Biological significance of *EPHA2* expression in neuroblastoma

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Abstract. Neuroblastoma is a pediatric solid tumor that exhibits striking clinical bipolarity. Despite extensive efforts to treat unfavorable neuroblastoma, survival rate of children with the disease is among the lowest. Previous studies suggest that EPHA2, a member of the EPH family receptor kinases, can either promote or suppress cancer cell growth depending on cellular contexts. In this study, we investigated the biological significance of EPHA2 in neuroblastoma. It was found that tumorigenic N-type neuroblastoma cell lines expressed low levels of *EPHA2*, whereas hypo-tumorigenic S-type neuroblastoma cell lines expressed high levels of *EPHA2* ($p<0.005$). Notably, inhibitors of DNA methylation and histone deacetylase enhanced *EPHA2* expression in N-type cells, suggesting that *EPHA2* is epigenetically silenced in unfavorable neuroblastoma cells. Furthermore, ectopic high-level expression of *EPHA2* in N-type neuroblastoma cell lines resulted in significant growth suppression. However, Kaplan-Meier survival analysis showed that high *EPHA2* expression was not associated with a good disease outcome of neuroblastoma, indicating that *EPHA2* is not a favorable neuroblastoma gene, but a growth suppressive gene for neuroblastoma. Accordingly, EPHA2 expression was markedly augmented *in vitro* in neuroblastoma cells treated with doxorubicin, which is commonly used for treating unfavorable neuroblastoma. Taken together, EPHA2 is one of the effectors of chemotherapeutic agents (e.g., gene silencing inhibitors and DNA damaging agents). EPHA2 expression may thus serve as a biomarker of drug responsiveness for neuroblastoma during the course of chemotherapy. In addition, pharmaceutical enhancement of EPHA2 by non-cytotoxic agents may offer an effective therapeutic approach in the treatment of children with unfavorable neuroblastoma.

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Introduction

Neuroblastoma is a neural crest-derived tumor and is the most common extracranial pediatric malignancy. The tumor accounts for 7-10% of all childhood cancers and is the cause for ~15% of fatalities in children with cancer. Neuroblastoma is unique because of its propensity to exhibit either a favorable or an unfavorable phenotype. Favorable neuroblastomas undergo spontaneous regression or maturation or are curable by surgical removal with or without adjuvant chemotherapy. In contrast, unfavorable neuroblastomas exhibit unrestrained growth despite the most intensive treatment (1).

EPHA2 is a member of the EPH (erythropoietin-producing hepatoma amplified sequence) family receptor tyrosine kinases. Like most EPH family receptor tyrosine kinases, EPHA2 is expressed during early development (2), but its expression is also detected in adult epithelial cells (3). Although specific cellular functions of EPHA2 in normal epithelia are not well understood, several studies suggest potential roles for EPHA2 in the control of cell growth, survival, migrations, and angiogenesis (4-8). EPHA2 is overexpressed and functionally altered in a variety of solid tumors, including colon cancer, glioma/glioblastoma, mammary cancer, melanoma and prostate cancer (5,9-17). EPHA2 expression is thus linked to the regulation of cellular behavior that confer a metastatic phenotype. In contrast, EPHA2 promotes apoptosis and suppresses growth of lung cancer, breast cancer, and melanoma cells (18-20). EPHA2 also acts as a tumor suppressor in a chemically-induced skin cancer model in mouse (21). In addition, *EPHA2* transcription is regulated by p53 (18,19). These observations suggest that EPHA2 can exhibit opposite biological effects: promotion or suppression of cell growth on cancer cells depending upon their cellular context. In this study, we investigated the biological significance of *EPHA2* in neuroblastoma. Our results show that *EPHA2* is a neuroblastoma growth-suppressive gene and that EPHA2 expression has potential therapeutic and clinical applications in neuroblastoma.

Materials and methods

Neuroblastoma cell lines. All neuroblastoma cell lines were grown in RPMI-1640 supplemented with 5% fetal bovine serum and 1% OPI (Gibco, Grand Island, NY). These cell lines were tested negative for mycoplasma, and their identity was validated by the original source or by microsatellite analysis. NBL-S was obtained from Dr Susan L. Cohn (University of Chicago). OAN, SKNAS, LHN, KAN, SAN, LAN5, KPN, LA1-55N, LA1-5S, KCN, and KCNR were from Dr C. Patrick Reynolds (Children's Hospital Los Angeles, CA). Nb69, IMR5 (a clone of IMR32), and CHP134 were from Dr Roger H. Kennett (Department of Biology, Wheaton College, Wheaton, IL; a former faculty member of Department of Human Genetics, The University of Pennsylvania School of Medicine). SY5Y and SHEP were from Dr Robert Ross (Fordham University, Bronx, NY). NGP, NMB, and NLF were from Dr Garrett M. Brodeur (The Children's Hospital of Philadelphia). CHP902 was established by Dr Hiro Kuroda (The Children's Hospital of Philadelphia). CHP901 and CHP902R were established by Dr Naohiko Ikegaki.

Primary neuroblastoma tumor samples. Fifty neuroblastoma tumor specimens were obtained from the Tumor Bank of the former Pediatric Oncology Group, the Tumor Bank of the Children's Hospital of Philadelphia, and Memorial Sloan-Kettering Cancer Center. The neuroblastoma cohort included 10 of stage 1, 8 of stage 2, 5 of stage 4S, 12 of stage 3, and 15 of stage 4. Among these, 9 are *MYCN*-amplified (18%). Of 50 neuroblastoma specimens, 49 had survival data. The neuroblastoma cohort was verified by the results of Kaplan-Meier analyses, which demonstrated that the established prognostic markers of neuroblastoma (age, stage, *MYCN* amplification, *EPHB6*) predict disease outcome in this cohort. The clinical correlative studies were performed at the Children's Hospital of Philadelphia, and the use of human tumor samples for this study was reviewed and approved by its institutional review board.

Quantitative reverse transcription-PCR (RT-PCR). RNAs were isolated from neuroblastoma cell lines or primary neuroblastoma tumors using the Qiagen RNeasy kit. Experimental procedures for the quantitative RT-PCR were previously described elsewhere (22,23). Primer sequences for *EPHA2* are 5'-TGCAGCAGTATACGGAGCAC-3', and 5'-TTCACC TGGTCCTTGAGTCC-3'.

Preparation of 5AdC, 4PB, and doxorubicin. 5-Aza-2'-deoxycytidine or 5AdC (Fluka) and sodium 4-phenylbutyrate or 4PB (Aldrich) were prepared as previously described (23). Doxorubicin (Sigma) was prepared by dissolving in acidic H_2O at the concentration of 2.5 mg/ml as a stock.

Western blot analysis. Western blot was performed according to the method previously described (24) except SuperSignal West Dura Extended Duration Substrate (Pierce) was used. Light emission signals were captured by either a Versadoc 5000 (Bio-Rad) or a LAS-3000 (Fuji) digital image analyzer. Cell extracts were made in the 2D gel sample buffer (9 M urea, 2% Nonidet-P40, 2% 2-mercaptoethanol, and 0.32% pH 3-10 2D Pharmalyte) and the protein content of the samples was determined by the Bio-Rad protein assay kit using bovine serum albumin as a standard and the sample buffer as the blank. The anti-EPHA2 mouse monoclonal antibody D7 was purchased from Upstate USA, Inc. The monoclonal antibody specific for p53, PAB1801, was purchased from Santa Cruz Biotechnology. The monoclonal antibody specific for $p21^{waf1}$, EA10, was purchased from Calbiochem.

Transient transfection of neuroblastoma cells with EPHA2. A cDNA clone of human *EPHA2* (3) was subcloned into pCI-neo mammalian expression vector (Promega). Neuroblastoma cell lines were transfected with pCI-neo or pCI/*EPHA2* by electroporation using a Gene Pulser Xcell electroporator (Bio-Rad) (120 V, 25 msec, a single square wave).

MTT assay. One and a half million SY5Y or IMR5 cells were transfected by electroporation with either pCI-neo eukaryotic expression vector (Invitrogen) alone or the vector containing a human *EPHA2* cDNA. The resulting transfectants were plated into 6 wells of a 24-well plate and selected for 5 days with 500 μg/ml neomycin. After selection, the cells were treated with 0.5 μg/ml MTT for 4 h to stain viable cells and to examine the effect of *EPHA2* on growth of neuroblastoma cells.

Statistical analysis. A χ^2 test with Yates' correction was employed to examine statistical significance of *EPHA2* expression in neuroblastoma cell lines. Survival probabilities in neuroblastoma subgroups were estimated according to the methods of Kaplan and Meier (25). Survival distributions were compared using log-rank tests (26). $P<0.05$ was considered statistically significant.

Results

Neuroblastoma cell lines derived from unfavorable neuroblastoma express low levels of EPHA2. We first examined *EPHA2* expression in 23 neuroblastoma cell lines and determined its expression pattern. As shown in Fig. 1, among the cell lines, N-type neuroblastoma cells expressed low levels of *EPHA2*. In contrast, S-type neuroblastoma cell lines (SHEP and LA1-5S) expressed high levels of *EPHA2*, and this differential expression was statistically significant ($p<0.005$). The above observation was intriguing because S-type neuroblastoma cells are known to be hypo-tumorigenic in mouse xenograft models and are considered more benign than N-type cells (27,28).

EPHA2 expression is silenced in N-type neuroblastoma cell lines. Our gene profiling study has shown that treatment of the neuroblastoma cell line IMR5 with inhibitors of DNA methylation and HDAC resulted in an increased *EPHA2* expression (Tang and Ikegaki, unpublished data). In this report, we confirmed the previous observation by quantitative RT-PCR. As shown in Fig. 2, *EPHA2* expression was significantly increased in neuroblastoma cell lines IMR5, CHP134 and SY5Y upon treatment with inhibitors of DNA

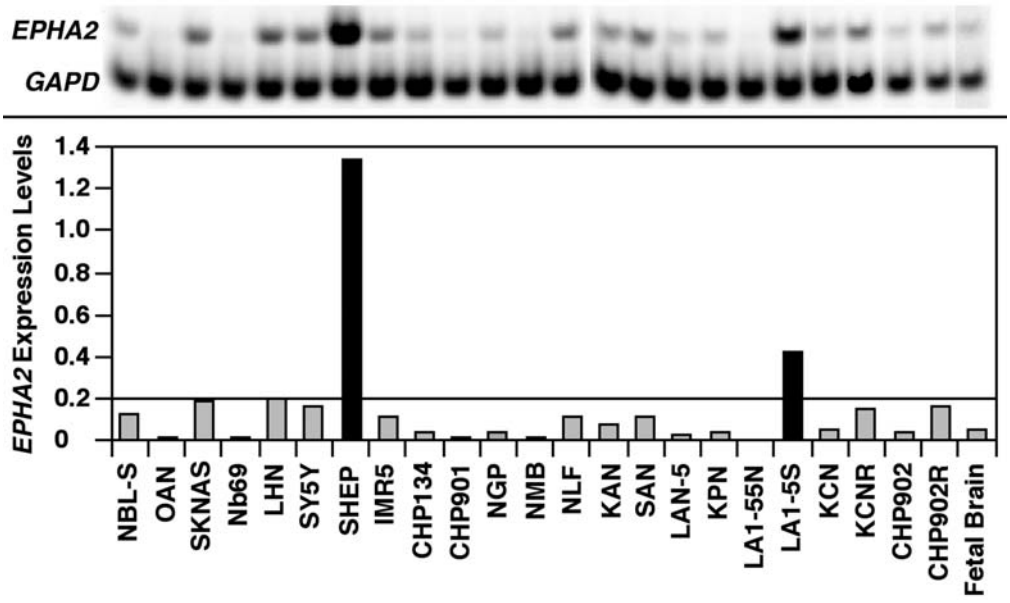

Figure 1. *EPHA2* expression in neuroblastoma cell lines. Quantitative RT-PCR was used to assess levels of *EPHA2* expression in neuroblastoma cell lines. Neuroblastoma cell lines derived from unfavorable neuroblastoma express low levels of *EPHA2*. However S-type neuroblastoma cells (SHEP and LA1-5S), which are more benign as they seldom form tumors in nude mice, express high levels of *EPHA2*. Differential expression of *EPHA2* was examined by a χ^2 test with Yates' correction.

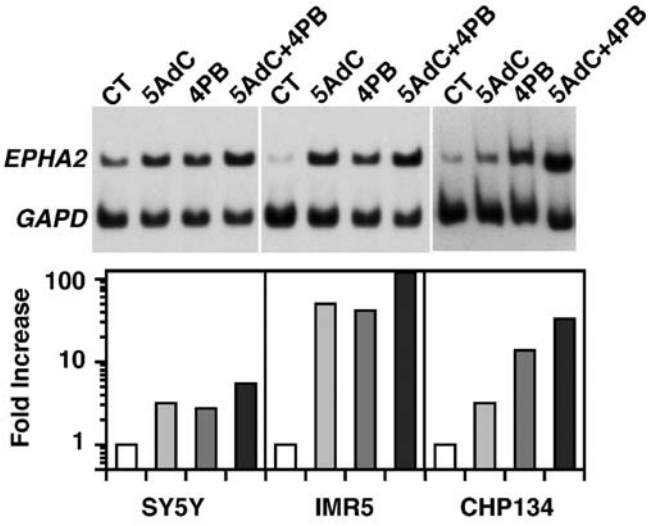

Figure 2. Inhibitors of DNA methylation and HDAC enhance *EPHA2* expression in neuroblastoma cells. *EPHA2* expression is enhanced by 5AdC and 4PB in neuroblastoma cell lines. *EPHA2* expression in neuroblastoma cell lines was examined using quantitative RT-PCR. SY5Y is a *MYCN* non-amplified cell line. IMR5 and CHP134 are a *MYCN*-amplified cell lines. These cells were treated with 5AdC (2.5 μM) and/or 4PB (2.5 mM) for 4 days and subjected to the gene expression studies. Levels of *EPHA2* expression were presented as fold increase over control (no drug treated cells).

methylation (5-Aza-2'-deoxycytidine or 5AdC) and HDAC (sodium 4-phenylbutyrate or 4PB).

Forced expression of EPHA2 in unfavorable neuroblastoma cells results in growth suppression. The facts that *EPHA2* expression is low in N-type neuroblastoma cells, and that gene silencing inhibitors suppress growth of these neuroblastoma cells (23) and can increase *EPHA2* expression suggest that high EPHA2 expression confers a growth suppressive effect on tumorigenic N-type neuroblastoma cell lines. We therefore examined the effect of forced EPHA2 expression on two N-type neuroblastoma cell lines (SY5Y and IMR5). SY5Y is a *MYCN*-non-amplified neuroblastoma cell line and IMR5 is a *MYCN*-amplified line, which express low levels of endogenous *EPHA2* (Fig. 1). As shown in Fig. 3A, transfection of SY5Y and IMR5 with an *EPHA2* cDNA, in fact, significantly inhibited the clonogenicity *in vitro*. The expression of EPHA2 protein in the transfectants was also confirmed by Western blot analysis (Fig. 3B). These *EPHA2* transfectants expressed EPHA2 protein at similar levels to that in SHEP cells.

EPHA2 expression is not associated with neuroblastoma disease outcome. The above results indicate that *EPHA2* shares several characteristics with favorable neuroblastoma genes (*EPHB6, EFNB2, EFNB3, TrkA, CD44* and *MIZ-1*) (22,23,29). Their expression is low in neuroblastoma cell lines and can be enhanced by gene silencing inhibitors. In addition, forced expression of these genes in neuroblastoma cell lines results in growth suppression. We therefore investigated whether or not *EPHA2* expression would be associated with disease outcome of neuroblastoma in a cohort of 50 primary tumors. This cohort was representative of the general neuroblastoma population as the expression of known favorable neuroblastoma genes such as *EPHB6* and *TrkA* was associated with disease outcome (data not shown). As shown in Fig. 4, the Kaplan-Meier analysis demonstrated that high *EPHA2* expression was not associated with a good disease outcome of neuroblastoma. Hence, *EPHA2* is not a favorable neuroblastoma gene (see Discussion), but a growth suppressive gene for neuroblastoma.

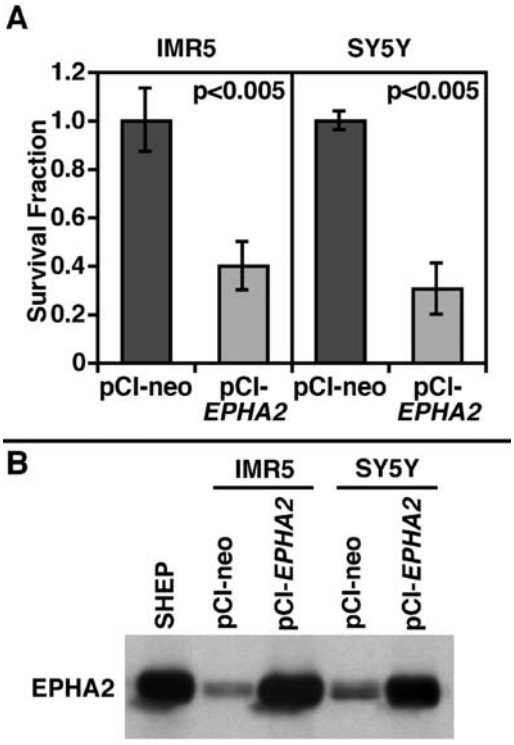

Figure 3. Forced expression of *EPHA2* in neuroblastoma cell lines results in growth suppression. (A) A full-length cDNA of *EPHA2* was cloned into a eukaryotic expression vector, pCI-neo (Promega). IMR5 and SY5Y cells were transfected with either the vector control or the pCI/*EPHA2* cDNA construct by electroporation. The resulting transfectants were selected by neomycin (500 μg/ml) for 4 days. MTT assay was used to assess viable cells in each culture on the seventh day. (B) Expression of EPHA2 protein in the EPHA2 transfectant neuroblastoma cells. SHEP was used as a positive control.

Doxorubicin augments the expression of EPHA2 in neuroblastoma cells. The growth suppressive effect of EPHA2 on tumorigenic N-type neuroblastoma cells suggests that EPHA2 is potentially involved in the drug responsiveness of unfavorable neuroblastoma. We therefore examined EPHA2 expression in neuroblastoma cell lines treated with doxorubicin, a commonly used chemotherapeutics for patients with neuroblastoma. As shown in Fig. 5A, treatment of SY5Y and IMR5 neuroblastoma cells with doxorubicin resulted in a significant increase in *EPHA2* transcript expression. Thus, *EPHA2* is a doxorubicin-inducible gene. Furthermore, Western blot analysis showed that EPHA2 expression was up-regulated in the doxorubicin-treated IMR5 cells in a time-dependent fashion. The increase in EPHA2 expression was accompanied by a similar time-dependent increase in p53 as well as $p21^{WAF1}$, a p53 target in these cells (Fig. 5B). As *EPHA2* is a known target of p53 (18,19), this result suggests that the effect of doxorubicin on the increase in EPHA2 expression is mediated by p53. To address this question, IMR5 cells were treated with $CoCl_2$, which is known to mimic hypoxia and increases p53 expression (30). The expression of EPHA2, p53 and $p21^{waf1}$ was then examined in the treated cells. As shown in Fig. 5B, the $CoCl_2$ treatment caused a marked increase in p53 and $p21^{waf1}$ expression in a time dependent fashion but resulted only in a slight increase in EPHA2 expression.

Discussion

We previously mapped the human *EPHA2* gene (or *ECK*) to 1p36.1 (31), a region where putative neuroblastoma tumor suppressor genes have been suggested (32). Extensive efforts from several laboratories have focused on mutation analysis on the genes localized to the 1p36 region to identify putative tumor suppressor genes of neuroblastoma. However, no consistent gene mutation in any gene in this region has been found so far. One exception to this is *KIF1B*, where mutations in this gene were found in a small number of familial neuroblastoma cases (33). Nevertheless, because of its chromosomal localization, mutation analysis was performed for the *EPHA2* gene in a dozen neuroblastoma cell lines. We found no mutation in *EPHA2* in the cell lines

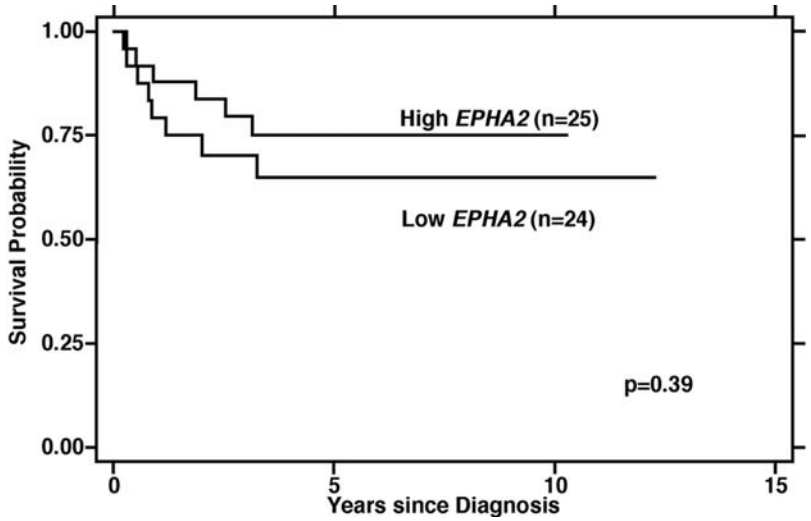

Figure 4. *EPHA2* expression is not associated with outcome of neuroblastoma. Survival probabilities of two groups of neuroblastomas with low- or high-level *EPHA2* expression were estimated by the method of Kaplan-Meier. The median expression value of *EPHA2* based on the entire cohort (n=50) was used as a cut-off to define high- and low-expression subgroups. Of 50 neuroblastoma specimens, 49 had survival data. The same analysis was performed for *TrkA* and *EPHB6* as a comparison. Five-year survival was calculated for each group, and the log-rank test was used to compare survival probabilities of the two groups.

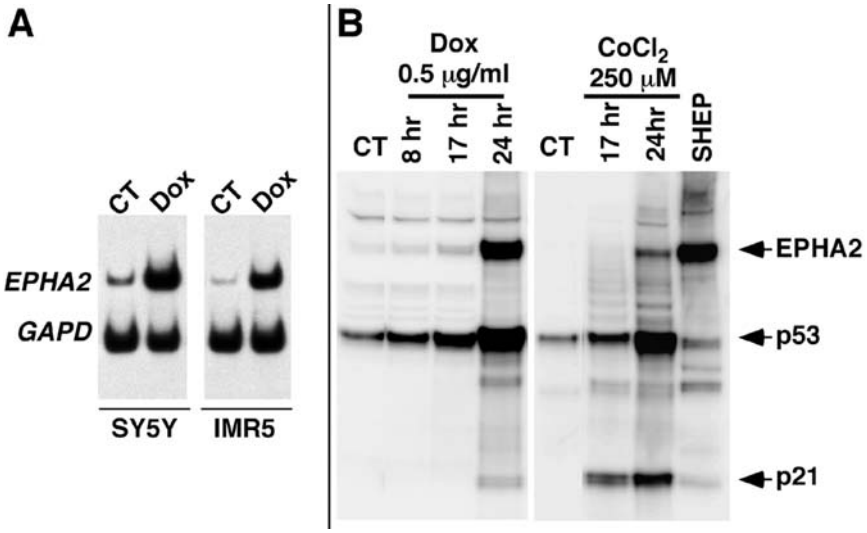

Figure 5. Chemotherapeutics enhances EPHA2 expression in neuroblastoma cells. (A) Doxorubicin enhances the expression of *EPHA2* in SY5Y and IMR5 neuroblastoma cells. SY5Y and IMR5 cells were treated with 0.5 μg/ml doxorubicin for eight hours, and then subjected to RT-PCR analysis for *EPHA2* expression. (B) Treatment of the neuroblastoma cell line IMR5 with doxorubicin or $CoCl_2$ results in concomitant up-regulation of EPHA2, p53 and $p21^{WAF1}$. IMR5 cells were treated with either 0.5 μg/ml doxorubicin or 250 μM $CoCl_2$ for the time indicated and are subjected to Western blot analysis using antibodies specific for the proteins indicated.

examined (Kung and Ikegaki, unpublished data), indicating that *EPHA2* is not a classic tumor suppressor gene. Rather, results of this study suggest that the low expression of *EPHA2* in neuroblastoma cell lines is due to an epigenetic mechanism. In fact, similar observations were previously made in genes residing in 1p36. These include *ZBTB17* (*MIZ-1*) (29) and *CHD5* (34,35).

As mentioned earlier, *EPHA2* is not a favorable neuroblastoma gene, as its expression does not predict neuroblastoma disease outcome. This is an unexpected finding because 1p deletion is associated with a poor disease outcome of neuroblastoma (36,37) and *EPHA2* resides in this region of chromosome (31) and is subjected to epigenetic silencing (this study). This inconsistency may be explained by the fact that S-type cells express *EPHA2* at very high levels in comparison to N-type cells. As has been reported, primary neuroblastomas are composed of a mixture of neuroblastic and Schwannian stroma cells of a common tumor progenitor cell origin, and their proportional representation may vary in given tumors (38). We speculate that the progressive neuroblastomas expressing high *EPHA2* at diagnosis may have included a greater number of Schwannian stroma cells, an equivalent to S-type cells *in vitro*, but neuroblastic and perhaps neuroblastoma stem cells, *in vivo* equivalents of N-type and/or 'I-type' cells (28), respectively (39), may eventually dominate, leading to a poor outcome.

The effect of ectopic overexpression of EPHA2 on growth of neuroblastoma cell lines (Fig. 4) and the augmentation on EPHA2 expression by doxorubicin in neuroblastoma cells (Fig. 5) have their own biological and clinical implications. Firstly, although EPHA2 expression was augmented along with a p53 up-regulation by doxorubicin, p53 expression induced by $CoCl_2$ had a small effect on the expression of EPHA2 in neuroblastoma. These observations suggest that EPHA2 expression can be augmented by both p53-dependent and p53-independent mechanisms in neuroblastoma cells. Secondly, our results suggest that high-level EPHA2 expression is incompatible with the aggressive growth of unfavorable neuroblastoma cells. Moreover, the fact that doxorubicin effectively induces EPHA2 expression in unfavorable neuroblastoma cells could explain why cytotoxic drugs are effective during the initial course of treatment in patients with neuroblastoma (Fig. 5). However, the cytotoxic drugs may also cause genetic changes that would ultimately lead to the drug resistance phenotype of unfavorable neuroblastoma cells (40,41). If so, pharmaceutical augmentation of EPHA2 by non-cytotoxic agents would be among the attractive therapeutic approaches in treatment for children with unfavorable neuroblastoma.

Our previous study has demonstrated that epigenetic silencing inhibitors suppress neuroblastoma growth *in vitro* and *in vivo* (23). In this study, we have shown that chemotherapeutic agents such as gene silencing inhibitors (Fig. 2) and doxorubicin (Fig. 5) markedly increase EPHA2 expression in neuroblastoma cells. In addition, we have observed that 13-cis-retinoic acid (a current maintenance therapy agent for neuroblastoma) alone or its combination with an HDAC inhibitor MS-275 enhances *EPHA2* expression in neuroblastoma cells (Ikegaki and Tang, unpublished data). Together these observations suggest that EPHA2 expression can be considered a biomarker of drug responsiveness during the course of chemotherapy of unfavorable neuroblastomas.

Acknowledgements

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