Notch Pathway Inhibition Significantly Reduces Rhabdomyosarcoma Invasiveness and Mobility In Vitro

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Abstract

**Purpose:** Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children and can be divided into two main subtypes: embryonal and alveolar RMS. Patients with metastatic disease continue to have very poor prognosis although aggressive therapies and recurrences are common in advanced localized disease. The oncogenic potential of the Notch pathway has been established in some cancers of the adult and in some pediatric malignancies.

**Experimental Design:** A real-time PCR assay was used to ascertain the expression of several Notch pathway components in a wide panel of RMS and cell lines. Four γ-secretase inhibitors (GSIs) were tested for pathway inhibition and the degree of inhibition was assessed by analysis of Hes1 and Hey1 expression. The putative effects of Notch pathway inhibition were evaluated by wound-healing, matrigel/transwell invasion, cell-cycle, and apoptosis assays.

**Results:** The Notch pathway was widely expressed and activated in RMS and underwent substantial inhibition when treated with GSIs or transfected with a dominant negative form of MAML1. RMS cells showed a significant decrease in its mobility and invasiveness when the Notch pathway was properly inhibited; conversely, its inhibition had no noticeable effect on cell cycle or apoptosis.

**Conclusion:** Pharmacological or genetic blockage of the pathway significantly reduced invasiveness of RMS cell lines, thereby suggesting a possible role of the Notch pathway in the regulation of the metastatic process in RMS. *Clin Cancer Res; 17(3); 505–13. ©2010 AACR.*

Introduction

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. Regarding histopathological criteria, RMS can be divided into 2 main subtypes: embryonal and alveolar RMS (eRMS and aRMS, respectively). The majority of aRMS (80% to 85%) contain one of the reciprocal chromosomal translocations: either t(2;13)(q35;q14), or t(1;13)(p36;q14). These translocations generate the novel fusion genes PAX3-FOXO1 and PAX7-FOXO1, respectively (1, 2). However, no characteristic translocations have been described in eRMS. The eRMS is typically characterized by loss of heterozygosity on the short arm of chromosome 11 (11p15.5, Ref. 3) and gains in chromosomes 2, 7, 8, 11, 12, 13, and 17 are also common in this subtype (4).

The Notch signaling pathway is an evolutionary conserved pathway that plays a critical role in tissue development in organisms ranging from nematodes to mammals. The highly conserved Notch gene family encodes 4 cell surface receptors (Notch1, Notch2, Notch3, and Notch4). When the Notch receptor is activated by its ligands (Delta and Jagged in vertebrates), the Notch intracellular domain (NICD) is released by the γ-secretase complex (5) and translocates to the nucleus where it binds to CSL (CBF1-Su(H)-Lag1) transcription repressors, converting them into transcriptional activators. The targets of these transcription factors in vertebrates are typified by *Hes* and *Hey* genes (6, 7).

The oncogenic potential of the Notch pathway was first described in acute T-cell lymphoblastic leukemia (T-ALL) in the late 1980s (8). An abnormal upregulation of the Notch pathway has also been reported in ovarian (9), breast (10), and other cancers (11). With respect to pediatric malignancies, Notch signaling appears to contribute essentially to osteosarcoma metastasis (12) and proliferation (13); Notch signaling also promotes medulloblastoma cancer stem cell survival (14) and contributes to angiogenesis in neuroblastoma (15).

In the last decade, the use of pharmacological inhibitors of the γ-secretase complex, which inhibit activation of the 4 Notch receptors, has been reported. γ-secretase inhibitors (GSIs) block the generation of the active form of Notch: the NICD (16). In recent decades, several GSIs have been actively studied as potential inhibitors of the generation of the β-amyloid peptide associated with Alzheimer’s disease (17). More recently, some...
GSIs have begun to be studied in phase I trials for patients with advanced breast cancer and acute T-cell leukemias (18, 19).

The possible role of the Notch pathway in RMS remains unknown. Since the regulation of Notch signaling is involved in satellite cell activation and in cell fate determination during postnatal myogenesis (20) and since RMS are embryonal tumors whose cells exhibit gene expression profiles resembling those of fetal muscle cells (21), we hypothesized that the Notch pathway could play a major role in maintaining the immature muscle status characteristic of RMS. In the present work, Notch pathway expression and activation profiles were characterized for the first time in a wide panel of RMS tumors and cell lines. The reduction by several γ-secretase inhibitors in the oncogenic potential of RMS was evaluated by wound-healing, matrigel/transwell invasion, cell cycle, and apoptosis assays.

**Methods**

**Tumor samples**

Samples were collected from a nonselected cohort of 37 children with RMS. Twenty-seven samples were from patients referred for diagnosis and/or treatment at the Vall d’Hebron Hospital Oncology Unit and 10 were a kind donation from Dr Albert Chetcuti (Tumour Bank, Oncology Research Unit, Children’s Hospital at Westmead, Australia). Informed consent has been previously obtained from all parents or legal guardians.

**Cell cultures and drug treatments**

Cells were cultured in MEM media with Earle’s Salts (PAA Laboratories), supplemented with 10% FCS (PAA), 2 mM L-glutamine (PAA), 1 mM sodium pyruvate (PAA), 1× NEAA (PAA), 100 U/mL penicillin (PAA) and 0.1 mg/mL streptomycin (PAA), and maintained at 37°C in a 5% CO2 water-jacketed incubator. All GSI inhibitors were purchased from Calbiochem and DAPT from Sigma-Aldrich. Cell lines RH-30, CW-9019, and HTB-82 were obtained from American Type Culture Collection (ATCC).

**RNA isolation, retrotranscription and real-time PCR**

Total RNA was isolated using a Quick-prep micro RNA isolation kit (Qiagen, Valencia); RNA integrity was quantified and verified by an ABI PRISM 7000 sequence detection system (Applied Biosystems). Samples of 2 μg of total RNA were reverse-transcribed using random primers (Invitrogen). The reaction mixture was incubated for 60 minutes at 37°C with 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). Real-time PCR was based on the TaqMan assay (Applied Biosystems). A 40-cycle PCR was performed to detect the 4 Notch receptors (assays HS01062014_m1, HS01050708_m1, HS0166432_m1, and HS0270200_m1 for Notch1, Notch2, Notch3, and Notch4, respectively) and the effectors Hes1 and Hey1 (assays HS00172878_m1 and HS00232618_m1, respectively). The housekeeping gene TBP (assay HS00172424_m1) was used as internal control. Quantification of relative levels of each mRNA analyzed was performed by the method of Livak and Schmittgen (22). All samples were tested in triplicate.

**Western blot analysis and immunohistochemistry**

Western blot: Cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSE, 1 mM EDTA, 5 mg/mL aprotinin, 5 mg/mL leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and incubated for 4 minutes at 90°C and the total protein content was measured (DC assay kit, Bio-Rad Laboratories). Following the addition of 5% β-mercaptoethanol, 0.001% bromophenol blue, and 20% glycerol, 12.5 μg of protein per lane were loaded in 12% SDS-PAGE and then blotted onto PVDF membranes (Bio-Rad Laboratories). Membranes were then incubated with rabbit polyclonal antibody anti-Hes1 AB5702 (Millipore) diluted 1:200. After incubation with anti-α-tubulin (Cell Signaling) diluted 1:2000 was used as a loading control.

Immunohistochemistry (IHC): Patient samples were obtained from the Vall d’Hebron Hospital Pathology Department (kindly provided by Dr. Nuria Toran). Paraffin-embedded tissues were sliced, deparaffinized, and rehydrated, followed by antigen retrieval in 10 mM citrate buffer (DAKO). Endogenous peroxidase activity was quenched using 1% hydrogen peroxide. Samples were blocked and incubated overnight with anti-Hes1 antibody (AB5702, Millipore) diluted 1:1000, and anti-α-tubulin (Cell Signaling) diluted 1:2000 was used as a loading control.

**Wound-healing and transwell assays**

For wound-healing assay, cells were grown until confluence in the presence of the appropriate GSI (48 hours). Cell monolayers were then scratched with a pipette tip and placed in complete growth medium with the appropriate
GSI. Over 37.5 hours, serial images of selected fields were acquired every 15 minutes by a live cell imaging cell-R workstation (Olympus) coupled to a bright-field microscope. The scratched area was measured using the ImageJ software (NIH, freely available at http://rsb.info.nih.gov/ij/) and the healing velocity was calculated as a quotient between the scratched area and time required for the wound to heal.

For the matrigel/transwell assay, cells were pretreated with GSI for 48 hours and harvested using trypsin. Once pretreated, 10^5 cells were resuspended in serum-free MEM media with the appropriate GSI and plated in the upper chamber previously coated with BD Matrigel™ (BD-Biosciences) in an 8 μm pore size transwell (Corning). Following incubation at 37°C, remaining cells were removed from the upper chamber with a cotton swab, and cells migrated to the lower surface of the membrane were stained in 5 ng/mL of Hoechst33342. Cells were then stained with 0.2% crystal violet, lysed in 10% acetic acid, and the absorbance at 590 nm was determined as a value proportional to the number of cells on each membrane lower surface. All analyses were made in triplicate.

Cell cycle and apoptosis

One million cells were grown for 3 days in MEM media supplemented with the appropriate GSI or DMSO (control) and harvested with trypsin. For cell cycle, cells were fixed in 70% ethanol, treated with 100 μg/mL RNase A, and stained for 1 hour at room temperature with 1 μg/mL propidium iodide. Apoptosis was detected using the annexin-V apoptosis kit (BD Biosciences) following manufacturer’s instructions. Cells were analyzed in a FacsCalibur cytometer (BD Biosciences).

Transfection with dnMAML1

Cells were transfected using FuGENE 6 transfection reagent (ROCHE) with dominant negative dnMAML1-GFP or GFP alone both cloned in the plasmidic vector pEGFP (Clontech Laboratories). Stably transfected cells were selected for vector incorporation over 3 weeks in MEM culture media containing 2.0 mg/mL G418 (Sigma-Aldrich).

Results

Notch pathway is consistently expressed and activated in RMS tumors

Expression of the 4 Notch receptors and 2 downstream effectors of the Notch pathway were studied by quantitative PCR in 37 RMS primary tumor samples. The results showed a wide distribution of the Notch receptors and downstream effectors in the RMS samples studied (Fig. 1). Levels of the RNAs studied in tumoral tissue (aRMS and eRMS) were compared with those of adult and fetal muscle (AM and FM, 2 pools of 3 samples were analyzed for both AM and FM). The most striking finding in tumors was the increase in the expression of Notch2, a receptor that showed very obvious upregulation in both aRMS and eRMS. Notch3 showed slight upregulation in both aRMS and eRMS not as clear as Notch2. Conversely, Notch1 and Notch4 showed no significant expression increase in RMS (Fig. 1A). Hes1 expression was slightly upregulated in eRMS compared with both adult and fetal muscle, while Hey1 showed an increase in aRMS and eRMS compared with adult muscle levels (Fig. 1A). IHC showed cytoplasmic and nuclear staining of Hes1 in a majority of RMS samples (Fig. 1B) compared with prostate carcinoma as positive control (23).

Notch pathway activation correlates with invasive phenotype in RMS cells

Although the RMS cell lines analyzed significantly expressed Notch1, Notch2, and Notch3, no significant association was observed between their expression and the oncogenicity of the 3 cell lines analyzed (data not shown). Conversely, when expression of the Notch downstream effectors Hes1 and Hey1 was studied in the 3 RMS cell lines, they showed low values in HTB-82 (derived from eRMS), moderate values in CW-9019 (aRMS with PAX7-FOXO1 translocation), and high values in RH-30 (aRMS bearing the PAX3-FOXO1 translocation). Thus, expressing results as a fold increase relative to the values found in HTB-82 (Fig. 2A), Hes1 showed 24.2-fold higher expression in CW-9019, and 37.5-fold higher in RH-30. The same increasing pattern was observed for Hey1 (12.3-fold higher in CW-9019 and 34.8-fold higher in RH-30). When the invasive capabilities of the 3 cell lines were tested by matrigel/transwell invasion assay (Fig. 2B), the results verified the correlation between the expression of the aforementioned Notch targets and the invasiveness of each cell line. Thus, invasiveness of the HTB-82 line was very low, with no significant invasion being found even at 48 hours. In contrast, the cell line CW-9019 showed noticeably invasive potential. At 8 hours, there was no significant invasion, but the number of cells migrated to the lower chamber rose considerably at 24 and 48 hours. Finally, the putatively most invasive cell line showed very high invasiveness detected as soon as at 8 hours. At longer times (24 and 48 hours), the invasion was so great that the lower chamber was saturated with cells and no significant differences were observed between 24 and 48 hours. When the wound-healing capability of the 3 cell lines was tested, the results revealed that while the RH-30 cell line completed the healing in approximately 18 hours, the CW-9019 cell line needed approximately 3 days to close the wound completely and the time required was even longer than a week for HTB-82. A significant image of each cell line was taken at 48 hours postwounding (Fig. 2C). At that time, cell line HTB-82 was very far from closing the wound, CW-9019 near to complete closure, and RH-30 was completely closed.

Notch pathway can be pharmacologically inhibited using γ-secretase inhibitors (GSIs) in RMS cells

The effects of the inhibitors on the Notch pathway were evaluated by quantification of Hes1 and Hey1 mRNA expression. Hes1 mRNA expression showed a significant
decrease in the 3 cell lines treated with DAPT (25 μM) GSI-XX (8 nM) and GSI XXI (2 nM). The GSI-X (200 nM) produced minor or null inhibition in the 3 cell lines analyzed (Fig. 3A). Hey1 expression showed a similar inhibition pattern, with a significant decrease in the 3 cell lines treated with GSI-XX and GSI-XXI, while DAPT rendered only a significant reduction in Hey1 expression in the cell lines RH-30 and CW-9019. The GSI-X rendered a minor or null reduction, only significant in the cell line RH-30 (Fig. 3B).
Although total inhibition was not achieved, Hes1 protein also decreased significantly when cells were treated with GSIs, especially with GSI-XXI (Fig. 3C). No significant effect was observed when cells were treated with GSI-X or DAPT (not shown).

**Notch pathway inhibition severely impairs cell mobility and invasiveness in RMS cells**

When the 3 RMS cell lines were treated with GSIs, a significant reduction was observed in their mobility that could be measured in a wound-healing assay (Fig. 4). The delay observed is illustrated in Figure 4A for the cell line RH-30. Two images per treatment taken immediately post-wounding and 18 hours later are shown. It can be clearly seen that while cells treated with DMSO (control) closed the wound in approximately 18h, cells treated with all GSIs, particularly with DAPT, GSI-XX, and GSI-XXI, did not. GSI-X, though presenting delay versus the control, showed a lesser effect on wound-healing time than the other 3 inhibitors tested. This lessening of wound-healing capability of cells treated with GSIs is demonstrated as a reduction in healing velocity to approximately half that of the control in the 3 cell lines treated with GSI-XX and GSI-XXI. DAPT produced significant inhibition; however, while the reduction in the RH-30 cell line was approximately half that of the control, the reduction in the other 2 cell lines was only moderate. Finally, with GSI-X treatment, the reduction was very slight or null for all 3 cell lines (Fig. 4B).

Furthermore, the 3 cell lines suffered severe impairment in the matrigel/transwell invasion assay when treated with...
some GSIs (Fig. 5), suggesting that the Notch pathway could also play a role in their invasiveness. In that assay, the observed effects of the 4 Notch inhibitors tested were also unequal. Thus, cells treated with GSI-X showed only a minor reduction in their invasiveness (slightly lower than 80% compared to the DMSO control). On the other hand, cells treated with GSI-XX and GSI-XXI showed a remarkable reduction in invasiveness in the 3 cell lines (all values under 30% compared to the control, respectively). Finally, DAPT produced cell–line dependent effects, with moderate effect on RH-30 invasiveness, but a remarkable effect on CW-9019 and HTB-82 (Fig. 5B).

**Downregulation of Notch pathway has no noticeable effect on cell cycle and apoptosis**

Notch pathway inhibition had no significant effect on cell cycling and apoptosis, and all the drugs applied (in a 3-day treatment) produced the same results in treated and nontreated RMS cells (data not shown), confirming that the inhibition observed in cell motility and invasion cannot be attributable to changes in cell proliferation or in apoptosis.

**Specificity (dnMAML1)**

Seeking to rule out the possibility of the effects observed in cells treated with GSIs being attributable to the involvement of other pathways and therefore demonstrate that these effects resulted from specific Notch inhibition, we genetically manipulated Notch signaling activity by transfecting a construct containing dominant negative MAML1 (dnMAML1) or empty vector (pEGFP). MAML1 is a co-activator that cooperates with NICD and CSL transcription factors (CBF1, Su(H) and Lag-1) to activate transcription of Notch target genes (24,25). It has been demonstrated that truncated versions of MAML-1 can maintain an association with the complex that behaves in a dominant negative fashion and depresses transcription of Notch target genes (26). In the RH-30 cell line stably transfected with dnMAML1, Hes1 mRNA expression fell to under 30% of the levels found in controls (Fig. 6A). Likewise, the levels of Hes1 protein underwent a decrease in cells transfected with dnMAML1 (Fig. 6B). This attenuation of Hes1 expression produced effects on cell mobility and invasiveness. Thus, RH-30 cells (not transfected) or transfected with GFP alone (control) closed the wound significantly faster than cells transfected with dnMAML1. This lessening of wound-healing capability in cells transfected with dnMAML1 is demonstrated as a reduction in healing velocity (Fig. 6C). Finally, and paralleling the results previously shown with GSI treatments, the RH-30 cell line expressing the dnMAML1 underwent substantial inhibition of its invasiveness on a matrigel/transwell assay (Fig. 6D) which was seen to fall to a level under 20% of that of control cells.

**Discussion**

Notch signaling is known to play a fundamental role in the normal development of multicellular organisms, particularly in processes that imply cell-fate determination. More recently, some advances have been made in elucidating the
role of the Notch pathway in adult cancer. However, little is known on the role of the Notch pathway in the oncogenicity of pediatric tumors. Only very recent works on medulloblastoma (14), neuroblastoma (15), and osteosarcoma (12, 13) have clarified some aspects of the Notch pathway in these neoplasias; however, the overall role of this pathway in pediatric malignancies remains unclear. In addition, the possible role of the Notch pathway on the oncogenicity of RMS remains unknown. RMS patients with metastatic disease continue to have a very poor prognosis, although aggressive therapies are indicated. Moreover, the major cause of death in these patients is the formation of distant metastasis. The cellular components that control metastasis in RMS should be identified before the development of targeted therapies focused on reducing metastasis in this neoplasia.

In this report, we provide evidence of Notch pathway expression and activation in RMS. One criterion to be met before a possible role of a pathway in a tissue can be considered is that this pathway must be expressed and...
activated in that tissue. It is noteworthy that all RMS tumors (n = 37) analyzed presented expression of several Notch receptors and downstream Notch targets. Upregulation of Notch2 was particularly striking while upregulation of Notch3, Hes1, and Hey1 was also significant.

The possible correlation between Notch pathway activation and the invasiveness and mobility of each RMS cell line analyzed is of particular interest. The HTB-82 cell line is derived from an eRMS, bears no translocation, and is associated with the most benign phenotype of the disease. Its invasiveness is minimal and Notch activation is practically absent. By contrast, the RH-30 cell line is derived from an aRMS bearing the PAX3-FOXO1 translocation (representing the most aggressive phenotype). This cell line presents a highly invasive phenotype in culture and is the cell line with the highest Notch pathway activation. Finally, the CW-9019 cell line is derived from an aRMS bearing the PAX7-FOXO1 translocation (moderately aggressive phenotype) and presents considerable invasiveness, although less than the RH-30 cell line. Its levels of Notch activation are also considerable and situated between the other 2 cell lines. These results raise the possibility that Notch pathway activation could be responsible for the triggering, at least in part, of metastatic mechanisms in the invasive cells, particularly in RMS cell lines. Using the 3 aforementioned RMS cell lines as models for the study of Notch activation and its possible role in the promotion of oncogenicity, some GSIs were used to test the effects of Notch inhibition on the oncogenicity of these cell lines. GSIs were first assayed for possible use as therapeutic agents in Alzheimer’s disease (27). However, the use of GSIs reveals a concomitant Notch proteolytic inhibition and, over recent decades, these inhibitors have also been tested for anti-oncogenic effects (28). In the present work, we demonstrate the usefulness of several GSIs for Notch pathway inhibition in 3 RMS cell lines. One of the 4 drugs tested (GSI-X) produced only moderate inhibition but the other 3 drugs (DAPT, GSI-XX and GSI-XXI) yielded better results. This GSI-mediated Notch inhibition in the 3 cell lines analyzed—although total inhibition was not achieved—led to severe lessen of cell mobility and invasiveness in vitro, suggesting that the Notch pathway is responsible, at least partially, for the control of the mechanisms that allow cells to migrate and invade, thereby pointing to a possible future use of the drugs tested—particularly GSI-XX and GSI-XXI—as a therapeutic agents in the fighting against metastasis in RMS. Treatment with GSIs produces considerable gastrointestinal toxicity (29) that could be reduced, at least in part, by concurrent treatment with corticosteroids (30). Moreover, GSI-XX and -XXI may have a more favorable therapeutic profile than the most commonly used DAPT, since they are effective at lower concentrations in vitro.

Around 35 membrane proteins are proteolyzed by γ-secretase (28), a fact to be borne in mind before the effects observed in γ-secretase inhibition are attributed to a single pathway. Therefore, in our opinion, all the results obtained should be validated using a γ-secretase independent inhibition methodology. For this reason, we provide a model in which Notch proteolysis remains untouched, while NICD cannot activate transcription of Notch target genes owing to the expression of a dominant negative MAML1 transcription factor. Using this methodology, the effects seen on cells were practically identical to those observed with GSI treatment, which confirms more specifically that Notch inhibition suffices to produce the effects described with GSI treatment, particularly on invasiveness.

Although our study demonstrates a critical role of the Notch pathway in the activation of the process that leads to cell mobility and invasiveness in cell culture, further study will be needed in RMS to elucidate its implications in the more intricate tumor microenvironment. In our opinion, one of the challenges for the future is to create RMS animal models to characterize the pathway in vivo and attempt the possible use of GSIs—while avoiding toxicity—as a possible complementary targeted therapy against RMS, particularly in cases with a high probability of relapse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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