Notch1 Expression Predicts an Unfavorable Prognosis and Serves as a Therapeutic Target of Patients with Neuroblastoma

Hsiu-Hao Chang1,9,12, Hsinyu Lee4,5, Ming-Kuan Hu11, Po-Nien Tsao1, Hsueh-Fen Juan4,6, Min-Chuan Huang10, Yu-Yin Shih5,7, Bo-Jeng Wang5,7, Yung-Ming Jeng4, Christina Ling Chang13, Shiu-Feng Huang14, Yeoou-Guang Tsay8, Fon-Jou Hsieh9, Kai-Hsin Lin1,9,12, Wen-Ming Hsu2,12, and Yung-Feng Liao7

Abstract

Purpose: Notch signaling has been implicated to play a critical role in the tumorigenesis of neuroblastoma (NB) and can modulate calreticulin (CRT) expression that strongly correlates with tumor differentiation and favorable prognosis of NB. We thus sought to determine how Notch regulates CRT expression and affects NB tumor behavior.

Experimental Design: The Notch-dependent regulation of CRT expression in cultured NB cells was analyzed by confocal microscopy and Western blotting. Notch1 protein expression in 85 NB tumors was examined by immunohistochemistry and correlated with the clinicopathologic/biological characters of NB patients. The progression of NB tumors in response to attenuated Notch signaling was examined by using a xenograft mouse model.

Results: We showed that CRT is essential for the neuronal differentiation of NB cells elicited by inhibition of Notch signaling. This effect was mediated by a c-Jun-NH2-kinase-dependent pathway. Furthermore, NB tumors with elevated Notch1 protein expression were strongly correlated with advanced tumor stages, MYCN amplification, an undifferentiated histology, as well as a low CRT expression level. Most importantly, the opposing effect between Notch1 and CRT could reciprocally affect the survival of NB patients. The administration of a γ-secretase inhibitor into a xenograft mouse model of NB significantly suppressed the tumor progression.

Conclusions: Our findings provide the first evidence that a c-Jun-NH2-kinase-CRT-dependent pathway is essential for the neuronal differentiation elicited by Notch signaling blockade and that Notch1 and CRT can synergistically predict the clinical outcomes of NB patients. The present data suggest that Notch signaling could be a therapeutic target for NB. Clin Cancer Res; 16(17); 4411–20. ©2010 AACR.
The present findings clearly showed that Notch1 is an unfavorable prognostic marker of neuroblastoma (NB) and that both Notch1 and calreticulin can synergistically predict the clinical outcomes of NB patients. Evaluation of Notch1 expression in tumor tissues of NB may provide complementary prognostic information for further subclassification of these tumors, which in turn may help determine the most appropriate strategy of treatment. Furthermore, because the expression of Notch1 inversely correlates with the differentiation of NB, inhibition of Notch signaling by a γ-secretase inhibitor could significantly promote neuronal differentiation in cultured NB cells and slow tumor progression in a xenograft NB mouse model. The present data strongly suggest that Notch1 is a novel prognostic marker and a potential therapeutic target of NB.

**Translational Relevance**

The present findings clearly showed that Notch1 is an unfavorable prognostic marker of neuroblastoma (NB) and that both Notch1 and calreticulin can synergistically predict the clinical outcomes of NB patients. Evaluation of Notch1 expression in tumor tissues of NB may provide complementary prognostic information for further subclassification of these tumors, which in turn may help determine the most appropriate strategy of treatment. Furthermore, because the expression of Notch1 inversely correlates with the differentiation of NB, inhibition of Notch signaling by a γ-secretase inhibitor could significantly promote neuronal differentiation in cultured NB cells and slow tumor progression in a xenograft NB mouse model. The present data strongly suggest that Notch1 is a novel prognostic marker and a potential therapeutic target of NB.

**Materials and Methods**

**Cell culture and cell lines**

Human NB cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum and 0.1 mg/ml penicillin-streptomycin. A stable transfectant stNB-V1 derived from human NB69 cell line that constitutively expresses a green fluorescent protein (GFP) tag (24) were grown in DMEM/F-12 supplemented with 10% fetal bovine serum and 400 μg/mL G418. Cells were incubated in a humidified incubator at 37°C in 5% CO₂.

**Authentication of cell lines**

The human NB cell line SH-SY5Y (ATCC CRL-2266) was obtained from the American Type Culture Collection in November 2007 and have been authenticated in our laboratory on a monthly basis. The SH-SY5Y cell line was last authenticated by microscopic morphology check and PCR-based microplasma test in October 2009. The generation of the human NB cell line stNB-V1 was previously described (24). This cell line is being authenticated in our laboratory on a monthly basis since March 2007. The stNB-V1 cell line was last authenticated by microscopic morphology check and PCR-based microplasma test in October 2009. The PCR-based microplasma testing was done as previously described (25).

**Neuronal differentiation of NB cells induced by the inhibition of Notch signaling**

Human SH-SY5Y NB cells that were plated onto two-well chamber slides and transfected with a CRT-specific siRNA or a nonspecific siRNA (mock) for 2 days were treated with 10 μmol/L 3,5-Difluorophenylacetyl-(S)-phenylglycine t-butyler ester (DAPT), a GSI that blocks Notch signaling (26), or vehicle alone (0.1% DMSO) in DMEM/F-12 containing 10% fetal bovine serum, followed by incubation at 37°C for additional 2 days.

**Transfection of siRNAs**

The chemically synthesized siRNA duplex oligoribonucleotides against human Notch1 (siNotch1) and a nonspecific scrambled siRNA (100 pmol) were transiently transfected into SHSY-5Y cells as previously described.

**Indirect immunofluorescence staining and confocal microscopy**

SH-SY5Y cells that were treated with DMSO (0.1%) or DAPT (10 μmol/L) for 5 days were analyzed by indirect immuno-fluorescence staining and confocal microscopy as described previously.

**JNK-dependent neuronal differentiation of NB cells**

To assess the role of JNK in the CRT-dependent neuronal differentiation downstream of the inhibition of Notch signaling...
signaling, human SH-SY5Y NB cells seeded onto six-well microplates were treated with 10 μmol/L DAPT in the presence or absence of the JNK inhibitor SP600125 (10 μmol/L) in DMEM/F-12 containing 10% fetal bovine serum at 37°C for 1 day. The neuronal differentiation of treated NB cells was assessed by the expression levels of CRT and GAP43 and the neurite length as previously described (19).

Patients and treatment
The clinical evaluation and use of tumor samples in each patient were approved by the Institutional Review Board.
During a period of 16 years (from December 1990 to December 2006), 85 histologically proven NB patients with complete follow-up were enrolled in this study. Additional information about this cohort can be found in the Supplementary Data.

Quantitative real-time PCR
The quantitative determination of Notch1 and CRT mRNA transcripts in NB tumor tissues was performed as previously described (20). Total RNAs from 18 tumor samples [6 UNBs, 7 differentiating NBs (DNB), and 5 GNBs] were subjected to real-time PCR analysis.

Immunohistochemical staining
Eighty-five tumor specimens collected before chemotherapy were fixed in formalin and embedded in paraffin. Expression of Notch1 and CRT was evaluated by using an avidin-biotin complex immunoperoxidase staining technique as described previously (20, 27). The immunoreactivity of Notch1 in NB tumors was classified into four categories: “−” (no expression, no stained cells or only isolated single stained cells seen), “1+” (weak expression, around 10–35% of cells stained), “2+” (moderate expression, around 35–70% of cells stained), and “3+” (strong expression, more than 70% of cells stained). Immunoreactivities of 1+ to 3+ were pooled as positive expression in contrast to that of − as a negative expression.
Tumor xenograft studies

Four-week-old female athymic nude mice were inoculated subcutaneously with $1 \times 10^7$ stNB-V1 cells, a NB cell line with stable GFP expression (24), with Matrigel (BD Biosciences). When tumors were grown to a volume of 500 mm$^3$, tumor-bearing mice were randomized into three treatment groups of four to six mice. Animals were treated by daily intraperitoneal injection with Jia142 (50 mg/kg, $n=6$), all-trans retinoic acid (ATRA, 5 mg/kg, $n=5$), or vehicle alone (control, $n=4$) for up to 14 days. Jia142 is a known GSI that can block Notch signaling to induce neuronal differentiation of NB cells (19).

Additional information regarding individual experimental procedures can be found in the Supplementary Data.

Results

Neuronal differentiation of NB cells induced by inhibition of Notch signaling is mediated by a JNK-CRT–dependent pathway

The expression of CRT is prominent in differentiated NB cells and can be induced by inhibition of Notch signaling in NB cells (19, 20). Using confocal microscopy, we first confirmed that the expression of CRT as well as another neuronal differentiation marker, MAP2, can be significantly induced in SH-SYSY cells in response to inhibition of Notch signaling by DAPT (Fig. 1A). In addition to its primary mode of action by transcriptional regulation of target genes, NICD can exert transcription-independent functions (11, 28, 29), such as blocking cytoplasmic JNK activation at the protein level (30). We thus investigated whether JNK activation is required for neuronal differentiation of NB cells induced by inhibition of Notch signaling. We showed that JNK activation is significantly enhanced in GSI-treated SH-SYSY NB cells compared with that in DMSO-treated cells, concomitant with augmentation in the expression of two differentiation markers, NSE and GAP43, as well as neurite length (Fig. 1B, C; Supplementary Fig. S3). Consistently, neuronal differentiation of NB cells was dramatically suppressed by pretreatment with the JNK inhibitor SP600125 (Fig. 1B, C; Supplementary Fig. S3) in response to inhibition of Notch signaling. To further confirm that specific downregulation of Notch1–dependent signaling is sufficient to drive increased expression of neuronal differentiation markers in NB cells, we used a RNA interference–mediated approach (siNotch1) to suppress Notch1 expression in SH-SYSY cells. This Notch1-specific siRNA targeted the exon 7 of endogenous human Notch1 gene that encodes part of its extracellular domain and efficiently downregulated the expression of endogenous Notch1 and its intracellular domain, but not NΔE (19). Our data showed that CRT expression is significantly increased in siNotch1-transfected cells compared with mock-transfected ones, and that the coexpression of a constitutively active mouse Notch1 (NΔE; ref. 31) in siNotch1-transfected cells can block the increase in CRT expression induced by Notch1 inhibition (Fig. 1D). Using a CRT promoter–driven luciferase reporter gene construct, we further showed that JNK activation elicited by Notch signaling blockade in NB cells can significantly enhance CRT promoter activity (Supplementary Data, Fig. S1), suggesting that activation of JNK could result in the transcriptional regulation of CRT expression. These results thus strongly favor a model in which JNK activation critically mediates neuronal differentiation of NB cells induced by inhibition of Notch signaling through modulating CRT expression at the transcriptional level.

Correlation between Notch1 expression and differentiation status of NB tumors

To determine the clinical significance of Notch signaling as well as its relationship with CRT and other clinicopathologic factors of NB tumors, we examined the protein expression of Notch1 and CRT in 85 NB tumors by immunohistochemical staining. Both Notch1 and CRT expression were vividly observed in NB cells but not in Schwannian stromal cells. Notch1 expression was usually found in undifferentiated NB cells of undifferentiated tumors (UNB), whereas CRT immunostaining was commonly noted in more differentiated NB cells of differentiated histologies (GNB or DNB; Fig. 2A). Our immunohistochemical data revealed that a positive expression of Notch1 protein could be detected in 46 of the 85 NB tumors (54.1%). The majority of NB tumors with evident

| Table 1. Notch1 expression and clinicopathologic and biological characteristics of NBs |
|-------|-----------------|-----------------|---|
| Case no. | Positive Notch1 expression (%) | $P$ |
| Age at diagnosis (y) | | | |
| $\leq$1 | 28 | 9 (32.1) | 0.004 |
| >1 | 57 | 37 (64.9) | | |
| Mass screen | | | |
| Yes | 8 | 2 (25.0) | 0.082 |
| No | 77 | 44 (57.1) | | |
| Sex | | | |
| Male | 46 | 26 (56.5) | 0.629 |
| Female | 39 | 20 (51.3) | | |
| Clinical stage | | | |
| I, II, IVS | 30 | 5 (16.7) | <0.001 |
| III, IV | 55 | 41 (74.5) | | |
| Tumor histology | | | |
| Undifferentiated NB | 36 | 30 (83.3) | <0.001 |
| Differentiating NB | 31 | 12 (38.7) | | |
| GNB | 18 | 4 (22.2) | | |
| MYCN | | | |
| Amplified | 23 | 19 (82.6) | 0.001 |
| Nonamplified | 62 | 27 (43.5) | | |
| CRT expression | | | |
| Positive | 40 | 6 (15.0) | <0.001 |
| Negative | 45 | 40 (88.9) | | |

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Notch1 protein expression exhibited undifferentiated histologies. The intensity and percentage of positive Notch1 immunostaining inversely correlated with the differentiation of histology (Fig. 2B, \( P < 0.001 \), \( \chi^2 \) test). Using quantitative real-time PCR analysis, we further confirmed that the expression levels of Notch1 mRNA in 18 NB tumor tissues, including 5 GNBs, 7 DNBs, and 6 UNBs, are tightly associated with undifferentiated histology (UNB) and inversely correlated with CRT mRNA levels (Fig. 2C). Together, our data showed that mRNA and protein levels of Notch1 coherently predict advanced tumor stages and unfavorable outcomes.

**Notch1 expression and clinicopathologic and biological factors of NB tumors**

The tumorigenic role of Notch signaling was further revealed by the association between the expression of Notch1 protein and the clinicopathologic and biological variables of NB tumors (Table 1). Positive Notch1 protein expression was significantly associated with adversely prognostic factors, including older patient age at diagnosis (>1 year old, \( P = 0.004 \)), advanced clinical stage (stage III, IV; \( P < 0.001 \)), undifferentiated tumor histology (\( P < 0.001 \)), and MYCN amplification (\( P = 0.001 \)). There was an inverse correlation between Notch1 and the favorable biomarker of CRT expression (\( P < 0.001 \)).

**Notch1 expression in NB tumors predicts an unfavorable outcome**

Kaplan-Meier analysis showed that patients with positive Notch1 expression have a predictive 5-year survival rate of 28.5% compared with a survival rate of 85% for those with negative Notch1 expression (Fig. 3A, \( P < 0.001 \), log-rank test), suggesting that positive Notch1 expression could predict an unfavorable outcome. Univariate analysis showed that, in addition to Notch1 protein expression, MYCN amplification consistently predicted a very poor outcome, whereas patient age \( \leq \) 1 year, early clinical stages (stage I, II,
or IVS), and differentiated histologies (including differentiating NB and GNB), as well as positive CRT expression, strongly correlated with better survival (Table 2). Multivariate analysis through the Cox proportional hazard model further confirmed that Notch1 protein expression, in addition to clinical stage and MYCN amplification, is an independent prognostic factor (Table 2). However, whereas expression of both Notch1 and CRT could serve as an independent prognostic factor when either one of them was put into the Cox hazard model, none of them could predict survival independently when both of them were in the model (data not shown).

The significance of Notch1 expression in prognostic discrimination was analyzed against clinical stage and MYCN status. Although Notch1 expression might not be suitable for the prognosis of most NB patients with early stage and favorable outcome (stages I, II, and IVS) due to a low rate of Notch1 protein expression, positive Notch1 expression can faithfully predict the unfavorable prognosis for patients with advanced-stage diseases (stage III or IV; Fig. 3B; \( P = 0.016, \text{log-rank test} \)). The prognosis of NB patients with MYCN amplification could not be distinguished by Notch1 expression, possibly due to the high frequency of positive Notch1 protein expression and very poor outcome for these high-risk patients. Nevertheless, among patients without MYCN amplification, positive Notch1 protein expression clearly predicted an unfavorable outcome (Fig. 3C; \( P < 0.001 \)). Together, these clinical analyses of this cohort strongly suggest that additional evaluation of Notch1 expression in tumor tissues could complement known prognostic factors for further subclassification of and tailored treatments to NB patients.

### Inhibition of Notch signaling by a GSI can suppress tumor progression in xenografted NB tumors

Given that GSIs could induce neuronal differentiation of cultured NB cells through inhibition of Notch signaling, we thus sought to determine whether GSIs could induce neuronal differentiation and reduce tumor growth in a mouse xenograft model of NB. This xenograft mouse model of NB was established by subcutaneously inoculating nude mice with stNB-V1 NB cells that constitutively express GFP reporter gene (24), allowing real-time tracking of tumor progression in animals by fluorescence imaging. To determine the \textit{in vivo} effect of attenuated Notch signaling on the progression of NB xenografts, NB mice were peritoneally injected with Jia142 for 2 weeks. Jia142 is a GSI with enhanced stability that can also induce neuronal differentiation of NB cells through inhibition of Notch signaling as previously reported (19). Based on body weight and behavior, this treatment regimen did not result in any significant toxicity. We found that tumor growth is significantly inhibited in Jia142-treated NB xenograft mice compared with vehicle-treated control animals, and that a statistically significant difference is observed after 4 days of treatment and sustained until the conclusion of treatments (day 14) as evidenced by dramatic reduction in tumor size or bioluminescent measurements (\( P < 0.05 \) by \( t \) test; Fig. 4A and B). A statistically significant suppression in tumor growth can be obtained from Jia142 therapy (48.9%) compared with ATRA therapy (24.7%) in terms of size ratio of tumors (day 14). Using Western blot analysis of lysates derived from harvested NB xenografts, we showed that Jia142 could effectively suppress Notch signaling (reduced NICD levels) and simultaneously induce neuronal differentiation (increased levels of CRT, GAP43, and NSE) in NB tumors (Fig. 4C, D). These findings suggest that inhibition of Notch signaling by a GSI could induce differentiation and suppress tumor progression of NB xenografts \textit{in vivo}. Our data also provide proof-of-principle for the first time that Notch signaling could serve as a therapeutic target of NB.

### Discussion

Our present study reveals a novel JNK-CRT–dependent pathway that is essential for the GSI-induced neuronal differentiation of NB upon blockade of Notch signaling. Within a cohort of 85 NB patients, we establish that Notch1 protein expression is tightly correlated with undifferentiated tumor histology and could independently predict poor outcomes of NB patients. The inverse correlation and counteracting effects between Notch1 and CRT further strengthen the notion that the Notch1-JNK-CRT pathway could govern cell differentiation and tumorigenesis of...
NB. Our results clearly suggest that Notch signaling could be a therapeutic target of NB and that GSI could represent a novel class of differentiation drugs for NB.

JNK activation is prominent in differentiated NB cells in response to inhibition of Notch signaling by GSI and induces expression of neuronal differentiation markers (CRT and GAP43; Fig. 1B). These results suggest that CRT could be a novel downstream target gene of Notch signaling. JNKs have been shown to regulate cell differentiation and apoptosis in the embryonic development of the neural tube (32–34) and also play a role in tumorigenesis and tumor suppression (35). Our finding that GSI-induced neuronal differentiation of NB cells is dependent on JNK activation provides the mechanistic explanation for how inhibition of Notch signaling can promote neuronal differentiation of NB. Both JNK signaling and CRT are responsive to stress stimulation and could mediate cell differentiation (20, 32, 36, 37). It is thus conceivable that JNK signaling and CRT expression could synergistically govern the differentiation of NB. This notion is corroborated by the finding that JNK activation can significantly augment CRT expression at the transcriptional level (Supplementary Fig. S1). Given that NICD, generated by γ-secretase cleavage of Notch, can inhibit JNK activation through direct interaction with JNK-interacting protein 1 (30), our data thus favor a model in which a JNK-CRT-dependent pathway could act concordantly with reduced Notch signaling in promoting differentiation of NB.

The prognostic role of Notch1 protein expression has been established in various solid tumors. Poorly differentiated breast tumors were associated with increased Notch1 protein levels and poor patient outcomes (38–42). Our present findings unequivocally establish Notch1 protein expression as a novel independent unfavorable prognostic

Fig. 4. Inhibition of Notch signaling by a GSI can suppress tumor progression in xenografted NB tumors. A, nude mice bearing stNB-V1 NB cells xenografts were treated daily with Jia142 (n = 6), ATRA (n = 5), or vehicle alone (control, n = 4) for 14 d. Tumor growth was assessed daily by metric measurement, and change in tumor growth was calculated as the tumor size on day x of treatment versus that on day 0 (before treatment). Data are shown as daily average ratio change (±95% CI) and analyzed by Student’s t test. Significant inhibition of tumor growth by Jia142 was noted starting from day 4 to day 14 after treatment (P < 0.05 versus control; P < 0.05 Jia142 versus RA). B, bioluminescent imaging of representative (one per treatment group) nude mice bearing NB xenografts treated after treatment for 14 d. C, Western blot analysis of NICD and GAPDH in NB xenografts harvested after 14 d treatment showed a significant suppression of Notch signaling by Jia142 (*, P < 0.05 versus control). D, Western blot analysis of CRT, GAP43, NSE, and GAPDH in harvested NB xenografts showed a significant increase in neuronal differentiation elicited by Jia142 (*, P < 0.05 versus control for CRT and NSE).
factor of NB (Fig. 3A). The prognostic power of Notch1 expression was further validated in an independent cohort of seven NBs (Supplementary Fig. S4). Clinical stage and MYCN status are two well-established prognostic factors of NB (43, 44). While NB patients with early stages have excellent outcomes but those with MYCN amplification carry a very poor prognosis, NB patients with advanced stages and those without MYCN amplification are actually two large groups showing prognostic heterogeneity. Additional factors are required to further discriminate these two groups of patients. Here, we show that positive Notch1 expression is capable of predicting an unfavorable prognosis among NB patients with either advanced stages or normal MYCN copy number (Fig. 3B and C). Therefore, assessing Notch1 protein expression in NB could provide complementary prognostic information to clinical stage and MYCN status, allowing clinicians to determine the most appropriate therapy for the NB patients.

The tumorigenic effect of activated Notch signaling in NB can be further substantiated by the animal study using a GSI that blocks Notch signaling. GSI-treated NB xenograft mice clearly exhibit a reduced tumor growth compared with DMSO-treated ones (Fig. 4). The tumor suppression effect of GSI is even more significant than that of retinoic acid, a differentiation agent of NB in clinical use (4). To our knowledge, this is the first study to show the GSI-induced tumor suppression of NB in an in vivo experimental model. Because GSIs have been effective in alleviating the malignancy of T-cell acute lymphoblastic leukemia and renal cell carcinoma (6, 45), our findings further strengthen the idea that the Notch signaling pathway not only affects cell differentiation in vivo but may also serve as a potential therapeutic target of NB.

In summary, our study shows that Notch1 protein expression is an independent prognostic factor and predicts poor outcomes in NB patients, complementary to clinical stage and MYCN status. Our findings could provide invaluable insights to determine the most appropriate therapy for NB patients. The present data also delineate a novel mechanism underlying the Notch-dependent regulation of neuronal differentiation through a JNK-CRT-dependent pathway. The in vivo efficacy of a GSI in a xenograft mouse model of NB also provides the basis for the development of novel therapeutics for NB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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