Juho Irjala

DEVELOPING RECOMBINANT ANTIBODIES FOR SORTING RECEPTOR SORLA

Advanced studies' thesis

Spring semester 2023

Juho Irjala

DEVELOPING RECOMBINANT ANTIBODIES FOR SORTING RECEPTOR SORLA

Abstract

Breast cancer is the most common malignancy and the leading cause of cancer-related death among women. About 15 to 20% of breast cancers has amplification of the HER2 oncogene. HER2 is a member of the epidermal growth factor receptor (EGFR) family that promotes cell survival, proliferation, motility, and resistance to apoptosis. Despite many advances in treatment options targeting HER2, anti-HER2 therapy resistance remains a relevant issue.

SorLA is a sorting receptor of the vacuolar protein sorting 10 proteins (vps10p) domain receptor family, a family of sorting receptors transporting cargo between cell surface and intracellular compartments such as the trans-Golgi network, endosomes, and lysosomes. A link between HER2 cancer signaling and the expression of SorLA has recently been discovered. SorLA was shown to promote HER2 recycling by preventing its lysosomal degradation. Depletion of SorLA decreased HER2 signaling, promoted HER2's accumulation in dysfunctional lysosomes and sensitized the cancer cells to a lysosome-targeting cationic amphiphilic drug ebastine. High SorLA expression correlated with poor patient outcomes in HER2-amplified breast cancer patients. These findings make SorLA an attractive target for drug therapy.

The aim of this study was to develop anti-SorLA antibody fragments (binders), the most promising of which will later be developed into a full-length humanized anti-SorLA antibody ultimately testable in patients. The binders were developed using phage display targeting either full-length SorLA or its binding domain. In the first round 576 clones were screened. In the second round 68 clones were screened and subsequently sequenced, producing 41 different sequences. These most promising 34 clones were then used to immunoprecipitated SorLA to further test for binding activity. Out of these 34 binders 23 successfully immunoprecipitated SorLA. The most promising clones will be tested in vitro in HER2 positive cancer cell lines to assess their cancer growth -halting capabilities. The most successful clone will be developed into a full-length human antibody that can be tested in patients.

Key words: antibody, breast cancer, HER2, immunoprecipitation, phage display, SorLA

1 Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related death among women.[1] About 15 to 20% of breast cancers has amplification of the HER2 oncogene.[2] HER2 is a member of the epidermal growth factor receptor (EGFR) family, a group of growth factor receptors whose cancer-promoting role has been widely established. HER2 signaling promotes cell survival, proliferation, motility, and resistance to apoptosis.[3] HER2 amplification is linked to increased risk for central nervous system metastasis.[4] Despite many advances in treatment options targeting HER2, anti-HER2 therapy resistance remains a relevant issue.[5]

The vacuolar protein sorting 10 proteins (vps10p) domain receptor family consists of five different sorting receptors in mammals, sortilin, SorLA, SorCS1, SorCS2 and SorCS3. Characterized by the N-terminal luminal/extracellular vps10p domain, a funnel formed by a ten-bladed β-propeller domain that serves as a key ligand binding site, this family of sorting receptors transports cargo between cell surface and intracellular compartments such as the trans-Golgi network, endosomes and lysosomes. Several ligands may bind to the vps10p domain and different receptors of the family may bind the same ligand. In sortilin, this vps10p domain makes up the entire luminal/extracellular moiety, whereas SorCS1, -2 and -3 have an additional leucine-rich domain that modulates protein-protein interactions (reviewed in [6]). SorLA has the most complicated extracellular domain consisting of the vps10p domain, a 10 CC motif, a β-propeller, an EGF-type repeat, complement-type repeats and a fibronectin-type III domain. The complement-type repeats (CR-C) domain is a major ligand binding site and the β-propeller facilitates pH-dependent release of ligands.[7] All vps10p family receptors have a short C-terminal cytoplasmic tail which contains binding motifs for different adaptor proteins that direct receptor traffic. Besides their cargo trafficking function, ectodomain shedding has also been demonstrated to constitute part of the actions carried out by this family of receptors.[6]

Vps10p family receptors have most extensively been studied in neurons in the brain and have subsequently been linked to neurodegenerative diseases, such as Alzheimer's disease and frontotemporal dementia, as well as being indicated in stroke and epilepsy.[6,8,9] In addition, SorLA, sortilin and SorCS1 have been associated in cardiovascular and metabolic pathologies including atherosclerosis, hypercholesterolemia, obesity and diabetes.[10-14] SorLA and sortilin are also involved in immune-related processes, SorLA being a regulator of CLF-1 and IL-6 signaling and sortilin regulating cytokine secretion through the control of IFN-γ and IL-6 exocytosis, indicating the potential role these receptors have in immune-related pathologies. For example, sortilin has been speculated to have a role in inflammatory processes promoting cancer (reviewed in [15]). Sortilin expression has been linked to multiple types of cancer, including breast cancer, colorectal cancer, pancreatic cancer and lung cancer.[16-19] SorLA seems to be important in the differentiation of hematological stem cells and the maintenance of normal hematological pool size.[20,21]

Recently an unexpected link between HER2 cancer signaling and the expression of SorLA has been discovered. SorLA levels correlated with high HER2 levels in multiple breast cancer cell lines and in a HER2 positive bladder cancer cell line. It was shown that SorLA co-localizes with HER2 on the

plasma membrane and endosomal compartments and promotes HER2 recycling. Depletion of SorLA decreased HER2 signaling, promoted HER2's accumulation in dysfunctional lysosomes and sensitized the cancer cells to a lysosome-targeting cationic amphiphilic drug ebastine. High SorLA expression correlated with poor patient outcome in HER2-amplified breast cancer patients.[22] In a follow-up study, it was shown that SorLA interacts directly with HER2 and HER3, forming a trimeric complex with HER2-HER3 and preventing their lysosomal degradation in a Rab4-dependent manner. In addition, HER3-signaling mediated by heregulin was discovered to induce SORL1 transduction via ERK-dependent induction of the SORL1 promoter, forming a feed-forward loop between SorLA and HER3. Silencing of SorLA sensitized metastatic anti-HER2 therapy resistant breast cancel cells to neratinib in vitro and in vivo in a zebrafish xenograft brain model.[23] These findings make targeting SorLA a novel and attractive possibility in the arsenal of treatments for HER2-positive breast cancer.

The aim of this study was to develop anti-SorLA antibody fragments (binders), the most promising of which can later be developed into a full-length humanized anti-SorLA antibody ultimately testable in patients.

Materials and methods

Development of binders using phage display

Two different targets were produced for binder production: full-length SorLA and SorLA complement-type repeats (CR-C) binding domain. The targets were biotinylated using Ez-link NHS-PEG4-biotin (Thermo) and biotinylation was tested using Eu-SA, Bio-BSA-Eu and LR11 Mab + Eu-RAM. Maxisorb-plates were coated with the biotinylated targets 1µg/ml, 200 µl/well. Three rounds of panning reactions were performed. The eluted phages were cloned into a pLK06FT production vector in XL1-Blue E.coli which encodes a scFv-AP-His6-FLAG fusion protein. Binder affinity screening was performed using ELISA. Maxisorb plates were coated with 50 ng of full-length SorLA or SorLA CR-C per well. BSA was used as control. Eu-anti-AP-pAb was used as secondary antibody. In the first round 576 clones were screened. In the second round 68 clones were screened and subsequently sequenced, producing 41 different sequences.

Immunoprecipitation

SorLA was immunoprecipitated with the purified binders following immunoprecipitation protocol. Cultured ARPE-n9 (high SorLA expression) and JIMT-1 (low SorLA expression) were lysed with Lysis buffer (40 mM HEPES with 75 mM NaCl, 2 mM EDTA, 1% NP-40, phosphatase and protease inhibitor cocktails). The lysed cells were incubated overnight at +4°C with 1 µg of purified SorLA binder and anti-FLAG ab per sample. Protein-G beads were incubated in 0,5% BSA-PBS buffer overnight at +4°C. In the next morning the beads and samples were mixed and incubated at +4°C for

2 hours and then centrifuged 3000 rpm for 6 minutes. Next 2x sample buffer was added. In Western blotting, 30 µl of sample were used per well. Anti-SorLA (mouse, BD Transduction Laboratories, Cat 612633) and anti-FLAG antibodies were used as primary antibodies. The following secondary antibodies were used: donkey anti-mouse IRDye 800CW (LI-COR, 926-32212), donkey anti-mouse IRDye 680RD (LI-COR, 926-68072), donkey anti-rabbit IRDye 800CW (LI-COR, 926-32213) and donkey anti-rabbit IRDye 680RD (LI-COR, 926-68073). Western blots were imaged using Odyssey infrared imaging system (LI-COR Biosciences).

Results

Second ELISA screening produced 67 clones, most of which showed stronger binding activity for either SorLA complement type repeats domain (CR-C) or full-length SorLA (the entire extracellular domain, ECD). Some clones showed strong binding for both. (**Figure 1**).
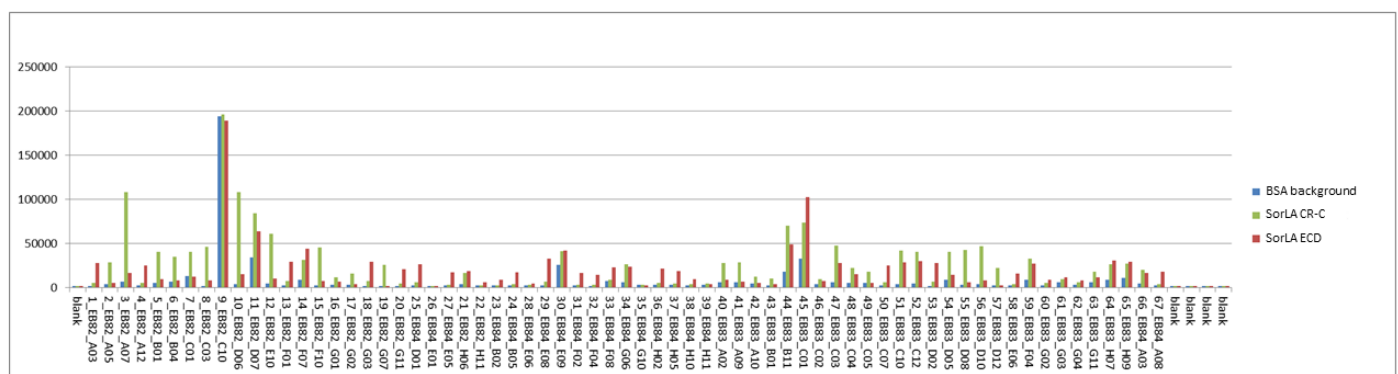

Figure 1. Second ELISA screening of SorLA binders including 67 clones. CR-C=complement-type repeats, ECD=extracellular domain.

The most promising 34 binders created through phage display were purified and their binding activity for SorLA was tested using immunoprecipitation. Out of these 34 binders 23 successfully immunoprecipitated SorLA (Figure 2 and 3).
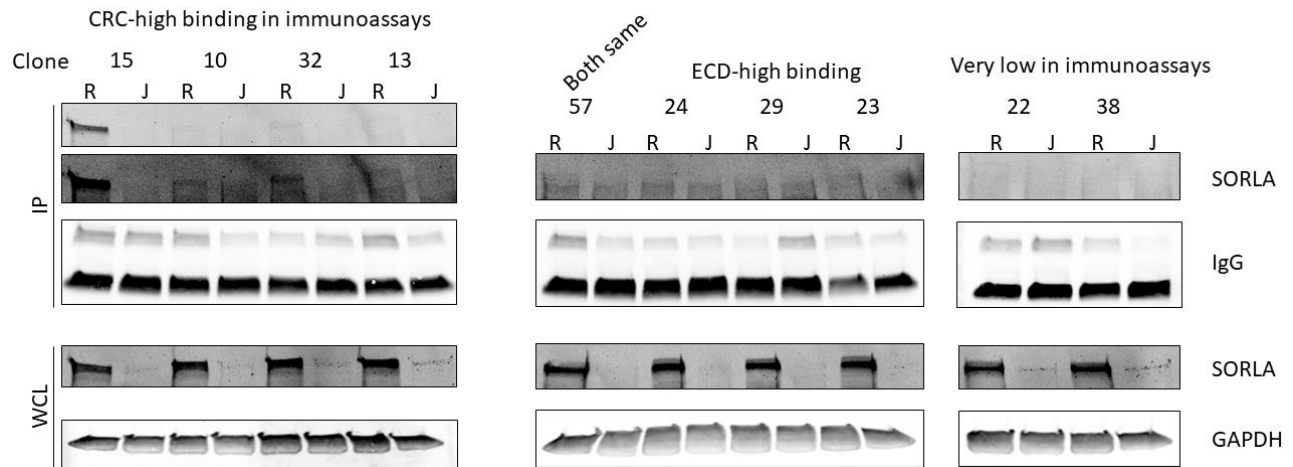
Figure 2. Immunoprecipitations for first set of SorLA binders, where clone 15 showed the strongest binding activity. R=ARPE-n9 cells with high SorLA expression, J=JIMT-1 cells with low SorLA expression. CR-C=SorLA complement-type repeats domain, ECD=SorLA extracellular domain. Rabbit IgG heavy chain was used as loading control.
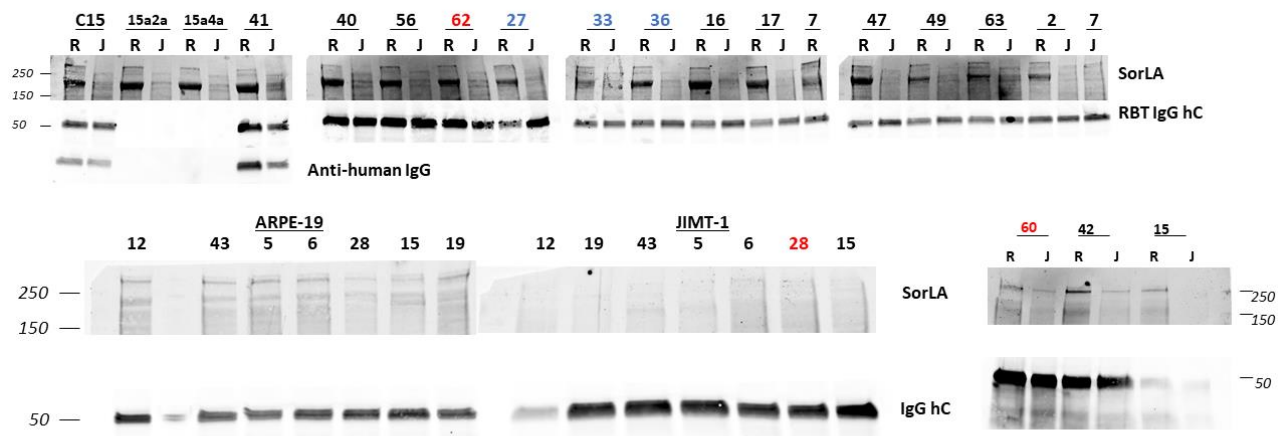

Figure 3. Immunoprecipitation for second set of SorLA binders. Multiple clones showed binding activity. Black clones bound SorLA CR-C in ELISA, blue clones bound SorLA extracellular domain and red clones bound both. R=ARPE-n9 cells with high SorLA expression, J=JIMT-1 cells with low SorLA expression. Rabbit IgG heavy chain was used as loading control.

Discussion

In this study we created multiple antibody fragments that successfully target either full-length SorLA, SorLA complement type-repeats (CR-C) domain or both. The most promising clones will be tested in vitro in HER2 positive cancer cell lines to assess their cancer growth -halting capabilities. The most successful clone will be developed into a full-length human antibody that can be tested in patients.

References

1. Global cancer statistics 2020. CA: A cancer journal for clinicans.
2. Wolff AC, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013 Nov 1;31(31):3997-4013.
3. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. Cell Mol Life Sci. 2008;65(10):1566-1584.
4. Pestalozzi BC, et al. Identifying breast cancer patients at risk for Central Nervous System (CNS) metastases in trials of the International Breast Cancer Study Group (IBCSG). Ann Oncol. 2006 Jun;17(6):935-44.
5. Zhang Y. The root cause of drug resistance in HER2-positive breast cancer and the therapeutic approaches to overcoming the resistance. Pharmacol Ther. 2021 Feb;218:107677.
6. Malik AR, Willnow TE. VPS10P Domain Receptors: Sorting Out Brain Health and Disease. Trends Neurosci. 2020 Nov;43(11):870-885.
7. O.M. Andersen, et al. Molecular dissection of the interaction between amyloid precursor protein and its neuronal trafficking receptor SorLA/LR11. Biochemistry, 45 (2006), pp. 2618-2628.
8. Campion D, Charbonnier C, Nicolas G. SORL1 genetic variants and Alzheimer disease risk: a literature review and meta-analysis of sequencing data. Acta Neuropathol. 2019 Aug;138(2):173-186.
9. Xu SY, Jiang J, Pan A, Yan C, Yan XX. Sortilin: a new player in dementia and Alzheimer-type neuropathology. Biochem Cell Biol. 2018 Oct;96(5):491-497.
10. Huang S, Yu X, Wang H, Zheng J. Elevated serum sortilin is related to carotid plaque concomitant with calcification. Biomark Med. 2020 Apr;14(5):381-389.
11. Demir İ, et al. Relation of Decreased Circulating Sortilin Levels With Unfavorable Metabolic Profiles in Subjects With Newly Diagnosed Type 2 Diabetes Mellitus. Am J Med Sci. 2020 Jan;359(1):8-16.
12. Yau B, Blood Z, An Y, Su Z, Kebede MA. Type 2 diabetes-associated single nucleotide polymorphism in Sorcs1 gene results in alternative processing of the Sorcs1 protein in INS1 β-cells. Sci Rep. 2019 Dec 19;9(1):19466.
13. Harada M, et al. Levels of circulating soluble LR11, a regulator of smooth muscle cell migration, are highly associated with atherosclerotic plaques in patients with carotid artery stenosis. Clin Chim Acta. 2019 Mar;490:69-76.
14. Whittle AJ, et al. Soluble LR11/SorLA represses thermogenesis in adipose tissue and correlates with BMI in humans. Nat Commun. 2015 Nov 20;6:8951.
15. Talbot H, Saada S, Naves T, Gallet PF, Fauchais AL, Jauberteau MO. Regulatory Roles of Sortilin and SorLA in Immune-Related Processes. Front Pharmacol. 2019 Jan 7;9:1507.

16. Roselli S, et al. Sortilin is associated with breast cancer aggressiveness and contributes to tumor cell adhesion and invasion. Oncotarget. 2015 Apr 30;6(12):10473-86.
17. Blondy S, et al. Overexpression of sortilin is associated with 5-FU resistance and poor prognosis in colorectal cancer. J Cell Mol Med. 2021 Jan;25(1):47-60.
18. Gao F, et al. The Membrane Protein Sortilin Can Be Targeted to Inhibit Pancreatic Cancer Cell Invasion. Am J Pathol. 2020 Sep;190(9):1931-1942.
19. Al-Akhrass H, et al. Sortilin limits EGFR signaling by promoting its internalization in lung cancer. Nat Commun. 2017 Oct 30;8(1):1182.
20. Nishii K, et al. The soluble form of LR11 protein is a regulator of hypoxia-induced, urokinase-type plasminogen activator receptor (uPAR)-mediated adhesion of immature hematological cells. J Biol Chem. 2013 Apr 26;288(17):11877-86.
21. Shimizu N, et al. G-CSF induces the release of the soluble form of LR11, a regulator of myeloid cell mobilization in bone marrow. Ann Hematol. 2014 Jul;93(7):1111-22.
22. Pietilä M, et al. SORLA regulates endosomal trafficking and oncogenic fitness of HER2. Nat Commun. 2019 May 28;10(1):2340.
23. Al-Akhrass H, Conway JRW, Poulsen ASA, Paatero I, Kaivola J, Padzik A, Andersen OM, Ivaska J. A feed-forward loop between SorLA and HER3 determines heregulin response and neratinib resistance. Oncogene. 2021 Feb;40(7):1300-1317.