Targeting neuropilin-1 interactions is a promising anti-tumor strategy

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Abstract

Neuropilins (NRP1 and NRP2) are multifunctional receptor proteins that are involved in nerve, blood vessel, and tumor development. NRP1 was first found to be expressed in neurons, but subsequent studies have demonstrated its surface expression in cells from the endothelium and lymph nodes. NRP1 has been demonstrated to be involved in the occurrence and development of a variety of cancers. NRP1 interacts with various cytokines, such as vascular endothelial growth factor family and its receptor and transforming growth factor β1 and its receptor, to affect tumor angiogenesis, tumor proliferation, and migration. In addition, NRP1^{+} regulatory T cells (Tregs) play an inhibitory role in tumor immunity. High numbers of NRP1^{+} Tregs were associated with cancer prognosis. Targeting NRP1 has shown promise, and antagonists against NRP1 have had therapeutic efficacy in preliminary clinical studies. NRP1 treatment modalities using nanomaterials, targeted drugs, oncolytic viruses, and radio-chemotherapy have gradually been developed. Hence, we reviewed the use of NRP1 in the context of tumorigenesis, progression, and treatment.

Keywords: Neuropilin-1; Anti-tumor; Immunotherapy; Tumor targeting

Introduction

Neuropilins (NRPs) is unique to vertebrates and is a highly conserved multifunctional type I single-pass transmembrane protein about 130,000 to 140,000 Da in size. It is involved in various physiological and pathological processes in the body.[1-5] NRPs include two subtypes, that is, NRP1 and NRP2. They regulate cell function by acting as co-receptors for multiple ligands. NRPs have been demonstrated to be involved in angiogenesis, cell migration, immune cell regulation, axon growth, and so on.[6-11] NRP1 is essential for the development of neurons and the cardiovasculature, while NRP2 plays a key role in neuronal patterns and lymphangiogenesis.[1,12-14]

Increasing evidence has demonstrated that high NRP1 expression is closely associated with tumor occurrence, progression, invasion, metastasis, and prognosis.[15-19] NRP1 can not only form complexes directly with vascular endothelial growth factor A (VEGFA) and vascular endothelial growth factor receptor 2 (VEGFR2) to enhance angiogenesis, but also promote RhoA activation after binding with VEGFA to directly affect the growth and metastasis of tumor cells and promote tumor development. In addition, NRP1 can also accelerate tumor progression by stabilizing the function of regulatory T cells (Tregs) and preventing tumor-associated macrophages (TAM) from entering the normoxic tumor area.[19] NRP1 has become a key therapeutic target for tumor therapy. Antagonists that target NRP1 have shown promise in several studies.[20]

Structure, Expression, and Function of NRP1 Protein

The structure of NRP1 protein

NRP1 was discovered in 1987 and was originally named A5. It was discovered as an antigen of a monoclonal antibody that was bound to neuronal cell surface proteins in the Xenopus nervous system.[6] The *NRP1* gene is 112 kb in length and is located on the human chromosome 10q12. It contains 17 exons and 16 introns.[21] NRP1 has an intracellular, transmembrane, and extracellular domain. Its intracellular domain is relatively small, lacks an inherent kinase domain, and does not participate in signal transduction. Its extracellular domain consists of five subdomains, that is, a1, a2, b1, b2, and c, with each subdomain associated with different molecular and/or cellular interactions [Figure 1].

The expression of NRP1 protein

NRP1 was originally found to be expressed in neurons, but later, was observed to be also expressed on the surface of

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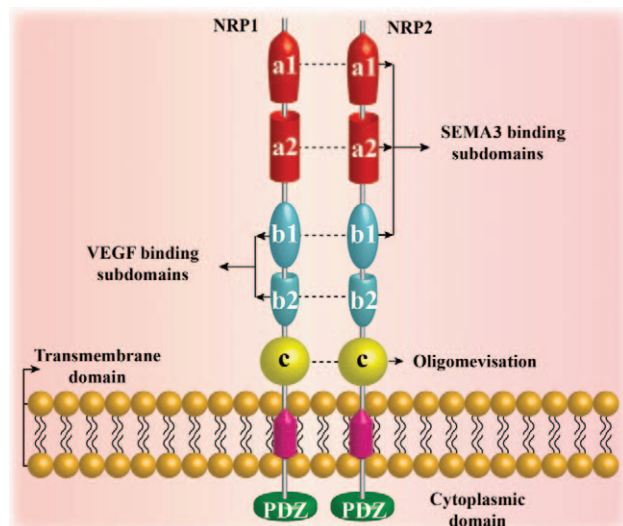E-Mail: yongxiang_zhao@126.com

Figure 1: Schematic of the neuropilin (NRP) molecular structure. NRP1 and NRP2 are unique transmembrane glycoproteins in vertebrates. In humans, *NRP1* is located on chromosome 10, and *NRP2* is located on chromosome 2. They have about 44% sequence homology at the amino acid level. The overall structure of the two NRPs is similar, including a large N-terminal extracellular domain, a short transmembrane domain, and a small cytoplasmic domain. The extracellular domain is divided into three domains: the complement protein binding homology domain (CUB domain or a1a2 domain), coagulation factor V/VIII homology domain (b1b2 domain), and the MAM domain (c domain). The a1a2 b1 domain binds to SEMA3, the b1b2 domain binds to vascular endothelial growth factor (VEGF), and the c domain is considered to play a role in NRP1 oligomerization. The C-terminus of NRP contains a three amino acid (Ser-Glu-Ala) sequence called SEA, which binds to the kinase through the PDZ domain.
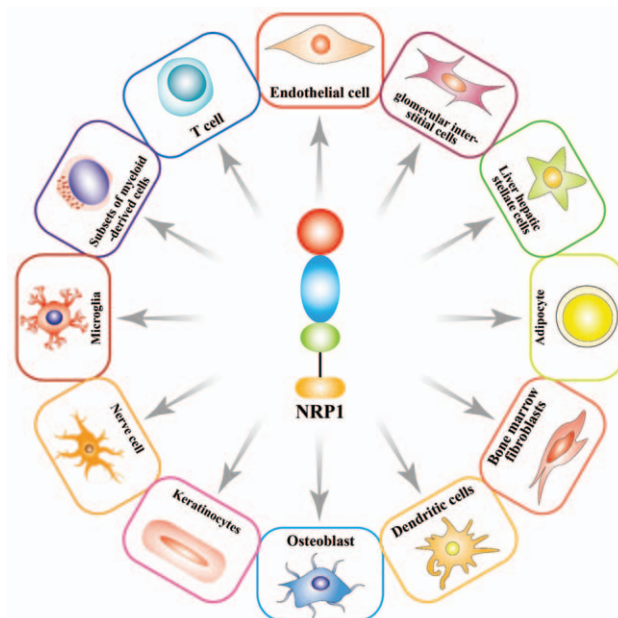

Figure 2: Cell types expressing neuropilin-1 (NRP1). NRP1 is expressed in several cell types, including endothelial cells, T lymphocytes, myeloid cell subsets, microglia, nerve cells, keratinocytes, osteoblasts, dendritic cells, bone marrow fibroblasts, fat cells, hepatic stellate cells, and glomerular interstitial cells.

several types of cells. High expression levels of NRP1 have been observed in osteoblasts, nerve cells, immune cells, adipocytes, glomerular stromal cells, endothelial cells, and hepatic stellate cells, and so on[22-25] [Figure 2]. Almost all tumor cells express NRP1 or NRP2 or both. These include certain leukemias, malignant melanomas, malignant gliomas, osteosarcomas (OSs), lung cancer, gastric cancer, and so on. Expression of both NRP1 and NRP2 has been associated with poor prognosis.[26,27]

The function of NRP1 protein

NRP1 was initially identified as a co-receptor for class 3 semaphorins (Sema3A). It forms a dimer with plexin A3 and is involved in axon guidance and nervous system development.[28] Later studies have found that NRP1 could form cis-acting complexes with the vascular endothelial growth factor (VEGF) family and its receptor (VEGFR) on the same cell to promote tumor angiogenesis.[29,30] Recent studies have shown that NRP1 could interact with glycosylation-dependent galectin-1 to activate transforming growth factor β1 (TGF-β1) and its receptors to accelerate liver fibrosis. In addition, NRP1 could promote cell migration induced by hepatocyte growth factor (HGF) or platelet-derived growth factor (PDGF) by phosphorylating p130Cas. Furthermore, NRP1 activates fibroblast growth factors (FGFs) and their receptors by interacting with heparin-binding proteins. NRP1 interacts with a variety of activated tyrosine kinase receptors and integrins to enhance tumor growth, survival, and invasion. NRP1 has been shown to play a regulatory role in the immune system. Overexpression of NRP1 on the

surface of dendritic cells (DC) and Tregs has been demonstrated to play a role in promoting tumor development.[31-33]

NRP1 Functions in a Variety of Immune Cells

NRP1 is widely expressed in lymphoid and myeloid cells. *In vitro* and *in vivo* studies have demonstrated its important role in the immune response, cell proliferation, chemotaxis, and cytokine production in DC.[34-36] The occurrence and development of tumors have been linked to immune cell function.

The role of NRP1 in Tregs

T cells, an important type of immune cell in the body, are involved in all aspects of tumor progression. A subset of T-cells, Tregs, are involved in inhibiting anti-tumor immunity. Tregs that infiltrate tumors inhibit the anti-tumor effects of CD4+ and CD8+ T cells through multiple pathways. This results in immune escape and tumor progression, that is, anti-cancer immunity of the micro-environment (TME).[37-40] In recent years, NRP1 has been demonstrated to play a role in the stability and function of Tregs. NRP1 interacts with the ligand Semaphorin-4a (Sema4a) expressed on Tregs to enhance the function and survival of Tregs in tumors. This in turn restricts the anti-tumor immune response.[41-44] In mouse models, knockout of the *NRP1* gene acting on Tregs could reduce tumor growth. This highlights the importance of NRP1 in suppressing anti-tumor immunity.

NRP1 has also been shown to act on DC. Sema4A secreted by DCs bind to NRP1 on Tregs and recruit PTEN to inhibit AKT phosphorylation. This in turn promotes the nuclear
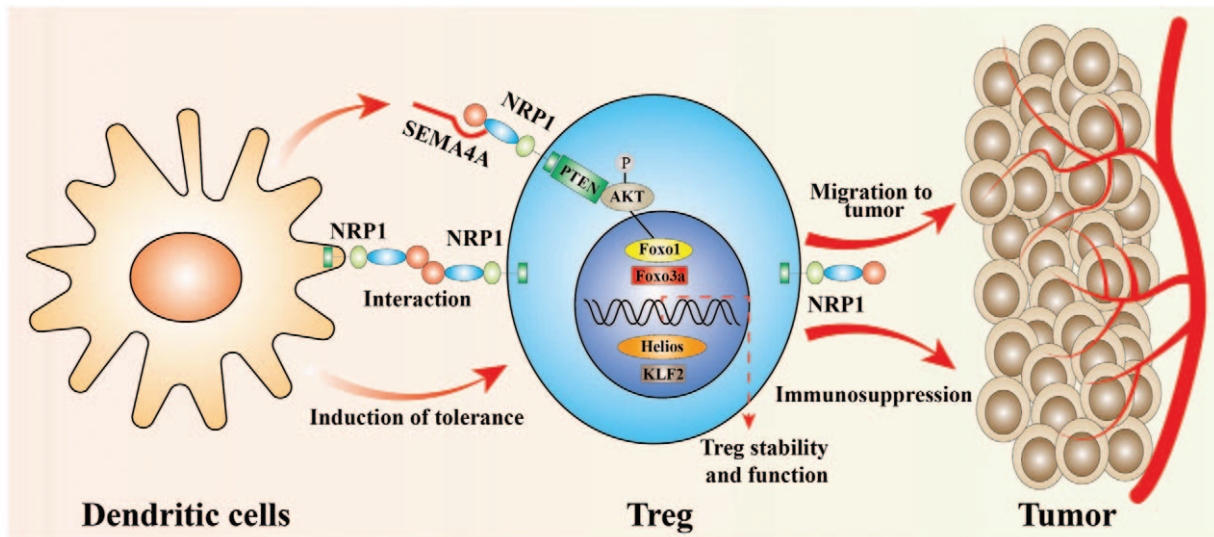
Figure 3: Schematic depicting the role of neuropilin-1 (NRP1) in dendritic cells (DC) and regulatory T cells (Tregs). NRP1 is mainly associated with the inhibitory function of Tregs. NRP1 is important for the formation of immune synapses between dendritic cells (DC) and T cells. Sema4A secreted by DC binds to NRP1 and recruits phosphatase and tensin homolog deleted on chromosome ten (PTEN) to inhibit protein kinase B (AKT) phosphorylation, thereby promoting the nuclear translocation of Forkhead box O3 (Foxo3a). This is important for the survival and stability of Tregs. NRP1 also plays an important role in the migration of Tregs into the tumor microenvironment in response to tumor cell-derived vascular endothelial growth factor.

translocation of Foxo3a, which is important for the survival and stability of Tregs[45] [Figure 3]. Jung et al[20] demonstrated enhanced anti-tumor activity by inhibiting the function of Tregs in a mouse tumor model using NRP1 antagonists. In addition, Overacre-Delgoffe et al[46] demonstrated that a high percentage of NRP1+ Tregs in patients with melanoma and squamous cell carcinoma of the head and neck were associated with poor prognosis.

Wang et al[47] found that NRP1 signaling-mediated accumulation of Tregs in tumors may play a key role in aggravating ischemic brain damage in tumor-bearing mice. When anti-NRP1 was combined with anti-PD-1 immunotherapy, it could enhance CD8+ T cell proliferation, cytotoxicity, and tumor control.[48] Hence, targeting NRP1 in combination with immunotherapy may be a promising approach.

The role of NRP1 in TAM

In addition to Tregs, TAM also play a role in promoting tumor progression. TAMs are macrophages in the tumor stroma. They participate in the process of tumorigenesis, growth, infiltration, and spread, and has been associated with tumor angiogenesis and lymphangiogenesis.[49-54] Deletions in the NRP1 gene in macrophages facilitate the entry of TAMs into the area of normoxic tumors. This reduces the pro-angiogenic and immunosuppressive functions of TAMs and inhibits the growth and metastasis of tumors.[44,55,56] Conversely, when TAM are recruited to avascular areas, tumor progression could be maintained.[55] These results were supported by the study conducted by Miyauchi et al[57] Hence, modulation of NRP1 in peripheral macrophages or microglia could make them more anti-tumorigenic, reduce neovascularization, and modulate glioma adaptive immune response.

Correlation Between NRP1 Expression and Tumor-initiating Cells (TIC)

Recent studies have demonstrated the relationship between NRP1 expression and TIC. TIC have the capacity for self-renewal and are responsible for the initiation and maintenance of a tumor.[19,58-61] TICs have been extensively investigated for their function.[62]

Recent studies have demonstrated that endothelial progenitor cells could be identified by their expression levels of NRP1. NRP1 is essential for the proliferation and cell migration of adult mesenchymal stem cells.[63-66] In addition, NRP1 maintains a tumor-initiating phenotype in gliomas and skin cancer cells.[67] Jimenez-Hernandez LE et al and others have also demonstrated that cells expressing NRP1 exhibit similar characteristics as TIC with high clonal ability. This suggests that NRP1+ lung cancer cells have tumor-initiating properties.[19] These findings provide new insights for cancer treatment and potential biomarkers for the study of TIC.

NRP1 Promotes Tumor Angiogenesis

Angiogenesis is essential during tumorigenesis and malignancy. Angiogenesis is a complex mechanism that induces new capillary formation from pre-existing vessels. The signaling pathways include the involvement of NRP1 and VEGF and their interactions with receptor VEGFRs.[68-71] Studies have confirmed that knocking out *NRP1* in mice can affect the development of nervous and cardiovascular systems.

VEGFA is the predominant VEGF and is one of the main stimuli to induce angiogenesis. Within the VEGFA family, VEGF165 has a major role in neovascularization. The
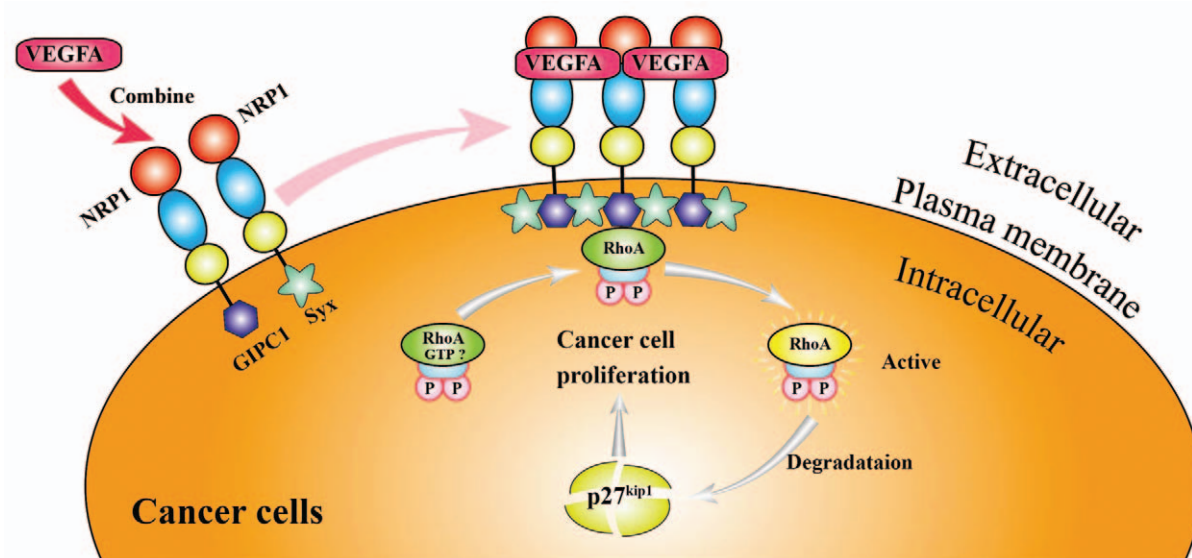
Figure 4: VEGFA induces RhoA protein activation through NRP1 to promote tumor cell proliferation. When VEGFA binds to NRP1, it promotes the interaction between NRP1 and GIPC1 (a scaffold protein) and enhances the assembly of the molecular complex of GIPC1 and Syx, resulting in GTP binding of RhoA. The active form is increased and activated RhoA contributes to the degradation of p27kip1. This promotes tumor cell proliferation. GIPC1 has anti-apoptotic effects in human breast cancer and colorectal cancer cells. Syx is involved in endothelial cell migration and endothelial cell connection integrity, barrier function, and vascular leakage. GTP: Guanosine triphosphate; NRP1: Neuropilin-1; RhoA: ras homolog family member A; Syx: Synectin-binding guanine exchange factor; VEGFA: Vascular endothelial growth factor A.

carboxy terminus of the gene encodes exons 7 and 8 and binds with the b1/b2 domain of NRP1.[72-75]

The formation of cis NRP1-VEGFA-VEGFR2 complexes within cells plays a crucial role in enhancing angiogenesis.[76-78] However, trans-NRP1-VEGFA-VEGFR2 complexes across cells play an inhibitory role in angiogenesis.[78] Pan et al[79] using a mouse xenograft tumor model, determined that antibodies that blocked VEGFA binding to NRP1 enhanced the anti-tumor effect of anti-VEGFA antibodies. Interestingly, in acute myeloid leukemia (AML), SEMA3A may partially reverse AML progression by inducing VEGFA overexpression. However, it is generally believed that SEMA3A binding to NRP1 plays a role in neurological development.[80] In addition, the VEGFR2/NRP1 complex plays a role in the early signaling of liver regeneration.[81]

In addition to interacting with VEGF, NRP1 also interacts with other pro-angiogenic cytokines, including FGF and HGF.[23,82-86] NRP1 binds to and promotes PDGF-β, as well as, TGF-β1 signaling pathway, thereby contributing to the activation and recruitment of perivascular cells.[22,87] Genetic studies have provided strong evidence that NRP1 is required for vascular morphogenesis. NRP1 deficiency leads to vascular reconstruction and branching defects. NRP1 expression has been shown to increase tumorigenicity in several tumor models such as murine hepatocellular carcinoma, human colon cancer, and non-small cell lung cancer. This may be by promoting VEGF-mediated angiogenesis.[42,88-91]

NRP1 Promotes Tumor Proliferation and Migration

Tumor infiltration and migration are important processes in tumor development and are the main reasons for poor prognosis. NRP1 promotes tumor cell growth, migration, invasion, and survival by interacting with several growth factors and their cognate signaling receptors.[92-96] Binding of VEGFA to NRP1 promotes RhoA activation and then, activated RhoA contributes to the degradation of p27kip1, which in turn, promotes tumor cell proliferation. This has been demonstrated in skin cancer, prostate cancer, and glioblastoma[60,97-99] [Figure 4]. In addition, PDGF and its receptor (PDGFR) are angiogenic factors closely associated with tumorigenesis and progression, and their over-expression is a common feature in different cancers.[100-102] Binding of NRP1 to PDGF and PDGFR promotes the phosphorylation of PDGF and consequently stimulates tumor growth.[22,103] Abelson tyrosine kinase (ABL), a non-receptor tyrosine kinase, itself promotes cell adhesion and migration, while NRP1 can promote endothelial cell migration through the NRP1-ABL1 pathway. This has been demonstrated in non-small cell lung and breast cancers.[24,104-106] In addition, NRP1 could affect the expression of the Bcl-2 protein family and block the mitogen-activated protein kinase signaling pathway. Inhibition of NRP1 has been shown to significantly inhibit the proliferation of glioma cells.[107] NRP1 is highly expressed in the metastatic MDA-MB-231 and MDA-MB-435 breast cancer cell lines, but not in the non-metastatic MDA-MB-453 breast cancer cell lines.[108] NRP1 not only directly promotes tumor growth and migration but also modulates the tumor microenvironment by interacting with integrins and remodeling the extracellular matrix to influence tumor growth.[4]

NRP1 in Cancer Treatment

Based on the function of NRP1 and its interactions with proteins involved in tumorigenesis, targeting NRP1 could have potent anti-tumor activity for several cancers. In

Table 1: Anti-tumor therapy targeting NRP1.

Items	Targeted association	Drugs or agents	Cancer models and cell lines	References
Block pathway	Block tumor angiogenesis	Bevacizumab EG00229 Nb-HS45	Glioma, squamous cell carcinoma, and so on	[109-113,114-116,117]
NRP1-Tregs	Release anti-tumor immune response	Fc(AAG)-TPP11		[20]
Reduce expression	Decrease the expression of NRP1	miR-130a, miR-130b miR-9–5p, miR-628 miR-1247 miR-9 5. NDGA	Epithelial ovarian cancer, Gastric cancer, Osteosarcoma, ALL, Adenocarcinoma, and so on	[125,15,126,127,128,4]
Competitive inhibitors of NRP1	Inhibition of NRP1 binding to its downstream targets	Combination of Sema3A protein and VEGFA inhibitor The SEMA3A point mutant	AML, Pancreatic cancer, and so on	[80,5]
NRP1 alternative splicing variants	Competitive NRP1 combination	s12NRP1, s11NRP1, sIIINRP1, sIVNRP1 NRP1-Δ7	Breast cancer, Prostate cancer, and so on	[130-132,17]
Multi-drug combination therapy	Enhance treatment effect	Nrp1 coupled multifunctional drug nanocarrier NRP1 complex iRGD+5-FU	Glioblastoma, Gastric cancer, and so on	[137,138,139]

EG00229: (S)-2-(3-(benzo[c][1,2,5] thiadiazole-4-sulfonamido)thiophene-2-carboxamido)-5- ((diaminomethylene)amino)pentanoic acid; Nb-HS45: Nanobody HS45; Fc(AAG)-TPP11: NRP1 antagonist; miR: MicroRNAs; NDGA: Nordihydroguaiaretic acid; ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; s12NRP1, s11NRP1, sIIINRP1, sIVNRP1, NRP1-Δ7: Soluble forms of NRP1; iRGD: Tumor homing peptide; 5-FU: 5-Fluorouracil.

recent years, NRP1 has been extensively studied, and the main therapeutic focus has been summarized in the following areas [Table 1].

Blocking the NRP1 pathway interaction to block tumor angiogenesis

NRP1 primarily promotes tumor angiogenesis by forming NRP1/VEGF/VEGFR2 complexes with the VEGF family and its receptors.

The anti-VEGFA antibody, bevacizumab, has been clinically used to treat patients.[109-113] To date, the most characteristic inhibitor of NRP1 is EG00229. It interacts with the extracellular b1b2 domain of NRP1 and has been identified as a specific inhibitor of NRP1 interaction with VEGFA. It has significant tumor-suppressive effects in gliomas and squamous cell carcinomas.[114-116] Rizzolio S et al were also successful in generating an NRP1-specific nanoantibody HS45 that showed high levels of affinity to human NRP1.[117]

Inhibiting NRP1 in Tregs to increase anti-tumor immune response

NRP1 is barely detectable in human peripheral Tregs, however, it is expressed in tumor Tregs. NRP1+ Tregs have been shown to significantly suppress anti-tumor immune responses.[20,115,118,119] The reduction of NRP1+ Tregs in cancer has been strongly associated with chemotherapy success.[22] Jung et al[20] synthesized an NRP1 antagonist, Fc(AAG)-TPP11, that selectively inhibits the function and survival of NRP1+ Tregs to enhance anti-tumor activity in TME. They validated their findings in a mouse model with no apparent toxicity.

Improving tumor efficacy by inhibiting NRP1 expression

There are several types of NRP1 inhibitors, and micro-RNAs, as one of them, can regulate gene expression at the post-transcriptional level by forming RNA-induced silencing complexes. This leads to translational repression or degradation of target genes. It has been shown that microRNAs targeting NRP1 could be used for the treatment of cancers.[120-124] NRP1 was a target of miR-130a and miR-130b and was the first to report that NRP1 was associated with multidrug resistance in ovarian epithelial carcinoma.[125] In gastric cancer cells, miR-9-5p and miR-628 bind to NRP1 and inhibit NRP1 expression to inhibit the proliferation and invasion of gastric cancer cells, while at the same time, increasing the sensitivity of gastric cancer cells to chemotherapeutic agents.[15,126] In OS, NRP1 was identified as a direct target of miR-1247 and has been shown to inhibit the viability and metastasis of OS cells.[127] In acute lymphoblastic

leukemia (ALL), it was demonstrated for the first time that NRP1 was a direct downstream target of miR-9 in ALL. These suggested that the development of novel therapeutic interventions targeting the miR-9/NRP1 signaling pathway could be a therapeutic option for ALL patients.[128] In the adenocarcinoma A549 cell line, miR-9 was found to directly target NRP1, and was found to enhance radiosensitivity in A549 cells.[129]

Certain drugs can also affect NRP1 expression. Nordihydroguaiaretic acid (NDGA) is a natural product that down-regulates NRP1 expression. NDGA could inhibit NRP1 expression and attenuate cell motility and adhesion of cancer cells to the ECM, in addition to attenuating tumor metastasis in a nude mouse model.[4]

Competitive inhibitors of NRP1 binding proteins

Inhibition of NRP1 binding to its downstream targets will inevitably lead to an attenuation of NRP1 oncogenic signaling. SEMA3A could partially reverse the binding of VEGFA to the NRP1 receptor. Combining the SEMA3A protein with a VEGFA inhibitor may be beneficial for the treatment of AML.[80] Similarly, Gioelli et al[5] designed and generated a safe, non-intestinal-delivery, non-NRP1-dependent subtype of the SEMA3A point mutant. This SEMA3A point mutant could bind with nanomolar affinity to PLXNA4 compared to the wild-type SEMA3A.

SEMA3A is a direct binding co-receptor for NRP1, which in turn, is associated with PLXN receptor signaling. However, PLXN receptor signaling is critical for cancer vasculature. SEMA3A point mutants can competitively bind with PLXNA4 and prevent NRP1 from binding to PLXNA4. This accelerates vascular normalization, reduces tissue hypoxia, and increases perfusion to inhibit tumor growth. The effectiveness of the SEMA3A point mutants for the treatment of cancer has been successfully demonstrated in a mouse model of pancreatic cancer.[5]

Application of recombinant sNRP-1 in tumor treatment

In addition to the anti-tumor therapy directly targeting NRP1, the emergence of NRP1 alternative splicing variants (sNRP1) is also a new direction of tumor treatment. At present, s12NRP1, s11NRP1, sIIINRP1, and sIVNRP1 are the most studied NRP1 variants. The proteins encoded by s12NRP1 and s11NRP1 mRNA contain a1a2 and b1b2 domains and some b/c junctions.[130,131] They are known as VEGF165 antagonists. s12NRP1 can inhibit the binding of VEGF165 to NRP1-expressing cells and inhibit the tyrosine phosphorylation of VEGFR-2 induced by VEGF165. In the rat model of prostate cancer, overexpression of s12NRP1 results in a high percentage of apoptotic cells, intratumoral hemorrhage, and few blood vessels. Both sIIINRP1 and sIVNRP1 contain a1a2 and b1b2 domains, but no c domain or the rest NRP1 sequence. It has been found that these two recombinant proteins sIIINRP1 and sIVNRP1 can inhibit the migration of MDA-MB-231 breast cancer cells mediated by NRP1.[132] Recently, Hendricks et al[17] characterized a novel splicing variant NRP1-Δ7, which lost seven amino acids on two residues downstream of

O-glycosylation site compared with NRP1. The proliferation, migration, and anchorage-independent growth of cells with increased NRP1-Δ7 expression decrease significantly in vitro, and NRP1-Δ7 inhibits the growth and angiogenesis of prostate tumor in vivo.

Multi-drug combination therapy targeting NRP1

Regarding cancer, a single drug often fails to achieve the desired therapeutic effect. Hence, a multi-drug combination therapy is generally used in clinical practice.[133-136] Teijeiro-Valino et al[137] coupled a multifunctional drug nanocarrier consisting of hyaluronic acid nanocapsules to NRP1. This significantly improved drug delivery capacity and demonstrated good efficacy. Benachour et al[138] generated polysiloxane nanoparticles chelated to NRP1 targeting peptides and 1,4,7,10 tetraazacyclododecane-N, N',N,N'-tetraacetic acid (DOTA) derivatives. This was therapeutically efficacious in eliminating intracranial U87 glioblastomas in a rat model Zhang et al[139] demonstrated that a novel tumor homing peptide, iRGD, increased tumor penetration of chemotherapeutic agents and that the NRP1 protein was the key mediator of iRGD. Hence, combining iRGD with 5-fluorouracil, the standard first-line chemotherapeutic agent for locally advanced or metastatic gastric cancer, maybe a novel and effective approach to improving tumor prognosis.

Conclusions and Future Directions

NRP1 plays a key role in the occurrence and development of tumors. It is involved in angiogenesis, cancer migration, and tumor immunity. Some of the NRP1 signaling pathways have been mentioned earlier in this report. Targeting these pathways may be efficacious in treating a variety of cancers. However, additional studies need to be performed to decipher the molecular mechanism of NRP1 as it relates to cancer progression and metastasis. For effective cancer therapy, inhibitors of NRP1 function have to be combined with other treatment modalities, including immunotherapy, radiotherapy, and chemotherapy to achieve a complete response in patients with cancers.

Acknowledgements

Conflicts of interest

None.

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