

# Overexpression of B-cell lymphoma 6 alters gene expression profile in a myeloma cell line and is associated with decreased DNA damage response

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## Key words

ATM, BCL6, DNA repair, IL-6, multiple myeloma

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B-cell lymphoma 6 (BCL6) attenuates DNA damage response (DDR) through gene repression and facilitates tolerance to genomic instability during immunoglobulin affinity maturation in germinal center (GC) B cells. Although BCL6 expression is repressed through normal differentiation of GC B cells into plasma cells, a recent study showed the ectopic expression of BCL6 in primary multiple myeloma (MM) cells. However, the functional roles of BCL6 in MM cells are largely unknown. Here, we report that overexpression of BCL6 in a MM cell line, KMS12PE, induced transcriptional repression of ataxia telangiectasia mutated (*ATM*), a DDR signaling kinase, which was associated with a reduction in  $\gamma$ H2AX formation after DNA damage. In contrast, transcription of known targets of BCL6 in GC B cells was not affected, suggesting a cell type-specific function of BCL6. To further investigate the effects of BCL6 overexpression on the MM cell line, we undertook mRNA sequence analysis and found an upregulation in the genomic mutator activation-induced cytidine deaminase (*AID*) with alteration in the gene expression profile, which is suggestive of de-differentiation from plasma cells. Moreover, interleukin-6 exposure to KMS12PE led to upregulation of *BCL6* and *AID*, downregulation of *ATM*, and attenuation of DDR, which were consistent with the effects of BCL6 overexpression in this MM cell line. Taken together, these results indicated that overexpression of BCL6 alters gene expression profile and confers decreased DDR in MM cells. This phenotypic change could be reproduced by interleukin-6 stimulation, suggesting an important role of external stimuli in inducing genomic instability, which is a hallmark of MM cells.

Multiple myeloma is a plasma cell neoplasm characterized by abnormal clonal plasma cell infiltration in the bone marrow. Genomic instability is one of the hallmarks of MM and affects the onset and progression of the disease.<sup>(1,2)</sup> However, the underlying mechanisms causing the instability of the genome in MM are largely unknown.<sup>(3–5)</sup> Recent whole genome sequencing studies have revealed infrequent mutations of DNA repair pathway genes in MM.<sup>(6,7)</sup> Thus, the question is still to be answered.

Abnormalities in DNA repair pathways have been extensively studied in B-cell neoplasms such as DLBCL. B-cell lymphoma 6 is a transcriptional repressor originally identified by its involvement in 3q27 chromosomal translocations associated with DLBCL.<sup>(8,9)</sup> It is expressed in GC B cells and facilitates simultaneously rapid proliferation and tolerance to the genomic instability, which occurs during the process of immunoglobulin affinity maturation mediated by *AID*.<sup>(10–12)</sup> B-cell lymphoma 6 directly suppresses the genes involved in DNA damage sensing and cell cycle arrest, such as *ATR*, *p53*, and *p21*,<sup>(13–15)</sup> and thereby attenuates the DDR.

Although BCL6 is generally downregulated as B cells differentiate into plasma cells, a recent study reported that BCL6 is upregulated in MM cells in the bone marrow microenvironment.<sup>(16)</sup> However, the functional role of BCL6 in MM has not yet been well established. Therefore, we investigated the effects of BCL6 expression in MM cells, focusing in particular on DDR and genomic instability.

In this study, we established an MM cell line (KMS12PE-BCL6) stably overexpressing BCL6, and examined the effects of BCL6 overexpression on the expression of DDR genes and  $\gamma$ H2AX formation on DNA damage. Additionally, mRNA sequence analysis was carried out to detect differentially expressed genes in KMS12PE-BCL6 cells. We found that BCL6 overexpression to KMS12PE led to upregulation of *AID*, downregulation of *ATM*, and attenuated DDR. Gene expression profiles in KMS12PE-BCL6 cells are suggestive of de-differentiation of plasma cells to GC B cells. These results imply that BCL6 overexpression is involved in genomic instability in MM cells.

## Materials and Methods

**Isolation of primary tumor cells.** Bone marrow samples obtained from 28 newly diagnosed MM patients were subjected to the study after written informed consent. This study was approved by institutional review boards of Gunma University Hospital (Gunma, Japan) and Nishigunma National Hospital (Gunma, Japan). CD138 positive bone marrow plasma cells were isolated as a purity of >95% using the EasySep human PE-positive selection kit (Stemcell Technologies, Vancouver, BC, Canada). These cells were used for the RNA isolation.

**Cell culture, plasmids, transfection, and retrovirus infection.** Human MM cell lines, KMS11, KMS12PE, KMS12BM, KMS18, KMS26, ARH77, and RPMI8226 were provided by Dr. Otsuki (Kawasaki Medical School, Okayama, Japan), and the human DLBCL cell line, CTB-1, was provided by the RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. These cell lines were cultured in RPMI-1640 medium (Life Technologies, Delhi, India) with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA). Overexpression of BCL6 in KMS12PE cells was achieved by infection of the cells with a retroviral vector encoding BCL6 (pMY-BCL6-IRES-EGFP). The virus was obtained and infected as previously described.<sup>(17)</sup> The EGFP-positive infected cells were isolated using a BD FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA).

**Real-time PCR.** RNA was extracted using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and cDNA was prepared using high-capacity RNA-to-cDNA kit (Life Technologies). Real-time PCR was carried out using a standard TaqMan PCR kit protocol on a 7300 Real-time PCR System (Applied Biosystems, Waltham, MA, USA). Gene expression was normalized to that of GAPDH. The data is shown as the mean  $\pm$  SD ( $n = 3$  per bar). The statistical significance was determined using Student's *t*-test.  $P < 0.01$  was considered significant.

**Quantitative ChIP.** Chromatin immunoprecipitation was carried out using ChIP reagents (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Anti-BCL6 (N-3) (sc-858; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-IgG control (ab46540; Abcam, Cambridge, UK) and Dynabeads Protein G (Life Technologies) were incubated at 4°C overnight. Primers for the each promoter region of *ATR* and *p53* genes and enhancer for *p21* have been reported.<sup>(13,14,18)</sup>

**Flow cytometry.** For BCL6 staining, we used Foxp3 Staining Buffer Set (eBioscience, San Diego, CA, USA) and Alexa Fluor 647-anti-BCL6 antibody (clone K112-91) (BD Biosciences) at 1:250 dilution. For  $\gamma$ H2AX, the cells were stained as previously described,<sup>(19)</sup> with FITC-anti- $\gamma$ H2AX (Merck Millipore, Darmstadt, Germany). After 3 h of incubation on ice,  $\gamma$ H2AX was measured. For the CD138 assay, PE-conjugated anti-CD138 antibody (Beckman Coulter, Brea, CA, USA) was used. All measurements were carried out on a FACSCanto II Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, San Carlos, CA, USA). The statistical significance was determined using the  $\chi^2$ -test by the population comparison platform of FlowJo.  $P < 0.01$  was considered significant.

**Treatment with BCL6 BTB domain inhibitor 79-6 and IL-6.** The BCL6 inhibitor 79-6 (Merck Millipore) was dissolved in DMSO. The BCL6-overexpressed KMS12PE (KMS12PE-BCL6) cells ( $5 \times 10^5$ /mL) were exposed to 50  $\mu$ M 79-6 or DMSO control for 8 h for RNA quantification. KMS12PE cells ( $2.5 \times 10^5$ /mL) were treated with 100 or 122 ng/mL

recombinant human IL-6 (R&D Systems, Minneapolis, MN, USA) or PBS supplemented with 0.1% BSA as the control for 16 h, then harvested for RNA extraction.

**DNA damage induction.** For induction of DNA damage, cells were exposed to 0, 3, 5, and 10 Gy IR using the RX-650 cabinet X-ray system (Faxitron X-ray, Tucson, AZ, USA) and then incubated at 37°C for 1 h before analysis. Cells were also treated with different concentrations of etoposide (0, 1, 5, 10, 50, and 100  $\mu$ M) for 30 min, washed with fresh media, and incubated at 37°C for 1 h before analysis. Formation of  $\gamma$ H2AX was assessed by flow cytometry and immunofluorescence staining. For real-time PCR and immunoblot analysis of DDR genes, cells were incubated at 37°C and collected 30 min after irradiation.

**Immunofluorescence staining.** After irradiation and incubation for 1 h at 37°C, cells were permeabilized with 0.5% Triton X and blocked and stained with anti- $\gamma$ H2AX antibody (ab22551; Abcam) at 1:800 dilution. Cy3 conjugated donkey anti mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as a secondary antibody, at 1:500 dilution for 1 h, and mounted with Prolong Gold with DAPI (Life Technologies). All the images were captured by a Leica DMLB fluorescent microscope (Leica Microsystems, Wetzlar, Germany). The mean density of  $\gamma$ H2AX expression per nuclei were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Immunoblot analysis.** Cells were harvested and lysed in RIPA lysis buffer (Santa Cruz Biotechnology), then frozen and thawed twice, centrifuged at 20 600 *g* for 10 min. The supernatant was collected as whole cell lysates. The protein (80  $\mu$ g) was used for the immunoblot, described previously.<sup>(17)</sup> Band densities were quantified with ImageJ software, and the relative protein amount was determined by comparison of the protein/ $\beta$ -actin ratios. The following antibodies were used for immunoblot analysis: ATM (2C1[1A1], ab78), phospho-ATM (Ser1981) (10H11.E12, ab36810), phospho-ATR (Ser428) (EPR2184, ab178407),  $\beta$ -actin (ab8227; all Abcam), BCL6 (N-3, sc-858), ATR (N-19, sc-1887), p53 (DO-1, sc-126), p21 (C-19, sc-397; all Santa Cruz Biotechnology), phospho-p53 (Ser15) (#9284; Cell Signaling Technology, Beverly, MA, USA), rabbit IgG-HRP, mouse IgG-HRP (both R&D Systems), and goat IgG-PO (Jackson ImmunoResearch Laboratories).

**Cycloheximide chase assay.** Cycloheximide (Wako Pure Chemicals, Tokyo, Japan) was dissolved in DMSO. KMS12PE-BCL6 cells were exposed to cycloheximide (80  $\mu$ g/mL) for 1 h at 37°C, and irradiated with 0 or 10 Gy. At 0, 0.5, 1, 2, and 4 h after IR, cells were harvested and lysed in RIPA lysis buffer, and supernatant was collected. Twenty-five micrograms of protein was separated by SDS-PAGE and used for immunoblot analysis as described above.

**Next generation mRNA sequencing.** The RNA concentration and purity were determined using a NanoPhotometer (Implen, Munich, Germany) and the integrity was verified using an Agilent RNA 6000 Pico Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity number for all samples was at least 9. Libraries were generated using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA). The libraries were quantified with EnSpire (PerkinElmer, Waltham, MA, USA) and validated using an Agilent 2100 Bioanalyzer for size, and then sequenced using NextSeq 500 (Illumina, San Diego, CA, USA). The count file was generated by RNA-express software version 1.0 (Illumina), and was moved onto EdgeR to identify differentially expressed genes. Genes with a false discovery rate <0.05

were defined as significantly different. The mRNA sequence data were submitted to the DDBJ/EMBL/GenBank databases under accession number DRA004263.

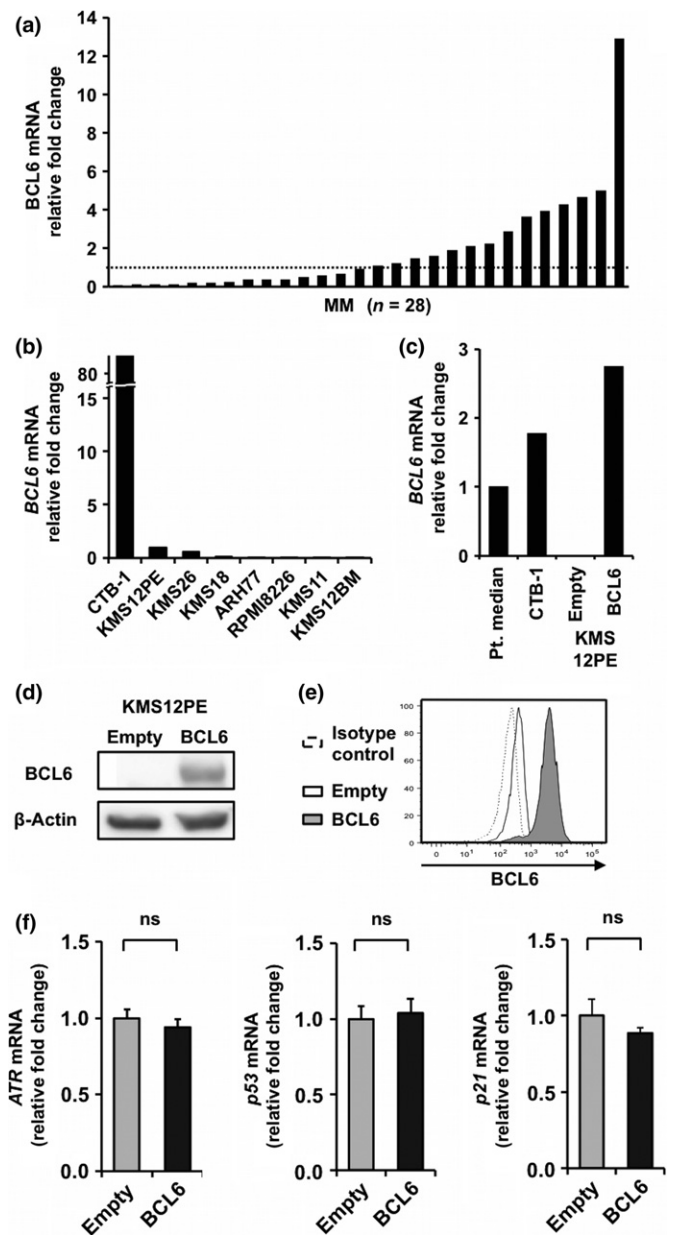
## Results

**Overexpression of BCL6 to KMS12PE cell line did not repress expression of DDR genes.** To determine the role of BCL6 in MM cells, we first examined the expression levels of BCL6 in tumor cells in the bone marrow of MM patients. We quantified the RNA expression levels by real-time PCR. Expression levels of BCL6 in CD138-positive cells from 28 newly diagnosed patients distributed in a wide range (Fig. 1a). Next we tested seven MM cell lines and a BCL6-positive DLBCL cell line, CTB-1, as the positive control. Unlike the tumor cells from the patients, all MM cell lines examined expressed very low levels of *BCL6* (Fig. 1b). To investigate the function of BCL6 in MM cells, we overexpressed BCL6 in KMS12PE cells, which expressed BCL6 at the highest level among the tested cell lines. We used a retrovirus system to obtain the cells overexpressing BCL6 (KMS12PE-BCL6) and the mock-infected cells (KMS12PE-Empty). The expression level of *BCL6* after retroviral infection was comparable to that of CTB-1 and the median of that in these patients (Fig. 1c). Increased protein levels of BCL6 were also confirmed by immunoblotting and flow cytometry analysis (Fig. 1d,e, respectively).

As BCL6 is known to directly repress the transcription of DDR genes, such as *ATR*, *p53*, and *p21*, by binding to the respective promoter/enhancer regions and recruiting transcriptional co-repressors in BCL6-positive B-cell lymphoma cell lines, we evaluated the expression levels of these genes in this cell line.<sup>(13–15,18)</sup> Unlike in DLBCL, none of these genes were repressed by BCL6 overexpression in KMS12PE (Fig. 1f). There were two possibilities: (i) BCL6 does not bind to these target genes; or (ii) BCL6 does bind to those regions, but cannot work as a transcriptional repressor. To answer this question, we investigated the binding of BCL6 to these genes.

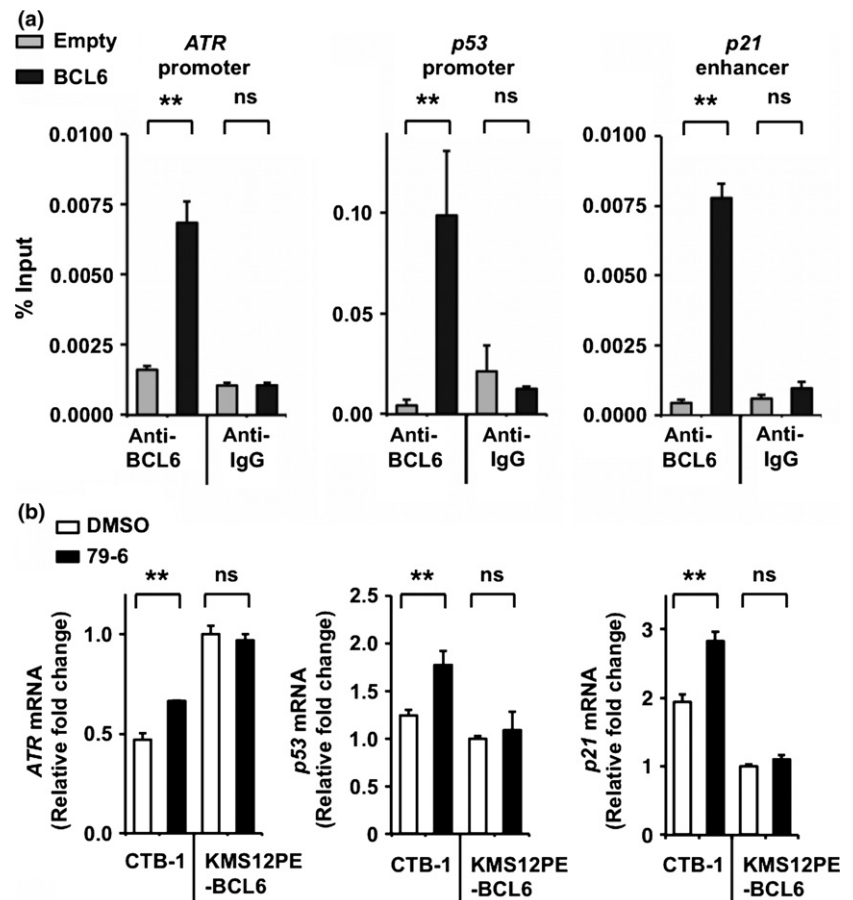
**B-cell lymphoma 6 binds to DDR genes in KMS12PE-BCL6 cells.** To examine whether BCL6 binds to the reported promoter or enhancer regions in KMS12PE-BCL6 cells, ChIP assay was undertaken for the promoters of *ATR*, *p53*, and the enhancer of *p21*, which encompasses the BCL6 binding sites in GC B cells.<sup>(13,14,18)</sup> Binding of BCL6 to each of these regions was clearly observed in KMS12PE-BCL6 cells (Fig. 2a). One of the major repressor functions of BCL6 is mediated by its dimerization and formation of the BTB domain interface, which serves as a docking site for co-repressors.<sup>(20,21)</sup> To further explore the functional effect of BCL6 expression against these DDR genes in KMS12PE-BCL6 cells, the cells were exposed to BCL6 inhibitor, 79-6, a small inhibitor molecule of BCL6, which was designed to bind to the groove of BCL6 BTB domain and disrupt the ability of BCL6 to recruit co-repressors SMRT and NCOR, and reactivate the expression of BCL6 target genes.<sup>(22)</sup> As a positive control, we used CTB-1, a BCL6-positive DLBCL cell line. Exposure to 79-6 did not upregulate the expression levels of *ATR*, *p53*, or *p21* in KMS12PE-BCL6, whereas the inhibitor upregulated these genes in the positive control cell line, CTB-1 (Fig. 2b). These results suggest that BCL6 binds to the reported DDR genes in MM cells, but unlike in B cells, it does not repress their transcription, which suggests the cell type-specific function of BCL6.

**Formation of  $\gamma$ H2AX after DNA damage was repressed by BCL6 overexpression in KMS12PE cells.** To further analyze the role of



**Fig. 1.** B-cell lymphoma 6 (BCL6) overexpression in the KMS12PE multiple myeloma (MM) cell line did not repress the expression of DNA damage response genes. (a) Expression of BCL6 RNA in CD138-positive cells from bone marrow samples of newly diagnosed MM patients was measured by real-time PCR and is presented as the fold change relative to the median (dotted line). (b) Real-time PCR was used to measure the expression levels of *BCL6* RNA in MM cell lines KMS11, KMS12PE, KMS12BM, KMS18, KMS26, ARH77, and RPMI8226; diffuse large B-cell lymphoma cell line CTB-1 was used as the positive control. The expression levels are presented as the fold change relative to that of KMS12PE. (c) Expression of *BCL6* RNA in CTB-1, KMS12PE-BCL6, and KMS12PE-Empty is presented as the fold change relative to that of the median of the MM patients. BCL6 protein expression levels of KMS12PE-BCL6 and -Empty cells were determined by immunoblot analysis (d) and flow cytometry (e).  $\beta$ -Actin was used as a loading control. (f) Real-time PCR results for *ATR*, *p53*, and *p21* in KMS12PE-BCL6 cells are presented as the fold change relative to each expression level of KMS12PE-Empty cells. ns, not significant.

BCL6 under DNA damaged conditions, we exposed the cells to IR or etoposide, both of which induce DNA double-strand breaks. Phosphorylation of Ser139 in  $\gamma$ H2AX by ATM is a



**Fig. 2.** B-cell lymphoma 6 (BCL6) binds to DNA damage response genes in KMS12PE-BCL6 multiple myeloma cells. (a) KMS12PE-BCL6 and -Empty cells were used for ChIP assay with antibodies against BCL6 and control IgG. Regions of the promoters of *ATR* and *p53*, and enhancer of *p21* encompassing the BCL6 binding sites were amplified by real-time PCR. (b) CTB-1 and KMS12PE-BCL6 cells were exposed to 50  $\mu$ M 79-6 or DMSO for 8 h and subjected to RNA quantification. RNA expression levels of *ATR*, *p53*, and *p21* are presented as the fold change relative to KMS12PE-BCL6 cells exposed to DMSO. \*\* $P < 0.01$ . ns, not significant.

primary event after DNA double-strand breaks.<sup>(23)</sup> Formation of  $\gamma$ H2AX is required for the assembly of DNA repair proteins at the sites of damage as well as for the activation of checkpoint proteins, which lead to cell cycle arrest. To examine whether BCL6 plays a role in DNA damage response, formation of  $\gamma$ H2AX was analyzed in KMS12PE-BCL6 cells. Quantitative analysis of  $\gamma$ H2AX foci formation by immunofluorescence staining indicated that the dose-dependent increase in the mean density of the  $\gamma$ H2AX signal following IR exposure was significantly suppressed by BCL6 expression (Fig. 3a,b). Analysis of  $\gamma$ H2AX by flow cytometry also showed similar results, in which the induction of  $\gamma$ H2AX was significantly lower in KMS12PE-BCL6 cells than KMS12PE-Empty cells in response to both IR and etoposide exposure (Fig. 3c,d, respectively). These results suggest that BCL6 attenuates the initial step of the DDR signaling pathway through repression of  $\gamma$ H2AX formation in KMS12PE cells.

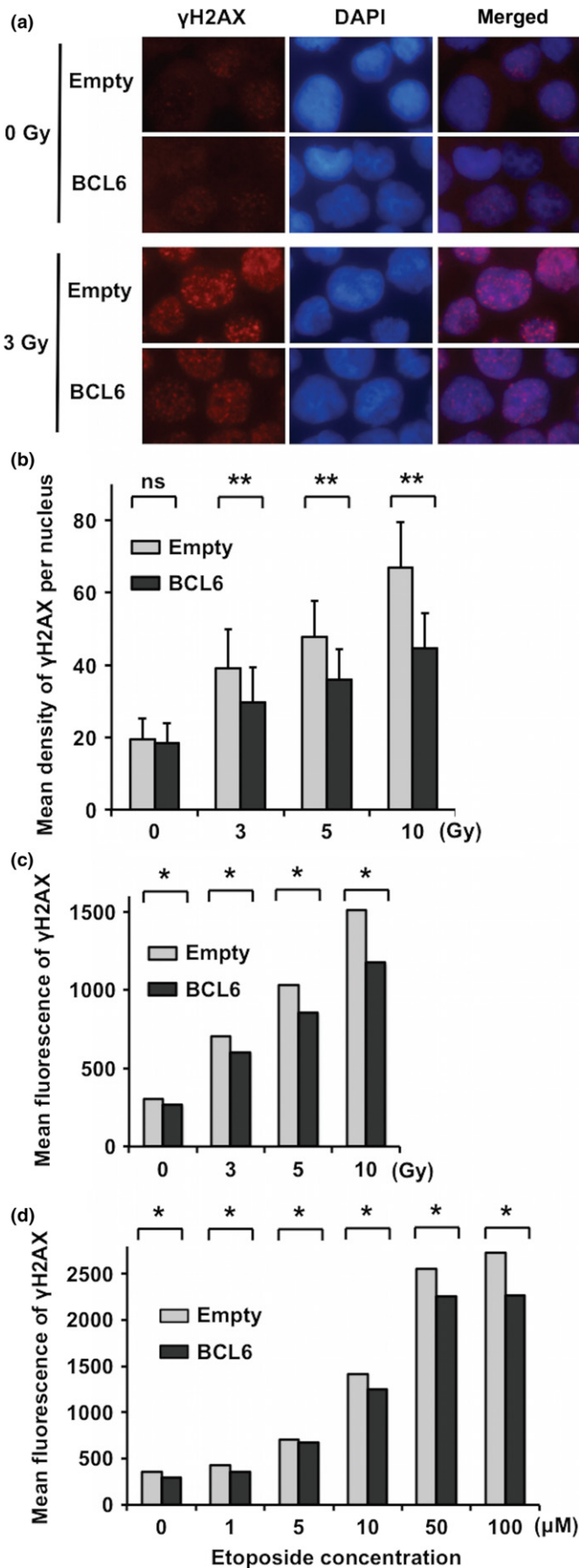
**Expression level of *ATM* repressed by BCL6 overexpression in KMS12PE cells.** Ionized radiation-induced  $\gamma$ H2AX foci formation is known to depend on ATM, but not on ATR.<sup>(23)</sup> We have already shown that overexpression of BCL6 failed to suppress *ATR* expression (Fig. 1e). To further examine the possibility that the repression of  $\gamma$ H2AX formation in KMS12PE-BCL6 is mediated by ATM, the total protein expression and phosphorylation form of ATM was assessed by immunoblotting before and after IR exposure. The reproducible results showed that overexpression of BCL6 caused a reduction in both the total ATM proteins and its phosphorylated forms after DNA damage induced by IR (approximately 39% and 28% reduction for total protein and phosphorylated

forms, respectively). Phosphorylation of p53 was also reduced by BCL6 overexpression after IR exposure (approximately 30% reduction) (Fig. 4a,b), which is a reasonable result as ATM is responsible for Ser15 phosphorylation of p53.

Moreover, real-time PCR showed that the BCL6 overexpression repressed *ATM* transcripts both before and after IR at same level (Fig. 4c), whereas the *p53* gene was not affected (Fig. 4d). To investigate whether the repression of *ATM* by BCL6 was mediated through its BTB domain, the cells were exposed to 79-6. Exposure to 79-6 failed to affect the expression of *ATM* mRNA in KMS12PE-BCL6 cells, suggesting that the BTB domain is not involved in this process in MM cells (Fig. 4e).

To further investigate the reason for the discrepancy between transcriptional repression and protein level change before and after IR, we speculated that the reduction in *ATM* transcription may not have affected the protein levels in a steady state because of slow degradation. We looked at the protein degradation time course of ATM with and without IR using cycloheximide. As shown in Figure 4(f,g), the ATM protein level was steady without IR but rapidly reduced after IR, suggesting faster degradation following DNA damage.

**Overexpression of BCL6 led to upregulation of *AID* and down-regulation of *CD138* in KMS12PE cells.** To investigate the effects of BCL6 overexpression on KMS12PE cells, mRNA sequence analysis was carried out. The total number of differentially expressed genes by KMS12PE with BCL6 overexpression was 2274, including 1265 upregulated genes and 1009 downregulated genes (Fig. 5a). Pathway analysis by iPathwayGuide software (Advaita, <http://www.advaitabio.com/ipathwayguide>)



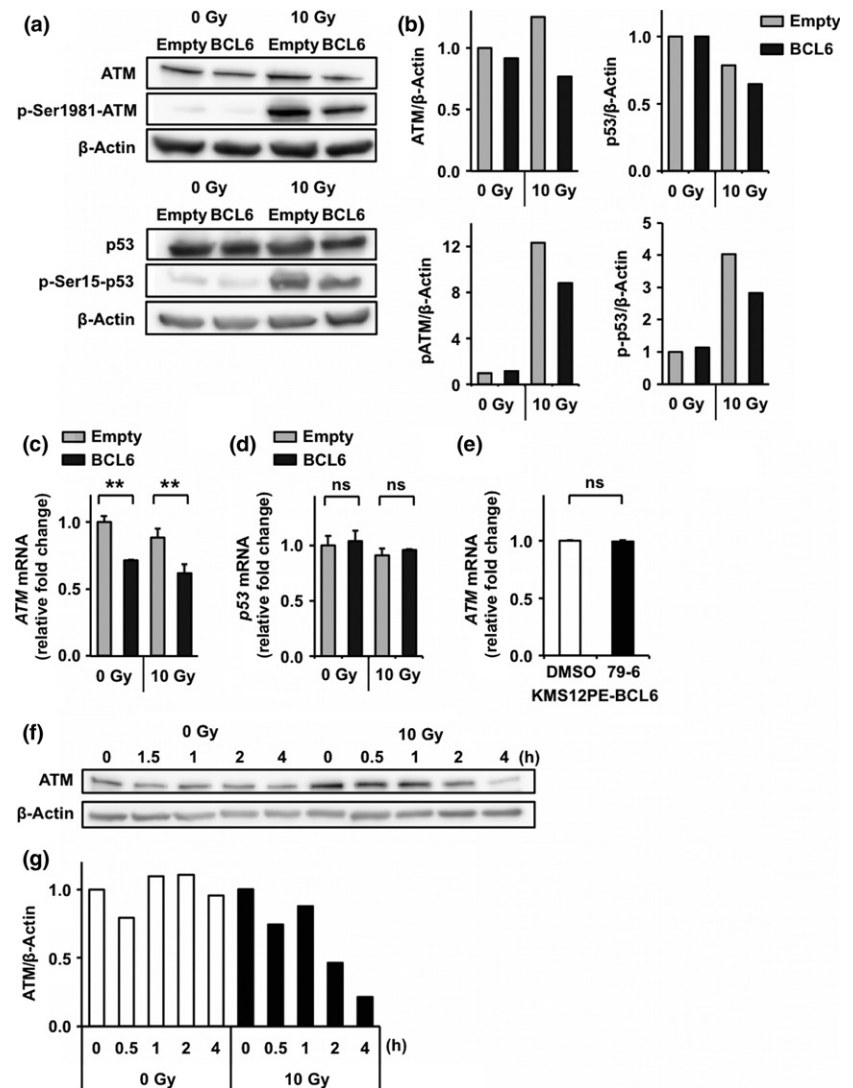
**Fig. 3.** Histone H2AX ( $\gamma$ H2AX) formation after DNA damage was repressed by B-cell lymphoma 6 (BCL6) overexpression in KMS12PE multiple myeloma cells. (a) KMS12PE-BCL6 and -Empty cells were irradiated by 3, 5, or 10 Gy and subjected to immunofluorescence staining for  $\gamma$ H2AX. Original magnification,  $\times 1000$ . Only the 3 Gy results are shown. (b) Mean densities of  $\gamma$ H2AX expression per nucleus in 100 cells of each group were measured by densitometry. The graph shows the data as mean  $\pm$  SD ( $n = 100$  per bar). \*\* $P < 0.01$ . ns, not significant.  $\gamma$ H2AX formation in KMS12PE-BCL6 and -Empty cells after several doses of ionized radiation (c) or etoposide (d) was measured by flow cytometry. The results are shown as the mean fluorescence intensity of  $\gamma$ H2AX. \* $P < 0.01$ .

revealed significantly impacted pathways, such as those associated with cell adhesion and cytokine–cytokine receptor interaction (both  $P < 0.001$ ). Neither the DNA repair pathway nor cell cycle checkpoints were extracted as a source of significantly different genes by BCL6 overexpression. Among the differentially expressed genes, we found an unexpected upregulation of the *AID* gene (Fig. 5a,b), which was verified by real-time PCR (Fig. 5c). As the expression of *AID* and *BCL6* is primarily restricted to GC B cells,<sup>(24,25)</sup> we tried to seek some other genes, which were known to be preferentially expressed in GC B cells or differentiated plasma cells from mRNA sequence data. As a result, we identified *SPIB* and *CIITA* as upregulated genes and *XBPI* and *SDCI* as downregulated genes (Fig. 5a,b). *SPIB* is required for normal GC reactions and prevents plasma cell differentiation,<sup>(26,27)</sup> and *CIITA* is required for the expression of MHC class II proteins and characteristics of B cells and is extinguished in terminally differentiated plasma cells.<sup>(28)</sup> *XBPI* is one of the essential transcriptional regulators for plasma cell differentiation,<sup>(29,30)</sup> and *SDCI* encodes terminally differentiated plasma cell marker CD138. These findings were verified by real-time PCR (Fig. 5d). Decreased CD138 expression in BCL6-transfected cells was confirmed by flow cytometric analysis (Fig. 5e). In addition, real-time PCR also showed BCL6 overexpression repressed expression of *PRDM1*, a master regulator of plasma cell differentiation (Fig. 5f), which is consistent with previous reports,<sup>(30)</sup> although the mRNA sequence analysis did not show the downregulation significantly. *PRDM1* has been shown to directly or indirectly control *CIITA* and *XBPI*.<sup>(31,32)</sup> Taken together, the gene expression profile of BCL6-transfected KMS12PE cells suggested the de-differentiation from plasma cells to B cells.

**Interleukin-6 exposure upregulated *BCL6* and *AID*, repressed *ATM*, and attenuated *DDR* in KMS12PE cells.** In the bone marrow microenvironment, IL-6 is reported to induce BCL6 expression in tumor cells of MM patients.<sup>(16)</sup> We also confirmed that *BCL6* was upregulated by treatment with recombinant IL-6 in KMS12PE and other cell lines except for RPMI8226 (Fig. 6a). Furthermore, IL-6 stimulation repressed *ATM* transcription in KMS12PE and KMS12BM (Fig. 6b), but not in other cell lines (data not shown). We also showed an attenuated  $\gamma$ H2AX formation induced by IR-dependent DNA damage in KMS12PE cells after IL-6 treatment (Fig. 6c). Moreover, IL-6 stimulation downregulated *SDCI* (CD138) (Fig. 6d) and upregulated *AID* (Fig. 6e) in this cell line. These phenotypic and functional properties in IL-6-treated KMS12PE cells resemble those of BCL6-transfected KMS12PE cells.

## Discussion

The expression levels of BCL6 in some of the patient samples were significantly higher than a B cell tumor cell line, which



**Fig. 4.** Expression level of *ATM* was repressed by B-cell lymphoma 6 (*BCL6*) overexpression in KMS12PE multiple myeloma cells. (a, b) KMS12PE-BCL6 and -Empty cells were irradiated by 10 Gy and analyzed by immunoblotting. *ATM* (c) and *p53* (d) RNA in KMS12PE-BCL6 and -Empty cells with or without ionized radiation (IR) are shown as the fold change relative to control cells without IR. (e) Expression levels of *ATM* of KMS12PE-BCL6 cells after exposure to 50  $\mu$ M 79-6 (*BCL6* inhibitor) are shown. (f, g) KMS12PE-BCL6 cells were treated with cycloheximide (80  $\mu$ g/mL) to block protein synthesis with or without 10 Gy IR. At 0, 0.5, 1, 2, and 4 h after IR, cell samples were collected and analyzed by immunoblotting. Relative band intensities were calculated by the protein/ $\beta$ -actin ratios and normalized to the control cells without IR in each protein (b), and at 0 h after IR (g). \*\* $P < 0.01$ . ns, not significant.

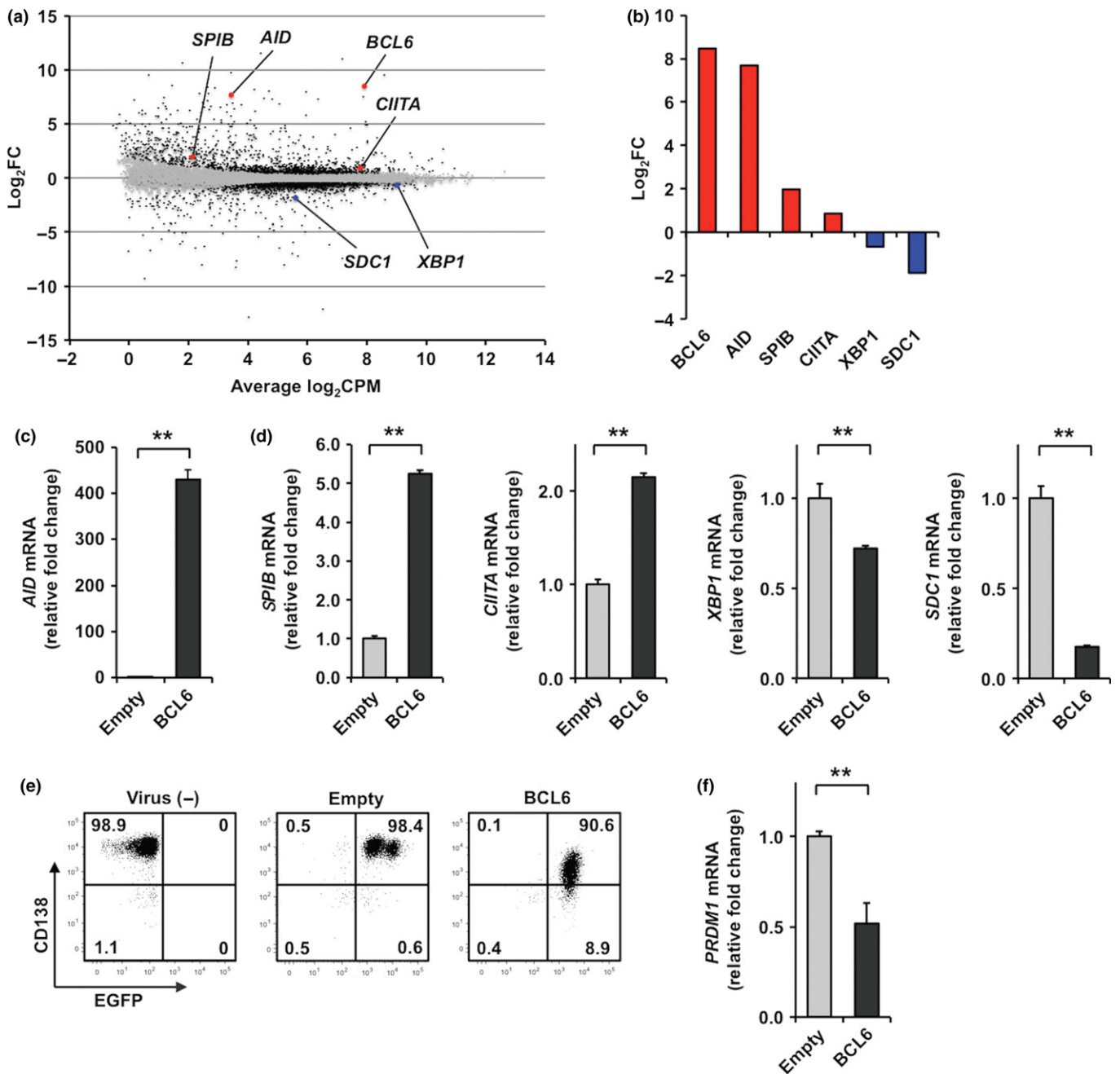
supports our experimental system using an MM cell line stably expressing *BCL6*. Using this model, the following results were observed. First, overexpression of *BCL6* induced downregulation of *ATM*, a major DDR signaling kinase, but not other DDR genes. *ATM* repression was associated with decreased  $\gamma$ H2AX formation following DNA damage. Second, decrease in the *ATM* protein levels became obvious in KMS12PE-BCL6 cells after irradiation due to the rapid degradation of *ATM* protein. Third, mRNA sequence analysis identified *AID*, primarily expressed by GC B cells, as one of the upregulated genes in KMS12PE-BCL6 cells. Upregulation in other GC B cell-related genes, *SP1B* and *CIITA*, and downregulation of plasma cell differentiation genes, such as *XBPI1*, *SDC1*, and *PRDM1*, were observed. Finally, IL-6 exposure to KMS12PE cells resulted in phenotypic and functional changes similar to those observed in KMS12PE-BCL6 cells: upregulation of *BCL6* and *AID*, downregulation of *ATM* and *SDC1*, and attenuation of DDR. These results suggest that *BCL6* overexpression in these MM cells leads to de-differentiation of plasma cells to GC B cells and has a significant impact on genomic instability.

In spite of the pivotal role of *ATM* in the genotoxic stress response, there are a few reports on its transcriptional regulation.<sup>(33–35)</sup> Although the detailed mechanism of this repression

is unknown, our study is the first to show that *BCL6* leads to the transcriptional repression of *ATM* in myeloma cells. As the *BCL6*-BTB domain inhibitor did not affect *ATM* expression in KMS12PE-BCL6 cells, further studies are necessary to determine the mechanisms of *BCL6*-dependent downregulation of *ATM* in MM cells.

In spite of the downregulation of *ATM* transcription in KMS12PE-BCL6 cells, we found no difference in the *ATM* protein levels before DNA damage. We also showed the fast degradation of *ATM* protein in DNA damage response, which is in concordance with the previous report that *ATM* cleavage occurs after DNA damage.<sup>(36)</sup>

Additionally, we found that *BCL6* overexpression in KMS12PE cells led to changes in the gene expression profile, implying de-differentiation from plasma cells to GC B cells. Our finding is consistent with a recent study that showed that exogenous expression of *BCL6* in a plasma cell line leads to repression of plasma cell-specific transcripts and re-expression of B-cell surface markers.<sup>(37)</sup> Furthermore, we found upregulation of *AID* in KMS12PE-BCL6 cells. *AID* is a member of the APOBEC family, a well-known DNA mutator, which targets not only immunoglobulin genes but also a number of others, such as oncogenes. *AID* is implicated in oncogenic mutations and chromosomal translocations, especially those involving

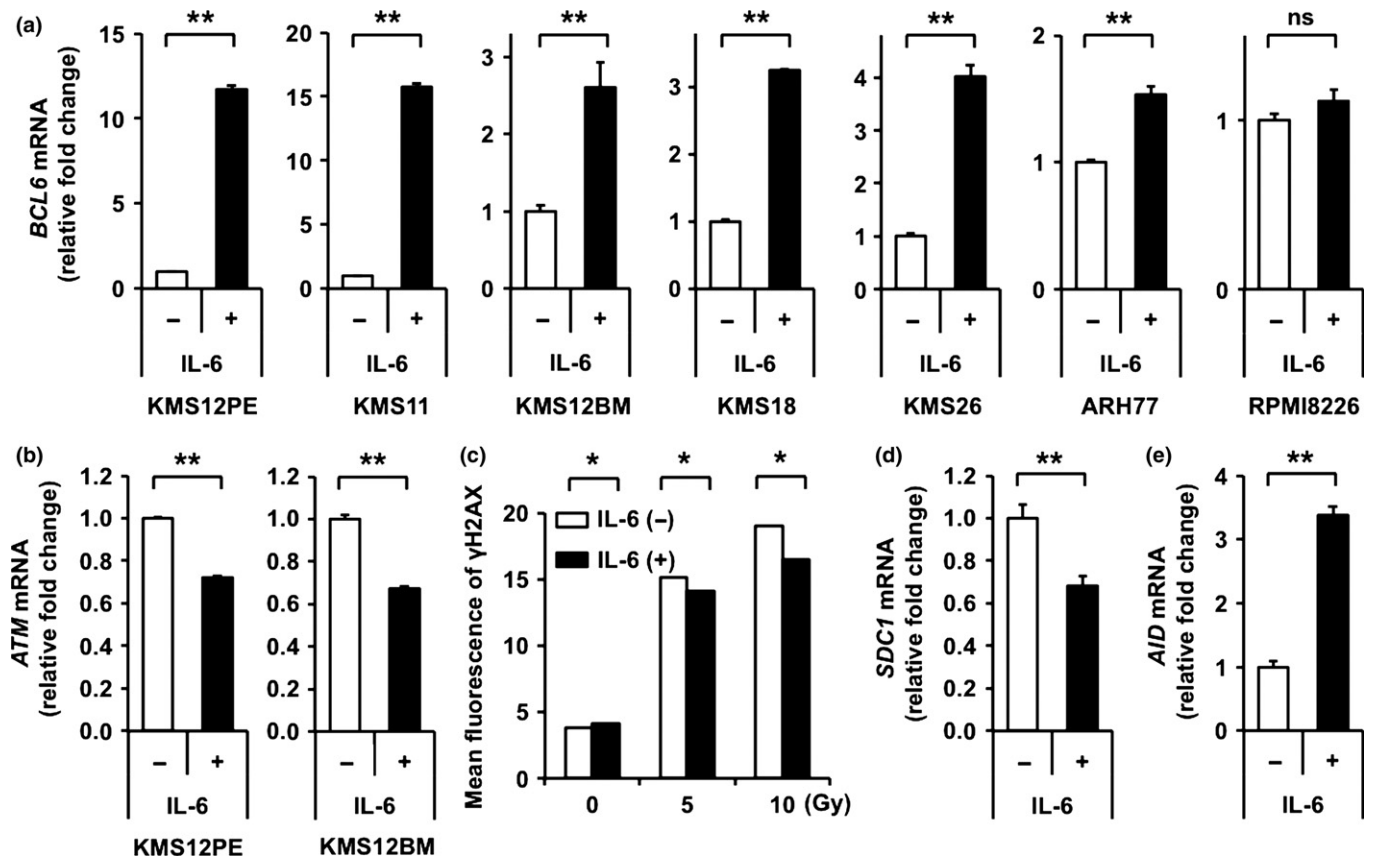


**Fig. 5.** B-cell lymphoma 6 (BCL6) overexpression led to upregulation of *AID* and downregulation of CD138 in KMS12PE multiple myeloma cells. (a) Global differential expression map of mRNA sequencing of KMS12PE-BCL6 versus -Empty cells. The Y-axis represents the logarithm of the fold change ( $\log_2FC$ ) and the X-axis represents the average logarithm of counts per million of reads ( $\log_2CPM$ ). Black dots represent differentially expressed genes ( $n = 2274$ ); gray dots, not significantly differentially expressed genes ( $n = 9889$ ); red dots, genes we focused on. (b)  $\log_2FC$  of genes which relate to GC B cell or plasma cell type are shown. Real-time PCR was used to measure RNA expression level of *AID* (c), *SPIB*, *CIITA*, *XBP1*, and *SDC1* (d), and *PRDM1* (f) in KMS12PE-BCL6 cells, presented as the fold change relative to that of KMS12PE-Empty. The data are shown as the mean  $\pm$  SD ( $n = 3$  per bar). \*\* $P < 0.01$ . ns, not significant. (e) We determined the expression level of CD138 in KMS12PE-BCL6 and -Empty cells and uninfected KMS12PE cells, referred to as virus (-), using flow cytometry.

immunoglobulin genes, which results in B-cell and plasma cell tumorigenesis.<sup>(38–40)</sup> It has been reported that the AID/APO-BEC-mediated mutational signature is associated with poor prognosis of myeloma patients.<sup>(41)</sup> Taken together, BCL6-dependent AID upregulation is a threat to the genomic integrity of myeloma cells and thus may affect disease progression.

We showed that IL-6 treatment of KMS12PE cells resulted in phenotypic and functional changes consistent with those

obtained by BCL6 overexpression. This report is the first to show that IL-6, possibly through the upregulation of BCL6, can affect DDR or genomic instability in MM cells. There are several reports suggesting that some cytokines attenuate DNA repair by disruption of nuclear localization of DNA repair proteins or upregulation of AID.<sup>(42,43)</sup> In MM cells, there is a report that IL-6 stimulation represses basal expression of excision repair genes.<sup>(44)</sup> Koduru *et al.*<sup>(5)</sup> showed that a tumor



**Fig. 6.** Interleukin-6 (IL-6) exposure upregulated *BCL6* and *AID*, downregulated *ATM*, and attenuated DNA damage response in KMS12PE multiple myeloma cells. Real-time PCR was used to measure RNA expression levels of *BCL6* (a) in multiple myeloma cell lines KMS11, KMS12PE, KMS12BM, KMS18, KMS26, ARH77, and RPMI8226, *ATM* (b), *SDC1* (d), and *AID* (e) in KMS12PE cells treated with 122 ng/mL recombinant human IL-6 for 16 h. The expression levels are presented as the fold change relative to each expression level of the cells without exposure to IL-6. (c) Histone H2AX ( $\gamma$ H2AX) formation in KMS12PE cells with or without exposure to 100 ng/mL IL-6 after exposure to 0, 5, or 10 Gy ionized radiation, and incubation for 1 h was assessed by flow cytometry. The results are shown as the mean fluorescence of  $\gamma$ H2AX. \* $P < 0.01$ .

microenvironment can upregulate *AID* in MM cells isolated from a patient's bone marrow. Our results indicating that IL-6 downregulated genomic guardian *ATM* and upregulated genomic mutator *AID* are in line with those of previous reports suggesting that genomic integrity could be threatened by extracellular factors. Thus, our results shed light on the mechanisms of the high frequency of somatic mutation in MM cells.<sup>(45)</sup> Given whole genome sequencing studies that showed that mutations of DNA repair pathway genes occur infrequently in MM patients, mechanisms other than cell intrinsic factors may operate in the dysregulation of DDR.<sup>(6,7)</sup>

There are several limitations to the present study. Our findings were obtained by examining a single cell line and the expression levels of *ATM* and *AID* in response to IL-6 were different. What makes the difference between these cell lines are unknown. Further studies are necessary to solve these questions.

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### Disclosure Statement

The authors have no conflict of interest.

### Abbreviations

AID	activation induced cytidine deaminase
APOBEC	apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BCL6	B-cell lymphoma 6
CIITA	class II major histocompatibility complex transactivator
DDR	DNA damage response
DLBCL	diffuse large B-cell lymphoma
GC	germinal center
IL-6	interleukin-6
IR	ionized radiation
MM	multiple myeloma
PE	phycoerythrin
PRDM1	PR/SET domain 1
SDC1	syndecan 1



SPIB Spi-B transcription factor  
XBP1 X-box binding protein 1

$\gamma$ H2AX phosphorylated H2AX

## References

- 1 Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. *Nat Rev Cancer* 2002; **2**: 175–87.
- 2 Neri P, Bahlis NJ. Genomic instability in multiple myeloma: mechanisms and therapeutic implications. *Expert Opin Biol Ther* 2013; **13**(Suppl 1): S69–82.
- 3 Shammas MA, Shmookler Reis RJ, Koley H, Batchu RB, Li C, Munshi NC. Dysfunctional homologous recombination mediates genomic instability and progression in myeloma. *Blood* 2009; **113**: 2290–7.
- 4 Herrero AB, San Miguel J, Gutierrez NC. Deregulation of DNA double-strand break repair in multiple myeloma: implications for genome stability. *PLoS ONE* 2015; **10**: e0121581.
- 5 Koduru S, Wong E, Strowig T *et al*. Dendritic cell-mediated activation-induced cytidine deaminase (AID)-dependent induction of genomic instability in human myeloma. *Blood* 2012; **119**: 2302–9.
- 6 Walker BA, Boyle EM, Wardell CP *et al*. Mutational spectrum, copy number changes, and outcome: results of a sequencing study of patients with newly diagnosed myeloma. *J Clin Oncol* 2015; **33**: 3911–20.
- 7 Chapman MA, Lawrence MS, Keats JJ *et al*. Initial genome sequencing and analysis of multiple myeloma. *Nature* 2011; **471**: 467–72.
- 8 Ye BH, Lista F, Lo Coco F *et al*. Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. *Science* 1993; **262**: 747–50.
- 9 Baron BW, Nucifora G, McCabe N, Espinosa R 3rd, Le Beau MM, McKeithan TW. Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas. *Proc Natl Acad Sci USA* 1993; **90**: 5262–6.
- 10 Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000; **102**: 553–63.
- 11 Nagaoka H, Muramatsu M, Yamamura N, Kinoshita K, Honjo T. Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. *J Exp Med* 2002; **195**: 529–34.
- 12 Huang C, Hatzi K, Melnick A. Lineage-specific functions of Bcl-6 in immunity and inflammation are mediated by distinct biochemical mechanisms. *Nat Immunol* 2013; **14**: 380–8.
- 13 Ranuncolo SM, Polo JM, Dierov J *et al*. Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat Immunol* 2007; **8**: 705–14.
- 14 Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-center B cells. *Nature* 2004; **432**: 635–9.
- 15 Phan RT, Saito M, Basso K, Niu H, Dalla-Favera R. BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. *Nat Immunol* 2005; **6**: 1054–60.
- 16 Hideshima T, Mitsiades C, Ikeda H *et al*. A proto-oncogene BCL6 is up-regulated in the bone marrow microenvironment in multiple myeloma cells. *Blood* 2010; **115**: 3772–5.
- 17 Takizawa M, Nobuhisa I, Igarashi K *et al*. Requirement of gp130 signaling for the AGM hematopoiesis. *Exp Hematol* 2003; **31**: 283–9.
- 18 Hatzi K, Jiang Y, Huang C *et al*. A hybrid mechanism of action for BCL6 in B cells defined by formation of functionally distinct complexes at enhancers and promoters. *Cell Rep* 2013; **4**: 578–88.
- 19 Muslimovic A, Ismail IH, Gao Y, Hammarsten O. An optimized method for measurement of gamma-H2AX in blood mononuclear and cultured cells. *Nat Protoc* 2008; **3**: 1187–93.
- 20 Ghetu AF, Corcoran CM, Cerchietti L, Bardwell VJ, Melnick A, Prive GG. Structure of a BCOR corepressor peptide in complex with the BCL6 BTB domain dimer. *Mol Cell* 2008; **29**: 384–91.
- 21 Ahmad KF, Melnick A, Lax S *et al*. Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Mol Cell* 2003; **12**: 1551–64.
- 22 Cerchietti LC, Ghetu AF, Zhu X *et al*. A small-molecule inhibitor of BCL6 kills DLBCL cells in vitro and in vivo. *Cancer Cell* 2010; **17**: 400–11.
- 23 Lobrich M, Shibata A, Beucher A *et al*. gammaH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization. *Cell Cycle* 2010; **9**: 662–9.
- 24 Muramatsu M, Sankaranand VS, Anant S *et al*. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 1999; **274**: 18470–6.
- 25 Basso K, Dalla-Favera R. Roles of BCL6 in normal and transformed germinal center B cells. *Immunol Rev* 2012; **247**: 172–83.
- 26 Su GH, Chen HM, Muthusamy N *et al*. Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B. *Embo J* 1997; **16**: 7118–29.
- 27 Schmidlin H, Diehl SA, Nagasawa M *et al*. Spi-B inhibits human plasma cell differentiation by repressing BLIMP1 and XBP-1 expression. *Blood* 2008; **112**: 1804–12.
- 28 Boss JM, Jensen PE. Transcriptional regulation of the MHC class II antigen presentation pathway. *Curr Opin Immunol* 2003; **15**: 105–11.
- 29 Shaffer AL, Shapiro-Shelef M, Iwakoshi NN *et al*. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* 2004; **21**: 81–93.
- 30 Lin KI, Tunyaplin C, Calame K. Transcriptional regulatory cascades controlling plasma cell differentiation. *Immunol Rev* 2003; **194**: 19–28.
- 31 Smith MA, Wright G, Wu J *et al*. Positive regulatory domain I (PRDM1) and IRF8/PU.1 counter-regulate MHC class II transactivator (CIITA) expression during dendritic cell maturation. *J Biol Chem* 2011; **286**: 7893–904.
- 32 Calame KL, Lin KI, Tunyaplin C. Regulatory mechanisms that determine the development and function of plasma cells. *Annu Rev Immunol* 2003; **21**: 205–30.
- 33 Kim WJ, Vo QN, Shrivastav M, Lataxes TA, Brown KD. Aberrant methylation of the ATM promoter correlates with increased radiosensitivity in a human colorectal tumor cell line. *Oncogene* 2002; **21**: 3864–71.
- 34 Berkovich E, Ginsberg D. ATM is a target for positive regulation by E2F-1. *Oncogene* 2003; **22**: 161–7.
- 35 Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* 2013; **14**: 197–210.
- 36 Smith GC, d'Adda di Fagnana F, Lakin ND, Jackson SP. Cleavage and inactivation of ATM during apoptosis. *Mol Cell Biol* 1999; **19**: 6076–84.
- 37 Fujita N, Jaye DL, Geigerman C *et al*. MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation. *Cell* 2004; **119**: 75–86.
- 38 Robbiani DF, Bunting S, Feldhahn N *et al*. AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. *Mol Cell* 2009; **36**: 631–41.
- 39 Chesi M, Robbiani DF, Sebag M *et al*. AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. *Cancer Cell* 2008; **13**: 167–80.
- 40 Takizawa M, Tolarova H, Li Z *et al*. AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. *J Exp Med* 2008; **205**: 1949–57.
- 41 Walker BA, Wardell CP, Murison A *et al*. APOBEC family mutational signatures are associated with poor prognosis translocations in multiple myeloma. *Nat Commun* 2015; **6**: 6997.
- 42 Tseng-Rogenski SS, Hamaya Y, Choi DY, Carethers JM. Interleukin 6 alters localization of hMSH3, leading to DNA mismatch repair defects in colorectal cancer cells. *Gastroenterology* 2015; **148**: 579–89.
- 43 Duan Z, Zheng H, Liu H *et al*. AID expression increased by TNF-alpha is associated with class switch recombination of Igalpha gene in cancers. *Cell Mol Immunol* 2016; **13**: 484–91.
- 44 Peng B, Hodge DR, Thomas SB *et al*. Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. *J Biol Chem* 2005; **280**: 4182–7.
- 45 Alexandrov LB, Nik-Zainal S, Wedge DC *et al*. Signatures of mutational processes in human cancer. *Nature* 2013; **500**: 415–21.