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Original Articles

Neuroblastoma patient-derived orthotopic xenografts reflect the microenvironmental hallmarks of aggressive patient tumours

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Treatment of high-risk childhood neuroblastoma is a clinical challenge which has been hampered by a lack of reliable neuroblastoma mouse models for preclinical drug testing. We have previously established invasive and metastasising patient-derived orthotopic xenografts (PDXs) from high-risk neuroblastomas that retained the genotypes and phenotypes of patient tumours. Given the important role of the tumour microenvironment in tumour progression, metastasis, and treatment responses, here we analysed the tumour microenvironment of five neuroblastoma PDXs in detail. The PDXs resembled their parent tumours and retained important stromal hallmarks of aggressive lesions including rich blood and lymphatic vascularisation, pericyte coverage, high numbers of cancer-associated fibroblasts, tumour-associated macrophages, and extracellular matrix components. Patient-derived tumour endothelial cells occasionally formed blood vessels in PDXs; however, tumour stroma was, overall, of murine origin. Lymphoid cells and lymphatic endothelial cells were found in athymic nude mice but not in NSG mice; thus, the choice of mouse strain dictates tumour microenvironmental components. The murine tumour microenvironment of orthotopic neuroblastoma PDXs reflects important hallmarks of aggressive and metastatic clinical neuroblastomas. Neuroblastoma PDXs are clinically relevant models for preclinical drug testing.
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Introduction

The high attrition of novel agents hampers oncology drug development [1,2], in part because conventional preclinical cancer models do not fully resemble the clinical disease and thus have low predictive power [2–4]. Patient-derived xenografts (PDXs) have recently attracted interest because established PDXs faithfully reflect the genetic and histopathological characteristics of human disease. As a consequence, PDXs better predict clinical outcomes than conventional cell line-derived xenografts, and PDXs have emerged as a promising strategy to personalise patient selection and treatment and for investigating the mechanisms of therapeutic resistance [3–6].

Neuroblastoma, a childhood tumour of the developing sympathetic nervous system, remains a therapeutic challenge. Patients with high-risk tumours often have a poor prognosis despite treatment with dose-intensive chemotherapy, surgery, radiotherapy, and/or immunotherapy [7,8]. We recently established orthotopic neuroblastoma PDXs by implanting undissociated high-risk neuroblastoma fragments into the para-adrenal space of immunodeficient NOD/SCID/gamma(c)(null) (NSG) mice [9]. The neuroblastoma PDXs accurately recapitulated the genomic and phenotypic features of the corresponding patient tumours and displayed widespread and robust metastases to lungs, liver, and bone marrow. Furthermore, we established *in vitro* cultures derived from neuroblastoma PDXs, and

Abbreviations: αSMA, α-smooth muscle actin; CAFs, cancer-associated fibroblasts; ECM, extracellular matrix; huCD31, human-specific CD31; IHC, immunohistochemistry; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; moCD34, mouse CD34; NCAM, neural cell adhesion molecule; NSG, NOD/SCID/gamma(c)(null); PDXs, patient-derived xenografts; SNP, single nucleotide polymorphism; TAMs, tumour-associated macrophages; TME, tumour microenvironment.

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these cells expressed neuroblastoma markers and retained tumourigenic and metastatic capacity *in vivo* after orthotopic injection into mice [9].

The undissociated tumour fragments used to establish PDX models contain both neuroblastoma cells and components of the tumour microenvironment (TME). The TME plays an important role in neuroblastoma progression and metastasis [10], tumour treatment responses, and therapeutic resistance [11,12]. Recent studies suggest that TME components can induce stem cell-like features in tumour cells [13,14]. The TME includes immune cells, cancer-associated fibroblasts (CAFs), blood vessels, lymphatic vessels, pericytes, and extracellular matrix (ECM) [15]. Aggressive neuroblastomas are typically highly vascularised with blood [16] and lymphatic [17] vessels, contain high numbers of tumour-associated macrophages (TAMs) [18,19] and CAFs [20], and are Schwannian stroma-poor tumours [21,22]. Furthermore, neuroblastomas contain lymphoid immune cells [19,23] and ECM components [24]. However, the extent to which co-engrafted human tumour stroma survives and contributes to neuroblastoma PDX growth and how murine stroma participates in this process are unknown. Therefore, and given the significance of the TME in neuroblastoma progression [10], we performed a comprehensive analysis of human and mouse stroma in neuroblastoma PDXs. Our aims were to establish: (i) whether co-engrafted patient-derived tumour stromal cells persist in neuroblastoma PDXs and (ii) examine the extent to which mouse-derived stroma replaces and reflects the TME in clinical neuroblastoma specimens.

Here we report that previously established [9] and two new PDXs (one of which lacks *MYCN* amplification) recapitulate clinically important TME hallmarks of the original neuroblastomas from patients. The rich vascularisation, pericyte coverage, TAM infiltration, and CAF and ECM composition resembled that of aggressive patient tumours. Patient-derived tumour endothelial cells were able to form blood vessels in PDXs, although tumour stroma was predominantly replaced with murine stroma. Little is known about differences in TME in different immunodeficient mouse strains. We show that the choice of mouse strain dictated the presence of TME lymphoid immune cells and lymphatic endothelial cells. Furthermore, spontaneous metastases from orthotopic neuroblastoma PDXs also contained TME components, albeit to a lesser extent than the primary tumours at the adrenal site. These results further support the use of neuroblastoma PDXs as clinically relevant models for drug testing in aggressive and metastatic neuroblastoma.

Materials & methods

Processing of viable neuroblastoma samples

Primary viable neuroblastoma samples were obtained from surgical neuroblastoma specimens. Intact tumour explants (approximately 2 × 2 × 2 mm) were cryopreserved in 50% foetal bovine serum (FBS), 40% DMEM high glucose cell media (Corning, New York, USA) and 10% dimethyl sulfoxide (DMSO) by stepwise cooling using CoolCell (BioCision, Larkspur, CA, USA) and stored at –80 °C. Tumour explants were thawed immediately before implantation. The Regional Ethical Review Board at Lund University (Dnr. 2011/289) and the Ethical Committee of the Clinical Hospital of Valencia and the ISCIII (reference: B0000339 26/11/2012) approved the study. Written informed consent was obtained from patients.

Animal procedures

Animal procedures are described elsewhere [9]. Briefly, four- to six-week-old female or male NSG mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Athymic nude mice were purchased from Taconic Biosciences Inc. (Hudson, NY, USA). Mice were housed under pathogen-free conditions and received autoclaved water and food. Mice were anaesthetised by 2.5% isoflurane inhalation. Intact patient-derived tumour fragments were incubated in Matrigel (BD Biosciences, San Jose, USA) for a minimum of 20 min at 4 °C before being placed in the para-adrenal space and immediately covered with Matrigel. PDX-derived cultured cells (1×10^6 cells) were injected into the adrenal gland of athymic nude mice (n = 5). Mice were sacrificed immediately when they exhibited symptoms of tumour growth. All animal procedures followed the guidelines set by the Malmö-Lund Ethical Committee for the use of laboratory animals and were conducted in accordance with the European Union directive on animal rights.

Immunohistochemistry (IHC) and histochemistry

Xenograft tumours and mice organs were formalin-fixed, embedded in paraffin, and 4 µm tissue sections were cut and analysed using single-marker IHC. A list of antibodies and their working dilutions is shown in Supplementary Table S1. Images were acquired using an Olympus BX63 microscope equipped with UPlanSApo objective lenses and a DP80 camera, and images were analysed using the cellSens Dimension imaging software (Olympus Life Sciences, Shinjuku, Japan). Slightly modified Gomori staining was used to detect reticulin fibres, Masson's trichome was used to visualise collagen type I fibres, and glycosaminoglycans were detected with Alcian blue at pH 2.5. Sections from all PDX models (n = 5) were analysed for each marker unless otherwise stated. Eight representative regions from each IHC slide (n = 1 per patient tumour/PDX) were chosen for manual quantitative analysis using cellSens Dimension. Average cell numbers were calculated for each slide and presented as the number of cells per mm^2. A quantitative analysis was performed for each ECM component in patient tumours and PDXs using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).

Multi-marker IHC staining and generation of composite images

Primary antibodies were sequentially applied to the tissue. Briefly, 2 µm thick paraffin-embedded tumour sections were subjected to heat-induced epitope retrieval (HIER) in a pre-treatment module (PT-link, Dako Cytomation, Glostrup, Denmark) with Tris buffer (pH 6) before IHC staining at room temperature with an automated IHC robot (AutostainerPlus, Dako). Sections were sequentially blocked with endogenous enzyme block (EnVision™ FLEX Peroxidase-Blocking Reagent, Dako) for 10 min and serum-free protein block (Dako) for 10 min before incubation with the first primary antibodies (Supplementary Table S2) for 60 min. Next, sections were incubated with secondary antibodies for 30 min, followed by incubation with standard HRP chromogen substrate for 10 min and counterstaining with Mayer's haematoxylin. The tissue sections were then digitalised using an Olympus VS-120 virtual slide microscope (Olympus). The procedure was then repeated for the other primary antibodies (Supplementary Table S2) and the tissue sections were digitalised after each staining cycle. Finally, the new positive chromogen staining for each marker was segmented out by computerised image analysis and combined into a multi-composite marker image. Cell numbers are presented as counts.

Single nucleotide polymorphism (SNP) analysis

Patient tumours and PDXs were snap-frozen and stored at –80 °C for SNP array analysis. Briefly, DNA was extracted from the cells and tissues using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The Affymetrix CytoScan HD platform was used for SNP array analysis as described elsewhere [9].

Results

Establishing novel neuroblastoma patient-derived orthotopic xenografts

We previously reported three metastasising neuroblastoma PDXs (PDXs #1–3) in NSG mice 59–102 days after tumour implantation [9]. Here we show successful engraftment of two additional neuroblastoma orthotopic PDXs (PDX #4–5). Tumour tissue for PDX #4 was obtained from a three-year-old child diagnosed with INSS stage III neuroblastoma who underwent surgery following chemotherapy. The SNP copy number profile (most importantly *MYCN* amplification and 17q gain; Supplementary Fig. S1A) and the neuroblastoma marker pattern (CD56/NCAM, tyrosine hydroxylase, chromogranin A, and synaptophysin; Supplementary Fig. S1B) demonstrated that the PDX retains clinically important molecular features of the corresponding patient tumour. We further used NCAM IHC to detect metastatic spread to distant organs. There were a few micrometastases in the lungs and liver (data not shown) but no evidence of macrometastatic disease and no tumour cells in the bone marrow. These findings were very similar to the clinical profile of the corresponding stage III patient, in whom no overt distant metastases were found. The metastatic behaviour of PDX #4 was different from that of the previously established stage IV tumour-derived PDXs, which showed robust distant metastatic spread [9].

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