SORT1/LAMP2-mediated Extracellular Vesicle Secretion and Cell Adhesion Are Linked to Lenalidomide Resistance in Multiple Myeloma

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Abstract:
Multiple myeloma (MM) is a hematopoietic malignancy whose prognosis has improved with the development of new agents such as lenalidomide over the last decade. However, long-term exposure to drugs induces the acquisition of resistance by MM cells and leads to treatment failure and poor prognosis. Here, we show the molecular and cellular mechanisms of lenalidomide resistance in MM. In a comparison between lenalidomide-resistant cell lines and the parental cell lines, the EV (Extracellular vesicles) secretion and adherence abilities were significantly elevated in the resistant cells. Whole-transcriptome analysis revealed that the SORT1 and LAMP2 genes were key regulators of EV secretion. Silencing of these genes caused decreased EV secretion and loss of cell adhesion in the resistant cells, resulting in increased sensitivity to lenalidomide. Analysis of publicly available transcriptome data confirmed the relationship between genes related to EV secretion and cell adhesion and patient prognosis. Together, our findings reveal a novel mechanism of lenalidomide resistance in MM mediated by EV secretion and cell adhesion via SORT1 and LAMP2.

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SORT1/LAMP2-mediated Extracellular Vesicle Secretion and Cell Adhesion Are Linked to Lenalidomide Resistance in Multiple Myeloma

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Key Points

- High expression level of SORT1/LAMP2 in lenalidomide resistant cells enhanced extracellular vesicle secretion and cell adhesion ability.
- Silencing the SORT1/ LAMP2 in lenalidomide resistant cells canceled cell adhesion and restored drug sensitivity.

Abstract

Multiple myeloma (MM) is a hematopoietic malignancy whose prognosis has improved with the development of new agents such as lenalidomide over the last decade. However, long-term exposure to drugs induces the acquisition of resistance by MM cells and leads to treatment failure and poor prognosis. Here, we show the molecular and cellular mechanisms of lenalidomide resistance in MM. In a comparison between lenalidomide-resistant cell lines and the parental cell lines, the EV (Extracellular versicles) secretion and adherence abilities were significantly elevated in the resistant cells. Whole-transcriptome analysis revealed that the SORT1 and LAMP2 genes were key regulators of EV secretion. Silencing of these genes caused decreased EV secretion and loss of cell adhesion in the resistant cells, resulting in increased sensitivity to lenalidomide. Analysis of publicly available transcriptome data confirmed the relationship between genes related to EV secretion and cell adhesion and patient prognosis. Together, our findings reveal a novel mechanism of lenalidomide resistance in MM mediated by EV secretion and cell adhesion via SORT1 and LAMP2.
Introduction

Multiple myeloma (MM) is a hematopoietic malignancy characterized by clonal proliferation of malignant plasma cells. MM develops as a result of the evolutionary process in which normal plasma cells transition through the premalignant stage to smoldering myeloma and myeloma that requires treatment (1). Although the understanding of the molecular drivers underlying the onset and progression of the disease has advanced, the course of clinical disease is highly heterogeneous due to the diversity in molecular mutations (2-5). In recent years, the prognosis of MM has been improved by the development of therapeutic drugs such as immunomodulatory drugs (IMiDs), proteasome inhibitors and antibody drugs; however, most patients eventually experience relapse and often exhibit multidrug resistance because of long-term exposure to these drugs. In particular, IMiDs have been used for long-term maintenance therapy after autologous bone marrow transplantation. Mechanistically, IMiDs directly bind cereblon (CRBN), which is their main target, leading to degradation of Ikaros (IKZF1) and Aioros (IKZF3), transcription factors that are essential for MM cell survival. Mutations in these genes have historically been thought to be the main cause of drug resistance (6); however, the mechanisms underlying the acquisition of resistance to IMiDs are not fully understood. For example, Dimopoulos K et al. showed that MM cells acquired resistance to ImiDs in a CRBN-independent manner (7). In MM, cell adhesion is also considered to be important for drug resistance (8-10). Kobune M et al. showed that the ability of MM cells to adhere to bone marrow stromal cells in an autocrine manner is
enhanced, which affects drug sensitivity (9). The existence of other drug resistance mechanisms independent of CRBN is suspected. Therefore, there is still an urgent need to define the molecular mechanisms of resistance to IMiDs.

It has been suggested that clones with drug resistance transmit information to other clones or cells in the bone marrow microenvironment, resulting in a change in the bone marrow microenvironment favorable for MM cell survival (11-13). Extracellular vesicles (EVs) have attracted attention as novel tools for cell-to-cell communication (14-16). EVs also play important roles in MM progression, by educating bone marrow surrounding cells for their favorable tumor microenvironment. (17-20). For instance, fibroblasts in BM uptake MM cell-derived EVs containing miR-27b-3p and miR-214-3p and then obtain proliferative capacity and resistance in apoptosis (17). In contrast, bone marrow stromal cells (BMSCs) also secrete EVs for interactions with MM cells (21-22). In addition, MM cell-derived EVs are known to be involved in bortezomib resistance (23-27); however, IMiDs resistance via EVs are not reported. Thus, a better understanding of EV-mediated intercellular communication could be helpful for elucidating MM pathophysiology. In this study, we investigated the contributions of EVs derived from resistant MM cells-derived to IMiDs resistance via enhancement of cell adhesion ability.

**Material and Methods**

**Cell culture**

The multiple myeloma cell lines KMS21, KMS27 and KMS34 were kindly
Lenalidomide-resistant cell lines, KMS21R, KMS27R, and KMS34R, were established by Hattori Y and have been exposed low dose lenalidomide for nearly one year (manuscript submitted). Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco), and cells were maintained in an atmosphere of 95% air and 5% CO₂. MM cell lines are also cultured on fibronectin (FN)-coating glasses (GG-18-FIBRONECTIN, Neuvitro corporation) or co-cultured with bone marrow stromal cells (PCS-500-012, ATCC). Also, we prepared FN-coated plates (10838039001, Roche). Coating plates were made according to the manufacturer’s instructions. Bone marrow stromal cells (BMSCs) were cultured with MesenPro medium (12746012, gibco) supplemented with 1% antibiotic-antimycotic (Gibco).

Reagents
The following antibodies were used for the ExoScreen assay: mouse monoclonal anti-human CD9 (clone 12A12, Cosmo Bio) and CD63 (clone 8A12, Cosmo Bio). These antibodies were used to modify either acceptor beads or biotin following the manufacturer’s protocol. The negative control siRNA (SI03650318) was purchased from Allstar, and the TSG101 siRNA (SI02655184) was purchased from Qiagen. The SORT1 and LAMP2 siRNAs (siGENOME SMART pool siRNA M-010620 and M-011715, respectively) were purchased from Dharnacon. Lenalidomide was purchased from Selleck.
**EV isolation**

Cancer cells were seeded in serum-free advanced RPMI 1640 medium supplemented with 2 mM L-glutamine and 1% antibiotic-antimycotic (Gibco). After incubation for 48 hours, conditioned medium was filtered through a 0.22 µm filter (Millipore) and centrifuged at 2,000 × g for 10 min to remove cell debris. For EV preparation, conditioned medium was ultracentrifuged at 110,000 × g for 70 min at 4 °C. The EV protein concentration was measured by using a Micro BCA protein assay kit (Thermo Scientific). This study was conducted according to MISEV2018 guidelines (28).

**Western blotting**

The following antibodies were used as primary antibodies: mouse monoclonal anti-human CD9 (clone 12A12, dilution 1:1000) and CD63 (clone 8A12, dilution 1:1000), from Cosmo Bio. Anti-SORT1 (Abcam, ab16640, dilution 1:1000), anti-LAMP2 (Sigma-Aldrich, HPA029100, dilution 1:1000), STAT-3 (CST, 9139s, dilution 1:1000), p-STAT3 (Y705) (CST, 9145s, dilution 1:1000), VLA-4 (Proteintech, 67040-1, dilution 1:1000), and Cereblon (Novus Biologicals, NBP1-91810, dilution 1:1000) were also purchased. Secondary antibodies (horseradish peroxidase-conjugated anti-mouse IgG, NA931; horseradish peroxidase-conjugated anti-rabbit IgG, NA934; both dilutions 1:5000) were purchased from GE Healthcare. Mini-PROTEAN TGX gels (4-20%, Bio-Rad) were used, and the same amount of protein was loaded in each lane. Bound antibodies were visualized by chemiluminescence using an ImmunoStar LD kit (Wako, Japan), and luminescence images were analyzed in a LuminoImager.
ExoScreen assay system

In brief, cancer cells \((2 \times 10^4)\) were seeded in 96-well plates in serum-free advanced RPMI 1640 medium supplemented with 1% glutamine and 1% antibiotic-antimycotic (Gibco). After 48 hours of incubation, an ExoScreen assay was performed. The plate was read in an EnSpire Alpha 2300 Multilabel Plate reader using an excitation wavelength of 680 nm and emission wavelength of 615 nm. Background signals obtained from filtered advanced RPMI medium or PBS were subtracted from the measured signals (29).

Cell proliferation assay

Cell viability was determined using a Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions, and the absorbance at 450 nm was measured using an EnVision Multilabel Plate Reader (PerkinElmer) and a SpectraMax iD3 plate reader (Molecular Devices). \(5 \times 10^3\) MM cells were seeded into 96-well plates and added lenalidomide each concentration. 72 hours later, the signal was measured.

Transwell assay

We used a coculture system with 0.4-μm pore size transwell membranes (Corning, 353095). Resistant cells were seeded into upper chamber, on the other hand, sensitive cells were seeded into lower chamber. The cells were co-cultured for 1 week. One week later, the cells were washed to except
non-adherent cells. The number of adherent cells and non-adherent cells were counted.

**The procedure of the experiments with ultralow-binding plates**

2 x 10^4 Lenalidomide resistant cells were seeded into normal 96-well plates (Thermo, 167008) or ultralow-binding 96-well plates (Corning, 3474). The cells were incubated for 96 hours with lenalidomide. After incubation, cellular proliferation was measured by cell viability assay.

**Caspase activity assay**

Caspase activity was determined using Caspase-Glo® 3/7 Assay System (Promega), according to the manufacturer’s instructions, and the luminescence was measured using an EnVision Multilabel Plate Reader (PerkinElmer) and a SpectraMax iD3 plate reader (Molecular Devices).

**siRNA transfection**

Neon System from Thermo Fisher was used for siRNA transfection. Optimization followed the manufacturer’s instructions. Briefly, 1 x 10^6 cells were resuspended in E buffer with each siRNA, then pulsed and seeded in culture dishes.

**Nanoparticle tracking analysis**

EVs were resuspended in PBS and further diluted for analysis in a NanoSight LM10-HS system according to the manufacturer’s protocol.
RNA Extraction

Total RNA was extracted from cultured cells using QIAzol reagent and a miRNeasy Mini Kit (Qiagen). RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher) and an Agilent bioanalyzer (Agilent Technologies).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cultured cells using QIAzol reagent and a miRNeasy Mini Kit (Qiagen). RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher). For qRT-PCR analysis, complementary DNA was reverse transcribed from total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The data were collected and analyzed using StepOne Software v2.3 (Applied Biosciences). All mRNA quantification data were normalized to the expression data for β-actin. TaqMan probes for SORT1 (Hs00361760_m1 SORT1), LAMP2 (Hs00174474_m1 LAMP2) and β-actin (Hs01060665_g1 ACTB) were purchased from Applied Biosystems. CRBN and β-actin primers were purchased from Eurofins (CRBN forward: TGTGTGCTTTCAACCATGT; reverse: AGCGAGGCCATGAAGTTAGA, β-actin forward: GGAGGAGCTGGAAGCAGCC; reverse: GCTGTGCTACGTCGCCCTG).

Knockdown of LAMP2, SORT1, and CRBN by using lentivirus

The short hairpin RNA (shRNA) targeting CRBN were purchased from Santa Cruz (control sh-RNA: sc-108060-SH; sh-LAMP2: sc-29390-SH; sh-SORT1:
sc-42119-SH). The plasmids were electroporated with the envelop plasmid and packaging plasmid by using Neon system.

**Reanalysis of available datasets in a public database**

scRNA-seq datasets deposited in the Gene Expression Omnibus (GEO) database (GSE106218 and GSE110499) (30) were used for expression analysis of the drug resistance signature genes. scRNA-seq analysis was performed with ‘Seurat’ in R version 3.6.1. (31). Log normalization was performed with the function ‘NormalizeData’ in Seurat. Samples were log normalized and scaled by the number of genes and percentage of mitochondrial reads. We performed principal component analysis (PCA) for dimensionality reduction and single-cell clustering with Seurat. The dimensionality-reduced cell clustering data are shown as a UMAP plot generated by the function ‘runUMAP’ in Seurat. Module analysis was performed with the function ‘AddModuleScore’ using the PC1/PC2 signature genes (Supplemental Table: Gene list of modules).

RNA-seq datasets of multiple myeloma cell lines were downloaded from the Cancer Cell Line Encyclopedia (CCLE, https://portals.broadinstitute.org/ccle). Expression data for SORT1 and LAMP2 were extracted from the RNA-seq data.

Clinical microarray datasets of MM patients were downloaded from the GEO database (GSE19784 and GSE136324) (32-33). Microarray analysis and RMA normalization were performed using ‘affy’ package. We subjected the clinical datasets to survival analysis which was executed by the ‘ggplot2’, “survminer,” and “survival” packages with R version 3.6.1., described previously as (34-35).
Data availability

RNA-seq data of Lenalidomide resistance MM cell lines generated for this study are included within this article and in the supporting information. GEO accession number in our RNA-seq is GSE165557.

Code availability

The source code used for scRNA-seq analysis is available on GitHub (https://github.com/JunNakayama/MM-project).

Statistical analysis

The data presented in the bar graphs are the mean ± s.d. of at least three independent experiments. Statistical analyses between two groups were performed by Student’s t test. p<0.05 was considered to be statistically significant. For multiple comparisons, the significance of differences in the average values was analyzed using one-way ANOVA with Dunnett’s post hoc test. The log-rank test in survival analysis was performed using ‘survminer’ package in R version 3.6.1.

Results

Increased EV secretion in lenalidomide-resistant MM cells

We established 3 different lines of lenalidomide-resistant MM cells, KMS21R, KMS27R and KMS34R, via long-term exposure to lenalidomide (Figure 1A;
Supplemental Figure 1A, and Methods). KMS21R was derived from KMS21, a highly sensitive MM cell line, and the IC50 of lenalidomide in KMS21R cells was approximately 400-fold greater than that in the parental cell line KMS21 (Figure 1A-B, left panel). KMS27R was derived from KMS27, a cell line with moderate sensitivity to lenalidomide (Figure 1A, B, right panel). KMS34, the parental cell line of KMS34R, was originally resistant to lenalidomide, and KMS34R cells also exhibited high resistance to lenalidomide. Interestingly, the resistant cells have an ability to attach onto non-coating culture dishes (Supplemental Figure 1A-C).

We also tried to culture MM cell lines on FN-coated plates (Supplemental Figure 1D) or in co-culture with bone marrow stromal cells (Supplemental Figure 1E), which are physiologically more similar to a bone marrow environment. In both conditions, not only the resistant cells but part of the parental cell lines also attached to the culture dishes. The number of adherent cells was counted (Supplementary Figure 1F).

As a first step to elucidate the molecular and cellular mechanisms of lenalidomide resistance in MM, we utilized KMS21, KMS21R, KMS27, and KMS27R cells because the parental cells clearly showed higher sensitivities to lenalidomide than the resistant cells, and the resistant cells had lower caspase activity than the parental cells (Figure 1B, C). As a similar trend was not observed for KMS34 and KMS34R (Supplemental Figure 1D, E), we excluded these cell lines from this experiment. Whole-transcriptome analysis showed differentially expressed genes, with 521 upregulated and 89 downregulated genes in the resistant cells (Figure 1D). Gene set enrichment analysis (GSEA) revealed that the gene sets of “Interferon (IFN)α response”, “IFNγ response”,...
“Mitotic spindle”, “Cell adhesion”, and “Snare interaction in vesicular transport” were significantly enriched in the resistant cells (Figure 1E; Supplemental Figure 2A). A heat map of the cell adhesion-related genes also showed high expression in the resistant cells (Supplemental Figure 2B and 2C). IFN signaling is well known to be related to MM progression (36). Cell adhesion is also considered to be one of the critical factors for drug resistance acquisition, and we observed that our resistant cell lines (KMS27R and KMS34R) attached tightly to culture dishes, while KMS21R cells exhibited weak attachment (Supplemental Figure 1A). Based on the GSEA results, we focused on the “Snare interaction in vesicular transport” gene set because this gene set is tightly associated with EV secretion, and EV secretion is well known to modulate drug sensitivity (37). As shown in Figure 1F, genes such as SNAP23 and VAMP3, which are important for EV secretion, were highly upregulated in the resistant cells. As expected, the ExoScreen assay and nanoparticle tracking analysis (NTA) showed that resistant cells secreted a greater number of EVs (Figure 1G, H; Supplemental Figure 3A). Expression of the EV markers CD9 and CD63 was confirmed by western blotting (Supplemental Figure 3B). Consistent with previous reports (38), the size of EVs was decreased (Figure 1I).

**EVs derived from lenalidomide-resistant MM cells influence lenalidomide resistance and cell adhesion in sensitive cell lines**

We next examined the effect of EVs secreted by lenalidomide-resistant MM cells on the parental MM cells. For this purpose, we used a coculture system with 0.4-μm pore size transwell membranes (Figure 2A). In this system, cells in the
upper chamber could not migrate into the lower chamber; only soluble proteins and EVs could move down to the lower chamber. The most resistant cell line, KMS34R, was seeded in the upper chamber, and the sensitive cell lines KMS21 and KMS27 were seeded in the lower chamber. After 1 week of coculture, the number of surviving cells in the presence of lenalidomide was determined. As shown in Figure 2B and Supplemental Figure 4A, B, when co-cultured with KMS34R, the number of KMS21 and KMS27 cells in the presence of lenalidomide were slightly increased. In contrast, KMS34 cells cultured with KMS34R cells did not show a clear change in drug sensitivity because KMS34 cells were initially resistant to lenalidomide (Figure 2B, right panel). Seeding the KMS21 and KMS27 cells in FN-coated also clearly increase the drug resistance when their resistant cells co-cultured. Unexpectedly, we found that the sensitive cells cocultured with resistant cells became adherent to the culture dishes (Figure 2C and Supplementary Figure 4C). We used EV-depleted conditioned medium (CM) as a control. The treatment of the EV-depleted CM did not influence cell adhesion (Supplementary Figure 4D). Thus, to further investigate the cell adhesion-related function of EVs derived from lenalidomide-resistant MM cells, we cocultured KMS21 and KMS27, with resistant cell lines (KMS21R, KMS27R and KMS34R) and quantified the numbers of adherent and non-adherent KMS21 cells (Figure 2D). Coculture with the resistant cell lines markedly increased the number of adherent cells (Figure 2D), suggesting that EVs might contribute to cell adhesion in MM.

Because a relationship between cell adhesion and drug resistance has been reported (9,10), we then examined the effect of cell adhesion on
lenalidomide resistance in MM cells (Figure 2E). Comparative analysis of lenalidomide sensitivity between normal and ultra-low attachment conditions revealed that KMS27R and KMS34R cells exhibited a significant decrease in viability in the presence of lenalidomide under the ultra-low attachment condition, suggesting that the cells became sensitive to lenalidomide under the nonadherent condition (Figure 2F, middle and right panels). In contrast, the sensitivity of KMS21R cells, which were very weakly attached, did not change under ultra-low attachment condition (Figure 2F, left panel).

Our transcriptome profiling of all cell lines revealed that lenalidomide-targeted pathways such as those mediated by IKZF1 and IKZF3 were significantly inhibited in resistant cell lines, and this inhibition was maintained under lenalidomide exposure (Supplemental Figure 5). Thus, in line with previous reports, our findings suggest that resistant MM cell lines acquire resistance to lenalidomide in a CRBN-independent manner, presumably related to EV secretion.

**SORT1 and LAMP2 regulated lenalidomide sensitivity and EV secretion in MM cells**

To identify key molecules responsible for lenalidomide resistance, EV secretion and cell adhesion, we compared transcriptome data between 4 resistant cell lines (KMS21R, KMS27R, KMS34 and KMS34R) and 2 sensitive cell lines (KMS21 and KMS27). As shown in Figure 3A, B, we narrowed down the dataset to 118 genes by fold change and p value (Figure 3C) and then selected 13 genes by a literature search. An siRNA targeting each of these 13 genes was
electroporated into KMS27R cells (Figure 3B), and after 72 hours, cell viability and caspase activity were evaluated in cells treated with lenalidomide at a concentration of 100 μm (Figure 3D, E). After this screen eight genes (SORT1, LAMP2, USP25, RAD51AP1, PRTFDC1, AASS, CASP7, and CLSTN3) were further analyzed to quantify EV secretion by the ExoScreen assay (Figure 3F). According to this screen, we focused on SORT1 and LAMP2 because their expression levels were low in sensitive cell lines (Figure 3C), and their silencing increased caspase activity under the lenalidomide exposure condition (Figure 3E) and decreased EV secretion (Figure 3F). The increased protein levels of SORT1 and LAMP2 in the resistant cell lines were confirmed by western blotting (Figure 3G, H). SORT1 protein levels were obviously increased in KMS21R and KMS27R cells compared with KMS21 and KMS27 cells, respectively, although we found a slight increase even in KMS34R cells in comparison with KMS34 cells (Figure 3H, right panel). In addition, previous studies have already shown that acquired lenalidomide resistant cells is associated with high expression of p-STAT3 or VLA-4. Although the protein level of STAT3 and p-STAT3 were up-regulated in the resistant cells, the protein level of VLA-4 is not changed (Figure 3G). We investigated the correlation between CRBN and LAMP2, SORT1 in both public database and data set of our cell lines (Supplementary Figure 6A and 6B). Furthermore, we established CRBN knockdown KMS27R cells (Supplementary Figure 6C and 6D). The protein level of CRBN was approximately 50% reduced; however, cell adhesion ability was not changed (Supplementary Figure 6E).
SORT1 and LAMP2 also influenced cell adhesion and lenalidomide sensitivity in MM cells

Since the relationship between EV secretion and cell adhesion was observed in MM cells (Figure 2), we examined whether the silencing of either SORT1 or LAMP2 also affects cell adhesion. To this end, we established stable knockdown of either SORT1 or LAMP2 by lentivirus-delivered shRNA in resistant MM cells (KMS21R, KMS27R and KMS34R) (Supplemental Figure 7A). The knockdown efficiency of SORT1 and LAMP2 was confirmed by western blotting (Figure 4A). Inhibition of EV secretion was verified by the ExoScreen assay in the silenced resistant cell lines (Figure 4B). Then, because KMS27R cells with silencing of SORT1 and LAMP2 exhibited marked phenotypic changes (Figure 4A-B), we used these cells and sought to determine whether attenuating SORT1 and LAMP2 gene expression influences cell adhesion (Figure 4C). When either SORT1 or LAMP2 was knocked down, we observed that most of the cells were detached from the culture dishes and the number of attached cells was significantly reduced (Figure 4D, E), while cell viability, including that of both attached and floating cells, was not changed in the absence of lenalidomide (Figure 4F). The same phenotype was observed when in the FN-coated plates (Figure 4G). This finding indicated that SORT1 and LAMP2 regulate cell adhesion in MM cells in a direct or an indirect manner. Finally, we investigated whether knockdown of these genes affects lenalidomide sensitivity. After knockdown of the SORT1 and LAMP2 genes in KMS27R cells, lenalidomide sensitivity was restored to a level comparable to that of the parental cell line KMS27 (Figure 4H top; normal plate, bottom; FN-coated plate). In addition,
we co-cultured KMS21 with KMS21R, sh-LAMP2 KMS21R, and sh-SORT1 KMS21R cells (Supplemental Figure 7B top; normal plate, bottom; FN-coated plate). As expected, KMS21 cells decreased the drug sensitivity when co-cultured with KMS21R, while the co-culture with sh-LAMP2 KMS21R and sh-SORT1 KMS21R cells did not. Furthermore, the knockdown of LAMP2 and SORT1 didn’t affect the CRBN expression (Supplemental Figure 7C, D).

When the sensitivity to lenalidomide was compared between normal and ultra-low attachment conditions, the effect of ultra-low attachment plate conditions was completely abolished in cells with silencing of SORT1 (Figure 4J), because SORT1 knockdown induced the detachment of KMS27R cells, and no significant differences in cell viability were observed between the normal and ultra-low attachment conditions (Supplemental Figure 7E). Collectively, these findings indicated that the SORT1 and LAMP2 genes were associated with lenalidomide sensitivity via cell adhesion and EV secretion. These secreted EVs may contribute to transferring drug resistance to bystander cells by conferring cell adhesion ability on MM cells.

**Contribution of EV secretion and cell adhesion-related genes to MM patient prognosis**

As a mutual interaction between the SORT1 and LAMP2 genes was reported (39), correlation analysis revealed a significant association between the expression levels of the SORT1 and LAMP2 genes in the cell lines used in this study (r = 0.98, p value; p < 0.001, Figure 5A). A similar trend was observed in MM cell lines from the Cancer Cell Line Encyclopedia (CCLE) database (Figure
The expression levels of SORT1 and LAMP2 were lower in the cell lines that had high sensitivity to lenalidomide, while the lenalidomide-resistant cell lines expressed higher levels of the SORT1 and LAMP2 genes (Figure 5A, B).

Next, we reanalyzed the single-cell transcriptome profiles of publicly available MM data sets (30) to examine whether EV secretion and cell adhesion are related to MM patient prognosis. The data set contained myeloma cells in bone marrow (BM) from 9 patients and in extramedullary sites (EM) from 4 patients (Supplemental Figure 8A). Thirteen patients were divided into two groups: 7 patients with a prognosis of less than two years and 6 patients with a prognosis of more than two years (Figure 5C). When we analyzed our bulk RNA-seq data of the 6 MM cell lines used in this study, the PCA plot revealed that principal component PC1 represented the difference in patients and that PC2 was considered to show the difference in patient state (Supplemental Figure 8B). The PC1 and PC2 signature genes are listed in Supplemental Figure 8C. We observed a group of PC1 and PC2 signature genes (Figure 5D, E) in the single-cell RNA-seq data of MM patients and found that a group of PC2 signature genes was significantly enriched in the patients with OS < 2 years (Figure 5E), while no significant difference was seen in a group of PC1 signature genes (Figure 5D). Because the PC2 signature included genes related to EV secretion and cell adhesion (Supplemental Figure 8C, bottom), this result highlighted that these genes are clinically associated with MM patient prognosis. In addition, LAMP2 was found to be significantly upregulated in the patients with OS < 2 years (Figure 5F), although the SORT1 expression level
merely tended to increase. In the PC2 signature, genes related to EV secretion, such as CAV1, CD9, TSPAN7, and AHNAK (Figure 5G), and cell adhesion, such as CD44 (Supplemental Figure 9), were also detected at high levels in the patients with OS < 2 years. Typical EV marker genes, CD63 and TSG101, which were not included in the PC2 signature, were also significantly elevated in the patients with OS < 2 years (Figure 5H). Furthermore, we reanalyzed the microarray dataset including about 300 MM patients (GSE19784) (32). The Kaplan-Meier analysis showed that high LAMP2 expression profile was significantly correlated with poor prognosis in both overall survival (OS) (Figure 6A) and progression free survival (PFS) (Figure 6B). In contrast, the patients with high SORT1 expression prolonged their OS and PFS (Figure 6A, B). Since the dataset used in GSE19784 did not include the patients who were treated with lenalidomide, we reanalyzed GSE136324, which contained more than 200 patients treated with lenalidomide (TT3b, TT4, TT5) (33). High SORT1 expression was significantly correlated with poor prognosis (Figure 6C). These suggested that the expression level of SORT1 might be directly related to lenalidomide resistance in the patients. Thus, analysis of this publicly available clinical data revealed that EV secretion and cell adhesion, which are likely mediated by SORT1 and LAMP2, are associated with patient prognosis in MM.

Discussion

Accumulating evidence has demonstrated that targeting EVs might be a new therapeutic strategy for cancer. Understanding the secretory mechanisms of
EVs in cancer cells may improve this strategy. However, the mechanism of EV biogenesis in cancer cells remains elusive because the secretory pathway is considered to be different depending on the cell of origin (15). In general, cancer cells secrete a greater number of EVs than normal cells (42). This characteristic indicates that inhibition of cancer cell-derived EVs will have a significant impact on the development of EV-targeted therapies. In this study, we identified novel EV secretion-related genes, SORT1 and LAMP2, which were highly expressed in lenalidomide-resistant cell lines and regulated EV secretion and cell adhesion in MM cell lines. Our findings indicated that EVs derived from lenalidomide-resistant MM cells partially conferred resistance on sensitive parental MM cell lines. Considering the effect of EVs, another important question is what kind of molecules in/on EVs are responsible for lenalidomide resistance in MM cells. For example, Safaei R et al. showed that ovarian cancer cells sorted lysosomal proteins abnormally and loaded cisplatin transporters into EVs, which could result in cisplatin efflux from cancer cells. Furthermore, since cargo transferred in EVs can induce a genetic change in the tumor microenvironment, drug-resistant cancer cells hijack this mechanism to confer resistance on sensitive cells (38, 43). In this way, EVs promote cancer cell survival through both autocrine and paracrine mechanisms that transform drug-sensitive cells into resistant cells. Although SORT1 and LAMP2 were identified to positively regulate EV secretion in MM, we did not focus on the cargo of EVs in resistant cells, which likely contained key factors for transferring drug resistance to sensitive cells. Thus, identification of the key molecules for EV-mediated drug resistance is important (44-45) and is the next challenge.
We also observed morphological changes in KMS27R and KMS34R cells from a round to a spindle shape. In MM, cell adhesion is important for cell survival (46-48). Cell adhesion is also considered to contribute to the efficient transfer of EVs to other cells in the bone marrow microenvironment. Kohmo S et al. showed that lung cancer cells expressed high levels of CD9, and increased cell adhesion capacity led to increased drug resistance (49). High level of CD9 was observed in our resistant cell-derived EVs and aggressive state of clinical samples. These results indicated that resistant cell-derived EVs were transferred to recipient drug sensitive cells, and then led to the CD9 expression onto the surface of lenalidomide sensitive cell membrane. Further investigations, such as animal studies and experiments in sophisticated coculture systems with BM stromal cells and immune cells, are required. Because the changes observed in vitro are considered unnatural, ideally, in vivo imaging of MM cells would provide better insight into how resistant cells emerge in MM.

SORT1 was originally known to be associated with endosomes. However, as circulating endothelial cells in metastatic cancer patients have been reported to highly express the SORT1 gene (50), SORT1 might also be related to cell extravasation. In MM, cancer cells are normally confined to the bone marrow; however, in some cases, MM cells are observed outside of the bone marrow. This condition is called extramedullary disease (EMD) and is tightly associated with poor prognosis in MM (32); the mechanism of EMD development is not fully elucidated. Because our data indicated that SORT1 positively regulated cell adhesion as well as EV secretion, high expression of SORT1 might be correlated with the EMD phenotype. LAMP2 is a well-known
key protein in the chaperone-mediated autophagy pathway, and its expression was reported to be elevated in breast tumor tissues (51). Qadir F et al. showed the relationship between LAMP2 and EVs (52). In a neck squamous cell carcinoma cell line, cancer cell-derived EVs, but not normal cell-derived EVs, were shown to modulate the expression of LAMP2 (52). Babuta M et al. reported the relationship between autophagy and exosome biogenesis and found that knockdown of LAMP2 increased EV release from hepatocytes and macrophages (53). Although their finding is ostensibly contradictory to our results, autophagy and exosome biogenesis could be reciprocally connected.

There are two limitations in this study. One is that we failed to examine whether the effect of SORT1/LAMP2 is specific or suppression of EV suffices to restore lenalidomide sensitivity. It is due to a lack of the optimal experimental model to adequately inhibit EV secretion in MM cells. We have tried a nSMase2 inhibitor, GW4869, which is broadly used for EVs suppression. However, GW4869 treatment caused only about 20% of EVs suppression in MM cells, and was considered not suitable for fully evaluating the effect of EVs suppression on restoration of lenalidomide sensitivity. Another is precise molecular mechanism on the relationship among EVs, cell adhesion and drug sensitivity. Previous studies have already shown that acquired drug resistant MM cells are associated with high expression of VLA-4, STAT3, p-STAT3 or CD44 (54-56). Our data confirmed higher expression of drug resistance-related genes, such as STAT3, p-STAT3 in all resistant cell lines, and silencing of LAMP2 or SORT1 decreased STAT3 and p-STAT3. In other words, JAK/STAT pathway was activated by long-term exposure to lenalidomide. In order to elucidate EV-related
drug resistance, the JAK/STAT pathway could be one of the therapeutic targets and would be further investigated for precise molecular mechanisms.

In conclusion, our comparative analysis between resistant and sensitive MM cell lines followed by whole-transcriptome analysis identified novel EV secretion regulators, SORT1 and LAMP2, which also regulated cell adhesion as well as lenalidomide resistance. In addition, continuous exposure of the EVs secreted by resistant cell converts sensitive cells to drug-resistant cells. This is a novel mechanism of lenalidomide resistance in MM patients (Figure 7). Finally, considering clinical applications, suppressing EVs by inhibiting their biogenesis or blocking secreted EVs with specific antibodies may be feasible approaches to prevent the acquisition of drug resistance in MM. Also, SORT1 could be a novel prognostic biomarker in MM.

Data Sharing Statement
RNA-seq data of Lenalidomide resistance MM cell lines generated for this study are included within this article and in the supporting information. GEO accession number in our RNA-seq is GSE165557. The source code used for scRNA-seq analysis is available on GitHub (https://github.com/JunNakayama/MM-project).

Authorship
Fundamental research project was launched by TO and YH. TY and JN designed the experimental plan. TY and YY performed experiments. TY performed revision. YH established the lenalidomide-resistant cell lines. TY, YY and JN analyzed data. TY, MK and TO wrote the manuscript with contribution
from all co-authors.

None of the authors has a relevant conflict of interest.

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**References**

1. Charlotte Pawlyn and Faith E. Davies. Toward personalized treatment in multiple myeloma based on molecular characteristics. Blood. 133, 7 ,2019.

2. Sonneveld P, Avet-Loiseau H, Lonial S, et al. Treatment of multiple myeloma with high-risk cytogenetics: a consensus of the International Myeloma Working Group. Blood. 2016; 127(24):2955-2962.

3. Manier S, Salem KZ, Park J, Landau DA, Getz G, Ghobrial IM. Genomic complexity of multiple myeloma and its clinical implications. Nat Rev Cancer. 2012 12;12(5):335-48.

4. Lohr JG, Stojanov P, Carter SL, Cruz-Gordillo P, et al., Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. Cancer Cell. 2014 13;25(1):91-101.
5. Bolli N, Avet-Loiseau H, Wedge DC, Van Loo P, et al., Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. Nat Commun. 2014;5:2997.

6. Eichner R, Heider M, Fernández-Sáiz V, et al., Immunomodulatory drugs disrupt the cereblon-CD147-MCT1 axis to exert antitumor activity and teratogenicity. Nat Med. 2016;22(7):735-43.

7. Dimopoulos K, Søgaard Helbo A, Fibiger Munch-Petersen H, et al., Dual inhibition of DNMTs and EZH2 can overcome both intrinsic and acquired resistance of myeloma cells to IMiDs in a cereblon-independent manner. Mol Oncol. 2018;12(2):180-195.

8. Kikuchi J, Koyama D, Wada T, et al., Phosphorylation-mediated EZH2 inactivation promotes drug resistance in multiple myeloma. J Clin Invest. 2015 26;125(12):4375-90.

9. Kobune M, Chiba H, Kato J, et al., Wnt3/RhoA/ROCK signaling pathway is involved in adhesion-mediated drug resistance of multiple myeloma in an autocrine mechanism. Mol Cancer Ther. 2007;6(6):1774-84.

10. Noborio-Hatano K, Kikuchi J, Takatoku M, et al., Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. Oncogene. 2009 15;28(2):231-42.

11. Sonneveld P, Brojil A. Treatment of relapsed and refractory multiple myeloma. Haematologica. 2016;101(4):396-406.

12. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer. 2007;7(8):585-98.
13. Oksana Z, Nicholas JH, Tarek HM, et al., Single-cell RNA sequencing reveals compromised immune microenvironment in precursor stages of multiple myeloma. Nature Cancer. 1, 493-506, 2020.

14. Pluchino S, Smith JA. Explicating EVs: Reclassifying the Rising Stars of Intercellular Communication. Cell. 2019 4;177(2):225-227.

15. Denzer K, Kleijmeer M, Heijnen H, Stoorvogel W, Geuze H. EV: from internal vesicle of the multivesicular body to intercellular signaling device. Journal of cell science. 2000, 19, 3365-74.

16. Yanez-Mo M, Siljander P, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. Journal of extracellular vesicles. 2015, 4, 27066.

17. Frassanito MA, Desantis V, Di Marzo L, et al., Bone marrow fibroblasts overexpress miR-27b and miR-214 in step with multiple myeloma progression, dependent on tumour cell-derived exosomes. J Pathol. 2019 ;247(2):241-253.

18. Zheng Y, Tu C, Zhang J, et al., Inhibition of multiple myeloma-derived exosomes uptake suppresses the functional response in bone marrow stromal cell. Int J Oncol. 2019;54(3):1061-1070.

19. Purushothaman A, Bandari SK, Liu J, et al., Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions. J Biol Chem. 2016 22;291(4):1652-1663.

20. Wang J, De Veirman K, Faict S, et al., Multiple myeloma exosomes establish a favourable bone marrow microenvironment with enhanced angiogenesis and immunosuppression. J Pathol. 2016 ;239(2):162-73.

21. Roccaro AM, Sacco A, Maiso P, et al., BM mesenchymal stromal cell–
derived exosomes facilitate multiple myeloma progression. J Clin Invest. 2013 123(4):1542-55.

22. Wang J, Hendrix A, Hernot S, et al., Bone marrow stromal cell–derived exosomes as communicators in drug resistance in multiple myeloma cells. Blood. 2014, 24;124(4):555-66.

23. Faict S, Oudaert I, D’Auria L, et al., The Transfer of Sphingomyelinase Contributes to Drug Resistance in Multiple Myeloma. Cancers (Basel). 2019 20;11(12):1823.

24. Bandari SK, Purushothaman A, Ramani VC, et al., Chemotherapy induces secretion of exosomes loaded with heparanase that degrades extracellular matrix and impacts tumor and host cell behavior. Matrix Biol. 2018 ;65:104-118.

25. Tang JX, Chen Q, Li Q, et al., Exosomal mRNAs and IncRNAs involved in multiple myeloma resistance to bortezomib. Cell Biol Int. 2021 ;45(5):965-975.

26. Rodrigues-Junior DM, Pelarin MFA, Nader HB, et al., MicroRNA-1252-5p Associated with Extracellular Vesicles Enhances Bortezomib Sensitivity in Multiple Myeloma Cells by Targeting Heparanase. Onco Targets Ther. 2021 15;14:455-467.

27. Xu H, Han H, Song S, EV-Transmitted PSMA3 and PSMA3-AS1 Promote Proteasome Inhibitor Resistance in Multiple Myeloma. Clin Cancer Res. 2019 15;25(6):1923-1935.

28. Théry C, Witwer KW, Aikawa E, et al., Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014
guidelines. J Extracell Vesicles. 2018 23;7(1):1535750.

29. Yoshioka Y, Kosaka N, Konishi Y, et al., Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. Nat Commun. 2014 7;5:3591.

30. Ryu D, Kim SJ, Hong Y, et al., Alterations in the Transcriptional Programs of Myeloma Cells and the Microenvironment during Extramedullary Progression Affect Proliferation and Immune Evasion. Clin Cancer Res. 2020 15;26(4):935-944.

31. Stuart T, Butler A, Hoffman P, et al., Comprehensive Integration of Single-Cell Data. Cell. 2019 13;177(7):1888-1902.e21.

32. Kuiper R, Broyl A, de Knegt Y, et al., A gene expression signature for high-risk multiple myeloma. Leukemia. 2012 ;26(11):2406-13.

33. Danziger SA, McConnell M, Gockley J, et al., Bone marrow microenvironments that contribute to patient outcomes in newly diagnosed multiple myeloma: A cohort study of patients in the Total Therapy clinical trials. PLoS Med. 2020 4;17(11):e1003323.

34. Han Y, Nakayama J, Hayashi Y, et al., Establishment and characterization of highly osteolytic luminal breast cancer cell lines by intracaudal arterial injection. Genes Cells. 2020 ;25(2):111-123.

35. Kuroiwa Y, Nakayama J, Adachi C, et al., Proliferative Classification of Intracranially Injected HER2-positive Breast Cancer Cell Lines. Cancers (Basel). 2020 6;12(7):1811.

36. Dimberg LY, Dimberg AI, Ivarsson K, et al., Ectopic and IFN-induced expression of Fas overcomes resistance to Fas-mediated apoptosis in multiple
myeloma cells. Blood. 2005 15;106(4):1346-54.

37. Qu L, Ding J, Chen C, et al., Exosome-Transmitted IncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. Cancer Cell. 2016 9;29(5):653-668.

38. Samuel P, Mulcahy LA, Furlong F, et al., Cisplatin induces the release of extracellular vesicles from ovarian cancer cells that can induce invasiveness and drug resistance in bystander cells. Philos Trans R Soc Lond B Biol Sci. 2018 5;373(1737):20170065.

39. Vázquez CL, Rodgers A, Herbst S, et al., The proneurotrophin receptor sortilin is required for Mycobacterium tuberculosis control by macrophages. Sci Rep. 2016 8;6:29332.

40. Dimopoulos K, Søgaard Helbo A, Fibiger Munch-Petersen H, et al., Dual inhibition of DNMTs and EZH2 can overcome both intrinsic and acquired resistance of myeloma cells to IMiDs in a cereblon-independent manner. Mol Oncol. 2018 ;12(2):180-195.

41. Zhu YX, Braggio E, Shi CX, et al., Identification of cereblon-binding proteins and relationship with response and survival after IMiDs in multiple myeloma. Blood. 2014 24;124(4):536-45.

42. Kosaka N, Iguchi H, Yoshioka Y, et al. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem. 2010, 285, 17442-52.

43. Safaei R, Larson BJ, Cheng TC, et al., Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol Cancer Ther. 2005 ;4(10):1595-604.
44. Shedden K, Xie XT, Chandaroy P, et al., Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. Cancer Res. 2003 1; 63(15):4331-7.

45. FJ, Carmona E, Le T, Mes-Masson AM, et al., The EV-mediated autocrine and paracrine actions of plasma gelsolin in ovarian cancer chemoresistance. Oncogene. 2020 ;39(7):1600-1616.

46. Sevilla-Movilla S, Arellano-Sánchez N, Martínez-Moreno M, et al., Upregulated expression and function of the α4β1 integrin in multiple myeloma cells resistant to bortezomib. J Pathol. 2020 5.

47. Noborio-Hatano K, Kikuchi J, Takatoku M, et al., Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. Oncogene. 2009 15;28(2):231-42.

48. Neri P, Ren L, Azab AK, et al., Integrin β7-mediated regulation of multiple myeloma cell adhesion, migration, and invasion. Blood. 2011 9;117(23):6202-13.

49. Kohmo S, Kijima T, Otani Y, et al., Cell Surface Tetraspanin CD9 Mediates Chemoresistance in Small Cell Lung Cancer. Cancer Res. 2010 15;70(20):8025-35.

50. Smirnov DA, Foulk BW, Doyle GV, et al., Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. Cancer Res. 2006 15;66(6):2918-22.

51. Han Q, Deng Y, Chen S, et al., Downregulation of ATG5-dependent macroautophagy by chaperone-mediated autophagy promotes breast cancer cell metastasis. Sci Rep. 2017 6;7(1):4759.

52. Qadir F, Aziz MA, Sari CP, et al., Transcriptome reprogramming by
cancer exosomes: identification of novel molecular targets in matrix and immune modulation. Mol Cancer. 2018 16;17(1):97.

53. Babuta M, Furi I, Bala S, et al., Dysregulated Autophagy and Lysosome Function Are Linked to Exosome Production by Micro-RNA 155 in Alcoholic Liver Disease. Hepatology. 2019 ;70(6):2123-2141.

54. Yingchun L, Huihan W, Rong Z, et al., Antitumor Activity of Asiaticoside Against Multiple Myeloma Drug-Resistant Cancer Cells Is Mediated by Autophagy Induction, Activation of Effector Caspases, and Inhibition of Cell Migration, Invasion, and STAT-3 Signaling Pathway. Med Sci Monit. 2019 20;25:1355-1361.

55. Fontana F, Scott MJ, Allen JS, et al., VLA4-Targeted Nanoparticles Hijack Cell Adhesion-Mediated Drug Resistance to Target Refractory Myeloma Cells and Prolong Survival. Clin Cancer Res. 2021 1;27(7):1974-1986.

56. Bjorklund CC, Baladandayuthapani V, Lin HY, et al., Evidence of a role for CD44 and cell adhesion in mediating resistance to lenalidomide in multiple myeloma: therapeutic implications. Leukemia. 2014 ;28(2):373-83.
Figure legends

Figure 1 Comparative analysis of parental and lenalidomide-resistant MM cell lines. (A) Illustration of the method for establishing lenalidomide-resistant cell lines. Relative resistance score (Rel. resistance) = (resistant cell IC\textsubscript{50} / parental cell IC\textsubscript{50}). (B) Cell viability assay with a CCK-8 kit. The values were normalized to those of the negative control. The error bars indicate the s.d. values. *, p<0.05. The cells were incubated for 72 hr. (C) Caspase activity was measured with a caspase3/7 assay kit. Cisplatin was used as a positive control. The cells were incubated for 72 hr. *, p<0.05. (D) Heat map showing gene expression in lenalidomide-resistant cells and parental cells. p < 0.05, FC > 1.5. (E) GSEA results showing the enriched pathways in resistant cell lines. (F) Heat map showing gene expression in the snare interaction in vesicular transport-associated genes gene set. (G) Measurement of EV secretion by an ExoScreen assay. The vertical axis in the graphs shows the ExoScreen signals normalized to the cell viability signals. The error bars indicate the s.d. values. *, p<0.05. Signal values were normalized to those of the negative control. The cells were incubated for 72 hr. (H) Nanoparticle tracking analysis. The error bars indicate the s.d. values. The particle count was normalized to the cell count. *, p<0.05. I. The particle size indicates the median size. The error bars indicate the s.d. values. *, p<0.05.

Figure 2 Relationship between cell adhesion ability and lenalidomide resistance. (A) Illustration of the coculture experiment setup. The resistant cells
were seeded in the upper chamber, and the parental cells were seeded in the lower chamber. After one week of coculture, the numbers of adherent and nonadherent cells were determined. After cell counting, cells were reseeded into a new 96-well plate to measure lenalidomide sensitivity. (B) Cell viability assay of cocultured cells with a CCK-8 kit. The values were normalized to those of the negative control. The error bars indicate the s.d. values. *, p<0.05. Parental cells (KMS21, KMS27 and KMS34) were cocultured with KMS34R cells. (C) Representative images of KMS21 and KMS27 cells after coculture with lenalidomide-resistant cells. Scale bar: 50 μm. (D) Fraction of cells from each sample after coculture (left). The total cell numbers in each sample (right). E. Illustration of the cell adhesion experiment setup with ultra-low attachment plates. Each lenalidomide-resistant cell line was seeded at 5,000 cells/well, and after 96 hours, cell proliferation was measured with cell viability assay. F. Cell viability assay of KMS21R, KMS27R, and KMS34R cells using ultra-low attachment plates. The values were normalized to those of the negative control. *, p<0.05.

**Figure 3 Screening of genes responsible for EV secretion in resistant MM cells.** (A) Flow diagram for the selection of candidate genes related to EV secretion. (B) Illustration of the method used for EV secretion-related gene screening. (C) Heat map showing the 118 genes highly expressed in lenalidomide-resistant cell lines (resistant: KMS21R, KMS27R, KMS34, and KMS34R; sensitive: KMS21 and KMS27). (D) Cell viability assay of cells with knockdown of the 13 selected genes in the presence of lenalidomide. (E) Caspase activity assay of cells with knockdown of the 13 selected genes in the...
presence of lenalidomide. (F) ExoScreen assay of cells with knockdown of the selected 8 genes. The values were normalized to those of the negative control. (G) Western blotting for SORT1, LAMP2, VLA-4, STAT3, p-STAT3 and β-actin in the 6 cell lines used in this study. Proteins were loaded at 15 μg/lane. (H) qRT-PCR analysis of the expression levels of LAMP2 and SORT1. Each expression value was normalized to that in the corresponding parental cell line.

**Figure 4 Functional analysis of LAMP2 and SORT1 knockdown in lenalidomide-resistant MM cells.** (A) Western blotting for SORT1, LAMP2 and β-actin in cells with stable knockdown of SORT1 and LAMP2 via shRNA. Proteins were loaded at 15 μg/lane. (B) EV secretion was measured with an ExoScreen assay. Each signal value was normalized to that of the control. *, p<0.05. (C) Illustration of the method used for the adhesion experiment. Seventy-two hours after seeding, cells were washed 5 times with PBS (-), and the numbers of attached cells were counted. (D) Picture of KMS27R cells with LAMP2 or SORT1 knockdown. Scale bar indicates 100 μm. (E) Cell counts of the adherent cells in Figure 4D. (F) The viability of nonadherent cells was estimated by trypan blue staining. The values were normalized to the total cell number. (G) Cell counts of the adherent cells using FN-coated, *, p<0.05. (H) Cell viability assay of KMS27R cells with LAMP2 or SORT1 knockdown in the presence of lenalidomide. The values were normalized to those of the negative control (0 μM lenalidomide). The error bars indicate the s.d. values. *, p<0.05. top; normal plate, bottom; FN-coated plate. (I) Comparative analysis of KMS27R cells with LAMP2 or SORT1 knockdown cultured in normal and ultra-low
attachment conditions via the cell viability assay. The signal values for the ultra-low attachment plate were normalized to those for the normal plate. The error bars indicate the s.d. values.

**Figure 5** Contribution of EV secretion and cell adhesion-related genes to MM patient prognosis. (A) A scatter plot showing the relationship between LAMP2 and SORT1 expression in the 6 cell lines used in this study. The X-axis indicates LAMP2 expression, and the Y-axis indicates SORT1 expression. (B) A scatter plot showing the relationship of LAMP2 and SORT1 expression in a publicly available CCLE dataset. Each dot indicates an MM cell line, red: cell lines in our dataset, blue: cell lines that are known to be partially resistant or resistant to lenalidomide. (C) UMAP plot classifying patient outcomes as aggressive or passive. (D) UMAP plot of MM cells focusing on the PC1 signature (left). Violin plot of PC1 signature genes among patient states (right). (E) UMAP plot of MM cells focusing on the PC2 signature (left). Violin plot for PC2 signature genes among patient states (right). (F) Violin plots for LAMP2 and SORT1 gene expression among patient states. (G-H) Violin plots for EV biogenesis-associated gene expression among patient states, among PC2 signature genes (G), typical EV marker (H).

**Figure 6** Survival analysis with SORT1 and LAMP2 expression profiles in clinical MM. (A, B) The Kaplan-Meier plots with SORT1 and LAMP2 expression profiles in GSE19784 datasets using endpoints of overall survival (OS) status (A) and progression-free survival (PFS) status (B). Low expression of SORT1
correlated to poor prognosis with OS (high: n = 54, low: n = 228) and PFS (high: n = 33, low: n = 247) endpoints. High expression of LAMP2 correlated to poor prognosis with OS (high: n = 31, low: n = 251) and PFS (high: n = 43, low: n = 237) endpoints. (C) The Kaplan-Meier plots with SORT1 expression profiles in GSE136324 datasets using endpoints of overall survival (OS) status (left panel) and progression-free survival (PFS) status (right panel). High expression of SORT1 correlated to poor prognosis with OS (high: n = 153, low: n = 48) and PFS (high: n = 153, low: n = 48) endpoints.

Figure 7 Schematic model of the regulation of drug resistance in MM cells.
Figure 1. Yamamoto T et al.

A) Parental cells
Resistant cells
Lenalidomide selection > 1 year

| Cell lines  | Type    | IC\(_{50}\) (μM) | Rel. resistance |
|-------------|---------|------------------|-----------------|
| KMS21       | Parental| 0.27             | 1.0             |
| KMS21R      | Resistant| 128             | 474.3           |
| KMS27       | Parental| 59              | 1.0             |
| KMS27R      | Resistant| N.S.           | N.S.            |

B) Relative cellular proliferation

C) Relative caspase activity

D) max min
p < 0.05, FC > 1.5

E) Snare interaction in vesicular transport
NES = 1.53
p = 0.0084
FDR-q = 0.102

F) ExoScreen

G) EV secretion (10\(^8\) particle/cells)

H) NTA

I) Particle size (nm)
Figure 2, Yamamoto T et al.
Figure 3. Yamamoto T et al.

A. Total: 15,293 genes
- Resistant vs. sensitive
  - FC > 1.5
  - p < 0.01
- Narrow down based on literature search
  - 118 genes
  - 105 genes
  - 13 genes
  - 5 genes

B. siRNAs based screening
- Transfect siRNAs
- 72 hours
- MTS assay and Caspase assay

C. Heatmap
- High
- Low

D. MTS assay
- Lenalidomide 100 μM
- Relative cellular proliferation

E. Caspase assay
- Lenalidomide 100 μM
- Relative caspase activity

F. ExoScreen assay
- Relative EV secretion

G. Western blot
- KMS21
- KMS27
- KMS34
- LAMP2
- SORT1
- VLA-4
- STAT3
- p-STAT3
- β-actin

H. Relative expression level of LAMP2
- Relative expression level of SORT1

P: parental  R: resistance
Figure 4. Yamamoto T et al.
Figure 6, Yamamoto T et al.

**A**

**SORT1 (OS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 54

n = 228

p = 0.029

**B**

**SORT1 (PFS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 33

n = 247

p = 0.019

**C**

**SORT1 (OS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 153

n = 48

p = 0.014

**LAMP2 (OS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 31

n = 251

p = 0.018

**LAMP2 (PFS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 43

n = 237

p = 0.028

**SORT1 (OS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 153

n = 48

p = 0.014

**SORT1 (PFS)**

| Survival probability | Time (months) |
|----------------------|---------------|
| 1.00                 | 0             |
| 0.75                 | 10            |
| 0.50                 | 20            |
| 0.25                 | 30            |
| 0.00                 | 40            |

High expression

Low expression

n = 153

n = 48

p = 0.017
Figure 7, Yamamoto T et al.

Continuous exposure of EVs secreted by lenalidomide resistant cells

Sensitive cells

Acquired resistant cells

Resistant cells

EVs

Lenalidomide exposure

SORT1/LAMP2

EV secretion

Cell adhesion

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