

MK2 is a therapeutic target for high-risk multiple myeloma

Multiple myeloma (MM) is an incurable plasma cell malignancy characterized by heterogeneous genetic diversity. Although the development of proteasome inhibitors and immunomodulatory drugs combined with autologous stem cell transplantation (ASCT) have achieved advanced improvement for MM treatment, the majority of MM patients ultimately relapse.^{1,2} One hypothesis for relapse is the cytogenetic evolution of drug-resistant MM cells and the generation of more aggressively proliferative subclones over the patients' disease course. Recent studies support this hypothesis and demonstrate that the existence of intraclonal heterogeneity in MM and genome of high-risk patients with poor outcome and survival present more changes over the disease course.³ The progression of modern high-throughput genomic and proteomic analytical technique such as gene expression profile (GEP) and whole exome or genome sequencing combined with bioinformatic and bio-statistic approaches has aided the investigation of these MM clinical samples.^{4,5} Based on GEP analysis of sequential MM primary samples during the disease course, characterized by serial cycles of response, remission, and relapse combined with health donor control, our group identified a serial of genes including *NEK2*, *RARα2*, which induce MM proliferation and drug-resistance resulting in MM relapse and poor outcome.⁶

MAPKAPK2 (MK2), a major substrate of p38, is regulated through direct phosphorylation by p38 MAP kinase, and participates in many cellular processes such as stress and inflammatory responses, cell proliferation and gene expression regulation.^{7,8} To date, abnormality of MK2 is associated with a broad range of cancers, including glioblastoma, lung and bladder cancer.⁹ Intriguingly, p38-

MK2-Hsp27 signaling maintains survival of cancer stem cells,¹⁰ which is regarded as an obstacle of MM treatment and the resource for MM relapse in clinics suggesting MK2 is a promising therapeutic target in MM. However, MK2 has received little attention in MM.

In order to explore the role of MK2 in MM, we examined MK2 expression of normal plasma cells (NP) (n=22), monoclonal gammopathy of undetermined significance cells (MGUS) (n=44) and newly diagnosed myeloma patient plasma cells (n=351) using our GEP database collected from the National Institutes of Health Gene Expression Omnibus GSE2658 and the result showed significantly increased MK2 expression in MM cells compared to NP and MGUS cells (*data not shown*).¹¹ Following analysis of array-based comparative genomic hybridization (aCGH) data, GSE4452, collected from 67 MM patients indicated that the *MK2* locus was frequently amplified in MM patient samples relative to normal control (*data not shown*).¹² We further observed elevation of MK2 expression in high-risk MM patients compared to low-risk patients (Figure 1A). The expression of MK2 in the PR (high proliferation) and MS (MMSET translocation) groups, the worst two subgroups in MM patients, was dramatically elevated compared to the other six groups¹³ (Figure 1B). Upon correlation analyses of MK2 with clinical characteristics, MK2 expression performed as an independent factor associated with parameters like C-reactive protein at least 4.0 mg/L ($P<0.05$), chromosomal abnormalities (by G-banding) ($P<0.05$), and magnetic resonance imaging focal bone lesions, at least three lesions, which were acknowledged as a poor diagnosed markers in MM (*data not shown*).

We further tested *MK2* mRNA expression in MM patients from APEX trials which evaluated the response to standard therapies (bortezomib or dexamethasone).¹⁴ A pronounced elevation of average *MK2* expression was observed in the no-response treatment group compared

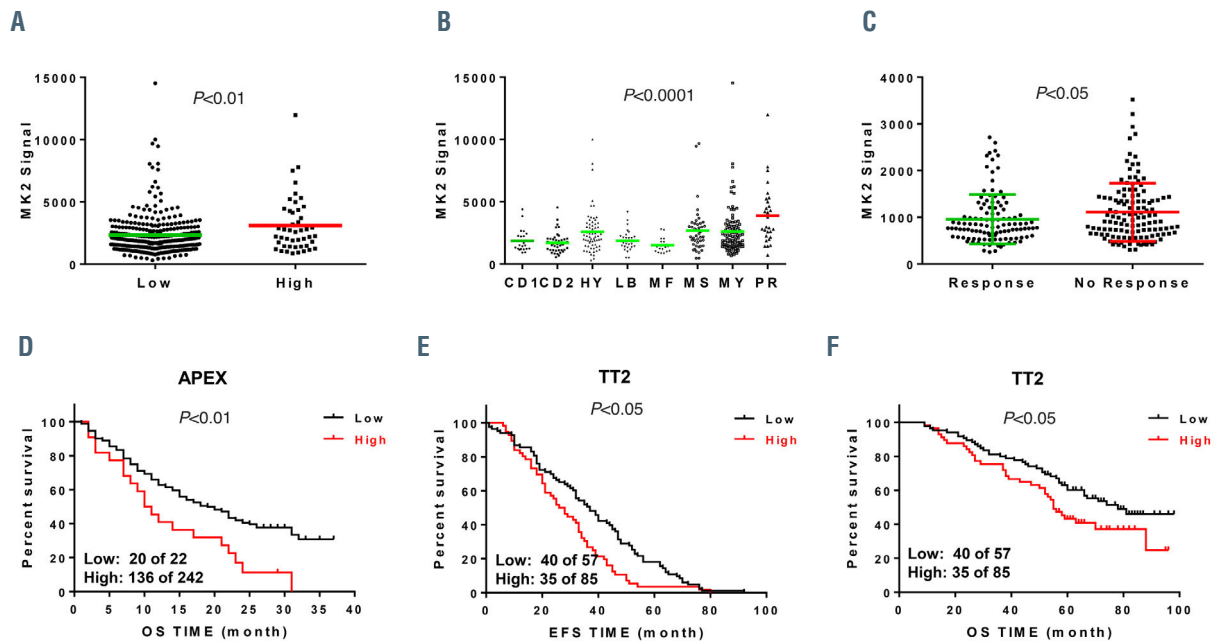


Figure 1. MK2 is a poor prognostic marker or a high-risk gene in multiple myeloma. (A) *MK2* expression in the TT2 high and low group based 70-gene model. (B) A box-plot exhibited the average *MK2* expression in eight multiple myeloma (MM) subgroups of the TT2 cohort. (C) A box-plot showed *MK2* expression in patients categorized by unresponsive (No Response) or responsive (Response) to treatment with dexamethasone and/or bortezomib. (D) Kaplan-Meier analysis on the MM patients' survival in APEX cohort divided by different *MK2* expression. (E and F) Kaplan-Meier curve on relapsed MM patients' event free survival (E) and overall survival (F) in the TT2 cohort divided by *MK2* expression.

to the response group indicating that MK2 may lead to drug-resistance in MM (Figure 1C). The distinction of MM patients with MK2 was clinically relevant and patients with high-MK2 expression had poor outcomes in the APEX cohorts (Figure 1D). Since the APEX cohort was comprised with relapsed MM patients, we also examined the patients who eventually relapsed in TT2 cohort. The results demonstrated that MM patients with higher MK2 expression were associated with poor survival, event free survival and overall survival (Figure 1E and F). These findings from two independent cohorts suggest that increased MK2 expression may lead to MM drug-resistance and relapse. Herein we propose that MK2 is a poor prognostic marker or a high-risk gene in MM.

In order to determine if MK2 plays a role as a high-risk gene in MM rather than a sequential phenomenon, we knocked down MK2 expression in MM cells using lentiviral single hairpin RNA (shRNA) transfection. We first detected the protein expression levels of MK2 in MM cells by western blot and found that all the nine MM cell lines, XG1, CAG, ARP1, U266, OMP2, H929, MM.1S, 8226 and OCI-MY5, used in this assay ubiquitously expressed MK2 (*data not shown*). Then we down-regulated MK2 expression in ARP1 and OCI-MY5 cells by lentiviral shRNA particles. As shown in Figure 2A, MK2 expression was remarkably knocked-down in MK2-shRNA transfected MM cells (KD) compared to the control (Ctrl). In order to expose the effect of MK2 on MM cell growth, KD and Ctrl cells were cultured for 5 days and cell numbers were counted daily. MK2-KD MM cells exhibited a significantly lower cell growth rate than the Ctrl cells in both ARP1 and OCI-MY5 cells (Figure 2B), which was also verified by MTT assay (*data not shown*). The growth inhibition effect of MK2-shRNA was further confirmed by a clonogenicity assay. As shown in Figure 2C, MK2-KD cells generated ample reduction of

colonies relative to corresponding control cells. The decreased growth rate of MK2-KD cells was ascribed to increased apoptotic cell death by MK2 inhibition, and flow cytometry showed that Annexin V positive cells significantly increased after MK2-shRNA transfection for 48h (Figure 2D). These results suggest MK2 expression is important for MM cell growth *in vitro*.

We further extended our findings to an *in vivo* study and injected both ARP1^{KD} and ARP1^{Ctrl} cells subcutaneously into the opposite side flanks of each NOD scid gamma mouse (NSG) mouse (n=4). Tumor diameters were measured and recorded twice a week to examine the growth rate of the tumor cells. After 4 weeks, the tumors produced by ARP1^{KD} cells were visibly smaller than their corresponding ARP1^{Ctrl} counterparts. The average weight of ARP1^{KD} tumors (0.39 g) was 25% lower than the control tumors (1.55 g; Figure 2E). Time course regression analyses of growth rates exhibited that the ARP1^{KD} tumors volume significantly fell behind the ARP1^{Ctrl} control tumors (Figure 2F). These results indicate that genetic knock-down of MK2 retards myeloma growth *in vivo*.

Inversely to the knockdown assay, we transfected MM cells with MK2 CRISPR lentiviral activation particles,¹⁵ and verified success of the transfection by western blot assay which showed a visible elevation of MK2 expression in the lentiviral-transfected (OE) cells compared with control cells (WT) (Figure 3A). The trypan blue cell number counting assay demonstrated that ARP1 and OCI-MY5 MK2-OE MM cells presented a higher growth rate than their WT counterpart after 5 days of culture (*data not shown*). Next, a colony formation assay was employed and indicated that regardless of experimental conditions, MK2-OE cells generated more colonies than WT cells. Initially, MK2-OE cells formed a higher number of colonies than WT cells. In addition, compared with WT cells, the growth capability of MK2-OE cells treated

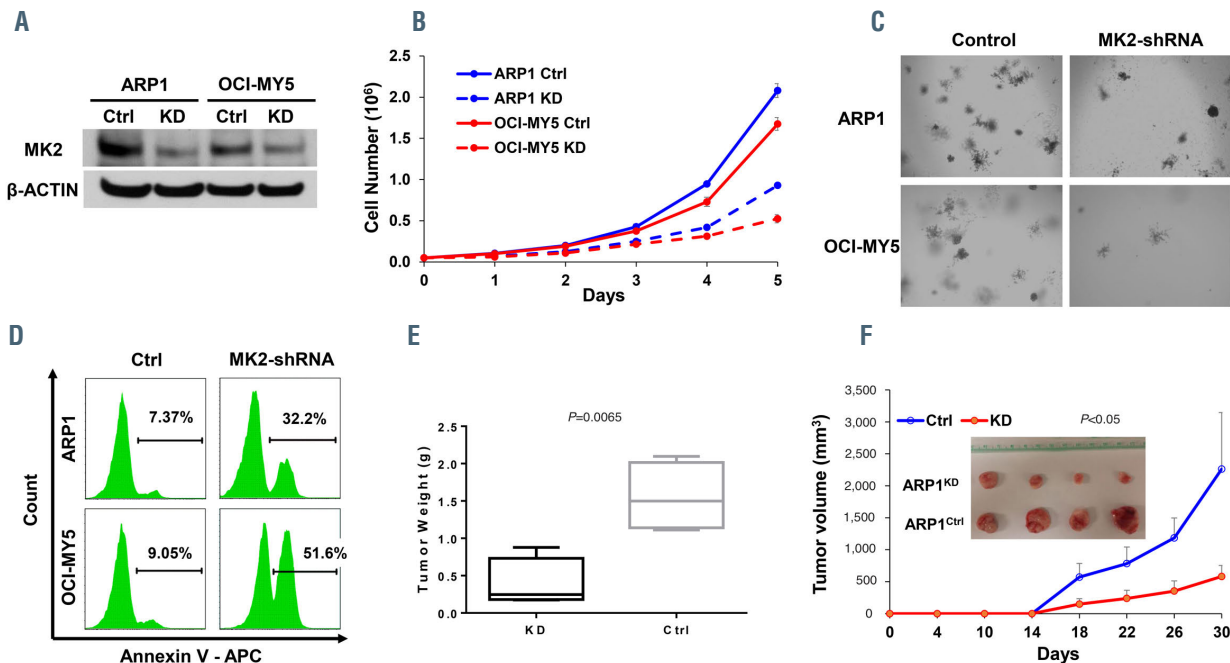


Figure 2. Decreased MK2 expression induces multiple myeloma cellular apoptosis and growth inhibition *in vitro* and *in vivo*. (A) MK2 expression in ARP1 and OCI-MY5 cells was measured by western blot after MK2-single hairpin RNA (shRNA) transfection. (B) Cell growth curve was drawn by trypan blue staining after observing ARP1 and OCI-MY5 MK2-knockdown (KD) and control (Ctrl) cells for 5 days. (C) Clonogenicity evaluation for the Ctrl and MK2-KD ARP1 and OCI-MY5 cells. (D) Flow cytometry for cellular apoptosis marker Annexin V in MM cells after MK2-shRNA lentivirus transfection for 48 hours. (E) Mean weight tumors derived from ARP1-Ctrl and ARP1-KD cells on day 30 post injection. (F) Tumor growth time course in NOD/SCID mice xenografted by ARP1-Ctrl and ARP1-KD cells in each flank respectively (n=4).

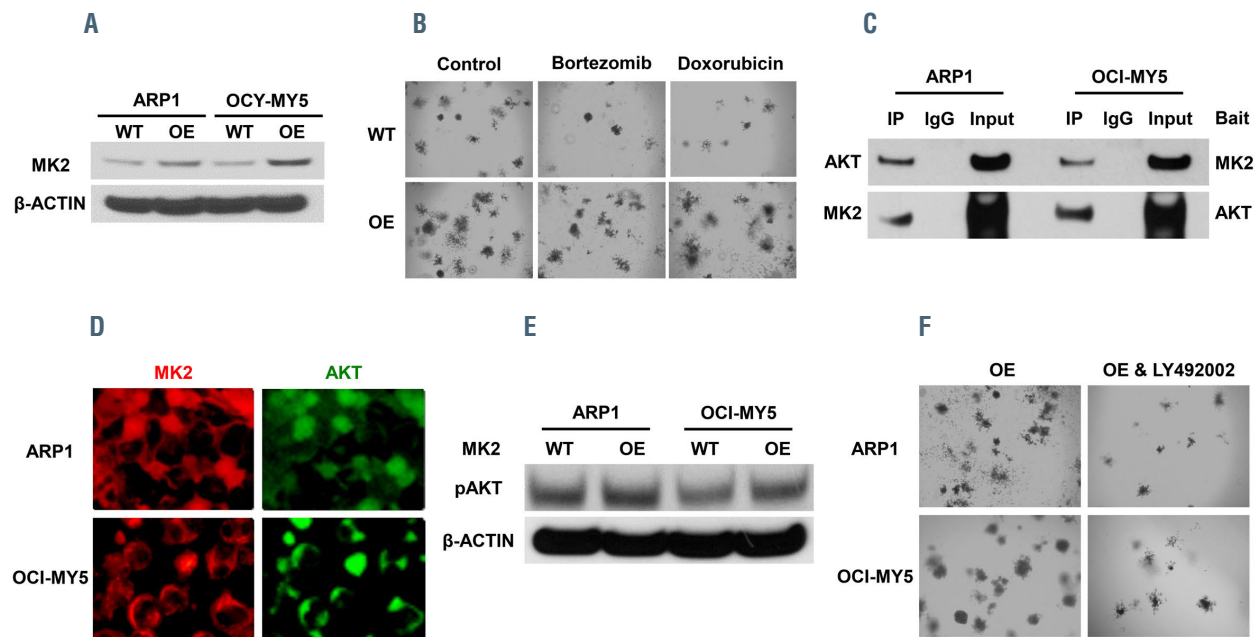


Figure 3. MK2 interacts with AKT to promote multiple myeloma progression. (A) Western blot assay on MK2 expression in ARP1 and OCI-MY5 wild-type (WT) and lentiviral-transfected (OE) cells. (B) Colony formation assay of ARP1 and OCI-MY5 MK2-WT and OE cells treated with or without bortezomib or doxorubicin. (C) Co-immunoprecipitation assay showed that MK2 interacted with AKT in MM cells. (D) Immunofluorescence staining on MK2, AKT and DAPI in ARP1 and OCI-MY5 cells. (E) Western blot assay on pAKT expression in ARP1 and OCI-MY5 MK2-OE cells treated with or without LY292002. (F) Colony formation of ARP1 and OCI-MY5 MK2-OE cells fed by medium in absence or presence of LY292002.

with bortezomib or doxorubicin was more prominent than that of MK2-OE cells without treatment (Figure 3B). Flow cytometric detection for Annexin V, a marker of apoptosis, illustrated the same trend, as treatment on cells with bortezomib (8 nM) or doxorubicin (100 nM) induced less death in the “OE” than “WT” samples (data not shown). These results support our proposal that MK2 promotes myeloma progression and drug resistance.

In order to analyze how MK2 mediates MM progression, a co-immunoprecipitation assay was performed to detect the down-stream target of MK2. We found that AKT could be immunoprecipitated by MK2 antibody. On the other hand, MK2 was pulled down using AKT antibody in both ARP1 and OCI-MY5 cells (Figure 3C). Further immunofluorescence study showed that the MK2 signal labeled by red color overlapped with green color representing the AKT signal (Figure 3D) in both ARP1 and OCI-MY5 cells. Both assays proved that MK2 directly bound with AKT in MM cells. As MK2 is a Ser/Thr protein kinase, we investigated whether MK2 could phosphorylate and activate AKT. Western blot results confirmed that pAKT(S473), the activated form of AKT, was up-regulated by MK2 overexpression compared to WT cells suggesting that MK2 phosphorylated AKT (Figure 3E). This interpretation was supported by the specific AKT phosphorylation inhibitor, LY292002, which overcame the MK2 activation induced MM cellular drug-resistance and profoundly suppressed clonogenicity in ARP1 and OCI-MY5 OE cells (Figure 3F). A plausible conclusion is, thus, that MK2 promotes MM progression through directly activating AKT. In addition, we also validated that MK2 inhibitor IV, a selective MK2 inhibitor had an inhibitory effect on MM cells both *in vitro* and in 5TGM1 MM mouse model (*data not shown*).

In summary, we first evaluated MK2 expression in MM cells relative to normal control cells, and correlated MK2 with MM patient outcomes in relapsed MM patients. We

also showed that MK2 mediated MM cellular growth and drug-resistance. Finally, we disclosed that MK2 regulates MM progression through activating AKT signaling. Our findings indicate that MK2 acts as a novel clinical marker for high-risk myeloma. Targeting MK2 in combination with current therapies may improve effectiveness and long-term patient response to treatment.

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