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This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/1524775 since 2017-01-11T22:18:41Z

Published version:
DOI:10.1158/0008-5472.CAN-15-0649

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The neuronal pentraxin-2 pathway is an unrecognized target in human neuroblastoma which also offers prognostic value in patients

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Running title: Targeting the NPTX2/NPTXR pathway in neuroblastoma

Keywords: Neuroblastoma, tumor microenvironment, neuronal pentraxins, target therapy

The authors declare no conflict of interest.

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Word count: 2775; Number of Figures: 4; Number of Supplementary Figures: 4
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 hetero-oligomerization of NPTX2 and NPTXR. We show that, in comparison to 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 vivo 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 crosstalk 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.
Introduction

Neuroblastoma (NB) 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 NB 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 1-3% 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 NB patients, representing potentially interesting targets. CD19 and GD2 membrane antigens (10) are being pursued for targeted radiation therapy and nanoparticle-driven drug delivery (11) as well as for antibody-mediated immunotherapy (12), with some success in the treatment and prevention of NB.

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 NB in the context of its native protein architecture, and characterized a set of specific peptide ligand motifs for NB-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 NB. 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 NB, and that blockage of the NPTX2 pathway inhibits the onset of NB by influencing the mutual recognition between tumor cells and their microenvironment. Finally, we propose the ligand NPTX2 as novel poor prognostic marker of human NB.
Materials and methods

Peptides and antibodies. The previously described HSYWLRS (NB-targeting) and LAKALHA (control) sequences (14) were synthesized by the Institute of Chemistry of Molecular Recognition (National Council of Researches, Milan, Italy) with additional YSHS and GGG sequences at the N- and C-terminal, respectively, and a further C-terminal cysteine residue, resulting in the YSHSHSYWLRSGGGC (TARG) and YSHSLAKALHAGGGC (CTRL) peptide, respectively. The following antibodies were used for (i) in vivo studies: anti-NPTX2 (NBP1-50275, Novus Biologicals, Littleton, CO), anti-NPTXR (NPR/B-2, sc-390081, Santa Cruz Biotechnologies, Santa Cruz, CA); (ii) immunostaining: anti-NPTXR (NBP1-86531, Novus Biologicals; HPA001079, Sigma Aldrich, Saint Louis, MO, images in Fig. 1B, S2B and S2E), anti-NPTX2 (PRS4573, Sigma Aldrich), anti-CD146 (EPR3208, Millipore, Vimodrone, Italy), anti-ephrin A1 (sc-911, Santa Cruz Biotechnology), anti-integrin α11 (sc-98740, Santa Cruz Biotechnology); (iii) in vitro studies: anti-NPTX2 (NBP1-50275), anti-NPTXR (NPR/B-2).

Cell lines and human samples. The human NB-derived cell lines GI-LI-N, HTLA-230, SH-SY5Y and IMR-32 and umbilical vein endothelial cells (HUVECs) were cultured as described (14, 17). Human dermal microvascular endothelial cells (HMECs) were from LGC-Promochem (Sesto San Giovanni, Italy). Human brain vascular pericytes (HBVP) were from ScienCell Research Laboratories (Carlsbad, CA). 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, Mountain View, CA). 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 NB were provided by BIT. 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 $10^6$ NB cells; for the pseudo-metastatic models, 4-week-old mice were injected into the tail vein with $4 \times 10^6$ NB 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 µM), 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 explanted.

Data Analysis. Statistical analyses were performed with Prism 5 software (GraphPad, La Jolla, CA). Depending on sample numbers, t-test or Fisher's 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 Chi-square test.
Results and Discussion

We have recently isolated a peptide motif as specific ligand for mouse models of human NB (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 NB tissues, as evaluated by IF staining followed by confocal microscopy and quantification (Fig. S1, fluorescent pixels × 10^6). 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 NB cell lines (i.e., GI-LI-N, HTLA-230, SH-SY5Y, IMR-32) in athymic mice (Fig. 1A, fluorescent pixels × 10^6; Fig. S2A, overview of the quantified images; Fig. S2B, controls). IF staining of tumor xenografts showed a non-homogeneous localization for these proteins (Fig. 1B) and, in association with a detailed morphological 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 NB cell lines, as evaluated by flow cytometry (Fig. S2C) and confirmed by immunoblot (Fig. S2D). Non-tumor tissues (kidney, adrenal gland, liver) from control animals were largely negative for NPTX2 and NPTXR expression (Fig. S3). These findings suggest that the onset and/or progression of NB 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 ~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 impact on NB 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 NB-targeting (TARG) peptide, or in the presence of either anti-NPTX2 or anti-NPTXR antibody. After 30 days, mice were killed and organs recovered for tumor burden analysis. We observed a reduction in tumor volume in mice receiving NB cells in the presence of the targeting peptide, as well as of the specific antibodies, compared to 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 to 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 impact the interaction of NB cells with normal cells from the tumor microenvironment. In a first set of experiments, we prepared mixed co-cultures of fluorescently labeled NB cells (GI-LI-N, HTLA-230, 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 NB-targeting peptide and antibodies. Under control conditions, NB cells were organized in large aggregates reminiscent of tumor masses. In contrast, NPTX2/NPTXR-targeting conditions caused NB cells to become sparse or organized in small clusters. Quantification of highly fluorescent areas (NB cell aggregates) confirmed 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 NB cell line on confluent layers of macrovascular (HUVECs), microvascular (HMECs) or perivascular (HBPV) cells, in either control or NPTX2/NPTXR targeting conditions. Adhesion of NB 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 (Fig. S2D-E), possibly accounting for the weaker inhibition observed. We also evaluated whether NPTX2 and/or NPTXR had a role in NB cell migration towards normal microenvironment cells. For these assays, we chose the SH-SY5Y cell line after extensive characterization of the migratory properties in all NB cell lines (Fig. S4). Interestingly, attraction of SH-SY5Y was increased by treatment with either targeting peptide or anti-NPTXR antibody, while 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 pro-adhesive effect, (ii) NPTXR, possibly activated by an alternative ligand such as NPTX1 (18), has an anti-migratory 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 NB in vivo and in vitro, we evaluated their expression in human NB 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 to the high amounts of both NPTX2 and NPTXR detected in NB, 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 (Fig. S5). These data demonstrate that both proteins are overexpressed in NB in clinical settings.
This finding led us to investigate a possible involvement for either protein during the progression of human NB. 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 ($P = 3.2e-06$). 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 biological 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 ($P = 6.1e-05$) (Fig. 3B).

In conclusion, we show that NPTX2 and NPTXR mediate tumor/normal cell recognition in NB and that interfering with this ligand/receptor system is a potential approach towards 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 NB patients.
Acknowledgments

This work was supported by Italian Association for Cancer Research (Associazione Italiana per la Ricerca sul Cancro, AIRC) - My First AIRC Grant (MFAG) (FP and SM) and AIRC-IG 14231 (MP), Piedmont Foundation for Cancer Research (Fondazione Piemontese per la Ricerca sul Cancro, FPRC) Intramural Grant 2010 - 5x1000 MIUR 2008 (SM), Umberto Veronesi Foundation (FP) and G Gaslini Institute Excellent Contract 2013-2014 (FP). AB is a recipient of a fellowship by FPRC, DDP is a recipient of a Umberto Veronesi Foundation fellowship. We thank BIT (Integrated Tumor BioBank, Gaslini Institute, Tissue Section) for providing human tumor samples.
References


Figure Legends

Figure 1. NPTX2 and NPTXR are functional markers in experimental mouse models of NB.
NPTX2 and NPTXR were evaluated by IF and IHC in the following tumor samples: G1-2, orthotopic GI-LI-N cells (G1, primary tumor; G2, lymph node metastasis); H1-4, i.v. HTLA-230 cells (tumor mass in: H1, left ovary; H2, right kidney; H3, right adrenal gland; H4, left adrenal gland); S1, orthotopic SH-SY5Y cells, primary tumor; I1-2, i.v. IMR-32 cells (tumor mass in: I1, bladder; I2, right adrenal gland). FFPE tissues were immunostained for NPTX2 and NPTXR. (A) Quantification of specific signals by ImageJ; graph bars represent mean ± S.D. from 6 pictures of each sample. (B) Representative IF pictures. Anti-NPTXR, HPA001079; nuclei, DAPI. (C) Subset of NB tissues IHC-stained and counterstained with hematoxylin. Black arrows, blood vessels; red arrows, tumor cells. (D-E) GI-LI-N or IMR-32 cells were implanted into the adrenal gland (n=5 mice per group) in the presence of CTRL, TARG, anti-NPTX2 or anti-NPTXR. Tumor volumes were evaluated after 30 days (D). Representative IHC staining of samples from the GI-LI-N model; arrows as in Fig. 1C (E). The experiment was performed twice with similar results.

*, P< 0.05; **, P< 0.01; ***, P< 0.001

Figure 2. NTPX2 and NPTXR drive the mutual interaction between NB and microenvironment cells. (A) Fluorescent NB cells were mixed with HUVECs and grown to confluence in the presence of CTRL or NPTX2/NPTXR-targeting agents. Fluorescent areas were quantified with ImageJ and have been processed in reverse-mode to be visualized in black. NB cells were evaluated for their adhesion on confluent layers of HUVECs, HMECs or HBPV cells in the same conditions as in A; adhered cells were counted with ImageJ (B). Migration of SH-SY5Y cells towards HUVECs, HMECs or HBPV cells (5 × 10⁴/well) was evaluated in a transwell system in the same conditions as in A; migrated cells were stained with crystal violet and counted under a light microscope (C). Graph bars represent mean ± S.D. from triplicate points of two independent experiments.

*, P< 0.05; **, P< 0.01; ***, P< 0.001
Figure 3. **NPTX2 and NPTXR are expressed in human NB, where NPTX2 is a prognostic marker of poor outcome.** IF and IHC staining of human specimens were performed as described in Fig. 1. Patient IDs: Patient #1, 07-B-1173 A1; Patient #2, 07-B-1312 A2; Patient #3, 07-B-1617 A2. Representative tissue staining is shown. White arrows, blood vessels; red arrows, tumor cells (A). The correlation between NPTX2, NPTXR and VE-cadherin expression and clinical outcomes in NB patients was evaluated on the SEQC dataset (B).

\[ X^2 = 21.70; \ P = 3.2 \times 10^{-6} \ (\text{NPTX2}); \ X^2 = 5.48; \ P = 0.019 \ (\text{NPTXR}); \ X^2 = 16.07; \ P = 6.1e-05 \ (\text{VE-cadherin}) \]

**Figure 4. The targeting of NPTX2 and NPTXR inhibits NB.** A highly conserved pentraxin domain is present in both NPTX2 and NPTXR, mediating homo- and hetero-oligomerization (likely, pentamerization) of these proteins. The NPTX2/NPTXR pathway is responsible for the reciprocal recognition between normal microenvironment cells and neuroblasts that promotes NB onset and progression. In the presence of a targeting agent, such as the TARG peptide, these interactions are disrupted, resulting in the inhibition of tumor onset and progression.
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Cancer Res  Published OnlineFirst August 20, 2015.

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