Notch1–JAG1 signaling induces aggressive myeloma cell behaviors

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The Notch signaling pathway plays a crucial role in the tumor microenvironment. We examined the expression of Notch receptors (Notch1, Notch2, Notch3, and Notch4) and its ligands (JAG1, JAG2, DLL1, DLL3, and DLL4) in multiple myeloma (MM) cells and the functions of Notch signaling in disease progression. *NOTCH1*, *NOTCH2*, and *JAG1* mRNA were highly expressed in 17 MM cell lines and primary MM cells from 28 patients, although mRNA levels of *NOTCH1* were significantly lower than those of *NOTCH2*. However, anti-Notch1 antibody inhibited MM cell proliferation and increased the percentage of bortezomib-induced apoptotic MM cells as compared with isotype control and cells cultured with anti-Notch2 antibody. Furthermore, Notch1+ MM cells showed greater proliferative potential and decreased drug susceptibility compared with Notch– cells. Aggressive disease behaviors in Notch1+ MM cells were suppressed by anti-JAG1 antibody. Furthermore, gene expression in Notch1+ cells showed upregulation of *p21* and *CCND1/2* and downregulation of apoptotic genes compared with those in Notch– cells. Those results demonstrate that the Notch1–JAG1 signaling pathway confers a high malignant potential, suggesting that its Notch signaling may be specifically associated with MM disease progression.

Key words: multiple myeloma, Notch signaling, Notch1, JAG1

Introduction

Multiple myeloma (MM) is an incurable hematological malignancy characterized by bone marrow (BM) infiltration of abnormal plasma cells, and most MM patients progress from an indolent (slowly developing) to a virulent (aggressive) form over many years [1, 2]. During MM progression, abnormal plasma cells (MM cells) gradually acquire malignant potential through multistep genetic and BM microenvironmental changes [2, 3]. Our previous study demonstrated that the signaling lymphocytic activation molecule family 3 (SLAMF3; also known as CD229 or Ly9) on MM cells transmits MAPK/ERK signals mediated by self-ligand interaction between MM cells and induces a high malignant potential in MM [4]. More-

over, the programmed death ligand 1 (PD-L1) on MM cells is involved in aggressive myeloma behaviors including cell proliferation capacity and drug resistance, and the interaction of PD-L1 with programmed cell death 1 (PD-1) molecules induces drug resistance in MM cells by antiapoptotic responses through the Akt signaling pathway [5, 6]. Therefore, the cell proliferation, drug resistance, and survival of MM cells are strongly facilitated by the interactions among several cytokines produced by MM and stromal cells as well as with other cell components in the BM niche [3, 7]. Increasing understanding of the mechanism of MM malignant formation in the BM niche is providing clues for the development of novel therapeutic strategies.

The Notch signaling pathway, which is one of the most commonly activated signaling pathways in various cancers, plays an important role in tumorigenesis as a major actor in both cancer cells and in each of the components of the tumor microenvironment [8, 9]. The Notch family has four transmembrane receptors, i.e., Notch1–4, that bind its ligands, i.e., Jagged1 (JAG1), JAG2, and Delta-like 1 (DLL1), DLL3, and DLL4, resulting in the modulation of cell-fate determination, tissue patterning, and morphogenesis. In the MM microenvironment, Notch receptors are expressed on MM cells, BM stroma cells, and osteoclasts, indicating that the activation of Notch signaling is involved in MM pathogenesis [10, 11]. In this study, we focused on Notch signaling in MM disease progression.

Thus, we examined the expression of Notch family molecules and their ligands in MM and the functions of Notch signaling in MM progression.

Materials and Methods

Human MM cell lines

Human MM cell lines were cultured in RPMI1640 medium (Wako Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C under 5% CO_2. KMS-5, KMS-11, KMS-18, KMS-20, KMS-27, KMS-28PE, KMS-28BM, and KMS-34 were kindly provided by Dr. Takemi Otsuki (Kawasaki Medical School, Okayama, Japan). U266, RPMI8226, and MM.1S were obtained from the American Type Culture Collection. OPM2 was purchased from DSMZ (Braunschweig, Germany). The human MM cell lines MOSTI-1, -2, -4, -6, and -40 were established in our laboratory using BM samples from MM patients [6]. Notch1$^+$ and Notch1$^-$ cell fractions were sorted using the FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA) after the cells were stained with PE-conjugated anti-Notch1.

Patient samples

BM samples were obtained from newly diagnosed MM patients for diagnostic purposes at Nippon Medical School after informed consent had been obtained according to the Institutional Review Board-approved protocol. Mononuclear cells (MNCs) were separated from BM samples with Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density centrifugation, and then CD138$^+$ plasma cells were purified from BM MNCs using the Human CD138 Positive Selection Kit (Stemcell Technologies, Vancouver, Canada). CD38$^+$CD138$^+$ and CD38^{dim}CD138$^-$ cells were sorted from BM MNCs of MM patients using the FACSAria II cell sorter (BD Biosciences) after the cells were stained with FITC-conjugated anti-CD138 (clone #MI15) and BV421-conjugated anti-CD38 (clone #HIT2; BioLegend, San Diego, CA, USA).

Reagents

Anti-human Notch1 (MAB5317; clone #527425), anti-Notch2 (MAB37351; clone #602845), and anti-JAG1 antibodies (MAB 1277; clone #188331; R&D Systems, Minneapolis, MN, USA) were used as blockade antibodies. Bortezomib and melphalan were purchased from Selleck Chemicals (Houston, TX, USA), reconstituted in dimethyl sulfoxide (DMSO), and stored at −20°C until use.

mRNA expression analysis

Total RNA was extracted from MM cell lines and CD138$^+$ plasma cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized from total RNA, and then quantification of mRNA using real-time PCR was performed [5, 6]. The relative expression was calculated by the formula $2^{\wedge}(-\text{delta Ct}) \times 1000$. The delta Ct value was calculated by subtracting Ct values (target gene − internal control gene); β-actin was used as an internal control. The primer sequences are shown in Supplementary Table S1.

Flow cytometry analysis

For flow cytometry (FCM) analysis, cells were suspended in 50 μl of fluorescence-activated cell sorter (FACS) buffer (PBS with 5% bovine serum albumin and 10 mM sodium azide) and blocked with Human TruStain FcX (BioLegend). Then the cells were stained with PE-conjugated anti-Notch1 (clone #MHN1-519) and anti-Notch2 antibody (clone #HMN2-35; BioLegend) for 30 min. Data acquisition was performed in a LSRFortess X-20 flow cytometer with FACSDiva software version 8.0.1 and analyzed using Flowjo software (BD Biosciences).

Cell proliferation and apoptosis assay

Bromodeoxyuridine (BrdU) analysis was performed using FCM as described previously [5, 6]. To assess drug-induced apoptosis, cells were exposed to melphalan and bortezomib for 24 hours at concentrations optimal for inducing apoptosis, and then the cells were stained with annexin-FITC (BD Biosciences) and propidium iodide (PI; Wako Chemical Industries) for FCM analysis.

Statistical analysis

The Student t test and Spearman's rank moment correlation coefficient were used to analyze the data. *P* values of less than 0.05 were considered to represent statistically significant differences. Statistical analyses were performed using GraphPad Prism version 8.3.0 software (San Diego, CA, USA).

Results

Expression of Notch family molecules and their ligands in MM

We first examined the expression levels of Notch family molecules, which include four receptors, Notch1, Notch2, Notch3, and Notch4, and their receptors JAG1, JAG2, DLL1, DLL3, and DLL4 in 17 MM cell lines and primary MM cells from 28 patients. *NOTCH1* and *NOTCH2* mRNA expression was detected in MM cell lines and primary MM cells, but only very low expression of *NOTCH3* and *NOTCH4* was detected in MM cell lines (Fig. 1A and 1B). Further, *NOTCH2* mRNA expression was higher than that of *NOTCH1* mRNA (Fig. 1A and 1B). Although the expression level of *NOTCH1* mRNA was low, it was detected
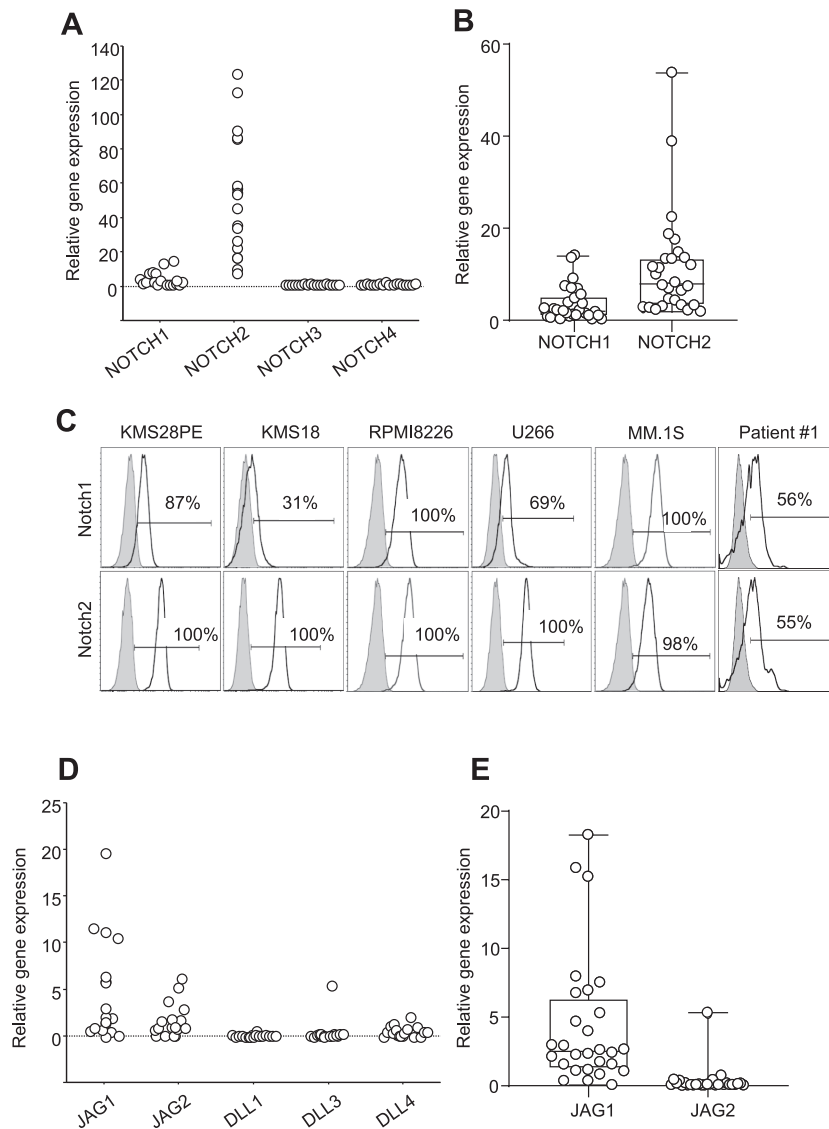
Figure 1. mRNA expression of Notch family molecules and ligand genes in MM cell lines and primary MM cells from patients. mRNA expression of Notch family genes *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4* and mRNA expression of ligand genes *JAG1*, *JAG2*, *DLL1*, *DLL3*, and *DLL4* were quantified using real-time PCR in MM cell lines (A and D) and primary MM cells from patients (B and E). (C) Cell-surface Notch1 and Notch2 expression in MM cell lines and primary MM cells analyzed using FCM. Solid line, staining with PE-conjugated antibody to Notch1 or Notch2; filled area, staining with isotype-matched control Ig. The numerical values represent the percentage of Notch1/Notch2$^+$ cells in each MM cell culture.

on the cell surface in MM cell lines (Fig. 1C). On the other hand, *JAG1* and *JAG2* mRNA was highly expressed in MM cell lines, but the expression levels of *DLL1*, *DLL3*, and *DLL4* were low (Fig. 1D). In primary MM cells, expression levels of *JAG1* mRNA were significantly higher than those of *JAG2* (Fig. 1E). The mRNA expression of *NOTCH1* and *NOTCH2* was not statistically correlated with its ligands *JAG1* and *JAG2* (data not shown). The *NOTCH1* mRNA level was significantly correlated only with corrected calcium levels (r = 0.598, *P* = 0.0113), and high *NOTCH1* mRNA levels in plasma cells from MM patients were not associated with poor survival times or other clinical characteristics (data not shown).

Aggressive myeloma behaviors associated with Notch1 molecules

We next investigated whether the cell proliferative potential and drug resistance of MM cells were associated with Notch1 or Notch2 molecule expression on the cells. BrdU incorporation in both U266 and MM.1S cells was significantly decreased after co-culture with anti-Notch1 antibody, but not after co-culture with the isotype control and anti-Notch2 (Fig. 2A). Furthermore, bortezomib-induced apoptosis was markedly increased by blockade of Notch1, but not of Notch2 (Fig. 2B). The mRNA of *NOTCH1* and *NOTCH2* was not changed by blocking of anti-Notch1 or anti-Notch2 antibodies in U266
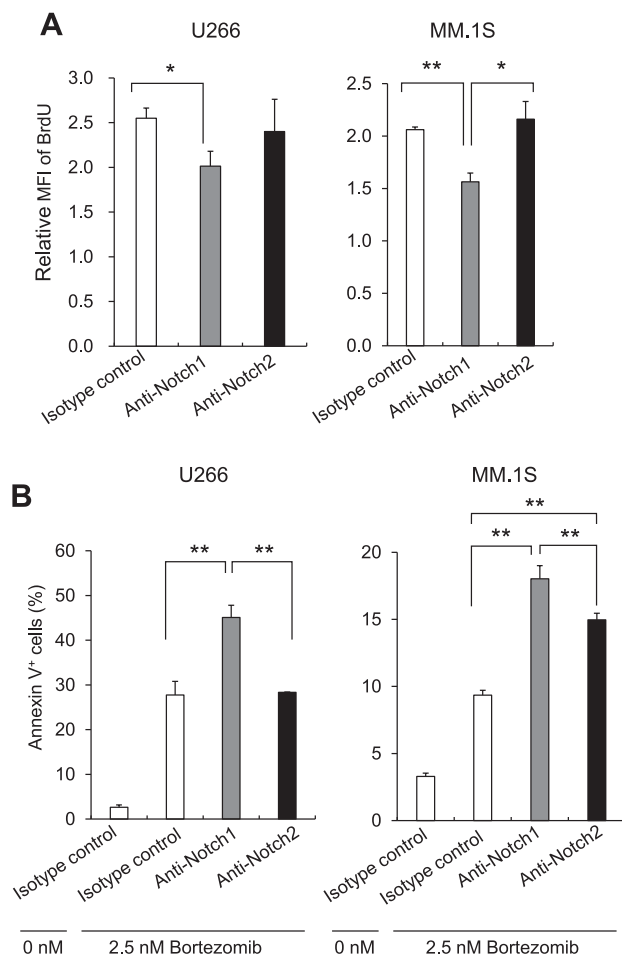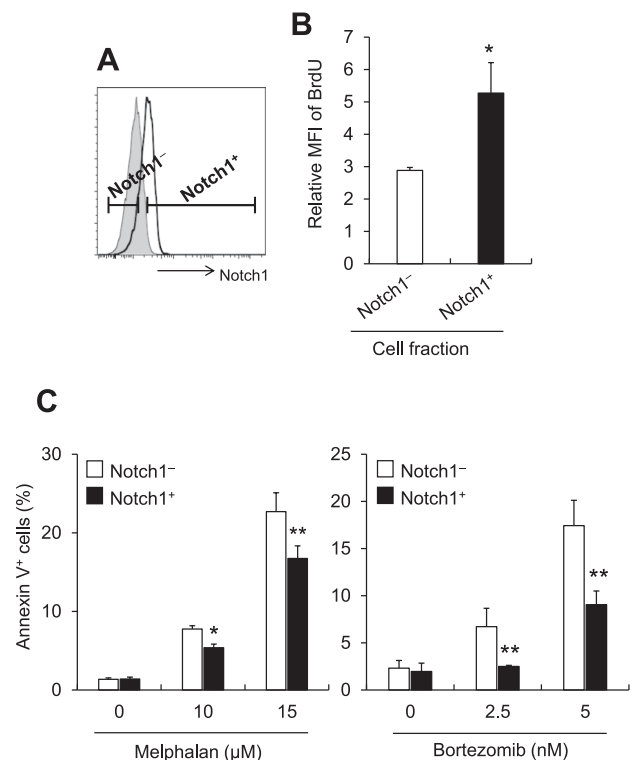
Figure 2. BrdU incorporation (A) and bortezomib sensitivity (B) in MM cells treated with anti-Notch1 and anti-Notch2. After 2-day cultivation with 20 μg/ml of control Ig or anti-Notch1 or anti-Notch2 antibodies, BrdU incorporation was examined. The relative mean fluorescence intensity (MFI) of the ratio between the MFI of antibody staining and the MFI of control IgG staining is shown. After 2-day cultivation with control Ig or antibodies, the cells were exposed to bortezomib overnight, and the percentage of annexin V$^+$ apoptotic cells was evaluated using FCM. The data are expressed as mean ± standard deviation of triplicate experiments. *$P < 0.05$, **$P < 0.01$.

Figure 3. Cell proliferation and drug sensitivity of Notch1$^+$ and Notch1$^-$ U266 cells. (A) Cell surface Notch1 expression in the U266 cell line analyzed using FCM. BrdU incorporation (B), and melphalan and bortezomib sensitivity (C) in Notch1$^+$ and Notch1$^-$ U266 cells. The data are expressed as mean ± standard deviation of triplicate experiments. *$P < 0.05$, **$P < 0.01$.

cells (data not shown). These data suggested that Notch1 molecules may be associated with cell proliferation signaling and antiapoptotic responses in MM cells.

### Cell characteristics of Notch1$^+$ and Notch1$^-$ U226 cells

Some MM cell lines contained both Notch1$^+$ and Notch1$^-$ cell fractions without any stimulation (Fig. 1C and Fig. 3A), and we thus evaluated the cell characteristics of Notch1$^+$ fractions. BrdU incorporation in Notch1$^+$ U226 cells was significantly higher compared with that in Notch1$^-$ cells (Fig. 3B). The percentage of apoptotic cells induced by melphalan and bortezomib was significantly lower in the Notch1$^+$ U226 cell fraction than in the Notch1$^-$ fraction (Fig. 3C). The mRNA

expression level of not only *NOTCH1* but also *JAG1* was elevated in the Notch1$^+$ cell fraction compared with that in Notch1$^-$ cells, and the *NOTCH2* mRNA expression level did not differ between the two MM fractions (Fig. 4A). The cell proliferative advantage and antiapoptotic responses induced by anti-MM agents in the Notch1$^+$ MM cell fraction was suppressed by anti-JAG1 antibody (Fig. 4B and 4C). *NOTCH1* mRNA levels in Notch1$^+$ and Notch1$^-$ MM cell fractions were not altered by treatment with anti-JAG1 antibody (data not shown). In BM samples of some MM patients, *NOTCH1* mRNA levels were higher in CD38$^+$CD138$^+$ plasma cell fractions than in other cell fractions (CD38^{dim}CD138$^-$ cells), while the expression pattern of *JAG1* mRNA differed in each sample (Fig. 5).

Consistent with these results, the mRNA expression of antiapoptotic gene *p21* (*cyclin-dependent kinase inhibitor 1A; CDKN1A*) and cyclin genes *CCND1* and *CCND2* was higher in the Notch1$^+$ than in the Notch1$^-$ U266 cell fraction (Fig. 6). On the other hand, the apoptotic genes *Bcl-2-associated X protein* (*BAX*), *BCL2-associated agonist of cell death* (*BAD*), *Fas-associated death domain protein* (*FADD*), *Caspase-3* (*CASP3*), *CASP8*, and *CASP9* were higher in the Notch1$^-$ cell fraction (Fig. 6).
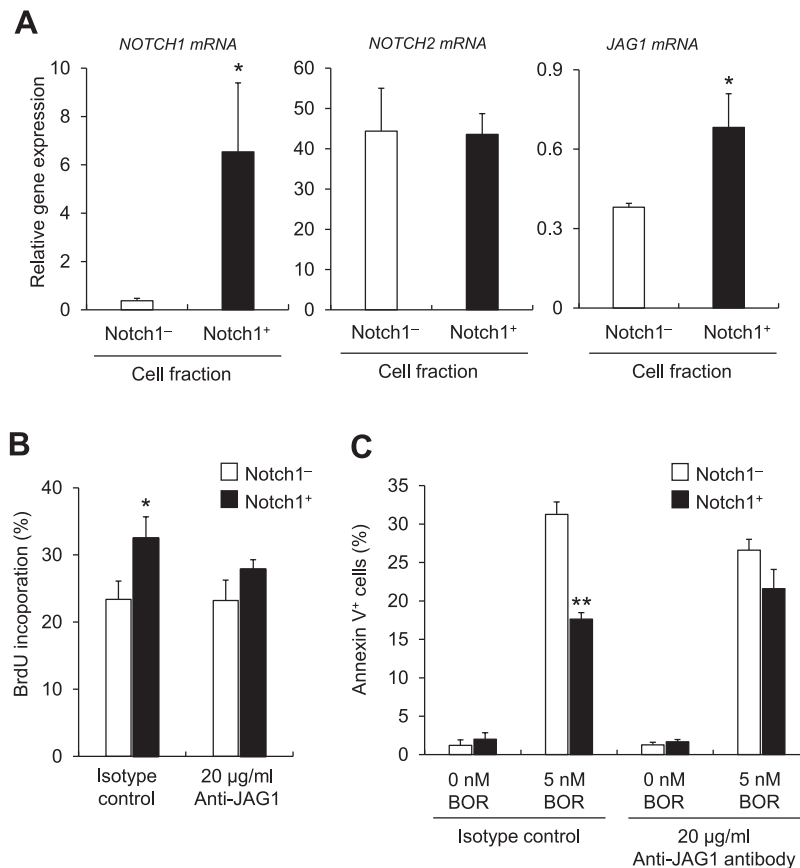
Figure 4. Cell proliferation and drug sensitivity of U266 cells treated with anti-JAG1 antibody. (A) The mRNA expression of *NOTCH1*, *NOTCH2*, and *JAG1* was quantified using real-time PCR in Notch1$^+$ and Notch1$^-$ U266 cells. BrdU incorporation (B) and bortezomib (BOR) sensitivity (C) in Notch1$^+$ and Notch1$^-$ U266 cells treated with anti-JAG1. After 2-day cultivation with 20 µg/ml of control Ig or anti-JAG1 antibody, BrdU incorporation was examined. Relative MFI in the ratio between the MFI of antibody staining and MFI of control IgG staining is shown. After 2-day cultivation with antibodies, the cells were exposed to bortezomib overnight, and the percentage of annexin V$^+$ apoptotic cells was evaluated using FCM. The data are expressed as mean ± standard deviation of triplicate experiments.

Discussion

Our results showed that Notch1, but not Notch2, on MM cells induces aggressive myeloma behavior including cell proliferative potential and antiapoptotic responses against anti-MM agents via binding with JAG1.
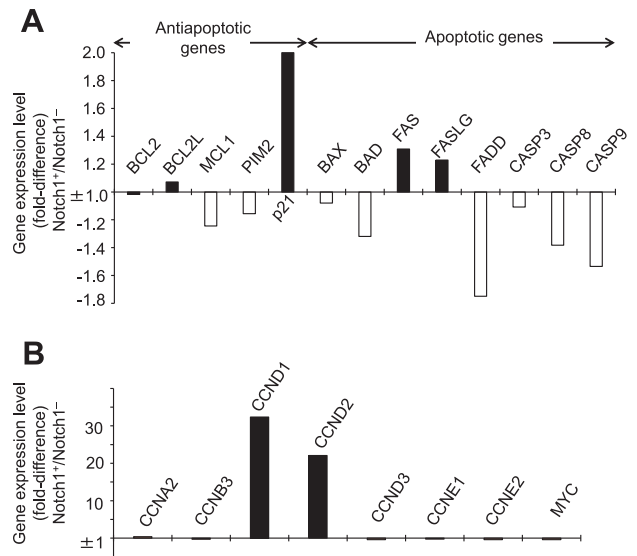
We first demonstrated that *NOTCH1* mRNA levels were significantly lower than those of *NOTCH2* in MM cell lines and primary MM cells, while cell-surface Notch1 expression was detectable. On the other hand, *JAG1* mRNA levels were higher in comparison with those of other Notch ligands. Other researchers reported that the protein expression of Notch family proteins and their ligands was observed in 8 different human MM cell lines, as shown by Western blotting analysis [12]. In immunohistochemical analysis, plasma cells from 92% and 78% of MM patients expressed Notch1 and JAG1, respectively, although cells from patients with monoclonal gammopathy of undetermined significance (MGUS) did not, suggesting that their expression may be associated with MM evolving from MGUS [13]. Further, Notch1, Notch2, and JAG1 over-

expression was observed in primary MM cells in BM biopsy specimens [14]. The blockade of both Notch1 and JAG1 inhibited the interaction between MM cells and suppressed aggressive myeloma behaviors including cell proliferative potential and drug resistance. When MM cells were co-cultured with JAG1-expressing 3T3 fibroblasts, activation of the Notch1 pathway protected MM cells from anti-MM agent-induced apoptosis via NF-κB activity [12, 15, 16]. Furthermore, the drug-induced apoptosis and cell proliferation in Notch1-positive MM cell lines, but not in the Notch1-negative cell line, were decreased and increased by JAG1-expressing 3T3 fibroblasts, respectively [12]. Notch1 molecules transmit positive signals to MM cells though homotypic and hetero-typic interactions with JAG1-expressing MM cells and stoma cells [10]. Notch activation leads to the transcriptional activation of Notch and Jagged, and the expression levels of these molecules go hand in hand (high Notch and high Jagged) [17]. Similarly, both *NOTCH1* and *JAG1* mRNA levels were higher in the Notch1$^+$ cell fraction of U266 cells than in the Notch1$^-$ cell fraction. Furthermore, our study showed that
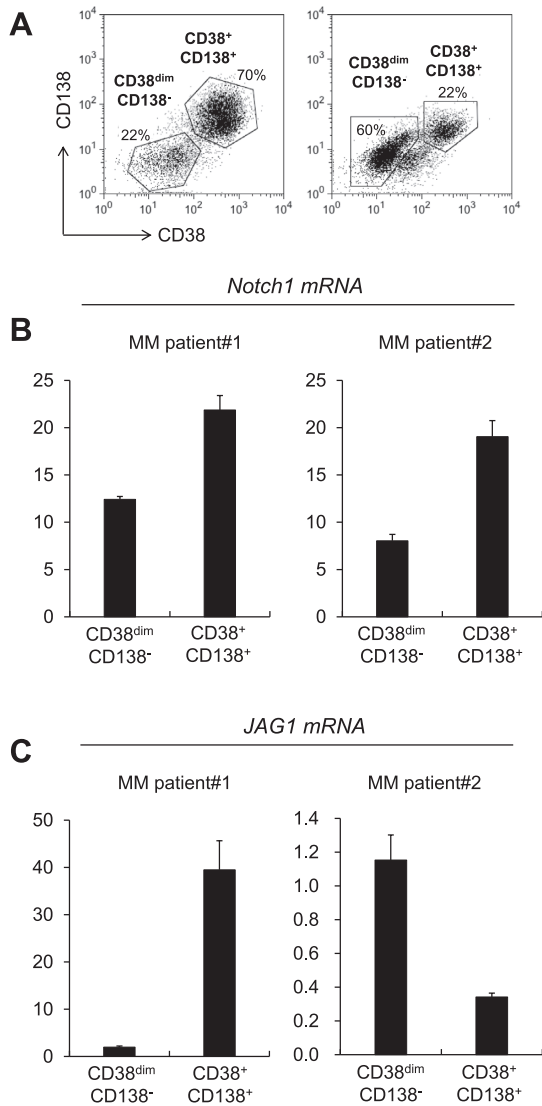
Figure 5. mRNA expression of *NOTCH1* and *JAG1* in BM cells. (A) Dot plots showing the percentage of CD38⁺CD138⁺ and CD38ᵈⁱᵐ CD138⁻ cells in BM cells from MM patients. The mRNA expression of *NOTCH1* (B) and *JAG1* (C) in CD38⁺CD138⁺ and CD38ᵈⁱᵐ CD138⁻ cell fractions.

Figure 6. Expression of apoptotic and antiapoptotic genes (A) and cell cycle-related genes (B) in Notch1⁺ U266 cells analyzed using RT-PCR. Data are represented as fold difference compared with the data for Notch1⁻ U266 cells.

NOTCH1 mRNA expression in plasma cells was higher than in other BM cells, whereas the expression pattern of JAG1 varied in some MM patient samples. The reason for this is unclear, and the differences in JAG1 expression patterns on MM cells or other BM cell components might reflect differences in the BM microenvironment in each MM patient. In actuality, Notch1–JAG1 signaling may be transmitted to MM cells via homotypic or heterotypic interactions, as shown in JAG1 expression patterns in the BM cells of individual MM patients. On the other hand, we and other researchers have not resolved the question of why Notch1, but not Notch2, signaling is associated with MM cell progression. MM cell-derived Jagged ligands boost Notch2, but not Notch1, signaling in monocytes and induce mature osteoclasts in the BM micro-

environment [11]. Therefore Notch1–JAG1 and Notch2–JAG1 interactions might operate as different pathways leading to aggressive tumor characteristics and induce aggressive MM cell behaviors and MM-associated bone disease, respectively.

Notch receptors are bound by its ligands, and its *C*-terminal fragment within the transmembrane domain is cleaved by γ-secretase, resulting in the release of the Notch intracellular domain (NICD) fragment [10, 16, 18]. The NICD fragment released by γ-secretase is translocated into the nucleus and subsequently induces the transcription of Notch target genes by interaction with the nuclear transcription factors, i.e., protein C protein binding factor 1 (CBF1; also known as RBP-Jκ or CSL for CBF1/Su[H]/Lag1) and Mastermind-like protein (MAML) [10, 16, 18]. Our data showed that the mRNA expression of cell cycle-related genes *CCND1/2* and antiapoptotic gene *p21* in Notch⁺ MM cells was upregulated in comparison to that in Notch1⁻ cells. Similarly, the expression of phosphorylated ERK and AKT, c-Myc, Bcl-2, p21, cyclin D1, and cyclin E was downregulated in larynx epithelioma cancer and non-small cell lung cancer cell lines by Notch1 knockdown [19, 20]. γ-Secretase inhibitor treatment decreased the expression of Notch-targeted genes in T-cell acute lymphoblastic leukemia cells [21]. When MM cell lines were co-cultured with JAG1-induced HtTA-jag10 cells pretreated with tetracycline, Notch signaling in MM cells promoted cell proliferation, and this phenomenon was suppressed by treatment with the γ-secretase inhibitor DAPT [14]. In addition, Notch signaling induced interleukin (IL)-6 production not only in MM cells but also in BM stroma cells, and autocrine and paracrine IL-6 supported MM

cell growth [22]. Moreover, Notch signaling in BM stroma cells induced the production of IL-6, vascular endothelial growth factor, and stromal cell-derived factor alpha (SDF1α; CXCL12) in the BM niche of MM, which play an crucial role in tumor growth, survival, and migration in MM progression [10, 23–26]. Therefore those studies suggested that Notch–JAG1 signaling might accelerate MM progression via the interaction with not only MM but also stromal cells in the BM niche.

In this study, we revealed that JAG1–Notch1 signaling is important in myeloma pathogenesis. This underscores the theory that Notch1–JAG1 may be a potential therapeutic target in MM patients.

Acknowledgments

Author Contributions

M.I. conceived the study, designed and performed the research, analyzed and interpreted data, and wrote and edited the manuscript. H.T. conceived the study, and designed and edited the manuscript. K.U. performed the research and analyzed and interpreted data. Y.I. discussed the data and gave conceptual advice. H.T. and K.I. collected patient samples and analyzed the clinical status of patients. M.R. gave conceptual advice.

Conflicts of Interest Disclosures

The authors declare no competing financial interest related to this work.

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