The Neuronal Pentraxin-2 Pathway Is an Unrecognized Target in Human Neuroblastoma, Which Also Offers Prognostic Value in Patients

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Abstract

Neuronal pentraxins (NPTX) and their corresponding receptors (NPTXR) have been studied as synapse-associated proteins in the nervous system, but their role in cancer is largely unknown. By applying a multidisciplinary, high-throughput proteomic approach, we have recently identified a peptide ligand motif for targeted drug delivery to neuroblastoma. Here, we report the sequence similarity between this peptide and a conserved portion of the pentraxin domain that is involved in the homo- and heterooligomerization of NPTX2 and NPTXR. We show that, in comparison with normal tissues, NPTX2 and NPTXR are overexpressed in vivo in mouse models, as well as in human Schwannian stroma-poor, stage IV neuroblastoma. Both proteins are concentrated in the vicinity of tumor blood vessels, with NPTXR also present on neuroblastic tumor cells. In vitro targeting of NPTX2 and NPTXR with the selected peptide or with specific antibodies reduces tumor burden in orthotopic mouse models of human neuroblastoma. In vitro interference with this ligand/receptor system inhibits the organization of neuroblastoma cells in tumor-like masses in close contact with vascular cells, as well as their adhesion to normal microenvironment-derived cells, suggesting a role in the cross-talk between tumor and normal cells in the early steps of neuroblastoma development. Finally, we show that NPTX2 is a marker of poor prognosis for neuroblastoma patients. Cancer Res; 1–7. ©2015 AACR.

Introduction

Neuroblastoma is the most frequent solid malignancy in the first year of life. Despite recent improvements in treatment, the cure rate for patients with high-risk neuroblastoma remains poor (1). The genetic nature of this disease has made it difficult to develop targeted therapies: only a few genes have been found mutated, and the occurrence of the corresponding genetic lesions is less than 10%, usually in the 1% to 3% range (2). Other potential targets have been identified by analysis of copy-number variation and epigenetic modifications. However, most of these targets [MYCN, AURKA (3), CHD5 (4), ATRX (5), ARID1A/B (6), ODC (7)] are cytoplasmic or nuclear, making them challenging to target therapeutically. The transmembrane proteins ALK (8) and TrkB (9) are mutated or overexpressed in a substantial number of high-risk neuroblastoma patients, representing potentially interesting targets. CD19 and GD3 membrane antigens (10) are being pursued for targeted radiotherapy and nanoparticle-driven drug delivery (11) as well as for antibody-mediated immunotherapy (12), with some success in the treatment and prevention of neuroblastoma.

While genetic and genomic analyses are powerful approaches for identifying potential therapeutic targets, they do not necessarily reflect the actual amounts, localization, and reciprocal interactions of the gene-encoded proteins. In addition, they provide data only on the tumor cells, without taking into account their microenvironment and tumor/normal tissue interactions, which play critical roles in tumorigenesis (13). Therefore, we have recently developed a multidisciplinary approach to investigate high-risk neuroblastoma in the context of its native protein architecture, and characterized a set of specific peptide ligand motifs for neuroblastoma-targeted drug delivery in vivo (14).

In the present work, we identify neuronal pentraxin-2 (NPTX2) and its receptor (NPTXR) as a functional ligand/receptor system in neuroblastoma. Neuronal pentraxins were originally described in the brain as synapse-associated proteins (15), although they are expressed also in few other tissues. Their role as cancer-promoting...
agents is unknown, with a single recent report showing that NPTX2 is overexpressed in clear cell renal cell carcinoma and induces survival and migration of tumor cells (16). Here we show that NPTX2 and NPTXR are upregulated in neuroblastoma, and that blockade of the NPTX2 pathway inhibits the onset of neuroblastoma by influencing the mutual recognition between tumor cells and their microenvironment. Finally, we propose the ligand NPTX2 as a novel poor prognostic marker of human neuroblastoma.

Materials and Methods

Peptides and antibodies
The previously described HSYWLRS (neuroblastoma-targeting) and LAKALHA (control) sequences (14) were synthesized by the Institute of Chemistry of Molecular Recognition (National Council of Researches, Milan, Italy) with additional YSH and GGG sequences at the N- and C-terminal, respectively, and a further C-terminal cysteine residue, resulting in the YSHSYWLRSGGGC (TARG) and YSHLAKALHAGGGC (CTRL) peptide, respectively. The following antibodies were used for (i) in vitro studies: anti-NPTX2 (NBPI-50275; Novus Biologicals), anti-NPTXR (NPR/B-2, sc-390081, Santa Cruz Biotechnology); (ii) immunostaining: anti-NPTX2 (NBPI–86531; Novus Biologicals; HPA001079, Sigma–Aldrich; images in Fig. 1B, Supplementary Fig. S2B and S2E), anti-NPTX2 (PRS4573; Sigma–Aldrich), anti-CD146 (EPR3208; Millipore), anti-ephrin A1 (sc-911; Santa Cruz Biotechnology), anti-integrin α11 (sc-98740; Santa Cruz Biotechnology); (iii) in vitro studies: anti-NPTX2 (NBPI–50275), anti-NPTXR (NPR/B-2).

Cell lines and human samples
The human neuroblastoma-derived cell lines GI-LI-N, HTLA-230, SH-SY5Y, and IMR-32 and umbilical vein endothelial cells (HUVEC) were cultured as described (14, 17). Human dermal microvascular endothelial cells (HMEC) were from LCG-Promochem. Human brain vascular pericytes (HBVP) were from ScienCell Research Laboratories. To obtain fluorescent cells for the in vitro assays, GI-LI-N, HTLA-230, and SH-SY5Y cell lines were stably transfected with a plasmid expressing the enhanced green fluorescent protein (pCMS-EGFP; Clontech). Cells were tested and proven negative for mycoplasma contamination and characterized by proliferation, morphology evaluation, and multiplex short tandem repeat profiling. Snap-frozen samples of Schwannian stroma-poor, stage IV neuroblastoma were provided by BJT. Collection and manipulation of human samples were approved by the Institutional Review Board (IRB). Informed written consent was obtained from each patient in accordance with the Declaration of Helsinki.

Mouse models
Athymic (nu/nu) female mice were purchased from Harlan Laboratories (Harlan Italy, S. Pietro al Natisone, Italy) and housed under pathogen-free conditions. Experiments were approved by the Institute Animal Care and Use Committee (IACUC, IRCCS University Hospital San Martino – National Institute for Cancer Research, Genoa, Italy) and by the Italian Ministry of Health. For the orthotopic models, 5-week-old mice were injected into the left adrenal gland with 10⁶ neuroblastoma cells; for the pseudometa-static models, 4-week-old mice were injected into the tail vein with 4 × 10⁶ neuroblastoma cells, as described (14). To evaluate the effect of NPTX2 and NPTXR targeting, GI-LI-N or IMR-32 cells were mixed with CTRL or TARG (100 μmol/L), or with anti-NPTX2 or anti-NPTXR antibody (5 μg/animal) immediately before orthotopic implantation. One month after tumor challenge, mice were killed and tumors were explanted.

Data analysis
Statistical analyses were performed with Prism 5 software (GraphPad). Depending on sample numbers, t test or Fisher exact test (two-tailed) were used to compare selected experimental points. Correlation between NPTXR, NPTX2, and VE-cadherin expression with patient outcome was explored using the “R2: microarray analysis and visualization platform” (http://r2.amc.nl) with default parameters for Kaplan–Meier survival graphs. The analysis was performed on the SEQC (Sequencing Quality Control) neuroblastoma project dataset, and the significance was evaluated by the χ² test.

Results and Discussion
We have recently isolated a peptide motif as specific ligand for mouse models of human neuroblastoma (14). To identify corresponding native ligands, we performed a BLAST analysis against the human and mouse proteomes. Four transmembrane or secreted proteins were retrieved with high homology scores. Of these, ephrin-A1 and α11 integrin were not further studied because their expression was barely detectable in neuroblastoma tissues, as evaluated by immunofluorescent staining followed by confocal microscopy and quantification (Supplementary Fig. S1; fluorescent pixels × 10⁶). On the other hand, NPTX2 and NPTXR were detected in samples from a panel (n = 9) of tumor models obtained by either orthotopic or i.v. injection of neuroblastoma cell lines (i.e., GI-LI-N, HTLA-230, SH-SY5Y, IMR-32) in athymic mice (Fig. 1A, fluorescent pixels × 10⁶; Supplementary Fig. S2A, overview of the quantified images; Supplementary Fig. S2B, controls). Immunofluorescent staining of tumor xenografts showed a nonhomogeneous localization for these proteins (Fig. 1B) and, in association with a detailed morphologic analysis by IHC, revealed that, while NPTX2 expression is confined to blood vessel-forming and/or surrounding cells (Fig. 1C, black arrows), NPTXR is present in high amounts also in neuroblastic cells (Fig. 1C, red arrows). These data are consistent with the in vitro expression levels of NPTX2 (low) and NPTXR (medium) in these same neuroblastoma cell lines, as evaluated by flow cytometry (Supplementary Fig. S2C) and confirmed by immunoblotting (Supplementary Fig. S2D).

Nontumor tissues (kidney, adrenal gland, and liver) from control animals were largely negative for NPTX2 and NPTXR expression (Supplementary Fig. S3). These findings suggest that the onset and/or progression of neuroblastoma induce the expression of both ligand and receptor in normal cells of tumor microenvironment, with a concomitant upregulation of the receptor in tumor cells.

NPTX2 and NPTXR represent a peculiar ligand/receptor system whose components share approximately 50% identity in the overall sequence, and 64% identity in the pentraxin domain where the homology with the targeting peptide is present. Because this domain is responsible for protein–protein interactions leading to functional homo- and hetero-oligomerization (18), we hypothesized that a targeted disruption of such interactions might affect neuroblastoma development. To investigate this hypothesis, we first used two mouse models obtained by orthotopic implantation of GI-LI-N and IMR-32 cells into the adrenal glands. We injected cells alone or in the presence of either control (CTRL) or neuroblastoma-targeting (TARG) peptide, or in the presence of

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either anti-NPTX2 or anti-NPTXR antibody. After 30 days, mice were killed and organs were recovered for tumor burden analysis. We observed a reduction in tumor volume in mice receiving neuroblastoma cells in the presence of the targeting peptide, as well as of the specific antibodies, compared with cells alone (not shown) or cells plus control peptide (Fig. 1D). This reduction was significant for all the experimental points in the GI-LI-N model, and for the anti-NPTXR experimental point in the IMR-32 model.
A higher affinity/blocking efficiency of the anti-NPTXR monoclonal antibody in comparison with both the peptide and the polyclonal anti-NPTX2 antibody, coupled with slightly different expression levels of the targets, might account for the different significance observed. After treatments, the overall amounts and distribution of both proteins, as well as the vascular architecture, were maintained (Fig. 1E), suggesting that interference with NPTX2 and NPTXR possibly affects early steps of tumor/normal tissue reciprocal recognition, rather than influencing successive tissue organization during tumor progression.

We therefore evaluated whether in vitro blockage of NPTX2 and/or NPTXR would affect the interaction of neuroblastoma cells with normal cells from the tumor microenvironment. In a first set of experiments, we prepared mixed cocultures of fluorescently labeled neuroblastoma cells (GI-LI-N, HTLA-230, and SH-SY5Y) and endothelial cells (HUVECs), and grew them in the presence of either CTRL, TARG, anti-NPTX2, or anti-NPTXR. The mixed cells globally reached confluence at the same time in all the experimental conditions; however, their distribution was influenced by the presence of neuroblastoma-targeting peptide and antibodies. Under control conditions, neuroblastoma cells were organized in large aggregates reminiscent of tumor masses. In contrast, NPTX2/NPTXR-targeting conditions caused neuroblastoma cells to become sparse or organized in small clusters. Quantification of highly fluorescent areas (neuroblastoma cell aggregates) confirmed that this redistribution was significant for almost all the experimental points (Fig. 2A). We investigated whether this effect was related to NPTX2/NPTXR-mediated cell–cell binding, by incubating each fluorescent neuroblastoma cell line on confluent layers of macrovascular (HUVECs), microvascular (HMECs), or perivascular (HBPV) cells, in either control or NPTX2/NPTXR-targeting conditions. Adhesion of neuroblastoma cells onto macrovascular and microvascular cells was impaired when NPTX2 and NPTXR...
were targeted; binding to perivascular cells was only slightly affected by interference with the NPTX2/NPTXR pathway (Fig. 2B). All these microenvironment cells express both NPTX2 and NPTXR; however, NPTXR levels are markedly higher in pericytes (Supplementary Fig. S2D and S2E), possibly accounting for the weaker inhibition observed. We also evaluated whether NPTX2 and/or NPTXR had a role in neuroblastoma cell migration toward normal microenvironment cells. For these assays, we chose the SH-SY5Y cell line after extensive characterization of the migratory properties in all neuroblastoma cell lines (Supplementary Fig. S4). Interestingly, attraction of SH-SY5Y was increased by treatment with either targeting peptide or anti-NPTXR antibody, whereas interfering with NPTX2 was ineffective (Fig. 2C). Together, these data show that NPTX2 and NPTXR affect the spatial organization and reciprocal recognition of neuroblasts and normal cells. They demonstrate that, in this setting, (i) the NPTX2/NPTXR pathway has a proadhesive effect, (ii) NPTXR, possibly activated by an alternative ligand such as NPTX1 (18), has an antimigratory effect, and (iii) both functions are reverted by a specific targeting of NPTX2 and/or NPTXR.

Having shown that NPTX2 and NPTXR are potential targets in neuroblastoma in vivo and in vitro, we evaluated their expression in human neuroblastoma samples. The overall tissue distribution of both proteins was similar to that seen in the mouse models, although more background was visible in the staining for NPTX2, due to technical issues (FFPE mouse samples versus snap-frozen human specimens) (Fig. 3A). Notably, in contrast with the high amounts of both NPTX2 and NPTXR detected in neuroblastoma, undetectable to low expression of these proteins is reported by the Human Protein Atlas (19) for the same normal tissues that we evaluated in mice (Supplementary Fig. S5). These data demonstrate that both proteins are overexpressed in neuroblastoma in clinical settings.

This finding led us to investigate a possible involvement for either protein during the progression of human neuroblastoma. For this purpose, we exploited public microarray expression data that we evaluated through the "R2 microarray analysis and visualization platform." Analysis of a large patient dataset (SEQC; n = 498) revealed that high levels of NPTX2 strongly correlated with poor overall survival ($P = 3.2 \times 10^{-6}$). Unexpectedly, NPTXR had a different trend, although with lower significance ($P = 0.019$; Fig. 3B). One might speculate a possible explanation for this paradoxical result is that while NPTX2 localizes prevalently to vascular compartments, NPTXR is expressed also by tumor cells distant from the blood vessels (Fig. 1C and E). It is therefore conceivable that a substantial amount of the receptor binds to an alternative pentraxin ligand (18, 20), with a different biologic outcome. Unfortunately, the SEQC dataset reports only a whole-tissue mRNA expression analysis, from which it is impossible to distinguish among the different species of NPTXR. The poor prognostic value of NPTX2 was not a surrogate for endothelial cell content: another endothelial expressed gene, VE-cadherin, followed an opposite trend and correlated with good overall survival ($P = 6.1 \times 10^{-5}$; Fig. 3B).

In conclusion, we show that NPTX2 and NPTXR mediate tumor/normal cell recognition in neuroblastoma and that interfering with this ligand/receptor system is a potential approach...
toward the development of an innovative targeted therapy. We summarize the proposed mechanism in Fig. 4. Finally, we show that NPTX2 is a novel poor prognosis tumor marker for neuroblastoma patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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