Targeting CD47/TNFAIP8 by miR-155 overcomes drug resistance and inhibits tumor growth through induction of phagocytosis and apoptosis in multiple myeloma

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Supplementary material and methods

Drugs and antibodies:

Bortezomib, Selleck Chemicals, Houston TX 77230 USA
TNFAIP8: Elabscience (E-AB-17126)
CD47: SAB Signalway antibody (32461)
Anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), Cell Signaling Technology, Inc.
Anti-β-Actin, Sigma-Aldrich, Saint Louis, Missouri 63103 USA
Anti- Poly- (ADP-Ribose) Polymerase, Roche, Inc. Indianapolis, IN, USA
Anti- Caspase-3, Cell Signaling Technology, Inc. Danvers, MA, USA
Anti- Cleaved Caspase-3, Cell Signaling Technology, Inc. Danvers, MA, USA
Anti- Caspase-8, Cell Signaling Technology, Inc. Danvers, MA, USA;
Anti- Cleaved Caspase-8, Cell Signaling Technology, Inc. Danvers, MA, USA;
Anti- Caspase-9, Cell Signaling Technology, Inc. Danvers, MA, USA;
Anti-Cleaved Caspase-9 (Asp330), Cell Signaling Technology, Inc. Danvers, MA, USA

Immunohistochemical (IHC) staining

CD138/CD47 immunohistochemical (IHC) staining was performed on bone marrow aspiration/biopsy specimens after decalcification (if needed) and paraffin-embedding, followed by cutting in sequential 4 μm–thick sections, mounting and dewaxing. The sections were treated with 3.0% hydrogen peroxide for 15 min. Antigen retrieval and incubation with anti-CD47 (Signalway Antibody, 32461, Citrate Buffer, pH:6.0, 1:3000 dilution, 1 hour) and anti-CD138 (Dako, Canada, TRIS-EDTA, pH:9.0, 1/200 dilution, 1 hour) were performed. Finally, sections were counterstained lightly with Hematoxylin. The external controls were used to ensure equal and adequate staining. The H-score method (0-300) based on the percentage and intensity of membranous CD47 staining was applied. The median H-score among our cohort was 155. High or low expressions were defined based on the H-score results which were above or below the median H-score, respectively (supplementary figure 1A). The results were evaluated by 2 independent pathologists who were blinded to the patient's status and clinical outcome. IHC was shown to be a reproducible method for semi-quantitative CD47 detection; the inter-observer agreement was assessed using the simple and weighted kappa (κ) statistic and 95% confidence interval (CI), and the intra-observer agreement was 96% (all of the cases were scored twice by

both observers). The results were then examined for any correlation with patient's clinical laboratory features and survival outcomes.

Cell culture and generation of stable cell lines

To generate cell populations stably expressing CD47 or TNFAIP8, lentiviral constructs harboring CD47 or TNFAIP8 gene (pLenti-GIII-CMV, Applied Biological Materials Inc. and EX-T1943-Lv122, GeneCopoeia, respectively) were co-transfected with packaging vectors into HEK-293T cells using lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. After 48 h of transfection, the MM cell lines were transduced with viral supernatant in the presence of 8 μg/mL polybrene (Sigma) by spinoculation method and the transduced cells were selected with 2.5 μg/mL puromycin (Sigma). To generate GFP tagged-MM cells, GFP-expressing lentiviral particles were produced by packaging of pGIPZ-GFP plasmid (empty backbone) into pMD2.G and psPAX2 vectors (Addgene plasmids, # 12259 and #12260, respectively, gifts from Didier Trono) using HEK293T cell line. 48 hours post transfection, HEK293T supernatant containing lentiviral particles was harvested and used for transduction of MM cells. Transduction efficiency (>95%) was evaluated by flow-cytometric GFP analysis.

miRNA mimics transfection

MM cell lines were transiently transfected with either miR-155 mimics or scrambled miRNA (GeneCopoeia) using HiPerFect transfection reagent (Qiagen, Germantown, MD) according to the manufacturer's instructions. Functional experiments were performed following transfection. The transfected cells were examined for qPCR or cell viability using the 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Biobasic) assay 72 h after transfection.

Cell viability and apoptosis assays

Cell viability was assessed by MTT colorimetric assay. MM cell lines were seeded in 96-well plates (Sarstedt, Inc.) in 100 μL complete medium at a density of 2.0×10^4 cells per well. MM cells were incubated at 37℃ and 5% CO2 with various concentrations of indicated drugs for 48 h. After the incubation, 10 μl of MTT (0.5 mg/mL) was added, and the cells were further incubated for an additional 4 h. Finally, precipitated dye was solubilized using acidified isopropanol and the absorbance of the wells was read through a microplate reader. Each experiment was performed in

triplicate and the mean value and SD were calculated. To examine apoptotic cell death, MM cells were treated with various concentrations of BTZ in combination with miR-155 for 48 h followed by annexin V-FITC/PI staining for apoptosis analysis in a FACS Calibur flow cytometer (Becton Dickinson). Captured events were analyzed using CellQuest software. The extent of apoptosis was quantified as percentage of annexin-V positive cells, and the extent of drug-specific apoptosis was assessed by the formula: percentage of annexin-V positive cells = (test - control) × 100/ (100 - control).

Protein extraction and western blotting

Cells were lysed in cold cell lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail or directly in 6×SDS sample buffer (250 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.5 M DTT, and 0.012% (w/v) bromophenol blue). Equal amounts of proteins were resolved by 8-15% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked by incubation in 5% nonfat dry milk in TBST (0.05% Tween-20 in PBS) and probed with primary antibodies mentioned above. Blots were then developed by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). To evaluate protein levels in tumor tissue, whole protein extracts were prepared from 3 representative tumors of each group using T-PER lysis solution (Pierce). Briefly, 50 mg of tissue samples were resuspended in 400 μL of lysis buffer supplemented with protease inhibitors. Samples were centrifuged at 15,000*g* for 15 minutes, and 50 μg of each supernatant was then analyzed by SDS-PAGE for protein level analysis.

Luciferase reporter assay

The 3’ UTR sequence of human CD47 and TNFAIP8 were cloned into the luciferase-expressing vector pEZX-MT01 to the downstream of the firefly luciferase gene. The mutant 3’UTR clone was constructed by introducing several mutations in miR-155 binding site in the 3’UTR of CD47 and TNFAIP8 by using QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies Canada Inc.) according to kit manual. The 293T cells were transiently co-transfected with the control (pEZX-MT01), mutant or wild type UTR luciferase reporter vectors together with miR-155 mimics or NC-miRNA with lipofectamine 3000 transfection reagent. Cells were harvested 48 h after transfection for measuring firefly and *Renilla* relative activities using the Dual-Luciferase

Reporter Assay System following the manufacture's instruction. Relative luciferase activities were analyzed as the activity of firefly relative to *Renilla*.

Quantitative real-time PCR

Total RNA including miRNA was extracted using miRNeasy mini kit (Qiagen). cDNA was synthesized by miScript II RT Kit (Qiagen) and applied to miRNA real time PCR using miScript SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. The expression of mature miRNAs was calculated relative to SNORD72 and fold changes in miRNA treatments relative to scrambled treatments were calculated by using the $2^{\Delta\Delta Ct}$ algorithm. The mRNA expression of target genes was determined by RT-qPCR for MM cell lines and sorted CD138+ cells from MM patients. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). cDNA synthesis and quantitative RT-PCR (qRT-PCR) were performed according to the manufacturer's instructions (Biorad). Ct values were extracted and after normalization to GAPDH and other housekeeping genes, fold changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ algorithm.

Immunostaining cell surface targets and indirect flow cytometry

To examine the level of cell surface CD47, MM cells were transfected with miR-155 mimics or scramble control. 3 x 10^5cells were harvested 48 h after transfection. The cells were washed twice with 1 ml FACS buffer (0.5% BSA- 0.1% Na-AZ in 1X PBS) and incubated with CD47 antibody (1:50) (Signalway Antibody LLC) for 1.5 h on ice and dark place. The cells were washed twice with 1 ml FACS buffer and stained with secondary Cy5-rabbit Ab (1:400) (Invitrogen) for 40 min on ice and dark place. The cells were washed twice with 1 ml FACS buffer and analyzed by flow cytometer.

Phagocytosis assay

THP-1 monocytes were seeded onto 24-well or 6-well plates at density 1 x 10^6 or 4 x 10^6 cell/well, respectively, in the presence of 50 ng/mL phorbol 12-myristate 13- acetate (PMA) for 3 days in order to induce differentiation into macrophage-like cells. MM cells were labeled with GFP by lentiviral transduction and transfected with either miR-155 mimics of scramble control as mentioned above. 5 × 10^4 macrophages were plated per well in a 24-well tissue-culture plate. Macrophages were incubated in serum-free medium for 2 h before adding 2 × 10^5 GFP-tagged MM cells and incubated for 2 h at 37°C. Macrophages were repeatedly washed four times with

PBS 1X and subsequently imaged with and fluorescent microscope. The phagocytic index was calculated as the number of phagocytosed GFP^+ cells per 100 macrophages.

Myeloma xenograft mouse model

Immunodeficient (SCID) mice (male, 6–8 weeks old; OCI) were housed in the animal care facility, under a 12/12 hours light/dark cycle at 22°C, and they received a standard diet and acidified water ad libitum. Using a protocol approved by the animal testing ethical committee of the UHN, mice were inoculated subcutaneously at their lower dorsum with 1×107 8226-R5 cells in matrigel basement membrane matrix (Becton Dickinson). When tumors were palpable (approximately 21 days after injection), mice were randomly assigned into 4 groups (n=5 in each), receiving intraperitoneal or intratumoral (for miRNA mimics) injection twice a week with 0.5 mg/kg BTZ alone or combined with miR-155 mimics, miR-155 mimics alone or an equal volume of vehicle in 3 days interval for 15 days. The shortest and longest diameters of the tumor were measured with external calipers every 3 days, and tumor volume (in mm3) was calculated using the following standard formula: V = 0.5a x b2, where “a” and “b” are the long and short diameter of the tumor. Survival was evaluated from the first day of tumor injection until death. In accordance with institutional guidelines, mice were sacrificed when their tumors reached 1.5 cm in diameter or in the event of paralysis or major compromise in their quality of life, to prevent unexpected suffering. For treating the mice with miRNA mimics, we have used lipid-based delivery of synthetic miRNA. Each dose contained 20 μg synthetic oligo, which equals 1 mg/kg per mouse with an average weight of 20 g. Administration of miRNA mimics was performed using the novel formulation of neutral lipid emulsion (NLE; MaxSuppressor in vivo RNA Lancer II, BIOO Scientific) according to the manufacturer's instructions. Treatments were performed intratumorally by using the formulation and dosage as described above. Tumor samples were snap-frozen in OCT medium (Sakura Tissue Tek). Immunohistochemical studies were performed on formalin-fixed Tumor samples using H & E, Ki-67 and TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) staining.

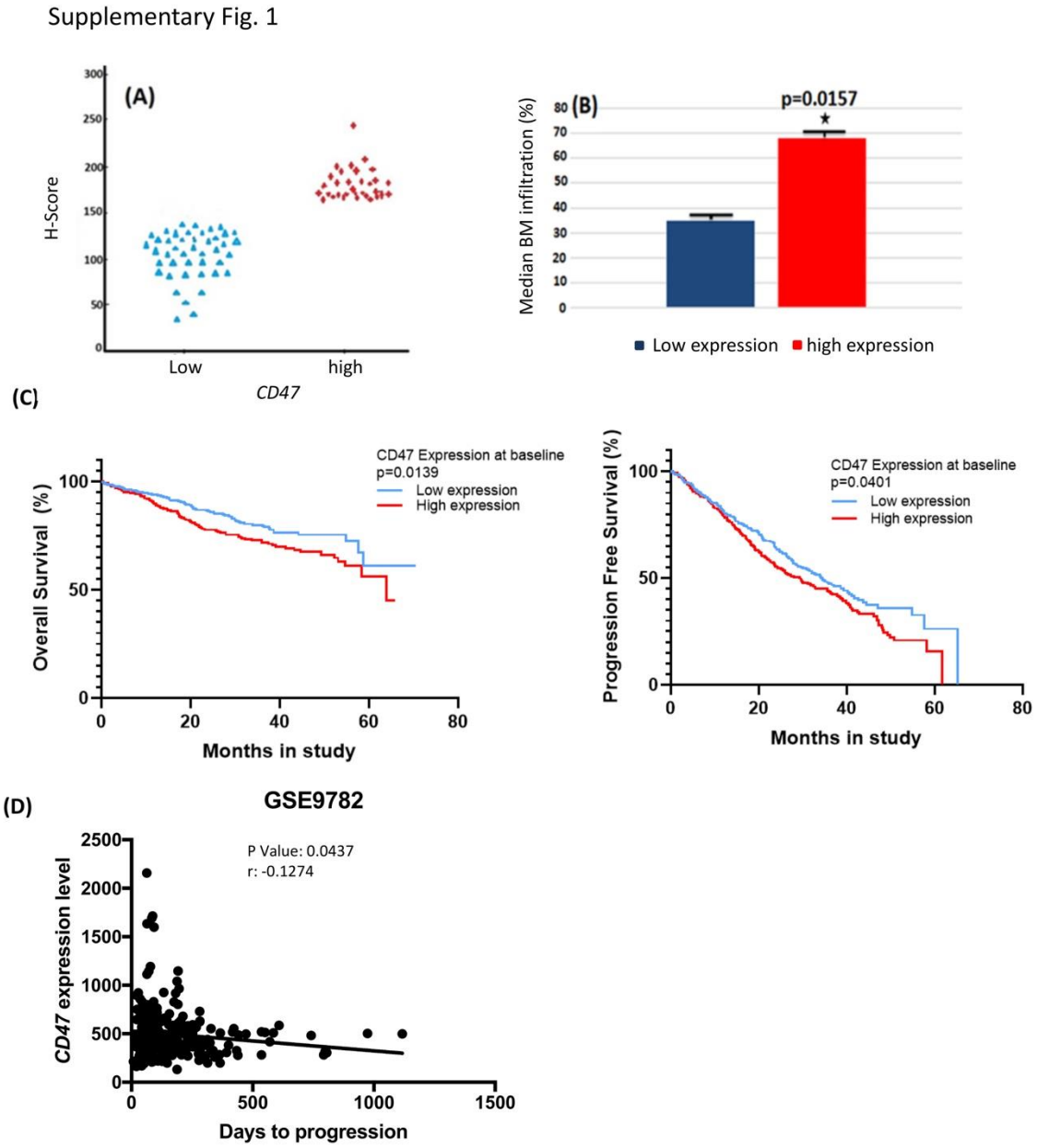
Statistical analysis

Categorical variables such as CD47 status, gender and vital status will be summarized with counts and percentages. The Continuous variable age at diagnosis and follow-up will be summarized with medians and ranges. Overall Survival (OS) and Progression Free Survival (PFS) rates were

calculated using the Kaplan-Meier product-limit method. Log-rank test was used as a univariate analysis to compare levels of patient characteristics and other potential predictive factors. All P-values were 2-sided and for the statistical analyses and $P < 0.05$ will be considered to indicate a significantly different result. Statistical analyses will be performed using SAS Version 9.4 (2002-2012 SAS Institute Inc., Cary, NC, USA).

All quantified data represent a mean of triplicate experiments ± SEM which analyzed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA) and comparisons between different groups were assessed by the Student's ***t*** test and one-way analysis of variance. The correlation between CD47, TNFAIP8 and miR-155 expression was determined using Pearson's coefficient test. Survival curves were estimated using Kaplan–Meier method and log-rank test was used to compute differences between the curves. Differences were considered significant at values of $P<0.05$.

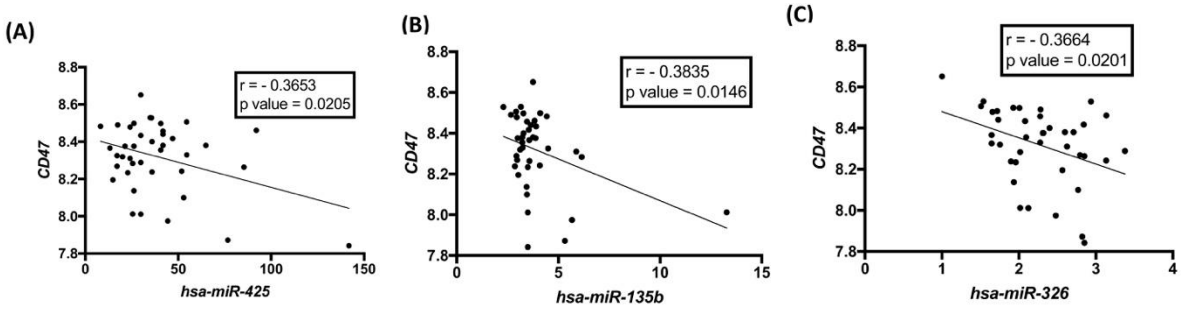
Supplementary Figures:



Supplementary figure 1: (A) Distribution of CD47 protein expression by H-score in our patients. (B) Median myeloma cells in BM as stratified by low or high CD47 expression. (C) Kaplan-Meier plots indicate the overall survival (OS) and progression free survival (PFS) for 767 MM patients

in the CoMMpass (Clinical Outcomes in Multiple Myeloma to Personal Assessment) IA13 study stratified by CD47 expression level (n=384 for low-CD47 group versus n=383 for high-CD47 group). Gene expression was determined by TPM (transcripts per million). Data available from (https://research.themmrf.org). (D) Correlation analysis of CD47 expression level with days to progression in patient dataset (GSE9782) presented as scatter plots. Linear regression with Pearson's correlation coefficients (r) and p value were presented in the graph.

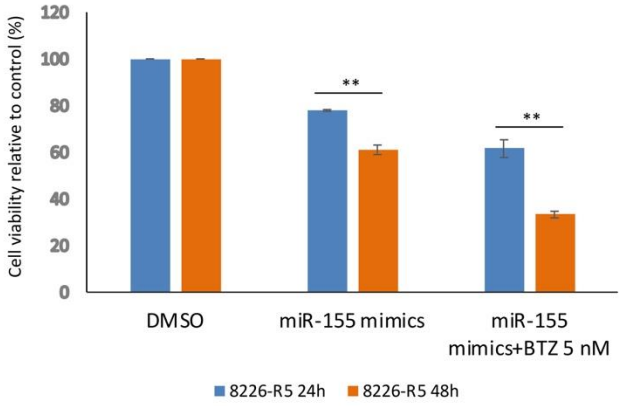
Supplementary Fig. 2

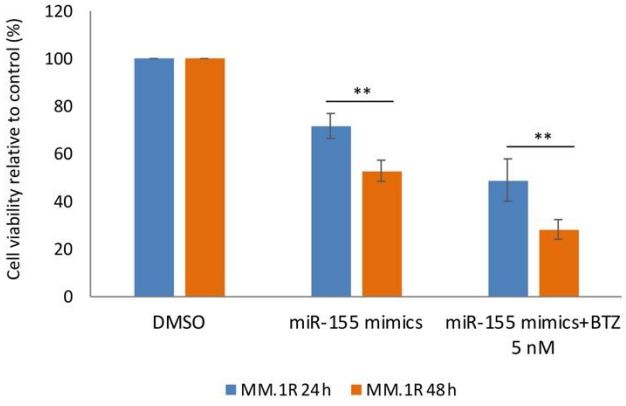


Supplementary figure 2: (A-C) negative correlation between CD47 and miRNAs expression level in MM patient samples. Correlation analysis of miRNA expression with CD47 expression in patient dataset (GSE70254, n=96 new diagnosed MM patients) presented as scatter plots. Linear regression with Pearson's correlation coefficients (r) and p value were presented in the graph.

Supplementary Fig. 3

(A)

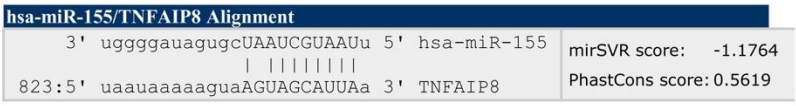




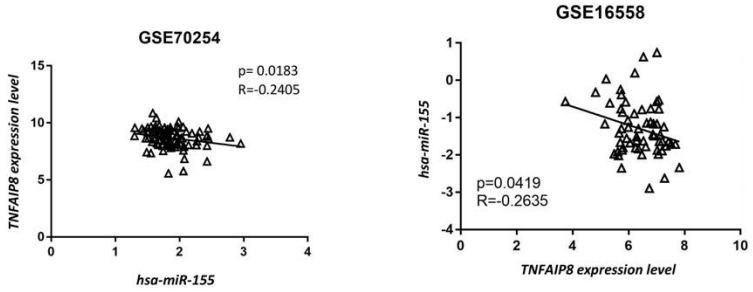
(B)

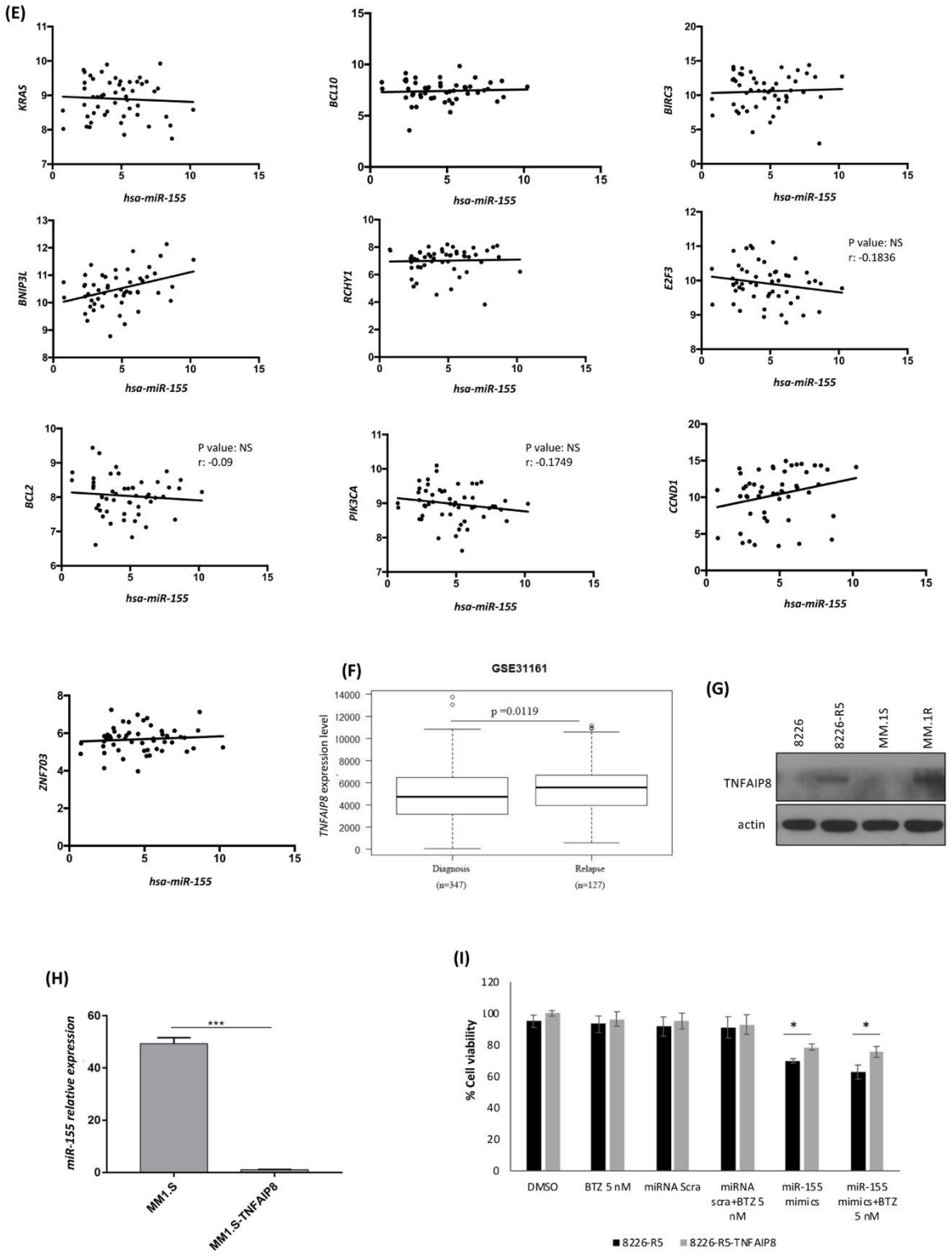
number of source	Gene	oncoscore
20	PIK3CA	87.83
19	CCND1	78.17
22	KRAS	77.54
21	ZNF703	76.68
20	BCL10	74.8
20	BNIP3L	73.27
22	RCHY1	71.23
17	E2F3	69.89
18	BIRC3	69.74
22	TNFAIP8	68.19
19	BCL2	65.68
18	CDC25A	64.77
17	EGFR	64.71
18	CACUL1	64.63
20	MIER1	64.48
20	KDM5A	64.32
19	CD109	64.11
18	TRAF3	64.01
20	PRKAR1A	63.88
20	PIK3R3	63.69

(C)



(D)





Supplementary figure 3. (a) The 8226-R5 and MM.1R cell lines were transfected with synthetic miR-155 or scramble control and treated with or without 5 nm BTZ for 24h or 48h and cell viability

was measured using MTT assay. (* indicates $p<0.05$, ** $p<0.01$ and ***: $p<0.01$ and significant difference based on the 95% of confidence intervals). (b) miR-155 target genes were analyzed by OncoScore. The list of 20 genes with the highest oncogenic score was shown in the figure. (c) The miR-155 binding site in the TNFAIP8 3′-UTR. Putative conserved target sites in the TNFAIP8 3′-UTR were identified using the TargetScan algorithm. Matched nucleic acid–base pairs were linked as "-" (d) Correlation analysis of endogenous miR-155 with TNFAIP8 expression in patient datasets (GSE70254 and GSE16558) presented as scatter plots. Linear regression with Pearson's correlation coefficients (r) and p value were presented in the graph. (e) Correlation analysis of endogenous miR-155 with its potential targets expression in patient dataset (GSE17306) presented as scatter plots. Linear regression with Pearson's correlation coefficients (r) and p value were presented in the graph. (f) The box whisker plot shows TNFAIP8 expression in primary multiple myeloma plasma cells from patients treated by total therapy 2, 3 and other protocols at baseline and relapse, GSE31161 dataset (g) The basal protein level of TNFAIP8 was assessed in 8226 vs. 8226-R5 and MM.1S vs MM.1R cells, respectively. (h) The total RNA was isolated from MM.1S cells and TNFAIP8 overexpressing MM.1S cells and the level of miR-155 was assessed by qPCR (i) The 8226-R5 cells with or without stable TNFAIP8 overexpression were transfected with synthetic miR-155 or scramble control. Cell proliferation was assessed 48 h after treatment in the absence or presence of 5 nM BTZ.

Table S1. Clinical and laboratory features of the MM patients according to CD47 expression

Clinical feature		Total (n=74)	CD47 negative (n=43)	CD47 positive (n=31)	P value
Sex(M/F)		42/32	26/17	16/15	0.4482
Age(y), median (range)		55(34-73)	54(38-67)	57(34-73)	0.2098
International Staging System (ISS), No (%)	I-II	23(31)	14(32.6)	9(29)	0.7855
	III	28(38)	17(39.5)	11(35.5)	
	N/A	23(31)	12(27.9)	11(35.5)	
Hemoglobin (g/L), median (range)		105(52-158)	105(64-158)	101(52-140)	0.3788
Calcium (mmol/L), median (range)		2.33 (1.62-4.21)	2.38 (1.62-4.21)	2.3 (1.9-2.86)	0.1163**
Creatinine (μmol/L), median (range)		85(46-1025)	88(46-1025)	70(51-400)	0.0807**
Bone lytic lesions, No (%)		38(51)	23(59)	15(56)	0.7823
B2-Microglobulin(mg/L), median (range)		2.22 (1.16-5.89)	1.76 (1.16-5.89)	3.28 (2.07-4.37)	0.0464**
Albumin(gr/L), median (range)		36.5 (27-49)	36.5 (29-49)	36.5 (27-49)	0.8377
Cyto-genetics, No (%) 13q deletion;	positive	25 (44.6)	15 (42)	10 (50)	0.5478
	negative	31 (55.4)	21 (58)	10 (50)	
17p (p53) deletion;	positive	3 (5)	0 (0)	3 (14)	0.0407
	negative	59 (95)	40 (100)	19 (86)	
t(4;14);	positive	8 (14)	5 (14)	3 (14)	1.000
	negative	48 (86)	30 (86)	18 (86)	
1p21 deletion;	positive	9 (13)	4 (44)	5 (17)	0.4713
	negative	62 (87)	38 (90)	24 (83)	
1q21 (CKS1B) amplification;	positive	16 (35)	8 (29)	8 (44)	0.2700
	negative	30 (65)	20 (71)	10 (56)	
BM myeloma cells infiltration, median (range)		40 (5-95)	35 (5-95)	68 (10-90)	0.0157