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Soluble SLAMF7 is generated by alternative splicing in multiple myeloma cells.

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Running title: Soluble SLAMF7 is produced by alternative splicing.

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Author Contributions: J.K. performed experiments, analyzed data, drafted, and finalized the manuscript; M.H. N.O. S.M. T.Ha. T.Hi. performed experiments; H.Y. provided cell lines; and A.S. S.K. M.A. H.N. and Y.F. designed and supervised research. All authors read and approved the manuscript before submission.

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

To the Editor:

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 and increases 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 is 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. The $sSLAMF7^{high}$ MM patients have a higher overall response rate to elotuzumab compared to the $sSLAMF7^{low}$ patients [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 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-positive MM cells and normal plasma cells (NPCs), 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 ten alternative splicing variants have been shown for SLAMF7, variant 1 fragment containing all exons (1008 bp) was present in both MM cells and NPCs. However, the MM cells harbored specific fragments with lower molecular weight (MW) (Fig.1a). 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 (Fig. 1b and Fig. 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 (Fig. 1b). We also analyzed the expression of the variant X transcript by semi-quantitative RT-PCR using specific primers (Fig. 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 (Fig. 2b). Furthermore, the expression of variant X transcript was positively correlated with the serum level of sSLAMF7 in MM patients (Fig. 2c). Although variant 1 was expressed at differential levels in all MM patients, we could not find a positive correlation with the variant X/variant 1 ratio (Supplementary Fig.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 interaction between SLAMF7 variant X, but not mutant SLAMF7 variant X, and SLAMF7 variant 1 through the IgV-domain (Fig. 3a and Supplementary Fig.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 (Supplementary Fig.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 (Fig. 3b, left panel) at levels similar to that in the serum of MM patients (Fig. 3b, right panel). In addition, the culture supernatant of cells expressing SLAMF7 variant X significantly increased the growth of the SLAMF7-positive KMS28-BM and MM.1S cells, but not of the SLAMF7-negative RPMI8226 and KMM.1 cells (Fig. 3c) [4,7]. Immunofluorescent staining revealed the binding of secreted SLAMF7 variant X on MM.1S cells but not on the KMM.1 cells (Supplementary Fig.S2B). Elotuzumab mitigated the effect of variant X on the growth of MM.1S cells (Supplementary Fig.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-positive and SLAMF7-negative 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 (Supplementary Fig. 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 MM patients is indicative of 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-positive 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 RRMM patients by reducing the abundance of SLAMF7-positive exhausted T cells and regulatory T cells [10]. Therefore, addition of elotuzumab to the treatment regimen of MM patients may improve prognosis, and elotuzumab may augment the response of RRMM patients 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 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 MM patients.

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Figure Legends

Figure 1. Soluble SLAMF7 is generated from alternative splicing in MM cells.

(a) The full length *SLAMF7* or *GAPDH* (internal control) was amplified from the cDNA of CD138-positive plasma cells derived from MM patients, normal plasma cells (NPCs) from healthy volunteers, and bone marrow mononuclear cells (BM-MNCs). 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 (896 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*.

Figure 2. Sequence of SLAMF7 variant X cDNA and the expression in MM 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 1-10 x 10^4 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 Supplementary Table S1, and TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). *SLAMF7* variant 1, variant X and *GAPDH* (internal control) mRNA expression in 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 ($CD34^-$), HEK293 cells (HEK293), stromal cell lines (UBE6T-7 and stroma-NK), 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) The 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). The

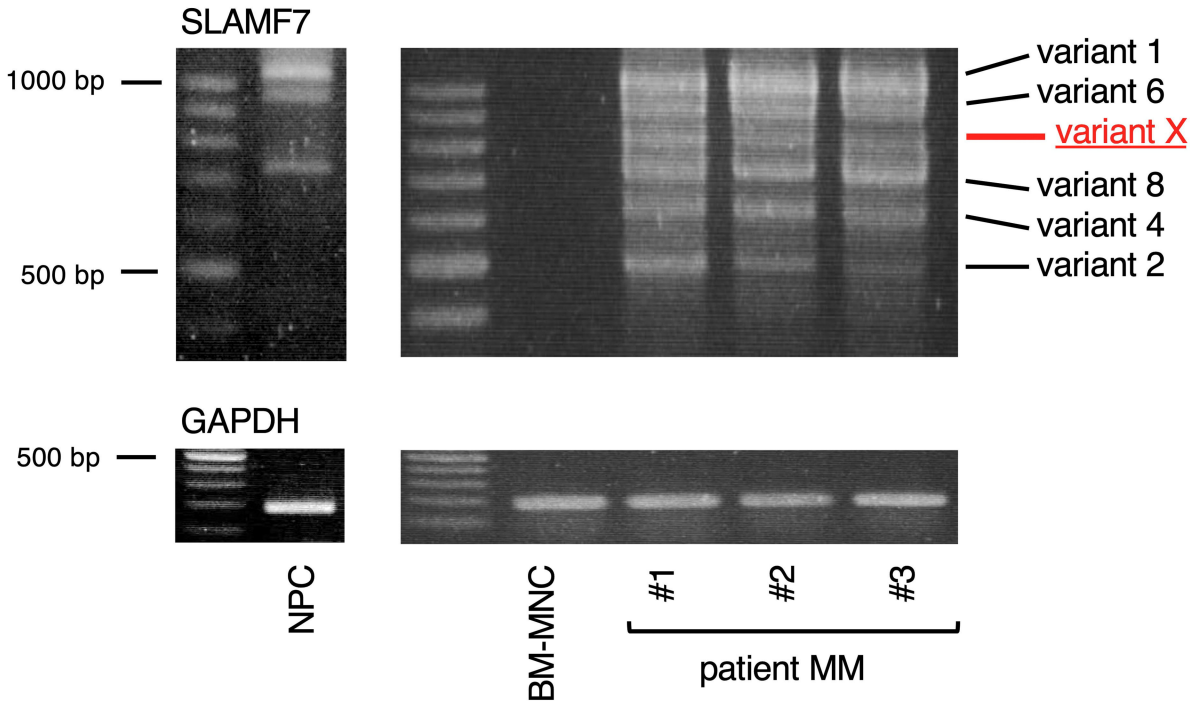
correlation between *SLAMF7* variant X expression and sSLAMF7 was evaluated by calculating Pearson's correlation coefficient.

Figure 3. Ectopic expression of SLAMF7 variant X secreted sSLAMF7 and enhanced MM 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* varinat 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 h 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); and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). 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 three 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 1 x 10^5 RPMI8226 cells transduced with *SLAMF7* variant 1 or *SLAMF7* variant X after 24 h of culture. Cell proliferation was assessed after 72 h using the MTT reduction assay and is shown relative to the variant 1 group. Bars indicate the means of three independent experiments. *P* value was calculated using a paired Student's *t* test.

Figure 1

A


SLAMF7
1000 bp
500 bp
variant 1
variant 6
variant X
variant 8
variant 4
variant 2
GAPDH
500 bp
NPC
BM-MNC
#1
#2
#3
patient MM

B

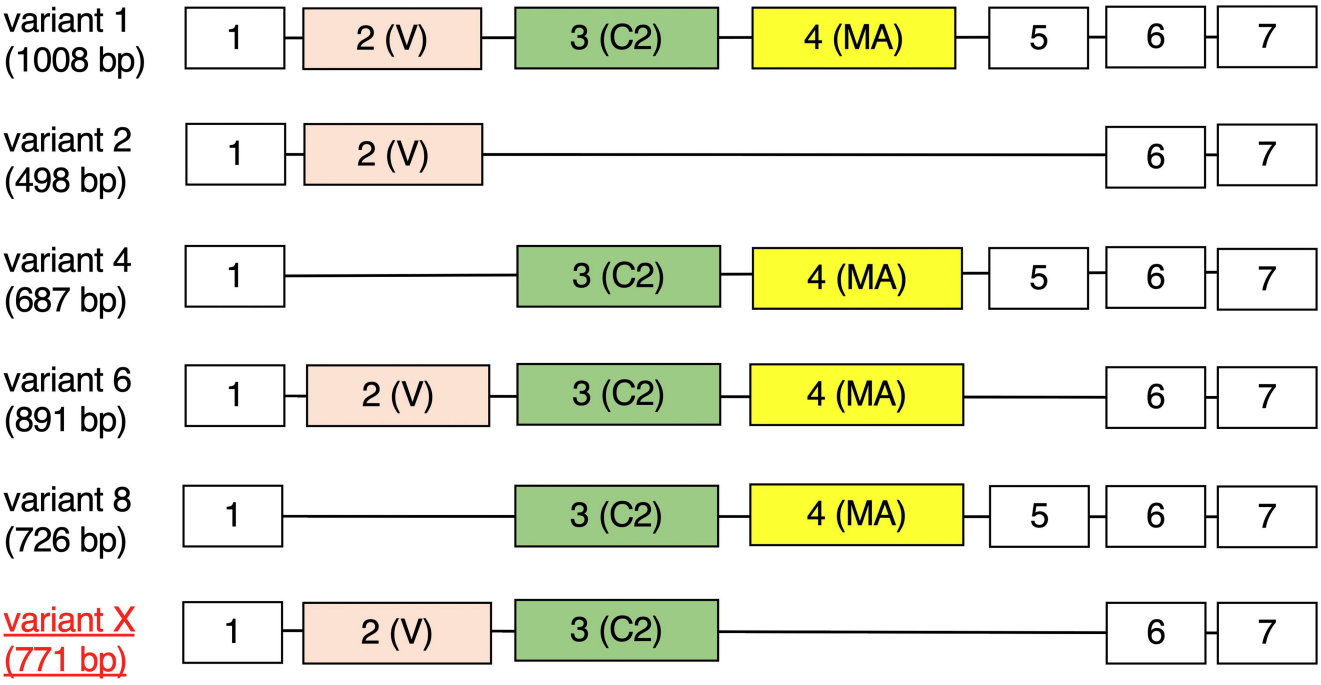

variant 1 (1008 bp)
1
2 (V)
3 (C2)
4 (MA)
5
6
7
variant 2 (498 bp)
variant 4 (687 bp)
variant 6 (891 bp)
variant 8 (726 bp)
variant X (771 bp)

Figure 2

A

Exon 1 | Exon 2

```
Atggctggttccccaacatgcctcaccctcatctatatcctttggcagctcacagggtca   60
 M  A  G  S  P  T  C  L  T  L  I  Y  I  L  W  Q  L  T  G  S    20

gcagcctctggacccgtgaaagagctggtcggttccgttggtggggccgtgactttcccc  120
 A  A  S  G  P  V  K  E  L  V  G  S  V  G  G  A  V  T  F  P    40

ctgaagtccaaagtaaagcaagttgactctattgtctggaccttcaacacaacccctctt  180
 L  K  S  K  V  K  Q  V  D  S  I  V  W  T  F  N  T  T  P  L    60

gtcAccatacagccagaaggggcactatcatagtgacccaaaatcgtaatagggagaga   240
 V  T  I  Q  P  E  G  G  T  I  I  V  T  Q  N  R  N  R  E  R    80

gtagacttcccagatggaggctactccctgaagctcagcaaactgaagaagaatgactca  300
 V  D  F  P  D  G  G  Y  S  L  K  L  S  K  L  K  K  N  D  S   100

gggatctactatgtggggatatacagctcatcactccagcagccctccacccaggagtac  360
 G  I  Y  Y  V  G  I  Y  S  S  S  L  Q  Q  P  S  T  Q  E  Y   120
```

Exon 3

```
gtgctgcatgtctacgagcacctgtcaaagcctaaagtcaccatgggtctgcagagcaat  420
 V  L  H  V  Y  E  H  L  S  K  P  K  V  T  M  G  L  Q  S  N   140

aagaatggcacctgtgtgaccaatctgacatgctgcatggaacatggggaagaggatgtg  480
 K  N  G  T  C  V  T  N  L  T  C  C  M  E  H  G  E  E  D  V   160

atttatacctggaaggccctggggcaagcagccaatgagtcccataatgggtccatcctc  540
 I  Y  T  W  K  A  L  G  Q  A  A  N  E  S  H  N  G  S  I  L   180

cccatctcctggagatggggagaaagtgatatgaccttcatctgcgttgccaggaaccct  600
 P  I  S  W  R  W  G  E  S  D  M  T  F  I  C  V  A  R  N  P   200

gtcagcagaaacttctcaagccccatccttgccagg aagctctgtgaagagaacaatcct  660
 V  S  R  N  F  S  S  P  I  L  A  R  K  L  C  E  E  N  N  P   220
```

Exon 6

Forward primer

Exon 7

```
aaaggaagatccagcaaatacggtttactccactgtggaaataccgaaaaagatggaaaa  720
 K  G  R  S  S  K  Y  G  L  L  H  C  G  N  T  E  K  D  G  K   240

tccccactcactgctcacgatgccagacacaccaaggctatttgcctaTGAcagcagtgc  780
 S  P  L  T  A  H  D  A  R  H  T  K  A  I  C  L               256
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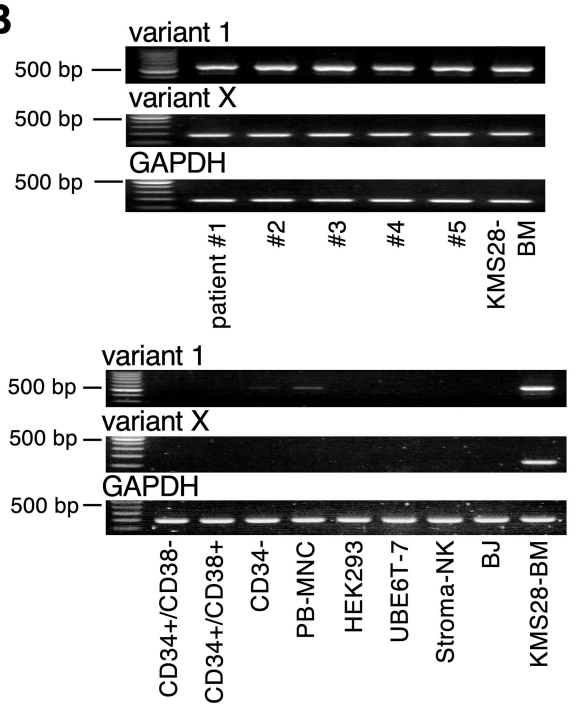
Reverse Primer

B



C

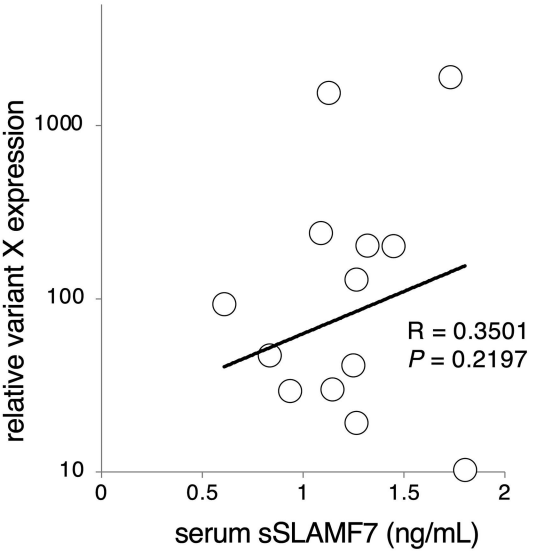


Figure 3

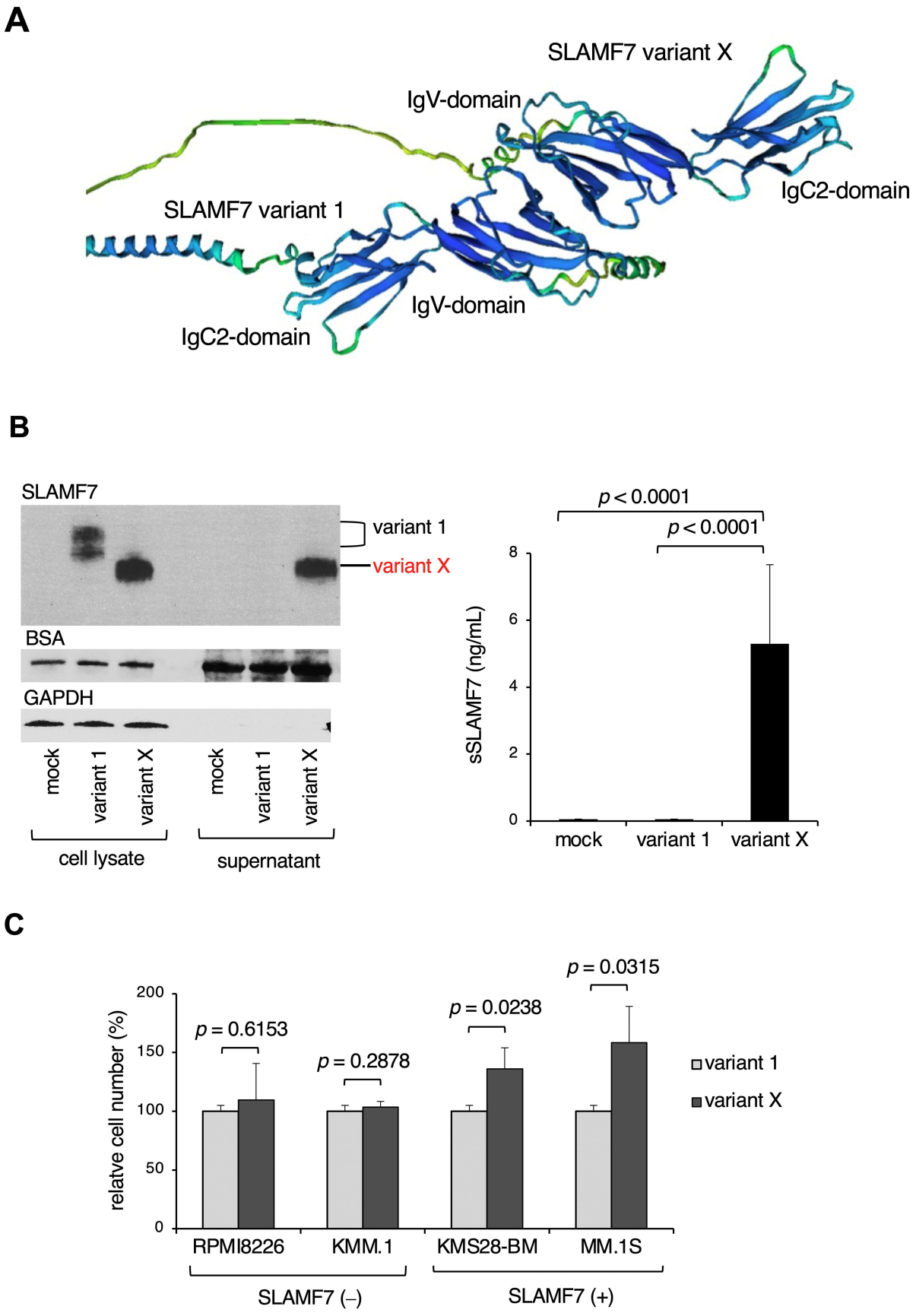
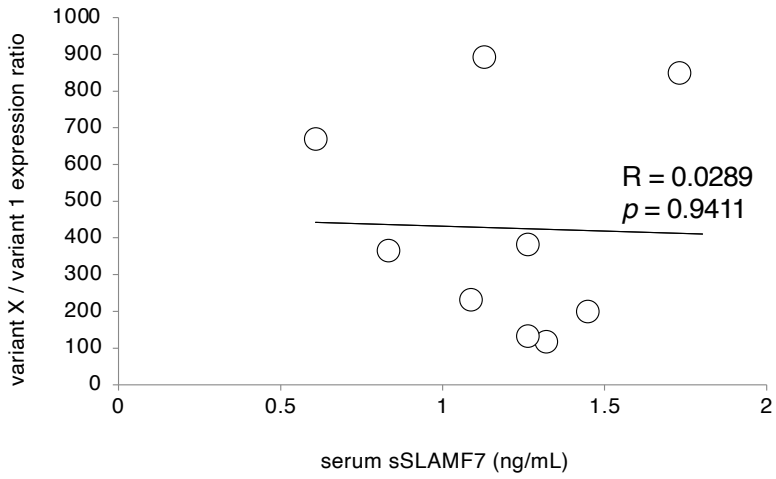

A
SLAMF7 variant X
IgV-domain
IgC2-domain
SLAMF7 variant 1
IgV-domain
IgC2-domain
B
SLAMF7
variant 1
variant X
BSA
GAPDH
mock
variant 1
variant X
mock
variant 1
variant X
cell lysate
supernatant
p < 0.0001
p < 0.0001
sSLAMF7 (ng/mL)
8
6
4
2
0
mock
variant 1
variant X
C
p = 0.0315
p = 0.0238
p = 0.6153
p = 0.2878
relative cell number (%)
200
150
100
50
0
variant 1
variant X
RPMI8226
KMM.1
KMS28-BM
MM.1S
SLAMF7 (–)
SLAMF7 (+)

Figure S1

A


variant X / variant 1 expression ratio
1000
900
800
700
600
500
400
300
200
100
0
0
0.5
1
1.5
2
serum sSLAMF7 (ng/mL)
R = 0.0289
p = 0.9411

B

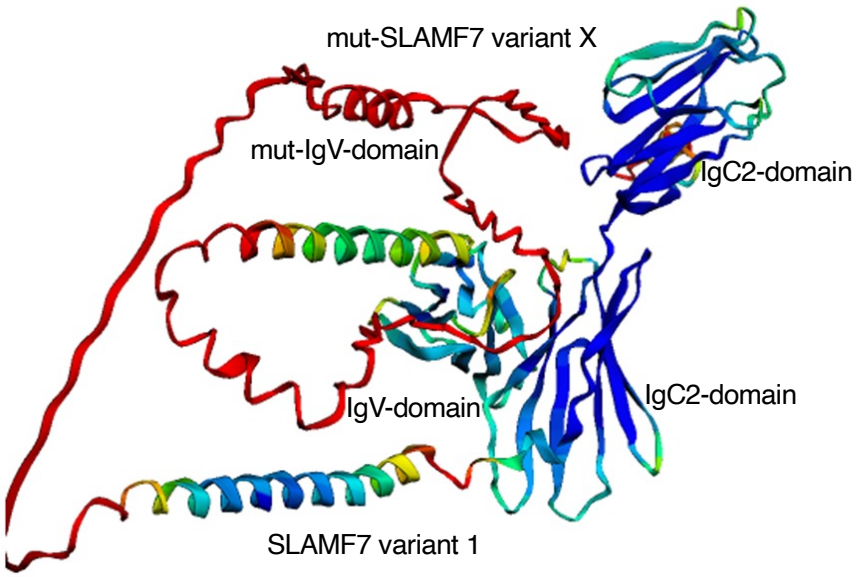
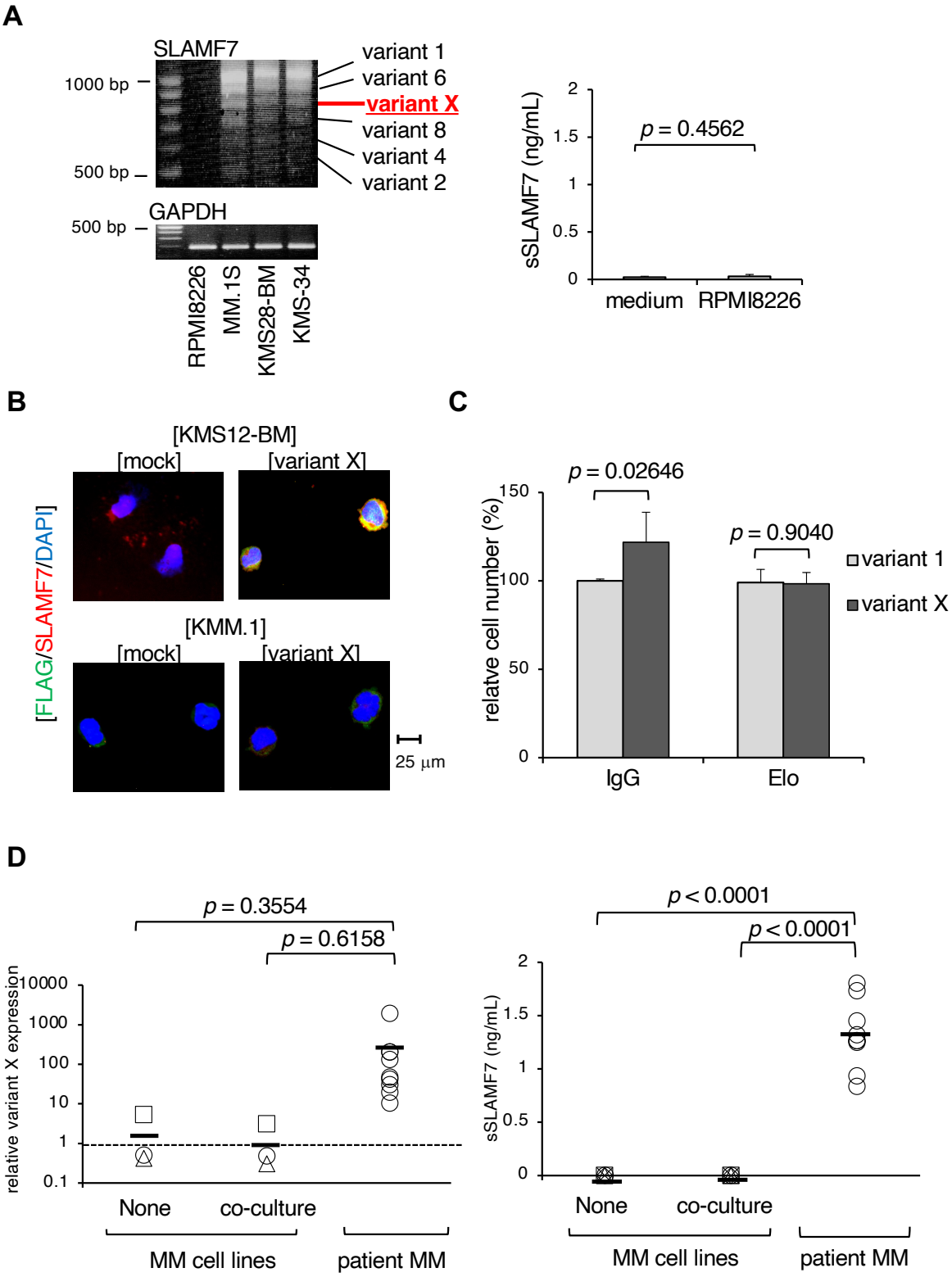

mut-SLAMF7 variant X
mut-IgV-domain
IgC2-domain
IgV-domain
IgC2-domain
SLAMF7 variant 1

Figure S1. (a) The expression levels of *SLAMF7* variant 1 in 10 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 the enzyme-linked immunosorbent assay (ELISA). The correlation between *SLAMF7* variant X/variant 1 expression ratio and sSLAMF7 was evaluated by calculating Pearson's correlation coefficient. (b) Structure-based prediction of SLAMF7 variant 1-mutant SLAMF7 variant X (mut-SLAMF7 variant X) interactions was performed using the AlphaFold2 program. The mutant SLAMF7 variant X was designed by randomly rearranging the amino acid sequence of the IgV domain.

Figure S2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1000 bp
500 bp
variant 1
variant 6
variant X
variant 8
variant 4
variant 2
GAPDH
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RPMI8226
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KMS28-BM
KMS-34
sSLAMF7 (ng/mL)
p = 0.4562
medium
RPMI8226
B
[KMS12-BM]
[mock]
[variant X]
[KMM.1]
[mock]
[variant X]
[FLAG/SLAMF7/DAPI]
25 μm
C
p = 0.02646
p = 0.9040
relative cell number (%)
variant 1
variant X
IgG
Elo
D
p = 0.3554
p = 0.6158
relative variant X expression
None
co-culture
MM cell lines
patient MM
p < 0.0001
p < 0.0001
sSLAMF7 (ng/mL)
None
co-culture
MM cell lines
patient MM

Figure S2. (a) Left panel: The full length *SLAMF7* or *GAPDH* (internal control) was amplified from the cDNA of RPMI8226, MM.1S, KMS28-BM, and KMS-34 cells. PCR was conducted for 40 cycles and the products were analyzed using agarose gel electrophoresis and ethidium bromide staining. Right panel: The supernatants were prepared from $1x10^5$ cells of RPMI8226 cells and the concentrations of sSLAMF7 were measured using the enzyme-linked immunosorbent assay (ELISA) (right panel). Bars indicate the means of three independent experiments. *P* value was calculated using a paired Student's *t* test. (b) Cells were mounted onto glass slides using a Cytospin device (Shandon Scientific, Cheshire, England). Immunostaining was performed using PE-conjugated anti-SLAMF7 (Thermo Fisher Scientific) and Alexa Fluor 488-conjugated anti-FLAG (MBL, Tokyo, Japan) antibodies at 1:200 dilution. The nuclei were counterstained with DAPI. Only merged images are shown. Data shown are representative of multiple independent experiments. (c) MM.1S cells were cultured with 5ug/mL of human IgG (IgG) or elotuzumab (Elo) in the supernatant derived from 1 x 10^5 RPMI8226 cells transduced with *SLAMF7* variant 1 or *SLAMF7* variant X after 24 h of culture. Cell proliferation was assessed after 72 h using the MTT reduction assay and is shown relative to the variant 1 group. Bars indicate the mean of three independent experiments. *P* value was calculated using a paired Student's *t* test. (d) Left panel: KMS12-BM, KMS26, and MM.1S cells were cultured with or without UBE6T-7 cells for 24 hours, respectively. The expression levels of *SLAMF7* variant X in KMS12-BM (△), KMS26 (◇), and MM.1S (□), and patient MM cells (○) 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. Right panel: The supernatants were prepared from $1x10^5$ cells of KMS12-BM (△), KMS26 (◇), and MM.1S (□) cells co-cultured with or without UBE6T-7 cells for 24 hours, respectively, and the concentrations of sSLAMF7 were measured using ELISA. Serum levels of sSLAMF7 in MM patients (○) were also measured using the ELISA. *P* value was determined by one-way ANOVA with Tukey's multiple comparison test.

Supplementary Table S1. PCR primers used in this study

gene	sequence (location)	product size
SLAMF7 variant 1 (full length)	forward: 5'- agggaagtggcttcatttcagtg -3'(exon 1) reverse: 5'- tctcataggcaaatagccttggt -3' (exon 7)	1100 bp
SLAMF7 variant 1	forward: 5'- acagctcatcactccagcagcc-3'(exon 2) reverse: 5'- agtgtgagggattgtgtcgtac-3' (exon 5)	547 bp
SLAMF7 variant X	forward: 5'- aagctctgtgaagagaacaatcc -3' (exon 3/6) reverse: 5'- gcagagacttaggggagtgc -3' (exon 7)	162 bp
GAPDH	forward: 5'-gagtcaacggatttggtcgt-3' reverse: 5'-gacaagcttcccgttctcag-3'	185 bp