Soluble SLAMF7 is generated by alternative splicing in multiple myeloma cells

Multiple myeloma (MM) is a malignant disease of monoclonal plasma cells. Recently, immunotherapeutic strategies were approved for clinical use and the anti-CD38 antibody daratumumab prolonged the 5-year overall survival rate of newly diagnosed MM (NDMM) to 66.3%.[1] Nevertheless, the prognosis of MM remains dismal due to the high incidence of relapse, thereby warranting effective treatment strategies for relapsed and refractory MM (RRMM).

Gain of chromosome 1q (1q gain) is an established risk factor in MM, and has been detected in 30-40% of the NDMM, increasing to over 70% of RRMM.[2,3] The upregulation of genes in the 1q region leads to drug resistance and immunosuppression, resulting in a high incidence of relapse.[3] The gene encoding the signaling lymphocyte activation molecule F7 (SLAMF7), an adhesion molecule highly expressed on MM cells, is also located on 1q, and likely associated with the worse prognosis of 1q gain-positive MM patients.[3,4] SLAMF7 comprises a distal immunoglobulin variable-like (IgV) and a proximal C2-like (IgC2) domain, which includes the recognition site of the anti-SLAMF7 antibody elotuzumab. In addition, SLAMF7 molecules interact homotypically with each other via the IgV-domains.[4] The extracellular domain of SLAMF7 is specifically detected in the serum of MM patients as a soluble form of the protein (sSLAMF7) and its concentration increases with disease progression.[5] In addition, sSLAMF7 can increase the proliferation of MM cells, and neutralization of sSLAMF7 with elotuzumab attenuates the growth of MM. Patients with sSLAMF7^{high} MM have a higher overall response rate to elotuzumab compared to patients with sSLAMF7^{low}.[4,6] While these findings suggest a pivotal role of sSLAMF7 in the pathophysiology of MM, the mechanisms underlying its production in MM cells remain unknown.

Previous studies revealed that soluble forms of cell surface antigens were generated by alternative splicing, and that MM-specific transcript variants play a crucial role in drug

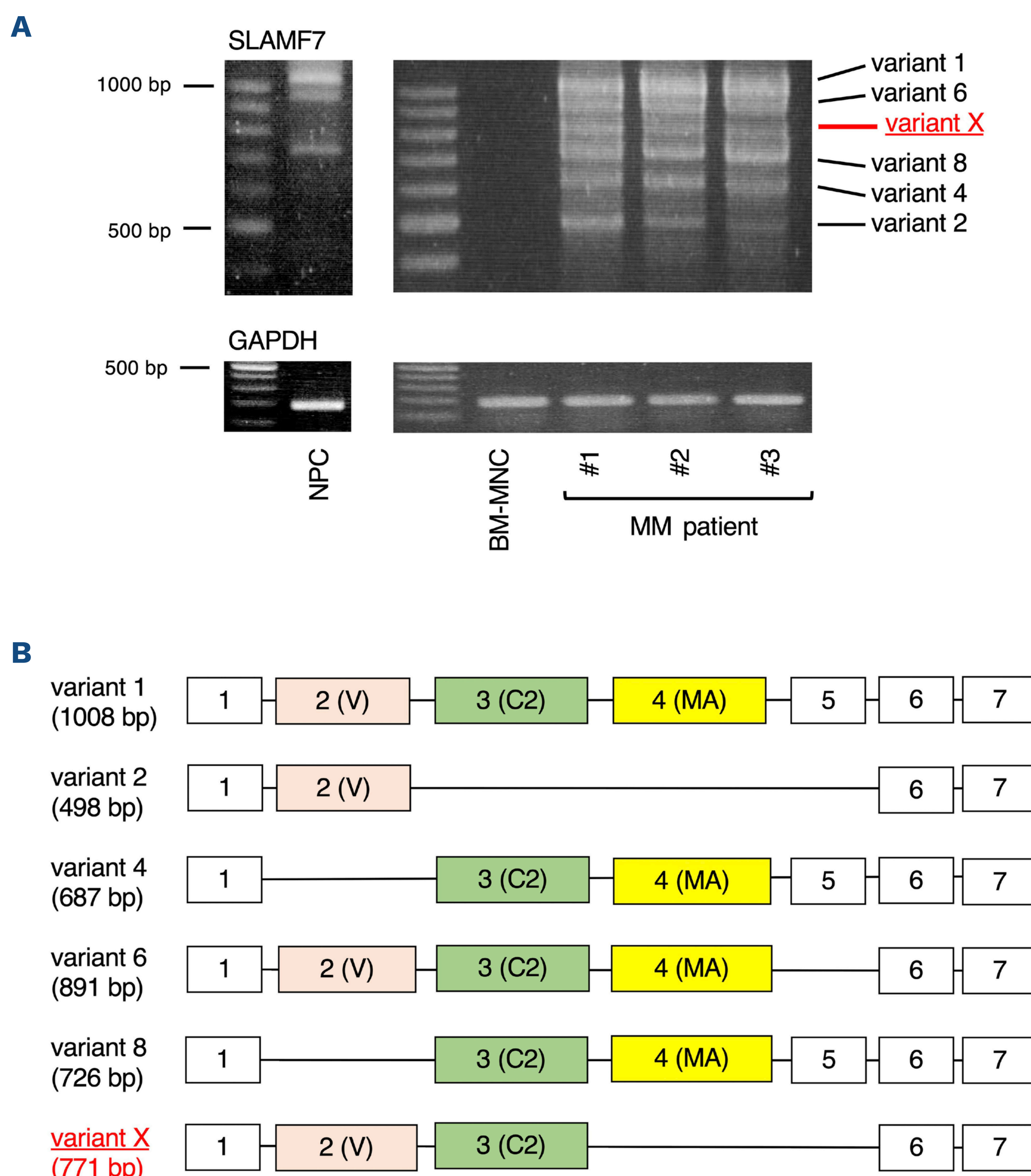
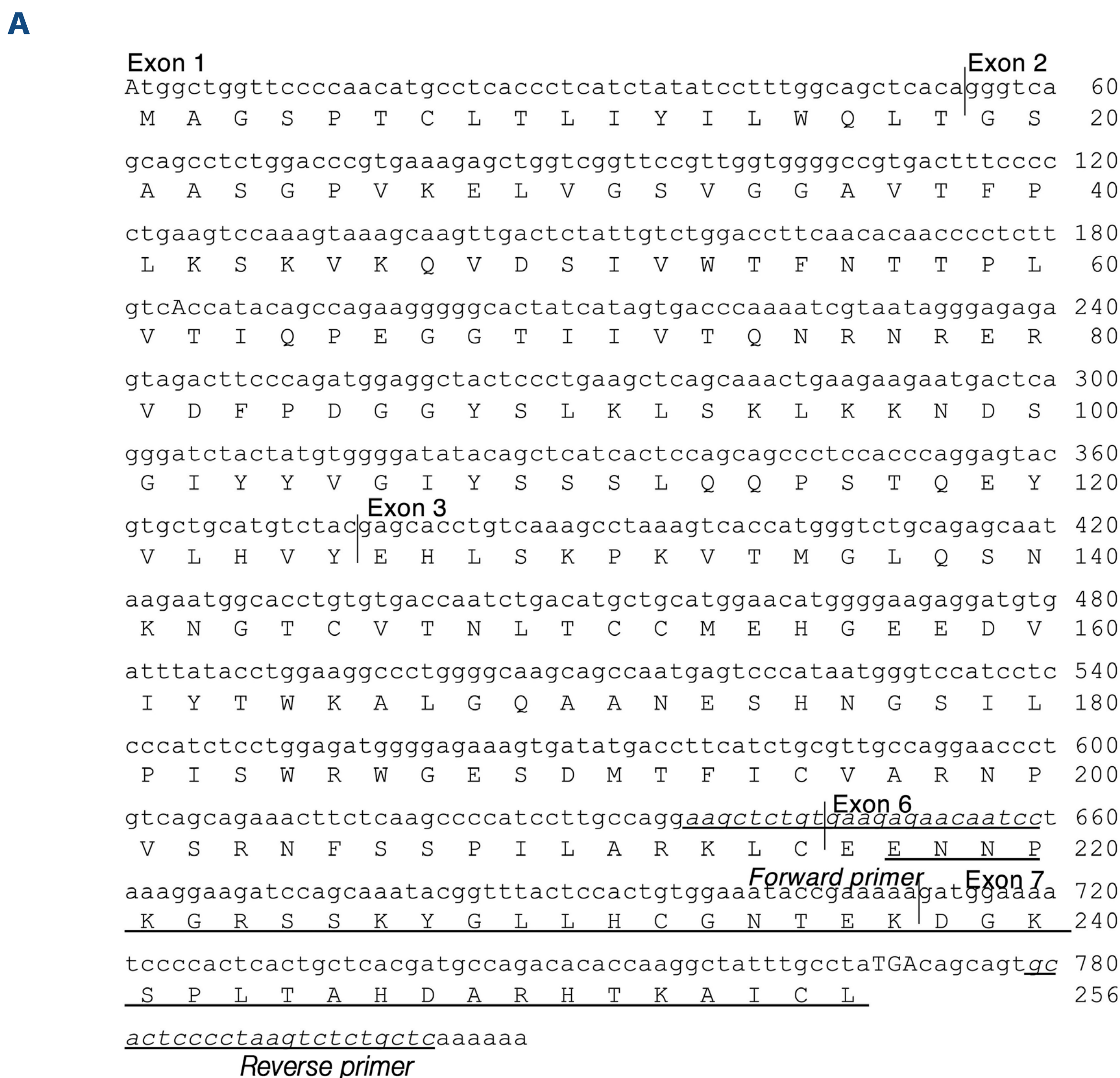


Figure 1. Soluble SLAMF7 is generated from alternative splicing in multiple myeloma cells. (A) The full length *SLAMF7* or *GAPDH* (internal control) was amplified from the cDNA of CD138$^+$ plasma cells derived from multiple myeloma (MM) patients, normal plasma cells (NPC) from healthy volunteers, and bone marrow mononuclear cells (BM-MNC). PCR was conducted for 30 cycles, and the products were analyzed using agarose gel electrophoresis and ethidium bromide staining. TA-cloning and complete sequencing of the PCR products identified the amplicons as variant 1 (1008 bp), variant 6 (891 bp), variant 8 (726 bp), variant 4 (687 bp), variant 2 (498 bp), and an unknown variant X (771 bp). (B) The schematics of the *SLAMF7* variants including exon numbers encoding the IgV-domain (V), IgC2-domain (C2), and membrane anchor portion (MA) of *SLAMF7*.

resistance and disease progression.[7] Therefore, we hypothesized that sSLAMF7 may be specifically translated in the MM cells from the variant transcript including IgV and IgC2 domains but lacking the membrane anchor portion.

To this end, we extracted polyA$^+$ mRNA from CD138$^+$ MM cells and normal plasma cells (NPC), and amplified the full length SLAMF7 cDNA. Informed consent was obtained in accordance with the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of Jichi Medical University. While 10 alternative splicing variants have been shown for SLAMF7, variant 1 fragment containing all exons (1008 bp) was present in both MM cells and NPC. However, the MM cells harbored specific fragments with lower molecular weight (MW) (Figure 1A).



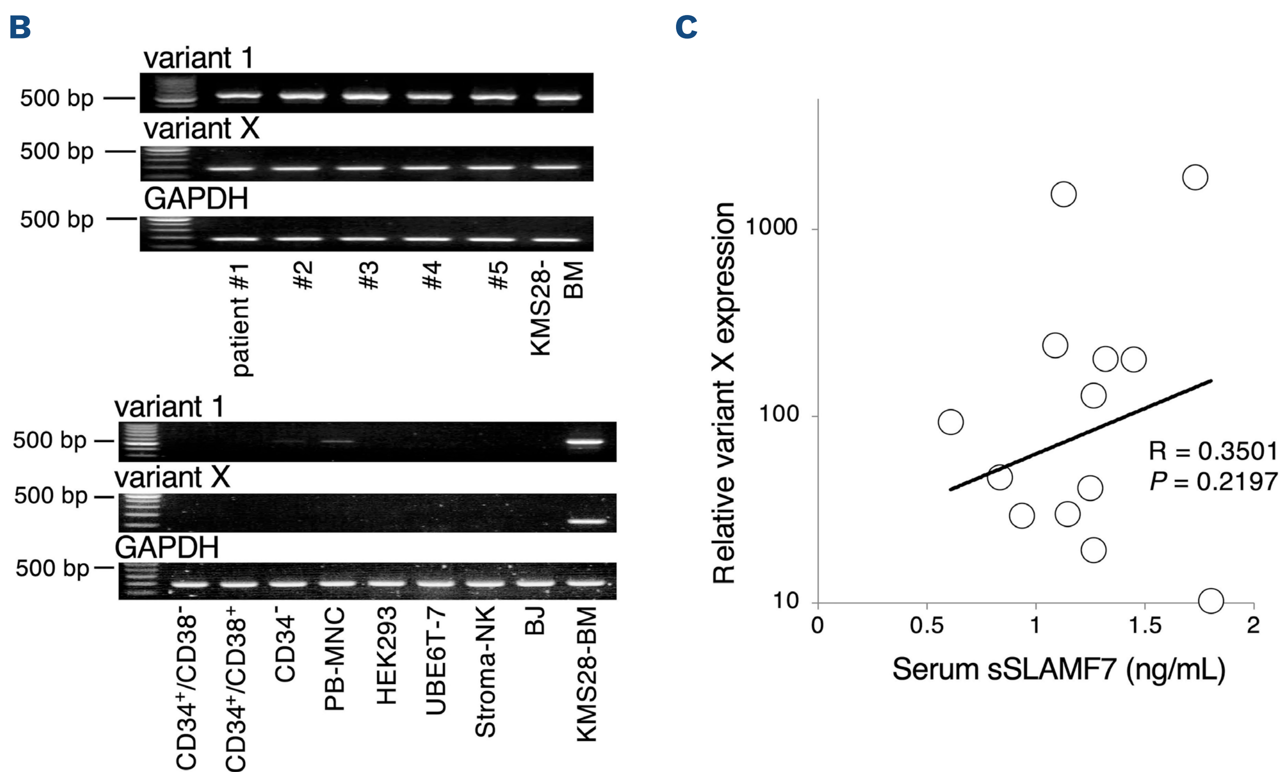


Figure 2. Sequence of SLAMF7 variant X cDNA and the expression in multiple myeloma cells. (A) Sequence of SLAMF7 variant X cDNA and its translation product. The C-terminus unique to the variant X protein is underlined. The variant X-specific forward and reverse primers are shown in italics. The primer pair was used for semi-quantitative and real-time quantitative RT-PCR (Q-PCR) analyses. (B) Total cellular RNA was isolated from 1-10x10^4 cells using an RNeasy Kit (Qiagen, Valencia, CA, USA), reverse-transcribed into complementary DNA using ReverTra Ace and oligo(dT) primers (Toyobo, Tokyo, Japan), and subjected to semi-quantitative RT-PCR or real-time quantitative RT-PCR (Q-PCR) using the specific primers described in *Online Supplementary Table S1*, and TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). *SLAMF7* variant 1, variant X and *GAPDH* (internal control) mRNA expression in multiple myeloma (MM) cells (patients 1-5) (upper panel), CD34$^+$/CD38$^-$ normal hematopoietic stem cells (CD34$^+$/CD38$^-$), CD34$^+$/CD38$^+$ normal hematopoietic progenitor cells (CD34$^+$/CD38$^+$), CD34$^-$ normal bone marrow cells (CD34$^-$), peripheral blood mononuclear cells (PB-MNC), HEK293 cells (HEK293), stromal cell lines (UBE6T-7 and stroma-natural killer), fibroblasts (BJ), and KMS28-BM were analyzed using semi-quantitative RT-PCR. The PCR products were analyzed using agarose gel electrophoresis and ethidium bromide staining. The results of suboptimal amplification cycles (35 cycles) are shown. KMS28-BM was used as a positive control. (C) Expression levels of *SLAMF7* variant X in 14 MM patients were determined by Q-PCR, normalized to that of *GAPDH*, and quantified using the $2^{-\Delta\Delta Ct}$ method with the values of BM-MNC set at 1.0. Serum levels of soluble SLAMF7 (sSLAMF7) were also measured using a SLAMF7 ELISA Kit (Aviva Systems Biology, San Diego, CA, USA). The correlation coefficient (R) between *SLAMF7* variant X expression and sSLAMF7 was evaluated by calculating Pearson's correlation coefficient.

TA-cloning and complete sequencing of these lower MW amplicons identified them as variant 6 (891 bp), variant 8 (726 bp), variant 4 (687 bp), and variant 2 (498 bp). In addition, there was an unidentified 771 bp PCR fragment that we designated as variant X (Figures 1B, 2A). As the variant X cDNA lacked Ex4 and Ex5, the predicted amino acid sequence included the IgV and IgC2 domains but not the membrane anchor portion (Figure 1B). We also analyzed the expression of the variant X transcript by semi-quantitative real-time PCR (RT-PCR) using specific primers (Figure 2A), and detected unique and expected PCR products (162 bp), as well as variant 1 in the MM cells but not in the normal cells (Figure 2B). Furthermore, the expression of variant X transcript was positively correlated with the serum level of sSLAMF7 in patients with MM (Figure 2C). Although variant 1 was expressed at differential levels in all patients with MM, we could not find a positive correlation with the variant X/variant 1 ratio (*Online Supplementary Figure S1A*). Altogether, these results suggest that the variant X cDNA encodes a truncated soluble form of SLAMF7.

Structure-based prediction of protein-protein interactions using the AlphaFold2 program revealed a possible interac-

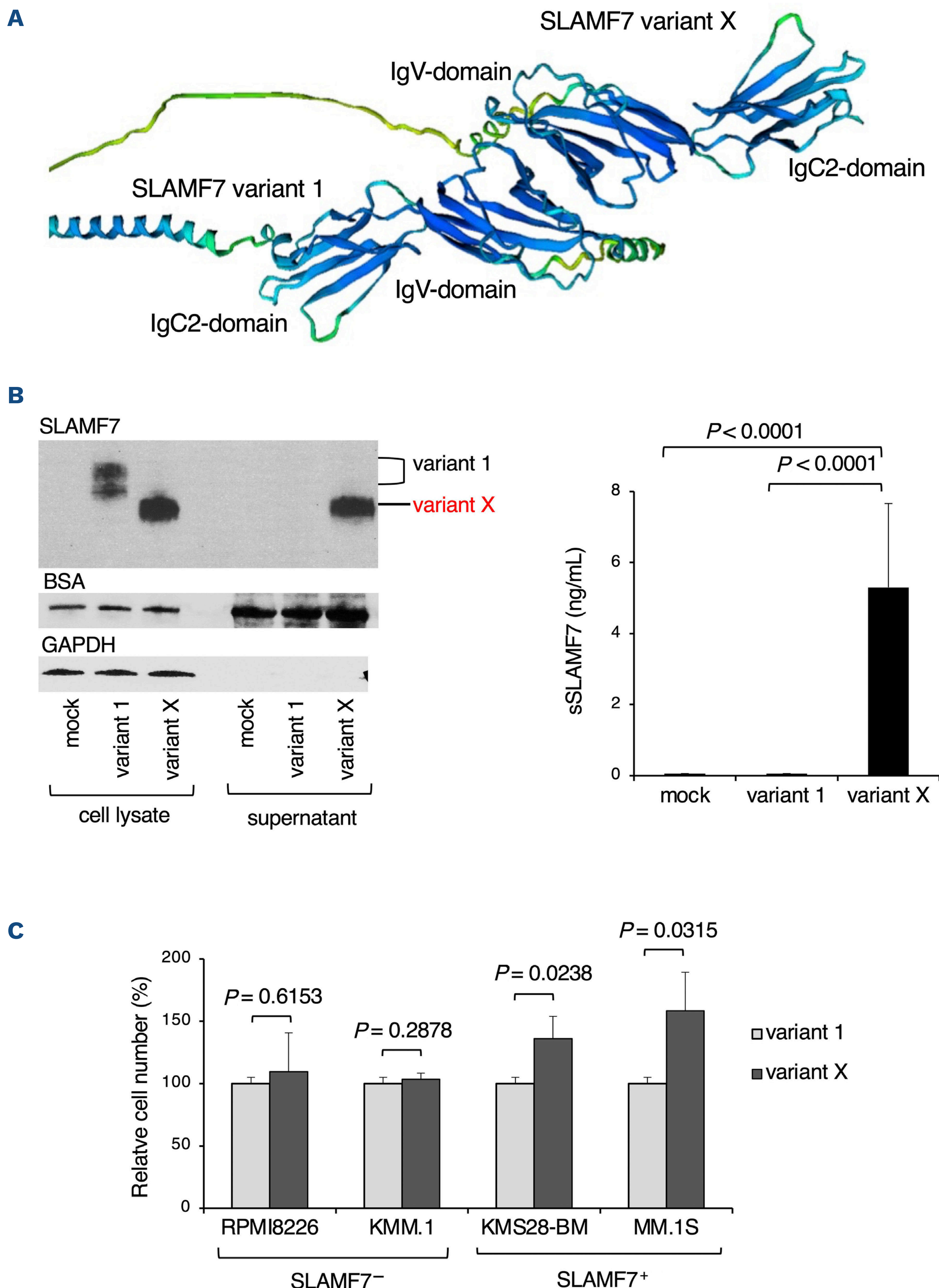


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Figure 3. Ectopic expression of SLAMF7 variant X secreted sSLAMF7 and enhanced multiple myeloma cell growth. (A) Structure-based prediction of SLAMF7 variant 1-variant X interactions using the AlphaFold2 program. (B) We used the lentiviral vector CSII-CMV-MCS-IRES-VENUS (provided by Dr. Hiroyuki Miyoshi, RIKEN BioResource Center, Ibaraki, Japan) containing the coding regions of *SLAMF7* variant 1 and *SLAMF7* variant X cDNA for gain-of-function experiments. Whole cell lysates and supernatants were prepared from RPMI8226 cells transduced with mock, *SLAMF7* variant 1, or *SLAMF7* variant X after 24 hours (hr) of culture. Immunoblotting was carried out according to a standard method using the following antibodies: anti-SLAMF7 (#98611), anti-BSA (#23053) (Cell Signaling Technology, Beverly, MA, USA); and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoblot shows the expression levels of SLAMF7 protein, with GAPDH as the loading control of cell lysate, and BSA as the loading control of supernatant (left panel). The sSLAMF7 levels in the culture supernatants were measured using ELISA (right panel). Bars indicate the mean of 3 independent experiments. *P* value was determined by one-way ANOVA with Tukey's multiple comparison test. (C) SLAMF7-negative (SLAMF7$^-$) RPMI8226 and KMM.1 cells and SLAMF7-positive (SLAMF7$^+$) KMS28-BM and MM.1S cells were cultured with the supernatant derived from 1x10^5 RPMI8226 cells transduced with *SLAMF7* variant 1 or *SLAMF7* variant X after 24 hr of culture. Cell proliferation was assessed after 72 hr using the MTT reduction assay and is shown relative to the variant 1 group. Bars indicate the means of 3 independent experiments. *P* value was calculated using a paired Student *t* test.

tion between SLAMF7 variant X, but not mutant SLAMF7 variant X, and SLAMF7 variant 1 through the IgV-domain (Figure 3A, *Online Supplementary Figure S1B*). To further explore this, we prepared whole cell lysates and supernatants from RPMI8226 cells transduced with mock, SLAMF7 variant 1, or SLAMF7 variant X, and analyzed the variant proteins through immunoblotting. Since RPMI8226 cells did not express any SLAMF7 transcript variants nor produced sSLAMF7 in the supernatant (*Online Supplementary Figure S2A*),[4] we used the cell line in this experiment. Both variant 1 and X were detected in the cell lysates, whereas only variant X was detected in the supernatant (Figure 3B, left panel) at levels similar to that in the serum of patients with MM (Figure 3B, right panel). In addition, the culture supernatant of cells expressing SLAMF7 variant X significantly increased the growth of the SLAMF7$^+$ KMS28-BM and MM.1S cells, but not of the SLAMF7$^-$ RPMI8226 and KMM.1 cells (Figure 3C).[4,7] Immunofluorescent staining revealed the binding of secreted SLAMF7 variant X on MM.1S cells but not on the KMM.1 cells (*Online Supplementary Figure S2B*). Elotuzumab mitigated the effect of variant X on the growth of MM.1S cells (*Online Supplementary Figure S2C*). Taken together, these results indicate that ectopically expressed *SLAMF7* variant X was translated to sSLAMF7, which enhanced the growth of MM cells via homotypic interaction.

In the present study, our results suggest that sSLAMF7 is generated from an MM-specific SLAMF7 variant, which lacks the membrane anchor portion and is transcribed from the *SLAMF7* gene via alternative splicing. Our findings reveal a potential mechanism by which sSLAMF7 levels increase in the sera of MM patients with disease progression, even though SLAMF7 is expressed in normal hematopoietic cells. In addition, we could not detect sSLAMF7 in the supernatants of SLAMF7$^+$ and SLAMF7$^-$ MM cell lines cultured with or without adhesion to stromal cells, despite high surface SLAMF7 expression. The expression of variant X transcript was also not up-regulated in MM cell lines co-cultured with stromal cells (*Online Supplementary Figure S2D*). These results suggest that sSLAMF7 is not generated from the cleavage of the extracellular domain of SLAMF7, thereby ruling out the possibility of antigen escape during elotuzumab therapy.

Previous studies have shown that high levels of sSLAMF7 in patients with MM is indicative of a poor prognosis, and sSLAMF7 is a potential biomarker of the response to elotuzumab.[5,6] In the present study, we showed the positive correlation between variant X expression and serum sSLAMF7. Therefore, variant X expression levels may also correlate with prognosis and antibody sensitivity, which will have to be validated on larger cohorts.

On the other hand, sSLAMF7 promotes the proliferation of SLAMF7$^+$ macrophages,[8] and these macrophages induce T-cell exhaustion via homotypic interaction of SLAMF7.[9] Thus, we surmise that sSLAMF7 secreted by the MM cells triggers immunosuppression, which can be reversed by elotuzumab via neutralization of sSLAMF7. Consistent with this hypothesis, Awwad *et al*. showed that elotuzumab improved the immune landscape in patients with RRMM by reducing the abundance of SLAMF7$^+$ exhausted T cells and regulatory T cells.[10] Therefore, addition of elotuzumab to the treatment regimen of patients with MM may improve prognosis, and elotuzumab may augment the response of patients with RRMM to chimeric antigen receptor-T cell therapy or bispecific T-cell engagers.

In conclusion, we have shown that a novel MM-specific splicing variant may be involved in disease progression. The specific splicing of the transcript should be studied further on a larger dataset, as well as in blood samples, to determine the range of expression and size of the RRMM segment. Although our findings will have to be validated through a randomized retrospective study, sSLAMF7 shows promise as an effective biomarker for predicting prognosis and response to elotuzumab in patients with MM.

Authors

Jiro Kikuchi,[1] Mitsuo Hori,[2] Naoki Osada,[1] Sae Matsuoka,[1] Atsushi Suzuki,[3°] Satoshi Kakugawa,[3°] Hiroshi Yasui,[4,5] Takeshi Harada,[6] Hirofumi Tenshin,[7] Masahiro Abe,[8] Hideki Nakasone[1] and Yusuke Furukawa[1,9]

[1]Division of Emerging Medicine for Integrated Therapeutics (EMIT), Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi; [2]Department of Hematology, Ibaraki Prefectural Central

Hospital, Kasama, Ibaraki; [3]Oncology Division, Bristol-Myers Squibb K.K., Shinjuku, Tokyo; [4]The Institute of Medical Science, The University of Tokyo, Minato, Tokyo; [5]Division of Hematology & Oncology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki; [6]Department of Hematology, University of Tokushima, Tokushima; [7]Department of Orthodontics and Dentofacial Orthopedics, Tokushima University Graduate School of Biomedical Sciences, Tokushima; [8]Department of Hematology, Kawashima Hospital, Tokushima and [9]Center for Medical Education, Teikyo University of Science, Tokyo, Japan

°AS current address: Hematology Medical Franchise, Japan Medical Affairs Division, Novartis Japan, Tokyo, Japan.
°SK current address: Japan Medical Affairs, GlaxoSmithKline K.K., Tokyo, Japan.

Correspondence:
J. KIKUCHI - kiku-j@jichi.ac.jp

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AS and SK are employees of Bristol-Myers Squibb K.K.

Contributions
JK performed experiments, analyzed data, and drafted and finalized the manuscript. MH, NO, SM, TH and HT performed experiments. HY provided cell lines. AS, SK, MA, HN and YF designed and supervised the research. All authors read and approved the manuscript before submission.

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Data-sharing statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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