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Inhibition of Mer and Axl receptor tyrosine kinases leads to increased apoptosis and improved chemosensitivity in human neuroblastoma

Yixin Li [a, *], Xiqian Wang [b], Shaojie Bi [c], Kun Zhao [a], Chao Yu [d]

[a] Department of Medical Imaging, The Second Hospital of Shandong University, Jinan 250033, China
[b] Department of Orthopedic Surgery, The Second Hospital of Shandong University, Jinan 250033, China
[c] Department of Cardiology, The Second Hospital of Shandong University, Jinan 250033, China
[d] Department of Clinical Laboratory, The Second Hospital of Shandong University, Jinan 250033, China

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ABSTRACT

Ectopic expression of Mer and Axl receptor tyrosine kinases (RTKs) are frequently found in various cancers as known to promote oncogenesis by activating antiapoptotic signaling pathways. However, the roles of these receptors in neuroblastoma remain unclear. We found Mer and Axl was co-expressed in neuroblastoma patient samples and cell lines. Ligand-dependent Mer or Axl activation led to an increase in phosphorylated ERK1/2, AKT and FAK indicating roles for these RTKs in multiple oncogenic processes. Furthermore, Mer and Axl knockdown led to apoptosis and inhibition of migration as well as a significant increase in chemosensitivity in response to cisplatin and vincristine treatment. Taken together, our results demonstrated that inhibition of Mer and Axl improved apoptotic response and chemosensitivity in neuroblastoma, providing new insights into development of novel therapeutic strategies by targeting these oncogenes.

1. Introduction

Neuroblastoma (NB), accounting for 6–10% of childhood cancers, is the most common type of pediatric tumors that arise from neural crest cells within the embryonic sympathetic nervous system and the adrenal medulla [1]. As a very complex disease which exhibits genetic and clinical heterogeneity, neuroblastoma has a worse prognosis responsible for 15% of childhood cancer mortality [2,3]. It has reported that some molecular genetic alterations determine the risk stratifications and the therapeutic outcomes. MYCN amplification, anaplastic lymphoma receptor tyrosine kinase (ALK) and paired-like homeobox 2b (PHOX2B) mutations only account for a few cases of neuroblastoma [4–6]. Identification of new actionable targets remains a large unmet need, which may offer new insights to targeted therapy against neuroblastoma.

Receptor tyrosine kinases (RTKs) are important transmembrane receptors which regulate key signal transduction such as cell proliferation, apoptosis, migration and invasion of many cancers [7,8]. The receptor tyrosine kinases Mer and Axl, belonging to the TAM receptor subfamily, are overexpressed in a spectrum of human tumors and have been characterized with oncogenic roles [9,10].

Abnormal overexpression or overactivated of Mer and Axl has also been reported in many solid tumors including lung cancer, kidney cancer, uterine cancer, prostate cancer, melanoma and rhabdomyosarcomas [11,12]. Mer and Axl co-expression also has been directly linked with higher rates of metastasis and poorer prognosis in gastric cancer [13]. However, the expression and functional consequences of Mer and Axl in neuroblastoma are poorly understood. Furthermore, roles for the co-expression of Mer and Axl in neuroblastoma have previously been unrecognized.

In this study, we found co-expression of Mer and Axl in neuroblastoma primary patient samples and cell lines. Inhibition of either Mer or Axl led to increased apoptosis, decreased cell proliferation, and higher sensitivity to chemotherapeutic agents. Our results elucidated the roles of Mer and Axl in neuroblastoma growth and resistance to chemotherapy, providing supports for these RTKs as attractive novel therapeutic targets for neuroblastoma.

2. Materials and methods

2.1. Tissue samples, cell lines and transfection

The collection of tumor tissues from patients with neuroblastoma was approved by our Institutional Review Board (IRB). A panel

* Corresponding author. Department of Medical Imaging, The Second Hospital of Shandong University, 247 Bei Yuan Street, Jinan 250033, China.
E-mail address: yixinli2020@163.com (Y. Li).

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of neuroblastoma cell lines (NMB, SKNF1, SHEP21N, GIMEN, SJNB8, SKNSH, SKNAS, SHSY5Y, SJNB1, SHEP2, SJNB6, AMC106, NGP, SMSKCNR and LAN1) was maintained per culture guidelines. Control and Mer/Axl specific siRNA were obtained from Santa Cruz. The transfection was done with Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

2.2. Immunohistochemistry (IHC) and Western blot

For IHC, the tissue samples were cut in 4 μm sections. After antigen retrieval, the sections were incubated with anti-Mer/Axl (Cell Signaling Technology) overnight, followed by a HRP-labeled second antibody. The staining was photographed under an inverted light microscope. For Western blot analysis, whole-cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma, St. Louis, MI, USA), and subjected to Western blot with the following primary antibodies: Mer, Axl, phospho-Axl (p-Axl, Tyr702), phospho-AKT (p-AKT, Ser473), AKT, phospho-ERK1/2 (p-ERK1/2, Thr202/Tyr204), ERK1/2, phospho-FAK (p-FAK, Tyr397), survivin and caspase3 from Cell Signaling Technology (Beverly, MA, USA), phospho-Mer (p-Mer, Tyr681/749) from Abcam (Cambridge, MA, USA). Equal loading was verified using an anti-Actin antibody (Abcam).

2.3. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues and cell lines using TRIzol reagent (Invitrogen). cDNA was obtained using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). The gene expression was validated using GoTaq qPCR Master Mix with SYBR green (Promega) with GAPDH as an internal control. Primers for genes were obtained from a pre-validated source, PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

2.4. Human phospho-kinase array

Whole-cell lysates were prepared from SHEP21N cells after treated with Gas6 (200 ng/ml) and anti-Mer antibody (10 μg/ml). The phosphorylation profile of kinases was analyzed using Human Phospho-Kinase Array Kit (ARY003, R&D Systems) according to the manufacturer's instructions.

2.5. Determination of cell growth and apoptosis

Cell growth was detected by MTS assay. Briefly, 3×10^3 cells were seeded into 96-well plates after transfection, then treated with increasing concentrations of cisplatin and vincristine (VCR) for 4 days. Cell growth was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Cell apoptosis was measured by annexin V-FITC/PI staining (BD Pharmingen) according to the manufacturer's protocol.

2.6. Cell migration assay

Cell migration assay was performed using transwell chamber (Corning, New York, NY, USA). Briefly, 5×10^4 cells were plated on the top chamber in serum-free medium. Medium supplemented with 1% serum was used as a chemoattractant in the bottom chamber. After incubation for 12 h, the non-migrated cells were removed from the upper surface of the membrane with cotton swabs. Cells on the lower membrane surface were fixed in 100% methanol, stained with 0.1% crystal violet and counted under a light microscope (Olympus microscope BX51). The assays were conducted three independent times.

2.7. Statistical analysis

All data were expressed as mean ± s.e.m.. The significance of differences between groups was examined by two-tailed t-test or one-way ANOVA using the GraphPad Prism program version 5 (GraphPad Software, USA). The asterisk indicates a statistically significant difference: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Mer and Axl receptor tyrosine kinases are co-expressed in human neuroblastoma and cell lines

Firstly, we evaluated expression of Mer and Axl in 18 human neuroblastoma tissues by immunohistochemistry, and found that 16 samples expressed Mer protein, 14 cases expressed Axl, and 14 samples co-expressed Mer and Axl, indicating the high frequency of coexistence of Mer and Axl in neuroblastoma (Fig. 1A). Further analysis showed that high Mer expression was correlated with high Axl expression with the coefficient of determination $r^2 = 0.505$ ($P < 0.001$) (Fig. 1B), suggesting positive correlation of Mer with Axl expression in neuroblastoma. We expand on that observation by reanalysis of two previously published datasets containing 88 and 51 human neuroblastoma samples, did find strong positive correlation between Mer and Axl expressions ($r^2 = 0.537$, $P < 0.001$ and $r^2 = 0.598$, $P < 0.001$, respectively) (Fig. 1C). The expression pattern of Tyro3 (the third TAM family member) was also examined and exhibited no correlation with Mer or Axl (data not shown).

Follow-up qPCR analysis for Mer and Axl expression was done in a panel of neuroblastoma cell lines, and found similar high levels of Mer and Axl expression (Fig. 1D). These results were further confirmed in a section of cell lines by Western blot as demonstrated that Mer protein was found in all cell lines tested, whereas Axl protein was expressed in 4 cell lines (Fig. 1D). The GIMEN and SHEP21N cell lines were chosen for further investigations as they contained both Mer and Axl RTK protein, and NMB cells with high Mer but no Axl expression were used as control.

3.2. Mer and Axl stimulated by Gas6 activate multiple signaling pathways

Previous reports have shown that Mer and Axl activation by their ligands, such as Gas6, could activate multiple pro-oncogenic signaling, include pathways promoting survival, inhibiting apoptosis and increasing migration. We found that high expression of Gas6, as a common ligand of Mer and Axl, was independently correlated with both high Mer expression ($r^2 = 0.597$, $P < 0.001$) and high Axl expression ($r^2 = 0.369$, $P < 0.001$) in our study (Fig. 2A). Reanalysis of the published datasets (GSE16476; n = 88) also demonstrated a significant positive correlation between the expression of Gas6 and that of Mer ($r^2 = 0.243$, $P < 0.001$) or Axl ($r^2 = 0.348$, $P < 0.001$) (Fig. 2B). To evaluate the roles of Gas6 in activating Mer and Axl signaling, we firstly examined the status of Mer and Axl activation and the response to Gas6 stimulation in a series of cell lines. As shown in Fig. 2C, both Mer and Axl exhibited increased phosphorylation following Gas6 treatment in SHEP21N and GIMEN cell lines, which highly expressed both Mer and Axl. Additionally, Gas6 induced activation of Mer but not that of Axl in NMB as no Axl expressed. These data suggested the activation of Mer and Axl by Gas6 in neuroblastoma.

To identify the downstream pathways that might be involved in ligand-dependent Mer and Axl activation specifically in neuroblastoma, we utilized a phosphokinase array to analyze extracts from SHEP21N cells stimulated with Gas6. As expected, an increase in phosphorylated ERK1/2, AKT and FAK were observed following

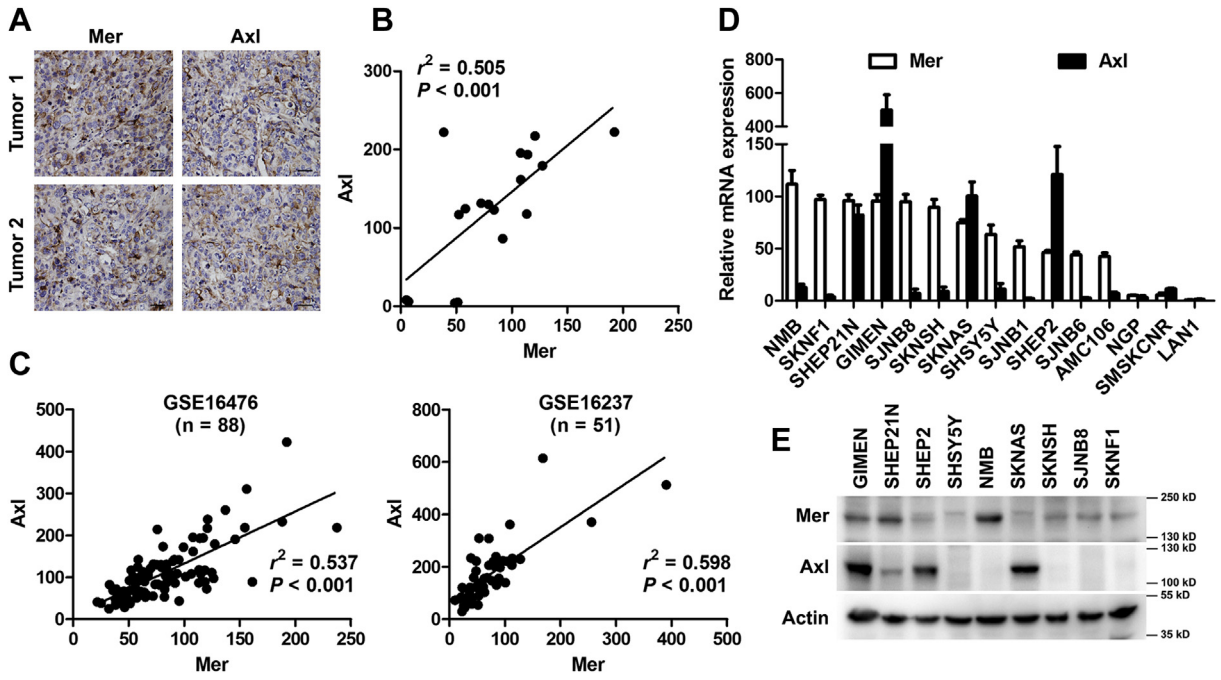

Fig. 1. Mer and Axl receptor tyrosine kinases are co-expressed in human neuroblastoma and cell lines. (A) Immunohistochemical staining of Mer and Axl in sections of neuroblastoma. Micrographs shown represent the range of staining observed in tumor cells. (B) qRT-PCR evaluating the expression of Mer and Axl in neuroblastoma samples, and the correlation was calculated by GraphPad Prism program version 5. (C) The correlation of Mer and Axl levels in two previously published datasets was analyzed. The dataset was downloaded from the NCBI Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov. The expression of Mer and Axl in a panel of NB cells was examined by (D) qRT-PCR and (E) Western blot, respectively.

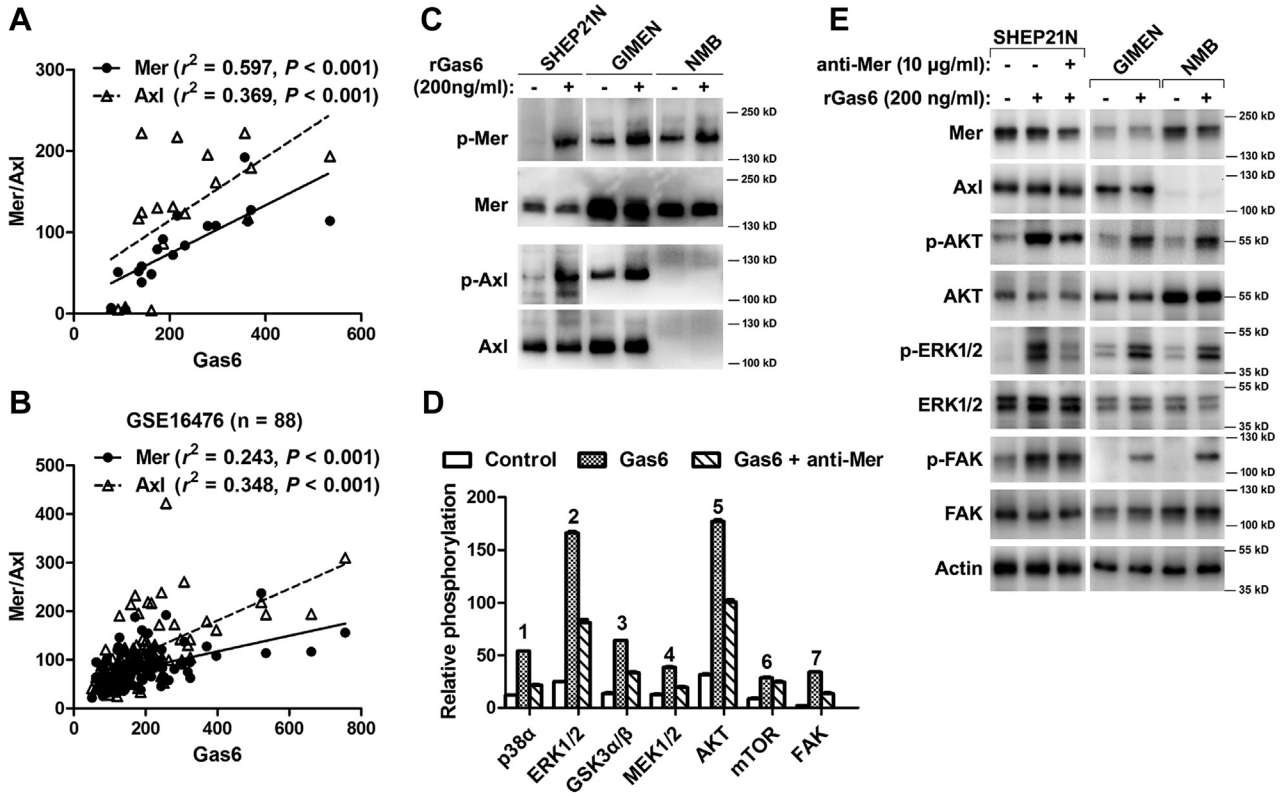

Fig. 2. Mer and Axl stimulated by Gas6 activate multiple signaling pathways. (A) The correlation between Gas6 and Mer/Axl levels in neuroblastoma tissues was showed. (B) Re-analysis of the expression of Gas6, Mer and Axl from previously published data. (C) Cells were treated with recombinant human Gas6 (200 ng/ml) for 6 h, and the activation of Mer and Axl were detected by Western blot. (D) The SHEP21N cells were treated with Gas6 and anti-Mer antibody, the whole-cell lysates of the indicated cell lines were subjected to phosphorylation analysis. And the relative phosphorylation was quantified by normalizing pixel density of each positive control to 100. (E) Cells were seeded into 6-well dishes and treated with Gas6 and anti-Mer antibody, the cell protein phosphorylation was detected by Western blot.

Gas6 treatment (Fig. 2D). These results were further verified by Western blot analysis in SHEP21N cells as well as two additional neuroblastoma cell lines (GIMEN and NMB) (Fig. 2E). Under Gas6 stimulation, the three cells commonly presented elevated activities in ERK1/2, AKT and FAK, suggesting the activation of the MAPK, PI3K/AKT and FAK signaling pathways by Gas6. As Mer and Axl are co-expressed and activated by Gas6 in SHEP21N cell lines, we roughly ruled out their contributions to these signaling pathways using a blocking antibody against Mer receptor (anti-Mer). The anti-Mer antibody only partly impaired ERK1/2, AKT and FAK phosphorylation induced by Gas6 (Fig. 2D and E), suggesting that Axl may also contribute to these signaling pathways. Taken together, these data suggest the roles of Mer and Axl in activating multiple oncogenic signaling pathways including cell growth, proliferation, survival and migration.

3.3. Knockdown of Mer or Axl expression leads to increased apoptosis and reduced migration

We further examined the effects of Mer and Axl depletion on cell survival and migration by transfection with two different target-specific siRNA sequences of Mer (si-Mer1 and si-Mer2) and Axl (si-Axl1 and si-Axl2). Both Mer- and Axl-specific siRNA markedly promoted the frequency of apoptosis in SHEP21N and GIMEN cell lines; and depletion of Mer but not that of Axl significantly induced cell apoptosis in NMB cells with no Axl expressed (Fig. 3A). These results were further validated by western blotting that knockdown of Mer or Axl expression led to increased cleavage of caspase3, a marker for cells undergoing apoptosis. It was also shown that depletion of Mer or Axl obviously inhibited ERK1/2, AKT and FAK phosphorylation as well as the expression of survivin (Fig. 3B).

The effect of Mer and Axl knockdown on neuroblastoma cell migration was also evaluated. As shown in Fig. 3C, whereas silencing Axl greatly attenuated migration of SHEP21N and GIMEN cells, no marked effects were observed by Mer inhibition, suggesting the regulation of migration by Axl in these cells with Mer and Axl co-expressed. Notably, Mer-specific siRNA markedly inhibited migration of NMB cells, which had no detectable Axl, indicating that Mer receptor tyrosine kinase may also influence this motility in these only Mer expressing cells. Taken together, these results indicated that Mer and Axl receptor tyrosine kinase contributed to cell survival and migration in neuroblastoma.

3.4. Inhibition of Mer and Axl increased the chemosensitivity of neuroblastoma cells

As previous studies have suggested that Mer and Axl mediate chemoresistance in several cancer types, we investigated whether Mer and Axl inhibition could improve the efficacy of cytotoxic agents that are currently used clinically in treatment against neuroblastoma. The control and Mer- or Axl-depleted cells were treated with increasing does of cisplatin and vincristine (VCR), and the cell proliferation was determined by MTS assay. The data showed that knockdown of Mer or Axl increased the sensitivity of SHEP21N to cisplatin and vincristine (VCR), with 2–40-fold reductions in the half-maximal inhibitory concentrations (IC50) relative to si-Control cells (Fig. 4A and Table 1). Exactly, the SHEP21N siMer1 and siMer2 cells were significantly more sensitive to cisplatin (IC50 = 4.86 and 2.91 μM) and vincristine (IC50 = 9.49 and 4.89 μM) compared with si-Control cells. Cells with Axl inhibition were also significantly more chemosensitive than control cells (6–18-fold for cisplatin, 26–42-fold for vincristine).

We further explored whether an increase in cell death existed in response to these agents, the frequency of apoptosis were analyzed by flow cytometry using annexin V-FITC/PI staining. As shown in Fig. 4B, a statistically significant increase in the percentage of apoptotic cells were observed in Mer or Axl knockdown cells after treatment with 5 and 10 μM vincristine (VCR) compared with the SHEP21N si-Control cells. Western blotting assay also confirmed these results as

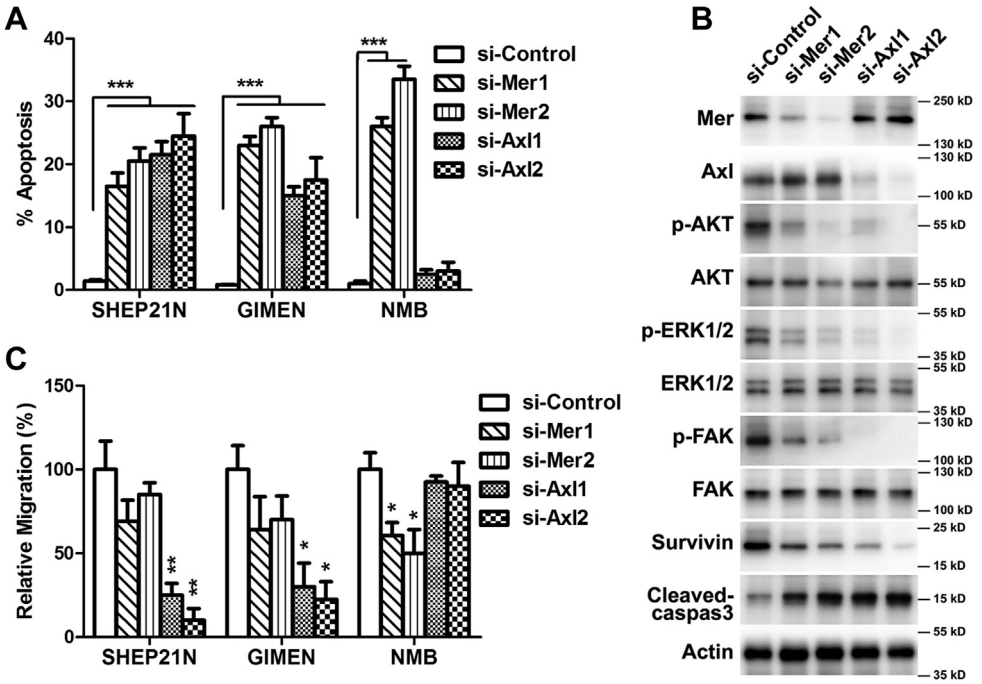

Fig. 3. Knockdown of Mer or Axl expression leads to increased apoptosis and reduced migration. (A) After transfected with Mer and Axl siRNA for 48 h, cell apoptosis was determined by flow cytometry. (B) And the levels of indicated protein in SHEP21N cells were analyzed by Western blot. (C) Cell migration was examined by transwell. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for the indicated comparisons.

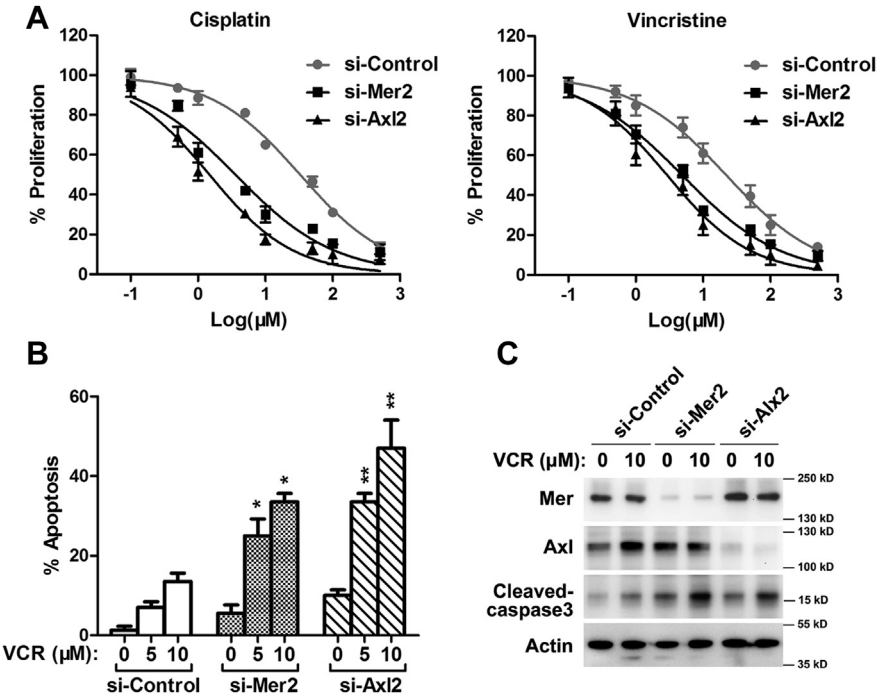

Fig. 4. Inhibition of Mer and Axl increased the chemosensitivity of neuroblastoma cells. (A) After transfected with Mer or Axl siRNA, cell proliferation after chemotherapy was determined in SHEP21N cells. (B) Cell apoptosis was determined after treated with increasing concentrations of vincristine in SHEP21N cells. (C) And the expression of Mer, Axl and cleaved caspase3 were detected by Western blot. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the si-Control cells.

demonstrated by obviously induction of cleaved-caspase3 by 10 μM vincristine (VCR) in Mer or Axl knockdown cells (Fig. 4C).

4. Discussion

Neuroblastoma is one of the few solid cancers which are only minimally responsive to conventional chemotherapeutic drugs, and the improvement in event-free survival (EFS) has relied on the success of surgical resection. Until recently, survival of high-risk patients has been about 35%, with only inconspicuous improvements in the past few years [14].

Mer and Axl, together with Tyro-3, belong to the TAM family of receptor tyrosine kinases (RTKs) [10]. Increasing evidences suggest that Mer and Axl overexpression occurs frequently in many human cancers, including various leukemias and numerous solid tumors, implicating a poor clinical prognosis [15–17]. However, the expression and the roles of Mer and Axl in neuroblastoma remain unknown. In this study, we found a highly expression of Mer and Axl in neuroblastoma tissues, and also found a significant positive correlation between them, indicating that Mer and Axl may play important roles in neuroblastoma.

Table 1
IC50 values for SHEP21N cells against chemotherapy agents.

	SHEP21N				
	si-Control	si-Mer1	si-Mer2	si-Axl1	si-Axl2
Cisplatin (μM)					
IC50 mean	35.29	4.86	2.91	1.33	0.85
s.d.	9.69	3.19	2.64	0.92	0.46
P[a]		***	***	***	***
Vincristine (μM)					
IC50 mean	23.93	9.49	4.98	4.04	1.31
s.d.	7.64	3.17	3.94	2.19	0.74
P[a]		*	**	**	***

Note: The IC50 values were determined by non-linear regression of MTS assay data.
[a] Significance versus si-Control was determined from mean and s.d. of at least three independent experiments, $^{*}p < 0.05$,$^{**}p < 0.01$ and$^{***}p < 0.001$.

Previous studies demonstrated that Mer and Axl could be activated by their ligands, implicating in the oncogenesis of many human cancers [18,19]. It has also been reported that both receptors share the ligand Gas6 [20]. Our results showed that the expression of Mer and Axl were highly correlated with that of Gas6 in neuroblastoma, and Gas6 markedly induced phosphorylation of Mer and Axl, suggesting that these RTKs were continuously activated in neuroblastoma via autocrine and/or paracrine mechanisms. Moreover, while Gas6-dependent signaling through Axl or Mer activates multiple oncogenic signaling pathways, including the FAK kinase, PI3K/AKT and MAPK pathways, blocking antibody to Mer partly suppressed such events, suggesting the combined action of both receptors. As MAPK and PI3K/AKT are known involved in cell growth, proliferation and survival, it therefore suggests that Mer and Axl contribute to these progress mainly though MAPK and PI3K/AKT signaling pathways. Additionally, regulation of neuroblastoma cell motility by Mer and Axl may be likely via altered FAK signaling, as the established roles for FAK in cell migration. The relevance of co-expression of these proteins to recurrence and progression of neuroblastoma still needed to be evaluated by further studies.

Signaling networks downstream of Mer and Axl contribute to various biologic processes such as cell survival and proliferation, migration and invasion, chemoresistance, and metastasis [21–23]. Studies to further validate targeting Mer and Axl as a potential therapeutic strategy have been quite promising. In our study, decreased expression of Mer or Axl using siRNA abrogated signaling through the FAK kinase, PI3K/AKT and MAPK pathways and functionally increased apoptosis and suppressed migration, suggesting that Mer/Axl targeted therapy may diminish neuroblastoma proliferation and metastasis. Furthermore, downregulation of either Mer or Axl expression markedly improved responsiveness to

chemotherapy, indicating that targeting Axl and Mer constituted a novel therapeutic strategy that may improve the efficacy of standard chemotherapy against neuroblastoma.

In conclusion, our work elucidated activation of Mer and Axl in neuroblastoma may be involved in the proliferation and metastasis. Inhibition of these RTKs significantly improved the efficacy of chemotherapy, providing additional treatment options in specific patient populations.

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Transparency document

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