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Journal of Cancer Immunology Commentary

Relevance of Neuropilin 1 and Neuropilin 2 Targeting for Cancer Treatment

Aurore Dumond[1], Gilles Pagès[1,2*]

[1]Scientific Centre of Monaco, Biomedical Department, 8 quai Antoine 1er, MC-98000 Monaco, Principality of Monaco

[2]University Cote D'Azur (UCA), Institute for research on cancer and aging of nice, CNRS UMR 7284; INSERM U1081, Centre Antoine Lacassagne, Nice, France

[*]Correspondence should be addressed to Gilles Pagès; Gilles.Pages@unice.fr

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Abbreviations

ccRCC: Clear cell Renal Cell Carcinoma; CTLA4: Cytotoxic T-lymphocyte Antigen-4; KO: Knockout; NRP: Neuropilin; PD1: Programmed cell death 1; PD-L1: Programmed death-ligand 1; SEMA: Semaphorin; shRNA: Short hairpin Ribonucleic Acid; TKi: Tyrosine-kinase inhibitors; VEGF: Vascular Endothelial Growth Factor; VEGFR: Vascular Endothelial Growth Factor Receptor

Introduction

Neuropilins (NRPs) are a class of transmembrane glycoprotein co-receptors including Neuropilin 1 (NRP1) and Neuropilin 2 (NRP2). They are the co-receptors of different families of receptors [1], thus they are involved in many hallmarks of cancer. Neuropilins, first described as neuronal co-receptors of Semaphorins (SEMA) [2], are the co-receptors of the Vascular Endothelial Growth Factors (VEGF) receptors (VEGFRs) [1]. NRP1, by forming a complex with the VEGFA, the main pro-angiogenic factor, and its receptors VEGFR1 and -2, is involved in angiogenesis pathway; NRP2, by binding to VEGFC, the main pro-lymphangiogenic factor, and to its receptor VEGFR3, is involved in lymphangiogenesis signaling pathway [3]. Our study was focussing on clear cell Renal Cell Carcinoma (ccRCC) [4]. ccRCC is one of the most vascularized cancer because of overexpression of the VEGFA. The current treatments of ccRCC are anti-angiogenics mainly tyrosine-kinase inhibitors (TKi), mTOR inhibitors or immunotherapies [5]. Sunitinib, a TKi targeting particularly the VEGFRs, has been the ccRCC reference treatment since 2007 [6]. Today, immunotherapies such as anti-PDL1 (avelumab, atezolizumab), anti-PD1 (pembrolizumab, nivolumab) or anti-CTLA4 (ipilimumab) combined with TKi such as axitinib or cabozantinib are the new reference treatments [7]. Despite an improvement of survival, only 30% of patients benefit of these new therapeutic strategies and the disease remains incurable for vast majority of patients. Hence, developing new treatments is still needed for patients that are still unresponsive to the current reference therapies. The relapse of patients observed after a few months of treatment depends on innate or acquired mechanisms of resistance that arise in the tumor such as the increase of redundant angiogenic pathways [8], or the overexpression of the pro-lymphangiogenic factor VEGFC [9]. Thus, our objective was to find new targets involved in different cancer hallmarks to counteract relapses on targeted therapies. NRPs, important mediators of angiogenesis and lymphangiogenesis, appear as relevant therapeutic targets. Furthermore, in ccRCC, VEGFRs are not expressed on most of the ccRCC cell lines as compared to NRPs [10,11]. Importantly, VEGFA produced by tumor cells acts autocrinally through Ras, a key signaling molecule downstream of NRP1, to promote NRP1-dependent cell proliferation [12]. Moreover, VEGFC is also produced by ccRCC cells and exerts a NRP2-dependent autocrine loop promoting extravasation and metastasis [10]. Thereby, inhibiting NRPs could result in the direct inhibition of tumor cell proliferation and migration, in addition to their effects on cells of the microenvironment. Furthermore, NRPs, through their expression by different immune cells drive an activation or an inactivation of the immune response depending on the tumor stage [13]. Thus, inhibiting NRPs might impact different cancer hallmarks including angio/lymphangiogenesis, tumor cell proliferation and migration, immune tolerance. To evaluate the role of NRPs in tumor aggressiveness, we inhibited NRPs' pathways through gene invalidation by CRISPR/

Cas9, and then with a pharmacological inhibitor, NRPa-308 [14]. The impact of such genetic and pharmacologic inhibition was investigated by evaluating proliferation, migration and invasion of tumor cells *in-vitro* and then on the growth of experimental tumors in mice generated with control or NRPs' invalidated cells.

Comments on the Results of the paper of Dumond et al. JECCR 2021.

NRPs inactivation by gene invalidation

The starting point of our study was the results of Cao Y. stating that NRPs' down-regulation by shRNA did not impact cell proliferation (NRP1/NRP2) and migration (NRP2) [10,11]. These results were surprising since they showed that NRP1 down-regulation inhibited AKT activity [11], a key signalling pathway involved in cell proliferation. Moreover, NRPs' signalling correlates with cell proliferation and migration in several tumors [15]. Therefore, we compared *in-vitro* the impact of NRP1 or NRP2 gene invalidation by CRISPR/Cas9 to those of a down-regulation of each of them by the same shRNA used by Cao Y. and colleagues and by additional shRNA to limit artefactual off target effects. We highlighted that the level of down-regulation (partial/shRNA or complete/CRISPR/Cas9) of NRP2 is an important criterion to obtain anti-tumoral effects. Indeed, a 60% inhibition of NRP2 by shRNA stimulated, but a 100% inhibition by CRISPR/Cas9 inhibited ccRCC cell proliferation. For NRP1, partial or complete down-regulation had the same trend in inhibiting cell proliferation. Importantly, the proliferation of ccRCC cells was slowed-down to a larger extent by the NRP2 knock-out as compared to the NRP1 knock-out. These results suggest that both NRP1 and NRP2 should be completely inhibited to obtain a maximal therapeutic effect.

NRPs' pharmacological inhibition

In collaboration with the University Paris-Descartes, we developed a NRPs' inhibitor, NRPa-308 [14]. NRPa-308 already showed anti-angiogenic and anti-proliferative abilities *in vitro* and anti-tumor effects on experimental breast cancer *in vivo* [14]. Our objective was to generalize the anti-tumor potential of NRPa-308 to experimental ccRCC. First, NRPa-308 is more efficient in decreasing the viability of ccRCC cells as compared to one of the reference treatments, sunitinib. NRPa-308 decreases ccRCC cell proliferation, migration, and invasion more efficiently than sunitinib. In our study, the IC50 for NRPa-308 was higher in cells knocked-out for NRP2 as compared to the IC50 of cells knocked-out for NRP1 suggesting that NRPa-308-dependent cytotoxic effects mainly depends on NRP2 and to a lesser extent on NRP1.

In vivo experiments

In our previous study [14], a dose of 50 mg/kg of NRPa-308 was required to delay the growth of experimental triple negative breast cancers. However, we were very surprised to observe that the same dose of NRPa-308 did not affect the growth of experimental ccRCC. In collaboration with the Imagine Institute in Paris, we showed that NRPa-308 inhibits the binding of VEGFC to NRP2 in a reverse dose dependent manner. This observation and the major anti-proliferative effects depending on NRP2 gene invalidation, incited us to evaluate the therapeutic effects of increasing doses of NRPa-308 (5 µg/kg, 500 µg/kg and 50 mg/kg) on the growth of experimental ccRCC in mice. This evaluation has been carried out in immunodeficient mice to investigate mainly, the direct effects of NRPa-308 on tumor cells, and in immunocompetent mice to highlight the role of NRPa-308 on the immune system. In both experimental models, at the lowest dose, mice survival increased, no mice weight loss was observed, and the number of tumors' capillaries decreased. A low dose of NRPa-308 *in vivo* also decreased the mRNA levels of pro-tumoral factors including pro-angiogenic (NRP1, VEGFR1), pro-lymphangiogenic (NRP2, Prox, VEGFC, VEGFR3) and immune tolerance factors (PD-L1, MET, HGF). Furthermore, we observed a decrease of M2 macrophages in immunodeficient mice.

Thus, our *in vivo* results correlate with our *in vitro* observations:

- NRP2 must be totally inactivated for a maximal therapeutic effect.
- NRPa-308 inhibits more efficiently NRP2 in a reverse dose-dependent manner.
- Better *in vivo* anti-tumoral effects at lower dose of NRPa-308 in ccRCC.

NRPa-308 efficacy in ccRCC versus breast cancer

As previously mentioned, NRPa-308 was first tested in triple negative breast cancer [14]. The main difference highlighted by our study was that NRPa-308 is efficient at a low dose (5 µg/kg) in ccRCC and at a higher dose (50 mg/kg) in breast cancer [14]. Therefore, why a higher dose of NRPa-308 was required for breast cancer? Thus, we investigated the levels of NRP1 and NRP2 and of their ligands VEGFA and VEGFC, in a large spectrum of ccRCC and breast cancer cell lines, including those used in the breast cancer study (MDA-MB231) [14] and in the present ccRCC study (786-O). NRP1 and VEGFA are expressed in most of the ccRCC and the breast cancer cell lines and particularly in MDA-MB231 and 786-O cells. VEGFC is expressed by all ccRCC cell lines but expressed to a different extent in breast cancer cell lines although present in MDA-MB231. However, in contrast to ccRCC cell lines,

NRP2 is not expressed or expressed at low levels in breast cancer cell lines. This difference in the expression of NRP2 may explain that the dose of NRPa-308 needed to inhibit the growth of experimental breast cancers is higher. Indeed, in breast cancer, NRPa-308 can only target NRP1. We showed in our *in vitro* experiments that the best anti-tumoral effects was obtained by completely inhibiting NRP2 whose affinity for NRPa-308 is better as compared to NRP1. Hence, higher doses of NRPa-308 are required to inhibit the growth of tumors only expressing NRP1.

Conclusion

Our study identified the involvement of NRP1 and NRP2 in different cancer hallmarks of ccRCC aggressiveness. Therefore, we highlighted the relevance of their targeting in these incurable cancers in the metastatic phase. The *in vivo* experiments on breast cancer and ccRCC showed that NRPa-308 is a "hit" molecule to target cancers expressing NRP2 and/or NRP1 such as ccRCC. However, for cancers that only express NRP1 such as breast cancers, the relevance of NRPa-308 is questioned since high doses are required to obtain anti-tumoral effects. Furthermore, the use of high doses, although efficient on tumor growth, may induce toxic side effects. In this case, new NRP1-specific inhibitors with a better affinity should be developed. In silico docking studies by the bioinformaticians from the CNAM in Paris strongly suggested that the NRP1 and NRP2 binding pockets for NRPa-308 are very similar but some amino acids are different. Thus, these differences are investigating to obtain specific inhibitors of NRP1 to improve the anti-tumoral effects on cancers expressing only NRP1. This work is carried out by our colleagues at Paris-Descartes University.

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Authors' Contributions

Writing, review: AD, GP.

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