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Inhibition of Axl improves the targeted therapy against ALK-mutated neuroblastoma

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ABSTRACT

Neuroblastoma (NB) patients harboring mutated ALK can be expected to potentially benefit from targeted therapy based on ALK tyrosine kinase inhibitor (TKI), such as crizotinib and ceritinib. However, the effect of the treatment varies with different individuals, although with the same genic changes. Axl receptor tyrosine kinase is expressed in a variety of human cancers, but little data are reported in NB, particularly in which carrying mutated ALK. In this study, we focus on the roles of Axl in ALK-mutated NB for investigating rational therapeutic strategy. We found that Axl is expressed in ALK-positive NB tissues and cell lines, and could be effectively activated by its ligand GAS6. Ligand-dependent Axl activation obviously rescued crizotinib-mediated suppression of cell proliferation in ALK-mutated NB cells. Genetic inhibition of Axl with specific small interfering RNA markedly increased the sensitivity of cells to ALK-TKIs. Furthermore, a small-molecule inhibitor of Axl significantly enhanced ALK-targeted therapy, as an increased frequency of apoptosis was observed in NB cells co-expressing ALK and Axl. Taken together, our results demonstrated that activation of Axl could lead to insensitivity to ALK inhibitors, and dual inhibition of ALK and Axl might be a potential therapeutic strategy against ALK-mutated NB.

1. Introduction

As the most common extracranial solid tumor, neuroblastoma (NB) is most commonly diagnosed in early childhood, accounting for 10% of all pediatric oncology deaths [1]. Increasing evidences have showed that activating mutations in the anaplastic lymphoma kinase (ALK) gene are considered to be the leading cause for most cases of hereditary neuroblastoma [2]. Activated ALK caused by ALK rearrangements, such as NPM-ALK, has been shown to influence several main signaling pathways, thus promoting cell growth and survival [3]. However, the situation appears to be more complex in neuroblastoma as mutations and amplification in ALK gene both result in constitutive phosphorylation, leading to oncogenic effects [4].

NB with ALK aberration (mutation or amplification) have been implied significantly more sensitive to ALK inhibitors than that with wild-type ALK. ALK is mutated in 8% of diagnostic tumor samples [5], and amplification of the ALK gene has only been described in 1.2–4.4% of NB patients [2,6]. In this study, we focused on the therapeutic effect of ALK-targeted therapy in ALK-mutated neuroblastoma. Growing evidences suggest that patients with ALK-mutated neuroblastoma might benefit from treatment with ALK tyrosine kinase inhibitors (TKIs), such as crizotinib and ceritinib, which have been approved by the US Food and Drug Administration (FDA) in 2011 and April this year [7,8].

Receptor tyrosine kinase Axl is a member of the TAM (Tyro3/Axl/Mer) family, and has reported been associated with a spectrum of human cancers [8]. Numerous studies have revealed that the oncogenic potential of Axl is attributed to the anti-apoptotic and proliferative signaling pathways triggered by activation of the tyrosine kinase domain [9,10]. Ligands that can induce Axl autophosphorylation have been described, such as GAS6 and protein S, potentially resulting in the activation of several canonical oncogenic signaling pathways [11]. Recent studies have shown that activation of Axl mediates resistance to chemotherapeutic drugs [12], as well as targeted therapy based on small-molecule inhibitor, such as erlotinib and lapatinib [13,14]. However, the expression and functional consequences of Axl expression in neuroblastoma and the role in targeted therapy against neuroblastoma by ALK-TKIs are poorly understood.

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Here, we focus on the role of Axl in ALK-mutated neuroblastoma, and provide support for this protein as attractive novel therapeutic target to design relevant therapeutic strategies for targeted therapy against ALK-mutated neuroblastoma patients.

2. Materials and methods

2.1. Tissue samples and immunohistochemistry (IHC)

The collection of tumor tissues from NB patients was approved by our Institutional Review Board (IRB). For IHC, the tissue samples from consenting patients were cut in 5-μm sections. After antigen retrieval, the samples were incubated overnight using a primary antibody, anti-ALK (Cell Signaling Technology; Denvers, MA, USA) and anti-Axl (Cell Signaling Technology). Then, signal was detected using a suitable HRP-labeled second antibody with DAB as the chromagen (Dako, Carpinteria, CA). The staining was photographed under an inverted light microscope (Olympus, Tokyo, Japan).

2.2. Cell culture and reagents

A panel of ALK-positive NB cell lines (NB1643, Kelly, SHSY5Y and NBSD) were maintained in DMEM (Invitrogen, Breda, The Netherlands) containing 10% heat inactivated fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 100 Units/ml penicillin, 100 mg/ml streptomycin (Hyclone Laboratories, Logan, UT, USA). NSCLC cell line H2228, which harbors the fusion protein EML4-ALK, was maintained in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 10% FBS. ALK inhibitors crizotinib (PF-02341066) and ceritinib (LDK378) as well as Axl inhibitor BGB324 were obtained from Selleck Chemicals (Houston, TX, USA), and stock solutions were prepared in DMSO. Recombinant human GAS6 was purchased from R&D Systems (Minneapolis, MN, USA) and reconstituted in PBS.

2.3. Western blot analysis

Whole-cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma, St. Louis, MI, USA). Antibodies for Western blot were: phospho-ALK (pALK, Tyr1507) from Abcam (Cambridge, MA, USA); phospho-Axl (pAxl, Tyr702), ALK, Axl and caspase3 Cell Signaling Technology (Denvers, MA, USA).

2.4. Cell proliferation assay

Cells were seeded in starvation medium in 96-well plates in a volume of 100 μl medium. After 12 h, cells were treated with crizotinib (100 nM) in the presence of GAS6 (200 ng/ml) for 3 days, and the cell proliferation were determined by the MTS assay (CellTiter 96 AQeous One Solution Cell Proliferation Assay, Promega, Madison, Wisconsin) and normalized to control. For determination of IC_{50} values, cells were transfected with control or Axl specific small interfering RNAs using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. Cells were then cultured for another 3 days in the presence of increasing concentrations of crizotinib or ceritinib. Each experiment was repeated 4–6 times. The IC_{50} values were analyzed using Prism 5 (Version 5, GraphPad Software, Inc.).

2.5. Colony formation assay

NB1643 cells were plated in 6-well dishes at 200 cells per well and cultured in medium containing crizotinib (100 nM) and GAS6 (200 ng/ml) for ten days. Colonies were washed with PBS and stained with crystal violet (0.5% w/v in 25% methanol). Stained plates were rinsed in PBS and allowed to dry at room temperature. Colony diameter was photographed under an inverted light microscope (Olympus, Tokyo, Japan).

2.6. Flow cytometric analysis of apoptosis

After treated with crizotinib (100 nM), ceritinib (100 nM) and BGB324 (300 nM) for 48 h, cells were resuspended and incubated with 5 μl FITC-conjugated annexin V (Invitrogen, Carlsbad, CA, USA) for 10 min in the dark. Then, cells were centrifuged and resuspended in 290 μl binding buffer and auditioned with 10 μl propidium iodide (PI) (Sigma-Aldrich). The Annexin-V-FITC and PI binding were determined using a flow cytometer (Beckman Coulter).

2.7. Animals and subcutaneous xenograft model

5×10^6 NB1643 cells were resuspended in 100 μl PBS and implanted into the back of female BALB/c nude mice. For the drug treatment, mice with a size of $\sim$100 mm^3 tumors were then treated with crizotinib alone (50 mg/kg), or BGB324 alone (125 mg/kg), or their combination. Tumor growth was measured by calipers in two dimensions, and the tumor volume was calculated as length $\times$ $width^2/2$. Tumor weight was also measured at the end of the study.

2.8. Statistical analysis

Data are expressed as mean ± s.e.m. All data are representative of three independent experiments unless otherwise noted. The significance of differences between groups was assessed by two-tailed *t*-test or one-way ANOVA. All analyses were all other data were performed using Prism 5 (Version 5, GraphPad Software, Inc.).

3. Results

3.1. Axl receptor tyrosine kinase is expressed in ALK-positive human neuroblastoma tissues and cell lines

Previous studies have revealed that Axl is expressed in a variety of human cancers, however, little data are reported in neuroblastoma, particularly in which expresses ALK. In this study, we focus on the roles of Axl in ALK-mutated neuroblastoma for investigating rational therapeutic strategy. We firstly examined the expression of Axl in 15 human neuroblastoma tissues by immunohistochemistry (IHC), and found that 11 tissues (73.3%) expressed Axl protein, including 2 tissues expressing ALK (Fig. 1A). We expand on that observation by analyzing Axl expression in a panel of neuroblastoma cell lines containing mutated ALK shown in Fig. 1B. Western blot analysis showed that 3 out of the 4 ALK-positive lines (75%) expressed Axl protein at varying levels (Fig. 1B), implicating the co-expression ALK and Axl in neuroblastoma. Additionally, NSCLC cell line H2228 carrying EML4-ALK fusion gene also presented a high level of Axl, indicating that this symbiotic relationship may also exist in other ALK-expressing tumors.

3.2. Ligand-dependent Axl activation promotes cell growth and impairs the efficiency of ALK inhibitor

Previous reports have showed that Axl can be activated by its ligand, such as GAS6, promoting multiple pro-oncogenic signaling, include pathways promoting survival, increasing migration, and inhibiting apoptosis. To investigate the role of activation of Axl by GAS6 in ALK-mutated neuroblastoma cells, the following NB cell lines were used: NB1643 (ALK mutation R1275Q), Kelly (ALK