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Short Communication

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Epidermal growth factor receptor is overexpressed in neuroblastoma tissues and cells

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Abstract

Neuroblastoma is the most common abdominal malignant tumor in childhood. Immunotoxin (IT) that targets the tumor cell surface receptor is a new supplementary therapeutic treatment approach. The purpose of this study is to detect the expression of epidermal growth factor receptor (EGFR) in neuroblastoma cell lines and tissues, and to explore if IT therapy can be used to treat refractory neuroblastoma. The EGFR expression in human neuroblastoma tissue samples was detected by immunohistochemistry staining. The positive rate of EGFR expression was 81.0% in neuroblastoma tissue and 50.0% in gangliocytoma, respectively, but without statistical significance between them ($P > 0.05$). The positive rate of EGFR expression in favorable type and unfavorable type was 62.5% and 92.3%, respectively, but they were not statistically different ($P > 0.05$). Results from pre-chemotherapy and post-chemotherapy samples showed that there was no significant statistical difference ($P > 0.05$) between them in the EGFR expression. Furthermore, the EGFR expression levels in five neuroblastoma cell lines were measured using cell-based ELISA assay and western blot analysis. The results showed that the expression of EGFR was higher in KP-N-NS and BE(2)-C than those in other cell lines. Our results revealed that there are consistent and widespread expressions of EGFR in neuroblastoma tissues as well as in neuroblastoma cell lines, suggesting that it is possible to develop future treatment strategies of neuroblastoma by targeting at the EGFR.

Key words: neuroblastoma, immunotoxin, epidermal growth factor receptor

Introduction

Neuroblastoma is the most common abdominal malignant tumor in children [1]. It often invades important organs or tissues with only 70% complete resection rate. Due to its hidden onset and rapid development, the disease is often diagnosed at stage 4 during its growth, yielding remote metastases in bone, bone marrow, liver, brain, and other sites. The remote metastases increase the difficulty in treatment and lead to unfavorable outcomes. Although comprehensive measures such as surgery [2], chemotherapy [3], radiotherapy [4], and biological treatment [5] have been used, small residual

foci deep in the body still cannot be eradicated completely, resulting in relapse and distant metastasis after termination of treatment [6]. The drug resistance of tumor and the development of bone marrow suppression during treatment can make regular therapy no longer effective. Furthermore, the toxic effects of high drug dosage used in regular therapies can damage the functions of the heart, lung, liver, and kidney. Ultimately, many children might even die of organ failure or infections instead of the tumor itself. Moreover, significant amount of drugs still remain in the body for a long time after treatment, which can cause chronic organ failure and secondary tumors. New methods of complementary treatment, which can increase tumor clearance and reduce the side effects of toxic drugs, would undoubtedly be of great importance.

Immunotoxin (IT) therapy is a type of auxiliary tumor therapy. ITs are targeted toxin proteins (monoclonal antibodies or peptides), which can specifically bind to tumor surfaces and selectively kill tumor cells but seldom harm normal cells [7]. Currently, bacterial toxins, such as diphtheria toxin or *Pseudomonas aeruginosa* exotoxin A [8], are widely used in IT therapies. In particular, a recombinant IT protein composed of a diphtheria toxin fragment and interleukin-2 fusion protein has been used to treat lymphoma [9,10]. Similarly, a recombinant IT fused with anti-CD22 monoclonal antibody and pseudomonas exotoxin segment has been successfully used in the treatment of hairy cell leukemia, with the complete remission rate of 60% [11]. Thus, for some refractory tumors, IT therapy can be a promising strategy.

Human epidermal growth factor receptor (EGFR) is a cell surface receptor for the extracellular protein ligands of the EGF family. EGFR is expressed in normal cells and participates in cell growth, proliferation, and differentiation. However, EGFR is overexpressed in many tumors [12,13], and has become a widely used tumor therapeutic target. Transforming growth factor (TGF)-α is a member of the EGF family and a ligand of EGFR. The specific binding of TGF-α with EGFR forms the theoretical basis for using TGF-α-conjugated anti-tumor drugs to target tumor cells that over-express EGFR and increase the specificity and tumor cell-killing activity of the drugs [14].

Therefore, if over-expression of EGFR is present on the surface of neuroblastoma cells, the application of TGF-α-conjugated IT as a targeted therapy of neuroblastoma will be possible and feasible. In this preliminary study, the expressions of EGFR in neuroblastoma tissues and in neuroblastoma cell lines were investigated to demonstrate the possibility of using IT therapy in the future treatment of neuroblastoma.

Materials and Methods

Cell culture

All cells were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in a humidified incubator at 37°C with 5% CO_2. A431 cells and KP-N-NS cells were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose (Hyclone, Logan, USA) supplemented with 10% FBS (Gibco, Gaithersburg, USA), 100 μg/ml penicillin, and 100 μg/ml streptomycin (Hyclone). SK-N-BE(2) cells, BE (2)-C cells, SK-N-SH cells, and SH-SY5Y cells were grown in DMEM/F12 medium (Hyclone) with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. HFL1 cells were grown in F-12K nutrient mixture (Gibco) with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Neuroblastoma tissue samples

Neuroblastoma tissue samples and clinical data were obtained from the Department of Surgery, Children's Hospital of Fudan University (Shanghai, China). The study was approved by the Local Research Ethics Committee of the hospital and written consent forms were signed by the parents of each patient. A total of 25 children who admitted to the hospital between August 2008 and April 2014 were included. There were 14 males and 11 females (0.1–12 years old) with average age of diagnosis around 4.3 years. Overall, 4 children were diagnosed with gangliocytoma, and the rest 21 children were diagnosed with neuroblastoma. Each tissue sample was sectioned, fixed in 10% neutral formalin and embedded in paraffin. Post-chemotherapy neuroblastoma tissue samples were also collected from 10 children with neuroblastoma. All the samples were examined by one pathologist. According to Shimada histopathologic classification, 8 of the 21 cases with neuroblastoma were favorable and 13 were unfavorable. Two liver biopsy samples of choledochus cyst were also obtained from the Department of Surgery, Children's Hospital of Fudan University (Shanghai, China) and included in the analysis as the control group [15].

Immunohistochemistry staining

The Formalin-Fixed and Parrffin-Embedded tissue was cut into 4-μm thick sections and affixed onto the slides. After being deparaffinized and rehydrated, the antigens were retrieved in boiled Tris-EDTA (pH 9.0) buffer for 15 min, cooled off for 1 h in the fume hood, and then blocked according to the protocol of DAB polymer detection kit (Gene Tech, Shanghai, China) for 10 min. The slides were incubated with primary antibody (anti-EGFR antibody, 1:500, ab137660; Abcam Biotechnology, Cambridge, UK) in 1% bovine serum albumin (BSA)/tris-base solution buffer at 4°C overnight. The next day, the slides were incubated with the secondary antibody and developed with DAB reagent according to the protocol of DAB polymer detection kit (Gene Tech). Finally, the slides were counterstained with hematoxylin. Omission and substitution of primary antibody with antibody diluent were used as the reagent blank to ensure the validity of the staining. The vascular epithelial cells in the same slide were considered as the positive control. The immunostainings were analyzed by one pathologist and scored as follows: 0 (−): <5% positive cells (no or weak staining intensity); 1 (+): between 5% and 25% positive cells (weak staining intensity); 2 (++): between 25% and 50% positive cells (moderate staining intensity); 3 (+++): $\geq$ 50% positive cells (strong staining intensity).

Cell-based ELISA

Cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated overnight at 37°C. On the next morning, the cells were washed with phosphate buffer saline (PBS), and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, cells were blocked by incubation with 3% BSA/PBS for 1 h at 37°C, followed by incubation with primary antibody (anti-EGFR antibody, 1:1000; ab137660; Abcam Biotechnology) for 2 h at 37°C. After being washed with 0.3% BSA/PBS, the cells were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (HRP-conjugated goat anti-rabbit antibody, 1:1000; Biotech Well, Oklahoma City, USA) for 1 h at 37°C. After being washed with PBS, the cells were incubated with 150 μl of 3,3′,5,5′-tetramethylbenzidine (TMB reagent; Biotech Well) for 30–60 min at room temperature in the dark. The reaction was stopped with the addition of

50 μl of 0.5 M HCl. Finally, the absorbance in each well was measured at 450 nm with a microplate reader (Thermo, Waltham, USA). A431 cell, a human epidermoid squamous cell carcinoma cell line, which over-expresses EGFR on the cell membrane [11], was used as the positive control [16]. HFL1 cell, a human normal fibroblast lung cell line, was used as the control group [17]. Omission and substitution of primary antibody with antibody diluent in each one of the six cell line parallel wells were utilized as the reagent blanks.

Western blot analysis

Whole cell extracts were prepared using radio immunoprecipitation assay lysis buffer containing proteinase inhibitors (Beyotime Biotechnology, Haimen, China). Total proteins extracts (30–60 μg) of each cell line were subject to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Invitrogen Life Technologies, Carlsbad, USA). The membrane was blocked with 5% non-fat milk for 2 h at room temperature and then incubated with the primary antibody (anti-EGFR antibody, 1:1000, ab137660; Abcam Biotechnology) overnight at 4° C. The membrane was then washed three times for 5 min in Tris-Buffered Saline with Tween at room temperature. Then the membrane was incubated with appropriate HRP-conjugated secondary antibody (HRP-conjugated goat anti-rabbit antibody, HRP-conjugated goat anti-mouse antibody, 1:1000; Biotech Well) for 1 h at room temperature. Finally, the membrane was detected using an enhanced chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate; Millipore, Billerica, USA). β-Actin was also detected using a mouse monoclonal anti-β-actin antibody (1:1000; Biotech Well) and used as the internal loading control.

Statistical analyses

All statistical analyses were carried out using ANOVA, Fisher's exact test, CMH $\chi 2$ test, and Wilcoxon matched-pairs signed-ranks test with Stata 10.1 statistical software (StataCorp, 4905 Lakeway Drive, College Station, USA). Data were expressed as the mean ± SD from at least three separate experiments. $P < 0.05$ was considered statistically significant.

Results

Expression of EGFR in neuroblastoma tissues

A total of 35 samples (10 of 21 children with neuroblastoma had both pre- and post-chemotherapy samples) from 25 children were investigated. As shown in **Fig. 1**, the immunohistochemistry (IHC) staining revealed that EGFR was localized both on the cell membrane and in the cytoplasm. Tumor samples from gangliocytoma showed rare positivity in some ganglion cells (**Fig. 1B**). In contrast, tumor samples from neuroblastoma showed significantly greater and more dispersive EGFR staining, especially in the immature cells (**Fig. 1A**), than the ganglion cells (**Fig. 1B**). **Figure 1C** and **D** showed the expression of EGFR in pre- and post-chemotherapy samples, where EGFR staining was seen on the cell membrane and in the cytoplasm of most immature cells. Two liver biopsy samples of choledochus cyst were shown as control group (**Fig. 1E,F**). There was no EGFR staining in the hepatocytes when compared with the vascular epithelial cells, which showed strong EGFR staining.

The scores of EGFR IHC staining of tissues from 21 neuroblastoma and 4 gangliocytoma were listed in **Table 1**. The positive rate of EGFR expression in neuroblastoma was 81.0% (17/21) and 50.0% (2/4) in gangliocytoma, but with no significant statistical difference between the positive rate of EGFR expression in neuroblastoma and gangliocytoma ($P > 0.05$, by Fisher's exact test). By contrast, EGFR IHC staining intensity in neuroblastoma tissue was significantly higher (8 with a score of 2 and 9 with a score of 3) than that in gangliocytoma tissue (2 with a score 1). There was significant statistical difference between the distribution of EGFR expression levels in neuroblastoma and gangliocytoma ($P < 0.05$, by CMH χ^2 test).

The scores of EGFR IHC staining of tissues from 8 favorable type neuroblastoma and 13 unfavorable type neuroblastoma were listed in **Table 2**. The positive rate of EGFR expression in favorable type and unfavorable type was 62.5% (5/8) and 92.3% (12/13), respectively, with no significant statistical difference in the positive rate of EGFR expression between these two types ($P > 0.05$, by Fisher's exact test). Moreover, the cases with different staining scores showed no significant statistical difference between the distributions of EGFR expression levels in these two types ($P > 0.05$, by CMH χ^2 test).

To explore the possible correlation between the expression of EGFR and chemotherapy, pre- and post-chemotherapy tissue samples and clinical data were collected from 10 children with neuroblastoma, and the expression of EGFR in pre-chemotherapy was compared with that of post-chemotherapy in self-control manner. The scores of EGFR IHC staining of tissues from 10 pre- and post-chemotherapy samples were listed in **Table 3**. There was no significant statistical difference in the EGFR expressions between the pre- and post-chemotherapy tissue samples revealed by the Wilcoxon matched-pairs signed-ranks test ($P > 0.05$).

Expression of EGFR in neuroblastoma cell lines

To further explore whether EGFR was expressed in cultured neuroblastoma cells, five different neuroblastoma cell lines were selected and cultured. The expression levels of EGFR were determined by cell-based ELISA. As shown in **Fig. 2**, five neuroblastoma cell lines exhibited different expression levels of EGFR ($P < 0.0001$). The expression in KP-N-NS cell was higher than that in BE(2)-C cell, and both of them were higher than those in the other three cell lines. EGFR expressions in all these five neuroblastoma cell lines were higher than that a normal human fibroblast lung cell line in HFL1.

Western blot analysis was used to further confirm the expressions of EGFR in different neuroblastoma cell lines (**Fig. 3**). All the five neuroblastoma cell lines expressed EGFR, with higher expression in KP-N-NS cells than in BE(2)-C cells and relatively weaker expressions in the remaining three cell lines (**Fig. 3A**). The EGFR expression in the normal human fibroblast lung cell line was very low. The histogram (**Fig. 3B**) showed the mean ± SD from three independent experiments. The result of western blot analysis was consistent with the result of cell-based ELISA, indicating that all neuroblastoma cell lines expressed EGFR, but with different expression levels.

Discussion

Targeted therapy can be a promising auxiliary therapy for some refractory tumors. It has been applied in lung cancer, ovarian carcinoma, breast cancer, melanoma, and so on. For neuroblastoma, Infarinato *et al.* [18] reported that heritable mutations of anaplastic lymphoma kinase (ALK) were the main cause of familial neuroblastoma, and a novel drug, PF-06463922, which targets ALK more precisely than crizotinib, could be an inspiring therapy for this lethal pediatric malignancy. Chipumuro *et al.* [19] reported that CDK7 inhibition may be a useful therapy for cancers that are driven by

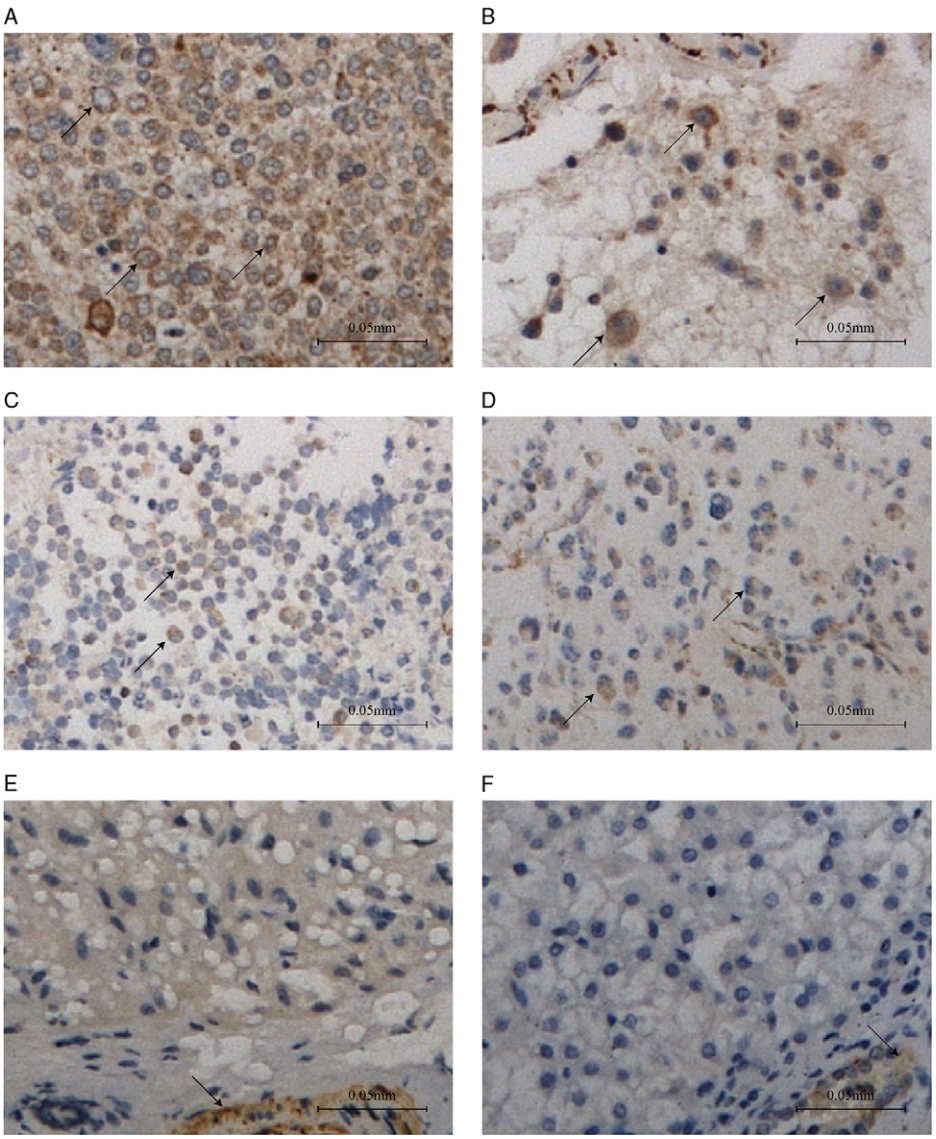

Figure 1. Representative IHC staining images of different tissues IHC staining was performed using anti-EGFR antibody. EGFR immunostained areas were shown at a magnification of ×100. Blue stains are indicative of cell nucleus stained with hematoxylin staining solution. Brown stains (indicated by arrows) are indicative of EGFR expression. (A) Neuroblastoma tissue, EGFR was stably expressed on the cell membrane and in the cytoplasm in most immature cells that are characterized by relatively high ratio of nucleoplasm to cytoplasm. (B) Gangliocytoma tissue, the EGFR was sporadically expressed in some ganglion cells. (C) Pre-chemotherapy neuroblastoma tissue and (D) post-chemotherapy tissue, EGFR was expressed on the cell membrane and in the cytoplasm in both samples with no significant difference. (E,F) Liver biopsy, EGFR was not expressed in hepatocyte, but strongly expressed in vascular epithelial cells.

Myc family oncoproteins like neuroblastoma, by selectively targeting the mechanisms that promote global transcriptional amplification in tumor cells. Previous reports also showed that ATF4 agonists and glutaminolysis inhibitors were potential cancer therapeutics against Myc-driven tumor, which opened a new field in target treatment for neuroblastoma [20,21]. These findings displayed a great potential in targeted therapy of neuroblastoma.

Because of the ligand–receptor relationship between EGFR and TGF-α, this preliminary study focuses on the determination the expression of EGFR on neuroblastoma's cell surface, which will help to explore the possibility of using a fusion protein formed by combining TGF-α with anti-tumor drug to kill neuroblastoma cells. If EGFR is expressed on the neuroblastoma's cell surface, neuroblastoma cell can receive TGF-α on the cell surface and the cancer cell can be killed by the anti-tumor drug connected to the TGF-α. Therefore, this new EGFR-mediated therapeutic method may represent original and novel-targeted treatment of neuroblastoma.

Our results showed that the positive expression rate of EGFR in neuroblastoma and ganglioneuroma was not statistically different, while the intensity of EGFR staining was significantly different between neuroblastoma and ganglioneuroma. In addition, the expression of EGFR was not significantly related to the Shimada Pathological Classification. Moreover, 10 cases with neuroblastoma were compared within themselves before and after chemotherapy, showing no significant difference in EGFR expression. These results suggested a wide expression of EGFR in neuroblastoma, and the positive rate of EGFR expression is independent of neuroblastoma or ganglioneuroma, pathological classification, or chemotherapy. However, the different intensities of EGFR staining between neuroblastoma and ganglioneuroma may suggest that the more immature

cells in the tumor tissue, the higher expression of EGFR. In addition, no significant statistical difference was found in the EGFR expressions between the groups of different age, gender, tumor stage or risk of neuroblastoma (data not shown).

Table 1. EGFR expression in different types of tumor tissues determined by IHC staining[a]

Tumor type	Negative cases (score)	Positive cases (score)			Total cases
Neuroblastoma	4 (−)	17[b]			21
		0 (+)[c]	8 (++)[c]	9 (+++)[c]	
Gangliocytoma	2 (−)	2[b]			4
		2 (+)[c]	0 (++)[c]	0 (+++)[c]	

[a]Data were expressed as the number of cases with staining scores in parenthesis. 0 (−): <5% positive cells (no or weak staining intensity); 1 (+): between 5% and 25% positive cells (weak staining intensity); 2 (++): between 25% and 50% positive cells (moderate staining intensity); 3 (+++): ≥50% positive cells (strong staining intensity).

[b]The positive rates of EGFR expression in neuroblastoma were 81.0% (17/21) and 50.0% (2/4) in gangliocytoma and gangliocytoma, respectively. There was no significant statistical difference between the positive rates of EGFR expression in neuroblastoma and gangliocytoma ($P > 0.05$, by Fisher's exact test).

[c]There was significant statistical difference between the distributions of EGFR expression levels in neuroblastoma and gangliocytoma ($P < 0.05$, by CMH χ^2 test).

Table 2. EGFR expression in favorable and unfavorable neuroblastoma tissues determined by IHC staining[a]

Tissue type[b]	Negative	Positive			Total case
Favorable	3 (−)	5[c]			8
		0 (+)[d]	3 (++)[d]	2 (+++)[d]	
Unfavorable	1 (−)	12[c]			13
		0 (+)[d]	5 (++)[d]	7 (+++)[d]	

[a]Data were expressed as the number of cases with staining scores in parenthesis. 0 (−): <5% positive cells (no or weak staining intensity); 1 (+): between 5% and 25% positive cells (weak staining intensity); 2 (++): between 25% and 50% positive cells (moderate staining intensity); 3 (+++): ≥50% positive cells (strong staining intensity).

[b]Tissue type was determined by Shimada Histopathologic Classification.

[c]The positive rates of EGFR expression in favorable type and unfavorable type were 62.5% (5/8) and 92.3% (12/13), respectively, with no significant statistical difference between the positive rates of EGFR expression in both types ($P > 0.05$, by Fisher's exact test).

[d]The cases with different staining scores showed no significant statistical difference between the distributions of EGFR expression levels in both types ($P > 0.05$, by CMH χ^2 test).

Both cell-based ELISA and western blot analysis results showed that EGFR was stability expressed in five neuroblastoma cell lines, three of which (i.e. KP-N-NS, SK-N-BE(2), and BE(2)-C) had not been investigated. We also found that EGFR expression levels in BE (2)-C cells and KP-N-NS cells were significantly higher than those in the other three cell lines, suggesting that these two cell lines may be used as the reference cell lines in future study of IT therapy against neuroblastoma. Our results were consistent with that of Ho *et al.* [22] who found that 13 neuroblastoma cell lines expressed EGFR, most at readily detectable levels.

It should be noted that this study was still preliminary and only 25 patients were included in the study. The results will be improved and confirmed by including more patients and more balanced cases between subgroups in the future study. In addition, more detailed clinical data such as MYCN gene amplification, clinical stages, and risk groups should be collected.

In summary, we proved the expression of EGFR protein in neuroblastoma tissues and cell lines, which is the basis for designing IT therapy in the treatment of neuroblastoma by targeting this protein. Our results also suggest that IT therapy may be applied as a possible supplementary treatment of refractory or relapsed neuroblastoma in the future. If IT can be designed to target EGFR, for example, using TGF-α as a ligand of EGFR, specific IT can act on neuroblastoma of any pathological types or any therapeutic states with targeted cell toxicity. However, the toxicity of any fusion protein drugs should be tested both *in vitro* and *in vivo*. One of the major challenges in this area that requires great attention is

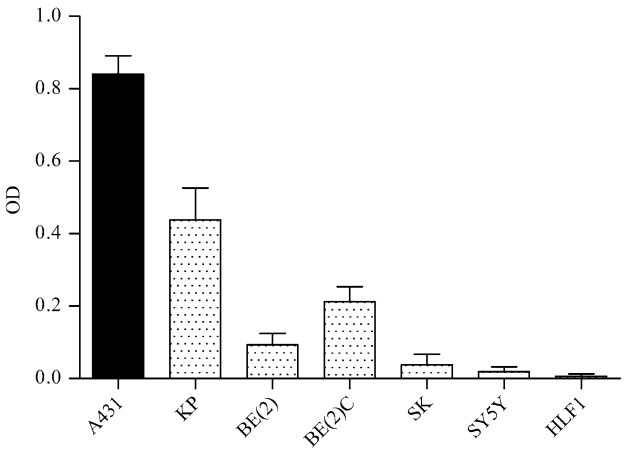

Figure 2. Expression levels EGFR in different cell lines detected by cell-based ELISA Data are expressed as the mean ± SD of the optical densities from three independent experiments. A431 is a human epidermoid squamous cell carcinoma cell line and used as a positive control. HFL1 is a normal human fibroblast lung cell line, thus used as control group. Omission and substitution of primary antibody with antibody diluent in each one of the six cell line parallel wells were used as reagent blanks.

Table 3. The expression of EGFR in the pre- and post-chemotherapy neuroblastoma tissues determined by IHC staining[a]

Case no.	1	2	3	4	5	6	7	8	9	10
Score of the pre-chemotherapy sample[b]	+++	+++	+++	++	+++	++	+++	+++	++	++
Score of the post-chemotherapy sample[b]	+++	+++	−	++	−	+++	++	++	+++	++

[a]Data are expressed as the scores of IHC staining. 0 (−): <5% positive cells (no or weak staining intensity); 1 (+): between 5% and 25% positive cells (weak staining intensity); 2 (++): between 25% and 50% positive cells (moderate staining intensity); 3 (+++): ≥50% positive cells (strong staining intensity).

[b]There was no significant statistical difference of the EGFR expression between the pre- and post-chemotherapy neuroblastoma tissue samples ($P > 0.05$, by Wilcoxon matched-pairs signed-ranks test).

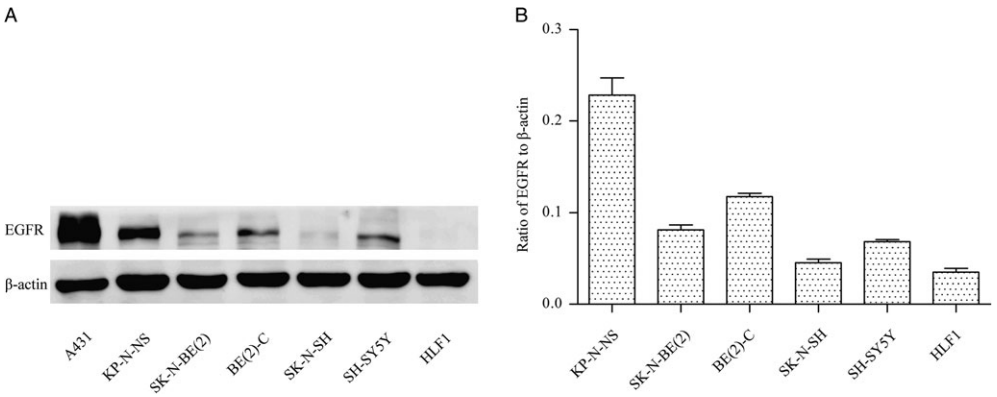

Figure 3. Expression levels of EGFR in different cell lines detected by western blot analysis (A) Western blot analysis of EGFR in different neuroblastoma cell lines. A431 was the positive control and HFL1 was the control group. (B) The ratio of EGFR to β-actin in different cell lines.

the activation of EGFR. Such activation may stimulate the intracellular signaling pathways, which may ultimately alter the proliferation, invasion, and survival rate of the cancer cells in the patient body.

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