Aberrant expression of Notch signaling molecules in patients with immune thrombocytopenic purpura

Daoxin Ma · Jianjian Dai · Xiaojuan Zhu · Shuxin Yan · Ping Zhao · Jingru Zhang · Yuanyuan Zhu · Jianzhi Sun · Jun Peng · Chunyan Ji · Ming Hou

Received: 3 January 2009 / Accepted: 30 June 2009 © Springer-Verlag 2009

Abstract To investigate the role of Notch signaling pathway in immune thrombocytopenic purpura (ITP), we measured the expression of 11 Notch pathway molecules in ITP patients and evaluated their clinical relevance. Real-time reverse transcriptase polymerase chain reaction results showed there was aberrant expression of some Notch molecules in ITP. Notch1 and Notch3 expression elevated, while Notch2 decreased statistically in ITP patients. As for Notch ligands, only DLL1 was found downregulated in ITP. The expression of Notch target gene, Hes1, was also upregulated. In accordance with the mRNA level, Notch1 and Hes1 protein expression was also found elevated by Western blot. Immunocytochemistry showed that Notch1 expressed highly in the cytomembrane, cytoplasm, and part of cellular nucleus for ITP while weak in cytomembrane for controls, and Hes1 of ITP was found expressed higher in cellular nucleus than that of controls. Our findings suggest that the aberrant expression profile of Notch pathway may be involved in ITP, and blockage of Notch1 pathway is likely a promising therapeutic concept.

Keywords Expression · Detection · Immune thrombocytopenic purpura · Notch

Introduction

Chronic immune thrombocytopenic purpura (ITP) is an autoimmune disease in which platelets are opsonized by antiplatelet autoantibodies and subsequently destroyed by the reticuloendothelial system [4]. Factors triggering autoimmune diseases are still poorly understood, although it is widely accepted that both genetic and environmental factors play important roles in the development of the disease. Despite that autoreactive B lymphocytes secreting antiplatelet antibodies are considered as the primary immunologic defect in ITP, dysfunctional cellular immunity is considered equally important in the pathogenesis of ITP [5, 6, 22, 28].

Notch signaling is an evolutionarily conserved pathway controlling diverse aspects of development and tissue homeostasis. Mammals express multiple receptors (Notch1–4) and ligands (DLL 1, 3, 4 and Jagged1, 2) [9]. Notch signaling requires cell–cell interactions leading to Notch cleavage via a γ-secretase. This frees the intracellular domain of Notch (Notch-IC), which translocates to the nucleus and forms a ternary complex with a highly conserved transcription factor, CSL (CBF1/Suppressor of Hairless/ Lag1) and transcriptional coactivators of the mastermind-like (MAML) family. This complex activates target gene transcription including the hairy/enhancer-of-split (Hes1) and Hey1 [3, 7].
The precise role of Notch signaling on lymphocyte activation, proliferation, and polarization remains unclear. T helper (Th) cell polarization and the profile of cytokine production may depend on the ligand interacting with the Notch receptor [1, 13, 15, 20]. It has been shown that Jagged1 promotes IL-4 expression and stimulates Th2-type responses [1], while DLL1 and DLL4 induce differentiation along a Th1-type pathway [15, 24]. The expression upregulation of Notch receptors after lymphocytes activation suggests that all Notch receptors may play a role in peripheral lymphocytes responses. Although the pathway seems to be common for all four Notch receptors, activation of individual Notch receptors may lead to different cell fate decisions.

Recent studies showed that the Notch pathway was involved in the development of several autoimmune diseases. In experimental autoimmune encephalomyelitis (EAE), blockade of both DLL1 and Jagged1 increased activation of peripheral lymphocytes, and Jagged1 signaling protected from EAE, while DLL1 was deleterious [8]. In mouse models, inhibiting Notch signaling was also found to be effective in autoimmune lymphoproliferative syndrome (ALPS) and systemic lupus erythematosus (SLE) [27]. However, there are no data to date about Notch pathway in patients with ITP. To improve our understanding of a role of the Notch signaling pathway in the pathogenesis of ITP, we examined the expression of Notch pathway molecules and evaluated their clinical relevance.

Materials and methods

Patients and controls

After approval by institutional review board, 22 adult chronic ITP patients (11 women and 11 men; mean age 37, range 16–83 years), six of them with untreated disease and 16 in recurrent status, were studied. There was no form of therapy for the patients at the time of investigation. Twenty milliliters of heparinized venous peripheral blood were collected from each patient or control subject. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden) and were preserved at −80°C in aliquots for examination and thawed only once to avoid degradation. The diagnosis of chronic ITP was based on the criteria reported previously which are consistent with the American Society of Hematology guidelines [10]. Only patients over 15 years old at diagnosis with platelet counts <100×10^9/L were included. Patients with systemic lupus erythematosus satisfying the American College of Rheumatology criteria [26] and/or antiphospholipid syndrome (APS) fulfilling the international consensus statement criteria [14, 29] were excluded from the study, as were pregnant patients or those with concomitant human immunodeficiency or hepatitis C virus infection. The control group consisted of 22 adult healthy volunteers (11 women and 11 men; median age 34, range 23–68 years). Informed consent was obtained from all participants. At the time of blood sample collection, the platelet count of ITP patients (median 19.5×10^9/L, range 2–29×10^9/L) was significantly lower than that of the controls (median 196.8×10^9/L, range 154–284×10^9/L; P<0.001).

Real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated by Trizol (Invitrogen) according to the manufacturer’s instructions. Approximately, 1 μg of total RNA from each sample was subjected to first-strand cDNA synthesis using RevertAid(TM) First Strand cDNA Synthesis Kit (MBI, Fermentas, USA). Reverse transcription reaction was done at 42°C for 1 h, followed by 95°C for 5 min. Real-time polymerase chain reaction (PCR) was conducted using the Light-Cycler rapid thermal cycler system 2.0 (Roche Diagnostics Ltd, UK) in accordance to the manufacturer’s instructions. The real-time PCR contained, in a final volume of 20μl, 10μl of 2× SYBR Green Real-time PCR Master Mix, 1μl of cDNA, and 1μl of the forward and reverse primers. The PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no by-products were formed. The relative concentrations of the PCR products derived from the target gene were calculated using LightCycler System software. The results were expressed relative to the number of GAPDH transcripts used as an internal control. All experiments were conducted in triplicate. The primers and annealing temperatures used for the amplification of human Notch pathway molecules were shown in Table 1.

Western blot analysis

Cells were lysed in lysis buffer [50 mmol/L Tris (pH7.5), 100 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10μl/ml protease inhibitor cocktail, and 1 mmol/L phenylmethylsulfonyl fluoride] for 20 min at 4°C. The protein concentrations were determined with the BCA Protein Assay reagent (Pierce, Rockford, IL, USA), according to the manufacturer’s instructions. Total proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked with blocking buffer [0.1 mol/L Tris (pH7.5), 0.9% NaCl, and 0.05% Tween-20 containing 5% nonfat milk powder], then
incubated with Notch1 intra, Hes1, and β-actin primary antibodies (Abcam, Cambridge, UK) at a 1:1,000 dilution, followed by incubation with anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies. The probed proteins were detected using the chemiluminescent reagents (SuperSignal West Pico West Chemiluminescent Substrate, Pierce, Rockford, IL, USA). The protein expression results were expressed by the ratio of the accumulated optical density value of target protein to that of β-actin.

Immunocytochemistry

Cells were placed on glass slides and fixed with 4% paraformaldehyde for 30 min, and nonspecific binding sites were blocked with diluted goat serum for 30 min at room temperature. Slides were then incubated with rabbit polyclonal primary antibodies raised against human Notch1 intra, Hes1 at 4°C overnight at a 1:300 dilution. Phosphate-buffered saline was used for all subsequent washes and for antiserum dilution. After extensive washing (3 × 5 min) to remove excess antibody, the slides were incubated with diluted HRP-labeled anti-rabbit antibody (Jingmei Co. Ltd, Beijing, China) for 1 h at room temperature. All the slides were then processed by the SP method (Zhongshan Co. Ltd, Beijing, China) for 30 min at room temperature. Negative controls for each slide were prepared by substituting the primary antiserum with non-immune IgG. For each experiment, all slides were stained in a single batch and thus received equal staining.

The modified monoclonal antibody immobilization of platelet antigens assay

The modified monoclonal antibody immobilization of platelet antigens assay of ITP patients using specific antiplatelet GPIIb/IIIa and/or GPIb/IX autoantibodies was carried out as previously described [11]. Briefly, platelets from healthy blood donors (blood group O) were sensitized with tested or control plasma, washed, and solubilized in Tris-buffered saline containing 1% Triton X-100. Diluted sensitized platelet lysate was added in duplicate into the wells of a microtiter plate coated with the mAbs SZ2 or P2. IgG bound to the captured glycoprotein (GP) was detected using alkaline phosphatase-conjugated goat anti-human IgG (Fc specific). p-Nitrophenyl phosphate was used as substrate, and the absorbance was recorded at 405 nm using a microtiter plate reader (Ultra-Mark; Bio-Rad, Hercules, CA, USA). Four normal plasma samples were analyzed on each plate, and an absorbance above the mean +3 SD recorded for the controls was considered as a positive reaction.

Statistical analysis

Statistical analyses were performed using SAS version 9 software. Because the data had nonnormal distribution or heterogeneity of variance, median (range) was shown. Comparison between groups was analyzed by Wilcoxon rank-sum test. Spearman's test was used for correlation analysis. P value <0.05 was considered statistically significant.

Results

Aberrant expression patterns of Notch pathway molecules in ITP patients

To examine whether Notch pathway molecules are involved in ITP, we first investigated the expression patterns of Notch pathway genes in ITP patients and controls using Table 1: Primers and annealing temperatures (Temp) for Notch pathway molecules

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5′ to 3′)</th>
<th>Reverse (5′ to 3′)</th>
<th>Size (bp)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>TCAGCGGGATCCACTGTGAG</td>
<td>ACACAGGCGAGTGAACTTG</td>
<td>104</td>
<td>62</td>
</tr>
<tr>
<td>Notch2</td>
<td>TGCAAAGCTCATGTTGGTTGTGA</td>
<td>TGCTAGGCTTGTGAGGTACG</td>
<td>132</td>
<td>60</td>
</tr>
<tr>
<td>Notch3</td>
<td>GGGGCCCACTCACCCCTTAC</td>
<td>GTGCAGCTGCTGCACTTG</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Notch4</td>
<td>CGGCCTCGGACTCAGTCA</td>
<td>CAACTCCATCCTACAACTCTTG</td>
<td>112</td>
<td>60</td>
</tr>
<tr>
<td>Jagged1</td>
<td>GCTGCCGGGCCGCTATGCTG</td>
<td>ACGGCCGGGCCGCTATGCTG</td>
<td>78</td>
<td>65</td>
</tr>
<tr>
<td>Jagged2</td>
<td>CACGCCGTCAGGCTGAGGAGA</td>
<td>ACCGGTGCGCCGCGCTGAGGAGA</td>
<td>84</td>
<td>65</td>
</tr>
<tr>
<td>DLL1</td>
<td>TGTTGGCTGATAAACAATCAGGAGGAG</td>
<td>GTGAAATGCGCGCTGAGGAGGAG</td>
<td>76</td>
<td>65</td>
</tr>
<tr>
<td>DLL3</td>
<td>GAGACACCAGGCTCAGCTTTCAG</td>
<td>CAGTGCAGCTGCTGCACTTG</td>
<td>61</td>
<td>65</td>
</tr>
<tr>
<td>DLL4</td>
<td>CCAGGAAAGTTTCACCACTTG</td>
<td>CCGACACTCTGGCTTTTCACT</td>
<td>82</td>
<td>65</td>
</tr>
<tr>
<td>Hes1</td>
<td>TGATTGGCATGCTGCTAAGAGGATA</td>
<td>GCTGCAAGTTGCTGCACTTG</td>
<td>98</td>
<td>65</td>
</tr>
<tr>
<td>Hey1</td>
<td>GCTGGTACCCAGGCTGCTTTCAG</td>
<td>TGCAGGACCTGGCCGCTTTCAG</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCACCGTGCAAGGCTGAGGAG</td>
<td>TGTTGAAGACGCCGCTGAGGAG</td>
<td>138</td>
<td>62</td>
</tr>
</tbody>
</table>
real-time reverse transcriptase polymerase chain reaction (RT-PCR) method. Though both ITP patients and controls were found to express Notch receptors (Notch1, 2, 3), ligands (Jagged1, 2, DLL1, 3, 4) and target genes (Hes1 and Hey1) at mRNA level, there were statistical differences for some of these molecules. Notch1 and Notch3 of ITP patients were statistically higher than those of controls ($P=0.001$), while Notch2 was lower ($P=0.001$). As for Notch ligands in ITP, only DLL1 was found significantly decreased ($P=0.041$), while Jagged1 was marginally lower ($P=0.071$), and Jagged2 was marginally higher ($P=0.051$). Hes1 of ITP was significantly higher than that of control, while no statistical difference was observed for Hey1 (Table 2).

To verify whether the alteration of Notch1 and Hes1 genes at the level of transcription ultimately results in alterations at the level of translation, Western blot analysis was conducted. Because of the limited samples, only seven ITP patients and seven controls were analyzed. In agreement with the real-time RT-PCR results, Western blot analysis showed that the Notch1 and Hes1 protein level of ITP patients was significantly higher than that of controls (Fig. 1, Table 3).

Among the 22 controls, significantly positive correlations were found between Notch1 and Notch2 ($r=0.55$, $P=0.009$), Notch2 and Jagged1 ($r=0.42$, $P=0.049$), Notch2 and DLL4 ($r=0.57$, $P=0.006$), Notch3 and Jagged1 ($r=0.46$, $P=0.033$), DLL1 and Jagged1 ($r=0.57$, $P=0.005$), and DLL4 and Jagged1($r=0.45$, $P=0.037$). However, because of aberrant expression of Notch molecules in ITP patients, statistically positive correlations were different from controls and were found between Notch2 and Jagged1 ($r=0.77$, $P=0.001$), Notch2 and DLL3($r=0.49$, $P=0.021$), Notch2 and Hey1($r=0.67$, $P=0.001$), Jagged1 and DLL3 ($r=0.69$, $P=0.001$), DLL1 and DLL3 ($r=0.45$, $P=0.036$), DLL1 and Hes1 ($r=0.43$, $P=0.044$), and Hes1 and Hey1 ($r=0.57$, $P=0.001$).

Aberrant cellular localization of Notch1 and Hes1 in ITP patients

We characterized the cellular localization of Notch1 and Hes1 in ITP patients or controls using immunocytochemistry. Notch1 expressed at very high level in cytomembrane, cytoplasm, and part of cellular nucleus for ITP while weak only in cytomembrane for controls, whereas Hes1, which was mainly found in cellular nucleus, expressed higher in ITP than in controls (Fig. 2).

Correlation of Notch pathway molecules with clinical and laboratory parameters in ITP patients

No significant differences of the Notch molecules tested were found between untreated and recurrent ITP patients ($P>0.05$). Correlations between platelets and Notch molecules were analyzed in ITP patients, and DLL1 was found to be negatively correlated with platelets ($r=-0.44$, $P=0.043$), while no statistical difference was found for all the other detected Notch pathway molecules.

Circulating antiplatelet autoantibodies are frequently detected in ITP patients. The most common targets of antiplatelet antibodies are the platelet glycoprotein GPIIb/IIIa complex and GPIb/IX. We examined the correlation of

![Fig. 1 Results of Western blot. The Notch1 and Hes1 protein levels in lymphocytes of ITP patients (I1, I2, and I3) were significantly higher than those of controls (C1, C2, and C3; $P<0.05$). The results are representative of seven ITP patients and seven controls.](image-url)
antiplatelet GPIIb/IIIa and/or GPIb/IX autoantibodies with the Notch pathway molecules in 12 ITP patients, but no statistical significance was found.

Discussion

Notch signaling regulates a variety of cellular processes, including stem cell maintenance, cellular differentiation, proliferation, and apoptosis. Besides being involved in carcinogenesis as showed by our previous study [30], Notch signaling is also important in regulating numerous physiological processes including lymphocyte activation and differentiation. Notch signaling governs multiple choices during thymic development [19]. Therefore, anomalies in Notch signaling, by way of abnormal expression of Notch receptors and ligands, are expected to influence the ontogeny of lymphocytes. However, the link between anomalies in Notch signaling during lymphocytes development and autoimmune disorders is still missing.

<table>
<thead>
<tr>
<th></th>
<th>ITP patients</th>
<th>controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1/actin</td>
<td>1.94 (1.13–4.36)</td>
<td>0.47 (0.03–0.71)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hes1/actin</td>
<td>0.48 (0.12–0.79)</td>
<td>0.03 (0.02–0.18)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Notch signaling is also involved in Th-cell development, particularly Th1/Th2 lineage choice [17]. Conflicting evidences have been reported on this issue. Some studies suggest that Notch signaling favors Th2 development by directly regulating IL-4 expression, and conditionally targeted deletion of a Notch-specific scaffold protein encoding gene, MAML, results in compromised Th2-type immune response against helminthic infection [2, 16]. Several other studies suggested that Notch signaling regulates Th1-type cytokine IFN-γ via direct regulation of the Th1 master regulator, T-bet, and inhibition of Notch activity could block IFN-γ production in peripheral blood cells [15, 18]. The questions addressed by these conflicting reports remain unresolved. ITP is generally considered a Th1-driven disorder [23]. Recent studies showed that the Notch signaling may be involved in the development of several other autoimmune diseases, and some of the results are conflicted. However, to date, there are no data about Notch pathway in patients with ITP. Investigating the Notch pathway profile in ITP could be an interesting area of further studies.

In this study, we first investigated the mRNA expression of Notch signaling pathway molecules in PBMCs of ITP patients and healthy controls. We have demonstrated that Notch1 and Notch3 were upregulated, while Notch2 and DLL1 ligand were downregulated in ITP patients. The expression of Notch1 target gene, Hes1, was also elevated, which showed an activation of Notch signaling pathway in controls (b), whereas Hes1, which was mainly found in cellular nucleus, was expressed higher in ITP (c) than in controls (d). The results are representative of seven ITP patients and seven controls.
ITP patients. In accordance with the mRNA level, Notch1 and Hes1 protein expression levels were also found elevated in ITP patients, which further certificated the aberrant expression of Notch molecules. The localization in cells is very important for the function of protein molecules, so immunocytochemistry was applied to characterize the location of Notch molecules in lymphocytes. We found that Notch1 was expressed higher in ITP patients and localized in cytomembrane, cytoplasm, and part of in cellular nucleus, which indicated that the Notch1 signaling was activated, and Notch-IC was freed from full-length Notch1 and translocated to the nucleus. The immunocytochemistry results also showed that Hes1 was mainly found in cellular nucleus and expressed higher in ITP patients, which further suggested the upregulation of Notch pathway.

Systemic autoimmune diseases are generally characterized by the production of autoantibodies that recognize a diverse array of cytoplasmic and nuclear antigens. Many studies provide strong evidence that factors in ITP plasma, possibly antiplatelet antibodies, are responsible for the platelet alterations in ITP. We further ask whether there are some associations of antiplatelet GPIIb/IIIa and/or GPIb/IX autoantibodies with Notch pathway molecules, but no significant association was found.

Our aberrant expression profile of Notch pathway signaling molecules in ITP patients is similar to some other reported autoimmune disease. In EAE, Notch1 expressed stronger during lesion evolution while weaker in the normal tissues [21], Jurynyczyk also reported that Notch1 and Notch3 mRNA expression increases after EAE-specific Ag stimulation of peripheral lymphocytes, and inhibition of Notch3 ameliorates the clinical course of passive EAE [12]. Inhibiting Notch signaling was also found to be effective in ALPS and SLE by reducing the production of abnormal double negative T cells (DNTs) and by blocking aberrant T-cell activation [27]. However, some reports were in contrast to our results to some degree. Sodsai reported that Notch1 was expressed higher in ITP patients and localized in cytomembrane, cytoplasm, and part of in cellular nucleus. We found that Notch1 was expressed higher in ITP patients and localized in cytomembrane, cytoplasm, and part of in cellular nucleus, which indicated that the Notch1 signaling was activated, and Notch-IC was freed from full-length Notch1 and translocated to the nucleus. The immunocytochemistry results also showed that Hes1 was mainly found in cellular nucleus and expressed higher in ITP patients, which further suggested the upregulation of Notch pathway.

Acknowledgments This study was partially supported by grants from the National Natural Science Foundation (30600680, 30471941, 30770922, 30470742, 30570779, 30600259, 30628015 and 30300312), 973 Project (2006CB503800), Key Clinical Research Project of Chinese Ministry of Health (2007–2009), Research Project of National Public Fare (200802031), and the Shandong Technological Development Project (2005BS03022, 2005GG4202018 and Q2008C07), and Taishan Scholar Foundation.

References


lymphocytes is associated with Notch-mediated human T cell suppression. J Immunol 178:6158–6163


