and it also activate receptor tyrosine
361   kinases (Davies 2012). However, whether the relationship between Sema4D and nivolumab
362   sensitivity is related to JAK-STAT signal pathway and other signal pathways needs further study.
363   In conclusion, we explored the role of Sema4D in the process of PD-1 inhibitor resistance. In
364   anti-PD-1 resistant cell line, upregulation of Sema4D, Plexin-B1 and PD-L1 was observed. When
365   Sema4D was silencing, it prevented the phosphorylation of PI3K and AKT, thus inhibiting the
366   PI3K/AKT pathway activity. Therefore, these results may have therapeutic implication and may
367   be exploited for the development of novel treatment for anti-PD-1 resistance melanoma in the
368   future.

Manuscript to be reviewed

369

Acknowledgments

371   Not applicable.

372

Authors' contributions

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

378

Funding

380   This study was supported by grants from Sichuan Provincial Science and Technology
381 Department Project(2022NSFSC0816, 2022YFS0619-C1), Project of Luzhou Science and
382 Technology Bureau(2021JYJ-82).

383

Availability of data and materials

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

387

Declarations

Ethics approval and consent to participate

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

393

Consent for publication

395   Not applicable.

396

Competing interests

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

399

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531

532 **Figure Legends**

533 Figure 1. | Sema4D, Plexin-B1 and PD-L1 expression in B16-F10 and B16-F10R cells. Cells were
534 derived into B16-F10R group(B16-F10R cells) and B16-F10 (B16-F10 cells), 24 hours after cell
535 culture, Sema4D, Plexin-B1 and PD-L1 expression were detected. (A) Protein bands of Sema4D,
536 Plexin-B1 and PD-L1. (B) Ratio to GAPDH of Sema4D. Image J V1.8.0 software(USA) was used
537 to measure the gray value of the value of the protein band, and then the relative density value was
538 obtained by comparing the value of GAPDH with the target protein, p = 0.0026. (C) Ratio to
539 GAPDH of Plexin-B1, p value = 0.0018. (D) Ratio to GAPDH of PD-L1, p value = 0.0016. (E)
540 mRNA expression of Sema4D, p value = 0.037. (F) mRNA expression of Plexin-B1, p = 0.0312.
541 (G) mRNA expression of PD-L1, p = 0.0212. Compared with B16-F10 group, Sema4D and Plexin-
542 B1 were significantly upregulated in B16-F10R group. * is p<0.05, ** is p<0.01, *** is p<0.001.
543 Repeat the experiment three times (n = 3), t test was used for statistical analysis.

544

545 Figure 2. | Sema4D silencing decrease PD-L1 expression. B16-F10R Cell were derived into
546 Sema4D-shRNA group and Sema4D-NC group, co-cultured with TIL at a 1:5 ratio and nivolumab
547 50 ng/ml 24 hours, collect the cell to detect the expression of PD-L1. (A) Correlation between PD-
548 L1 and Sema4D (B) Protein bands of PD-L1and Sema4D. (C) Ratio to GAPDH of Sema4D, p =
549 0.0002. (D)Ratio to GAPDH of PD-L1, p = 0.0016. (E) mRNA expression of PD-L1. Compared
550 with Sema4D-NC group, ** is p<0.01, *** is p<0.001. The experiment was repeated three times,
551 n=3, t test was used for statistical analysis.

552

553 Figure 3. | Sema4D **repression** renders B16-F10R cells sensitive to nivolumab treatment. (A)
554 Effects of nivolumab on cell viability of B16-F10 and B16-F10R. B16-F10 cells were derived into
555 B16-F10 and B16-F10+nivolumab group, B16-F10R cells were derived into B16-F10R and B16-
556 F10R+nivolumab groups, B16-F10 and B16-F10R groups were as control group without
557 treatment, B16-F10+nivolumab and B16-F10R+nivolumab groups were treatment with 50ng/mL
558 nivolumab and co-cultured with TIL at a 1:5 ratio (van den Berg et al. 2020). Compared with the
559 B16-F10+nivolumab group, ** is p<0.01, n = 3, one-way ANOVA was used to compare the
560 differences. (B) RNA expression of Sema4D overexpression. Cells were derived into B16-
561 F10+LV-Sema4D and B16-F10+LV-NC group, compared with the LV-NC group, ** is p < 0.01,
562 n = 3, t-test was used to compare the differences. (C) Effects of nivolumab on B16-F10 cell
563 viability after Sema4D overexpression. B16-F10 cells were derived into B16-F10, B16-
564 F10+nivolumab, B16-F10+LV-Sema4D and B16-F10+LV-NC group, B16-F10 is control group,
565 another 3 groups were treatment with 50ng/mL nivolumab and co-cultured with TIL at a 1:5
566 ratio, B16-F10+LV-Sema4D group was also treated with overexpression Sema4D, B16-
567 F10+LV-NC group was also treated with Sema4D negative control. Compared with B16-F10
568 group, **** is p < 0.0001; Compared with B16-F10+LV-NC group, #### is p < 0.0001, n = 3,
569 one-way ANOVA was used to compare the differences. (D) Effects of nivolumab on B16-F10R
570 cell after Sema4D silencing. B16-F10R cells were derived into B16-F10R, B16-F10R+nivolumab,
571 Sema4D-shRNA and Sema4D-NC group, B16-F10R is control group, another 3 groups were
572 treatment with 50ng/mL nivolumab and co-cultured with TIL at a 1:5 ratio, Sema4D-shRNA group
573 was knockdown Sema4D, and Sema4D-NC group was Sema4D-shRNA negative control group.

574 Compared with Sema4D-shRNA group, * is p<0.05, ** is p<0.01, n=3, one-way ANOVA was
575 used to compare the differences. (E) B16-F10R cell apoptosis rate after treatment with nivolumab.
576 B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-NC group, the treatment
577 is the same as C. Cell apoptosis was detected by PI and Annexin V/Alexa Fluor 647 double
578 staining, Alexa Fluor 647 was a fluorescent dye binding with Annexin V, reflecting early apoptosis
579 of cells. Compared with Sema4D-NC group, ** is p<0.01, n=3, t-test was used to compare the
580 differences. (F) Tumor volume of knockdown Sema4D with nivolumab treatment. Balb/C mice
581 were derived into Sema4D-shRNA group (n=6) and Sema4D-NC group(n=5), B16-F10R-
582 Sema4D-shRNA cells and B16-F10R-Sema4D-NC cells were intradermal injected into the right
583 side of mice, then intraperitoneally injected nivolumab 10 mg/kg once at day 1, t-test was used to
584 compare the differences. (G) Tumor weight of knockdown Sema4D with nivolumab, t-test was
585 used to compare the differences. Compared with Sema4D-NC group, *** is p<0.001, **** is
586 p<0.0001. (H) Mouse melanoma specimen. Major marks represent cm on the scale.

587

588 Figure 4. | Sema4D deficiency potentiates nivolumab inhibitory effects on B16-F10R cell
589 invasion and migration. B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-
590 NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50 ng/ml, cell invasion and
591 migration were detected. (A) cell migration. Representative image of cell migration in the
592 absence and presence of shRNA Sema4D at 0 h, 24 h, and 48 h. The scratched areas were
593 measured in three random fields in each group. Wound healing analysis showed a significant
594 difference in the cell-free area of Sema4D-shRNA group was significantly wider than that of
595 Sema4D-NC group at 24 and 48 hours (p < 0.0001). (B) Ratio of cell free area. (C) chamber
596 invasion of cells. Chamber invasion analysis showed a significant difference lower number of
597 invasive cells in Sema4D-shRNA group than Sema4D-NC group (×100) at 48h. (D) cell invasion
598 number per field. Compared with Sema4D-NC group, **** is p<0.0001. The expression was
599 repeated three times, n =3.

600

601 Figure 5. | Sema4D knockdown is significantly associated with inhibition of PI3K-AKT
602 signaling pathway. (A) Association of clustered modules with Sema4D expression. The brown
603 module was identified as the most relevant module for Sema4D expression. (B) Module-trait
604 associations. (C) Gene set variation analysis revealed several significantly dysregulation
605 malignancies events between different Sema4D expression population. (D) Protein-protein
606 interactions of Sema4D. (E) Pathway enrichment analysis showed that Sema4D is involved in
607 the PI3K-AKT signaling pathway. (F) PI3K-AKT signaling pathway proteins expression after
608 Sema4D knockdown. B16-F10R cells were derived into Sema4D-shRNA group and Sema4D-
609 NC group, co-cultured with TIL at a 1:5 ratio and nivolumab 50 ng/ml, PI3K, AKT protein
610 expression were detected. The experiment was repeated three times, n = 3. Western blotting
611 showed that Sema4D was significantly associated with inhibition of PI3K-AKT signaling
612 pathway. Compared with Sema4D-NC group, ** is p<0.01.

613

614

Figure 1

Figure 1

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. Image J V1.8.0 software(USA) was used to measure the gray value of the value of the protein band, and then the relative density value was obtained by comparing the value of GAPDH with the target protein, p = 0.0026. (C) Ratio to GAPDH of Plexin-B1, p value = 0.0018. (D) Ratio to GAPDH of PD-L1, p value = 0.0016. (E) mRNA expression of Sema4D, p value = 0.037. (F) mRNA expression of Plexin-B1 , p = 0.0312. (G) mRNA expression of PD-L1, p = 0.0212. 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. Repeat the experiment three times (n = 3), t test was used for statistical analysis.
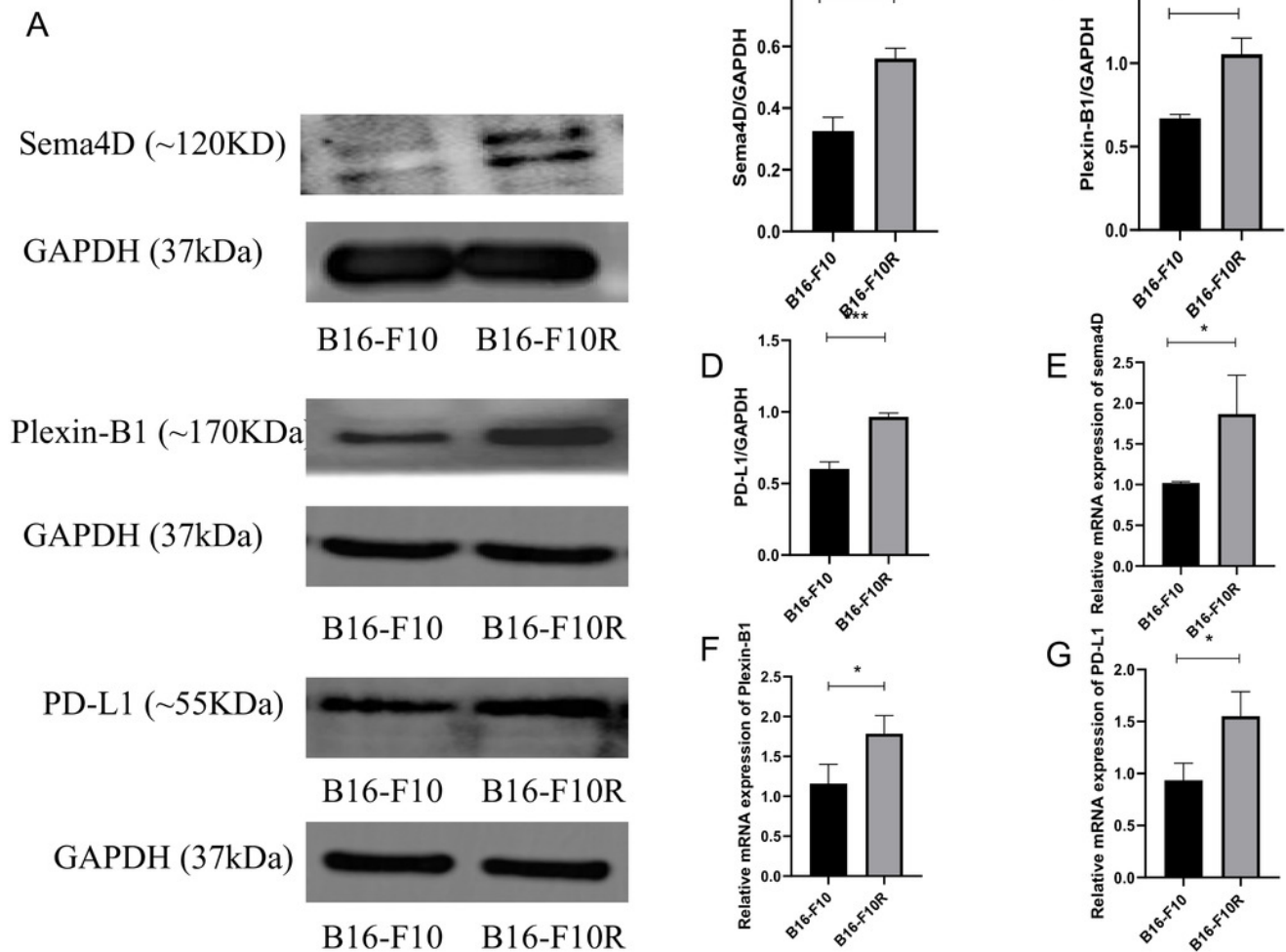
Figure 2

Figure 2

Figure 2. | Sema4D silencing decrease 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, p = 0.0002. (D)Ratio to GAPDH of PD-L1, p = 0.0016. (E) mRNA expression of PD-L1. Compared with Sema4D-NC group, [**] is p<0.01, [***] is p<0.001. The experiment was repeated three times, n=3, t test was used for statistical analysis.

A

B

C

D

E
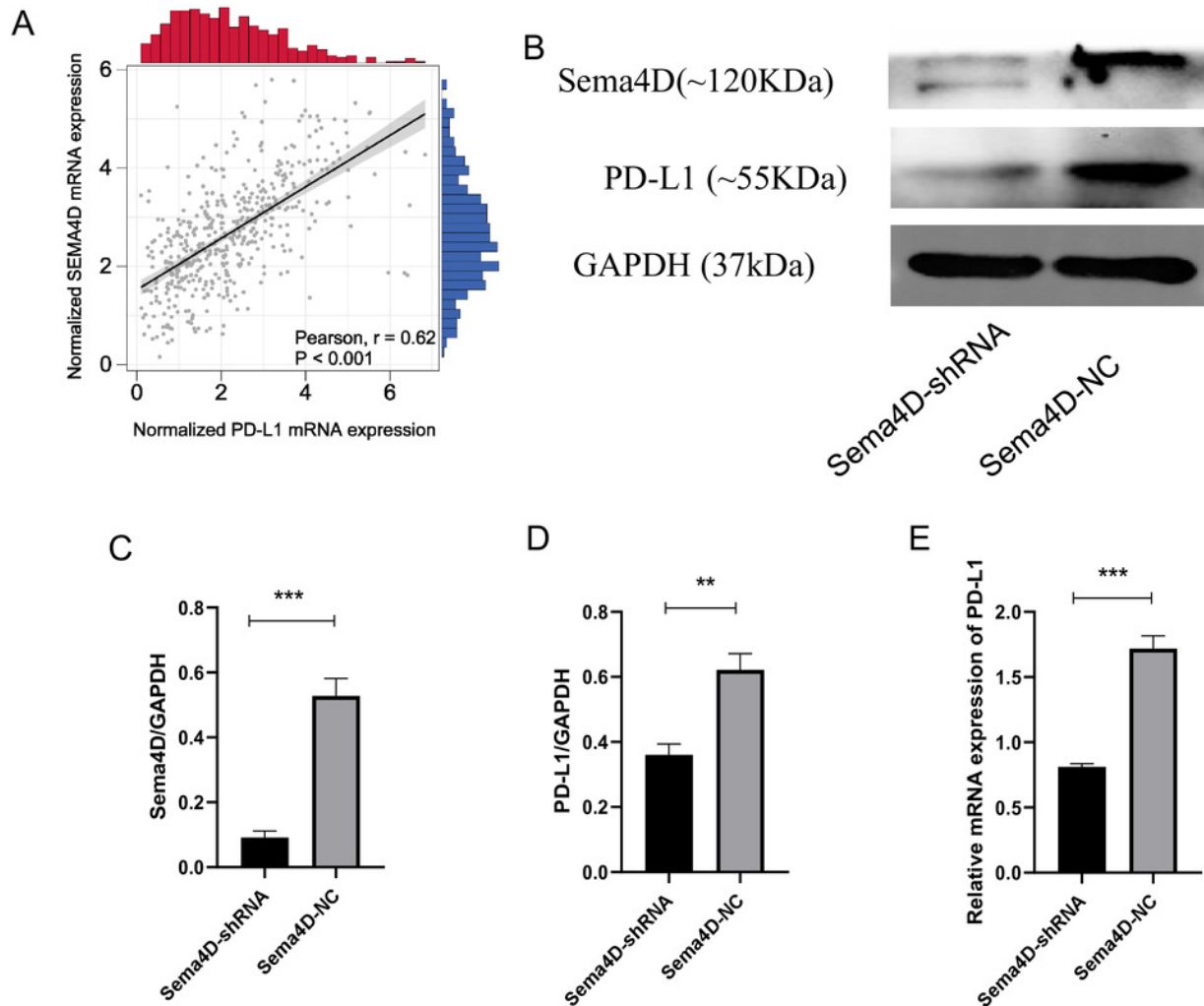
Figure 3

Sema4D repression renders B16-F10R cells sensitive to nivolumab treatment.

Figure 3. | Sema4D **repression** 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 the B16-F10+nivolumab group, [**] is p<0.01, n = 3, one-way ANOVA was used to compare the differences. (B) RNA expression of Sema4D overexpression. Cells were derived into B16-F10+LV-Sema4D and B16-F10+LV-NC group, compared with the LV-NC group, ** is p < 0.01, n = 3, t-test was used to compare the differences. (C) 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, n = 3, one-way ANOVA was used to compare the differences. (D) Effects of nivolumab on B16-F10R cell after Sema4D silencing. 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, n=3, one-way ANOVA was used to compare the differences. (E) 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. Cell apoptosis was detected byPI and Annexin V/Alexa Fluor 647 double staining, Alexa Fluor 647 was a fluorescent dye binding with Annexin V, reflecting early apoptosis of cells. Compared with Sema4D-NC group, ** is p<0.01, n=3, t-test was used to compare the differences. (F) Tumor volume of knockdown Sema4D with nivolumab treatment. Balb/C mice were derived into Sema4D-shRNA group (n=6) and Sema4D-NC group(n=5), 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, t-test was used to compare the differences. (G) Tumor weight of knockdown Sema4D with nivolumab, t-test was used to compare the differences. Compared with Sema4D-NC group, *** is p<0.001, **** is p<0.0001. (H) Mouse melanoma specimen. Major marks represent cm on the scale.
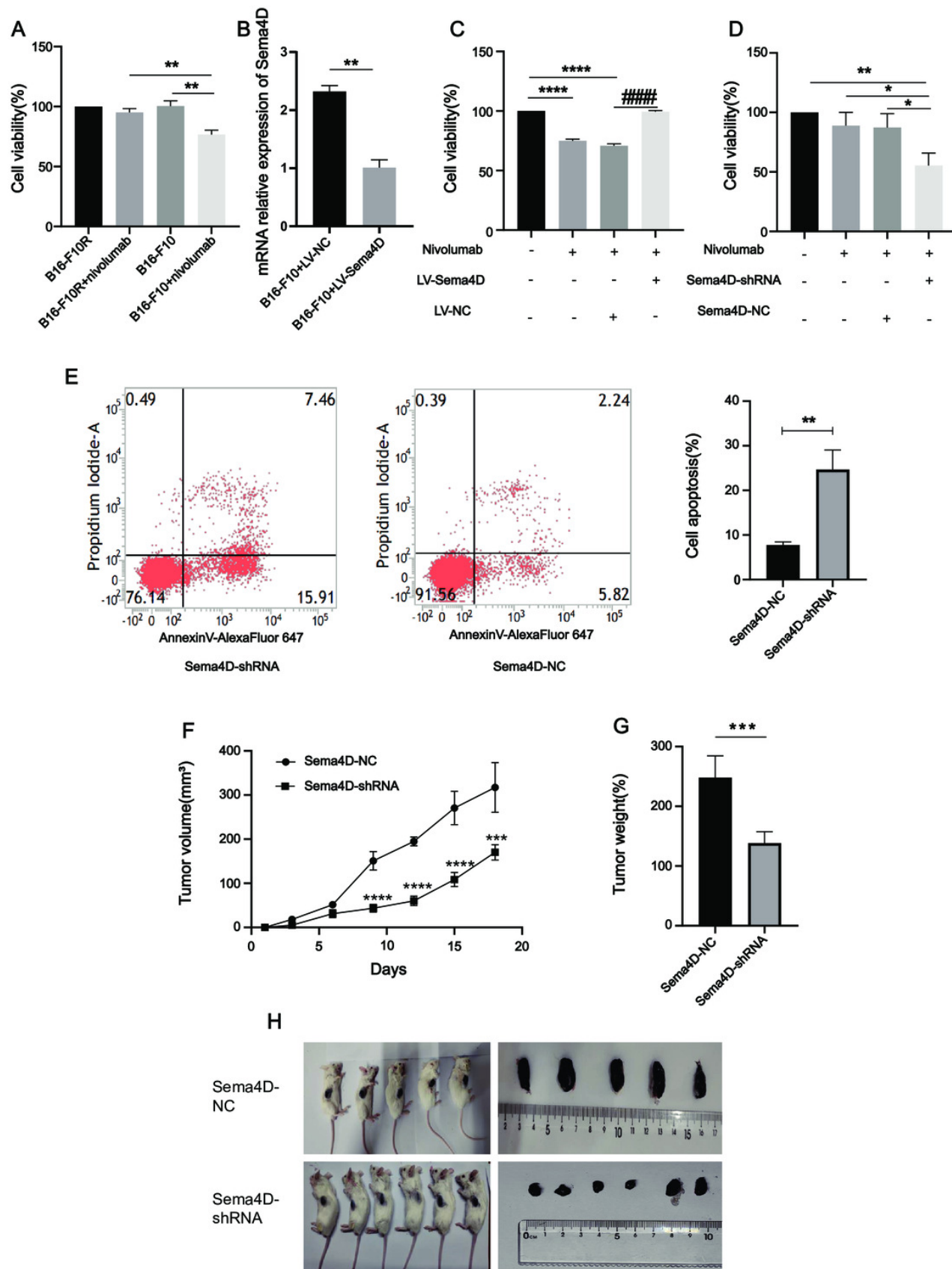
Figure 4

Figure 4

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 (×100) at 48h. (D) cell invasion number per field. Compared with Sema4D-NC group, [****] is p<0.0001. The expression was repeated three times, n =3.

A

0h

24h

48h

Sema4D-shRNA   Sema4D-NC

B

Sema4D-NC
Sema4D-shRNA

Ratio of Cell free area(%)

****

****

0h   24h   48h

C
48h

Sema4D-NC   Sema4D-shRNA

D

Cell invasion number per field (×200)

****

Sema4D-NC   Sema4D-shRNA
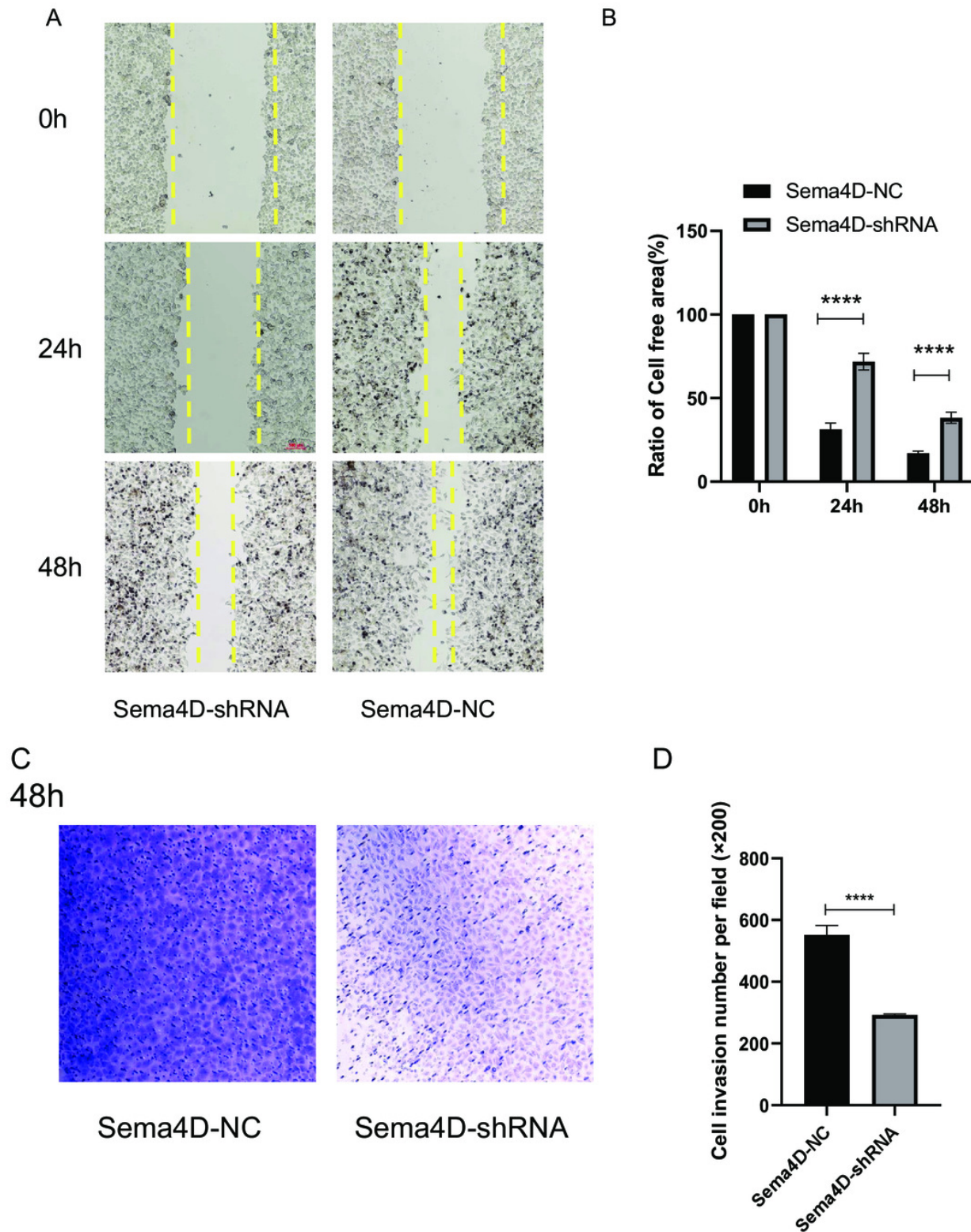
Figure 5

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. The experiment was repeated three times, n = 3. Western blotting showed that Sema4D was significantly associated with inhibition of PI3K-AKT signaling pathway . Compared with Sema4D-NC group, [**] is p<0.01.