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MK2 is a therapeutic target for high-risk multiple myeloma

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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 the intraclonal heterogeneity in MM and genomes of high-risk patients with poor outcome and survival presented more changes over 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 disease course, characterized by serial cycles of response, remission, and relapse combined with health donor control, our group have identified a serial of genes like NEK2, RARα2 et al, 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-MAPKAPK2-Hsp27 signaling maintains survival of cancer stem cells (10), which are regarded as obstacle of MM treatment and the resource for MM relapse in clinics suggesting MK2 is a promising therapeutic target in MM. However, MK2 received little attention in MM.

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 (NP, n=22) collected from NIH 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 is 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 6 groups (13) (Figure 1B). Upon correlation analyses of MK2 with clinical characteristics, MK2 expression performed as an independent factor associated with parameters like CRP at least 4.0mg/l (p<0.05), Chromosomal abnormalities (by G-banding) (p<0.05), and MRI focal bone lesions, at least three, which are acknowledged as a poor diagnosed marker 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 to the response group indicating that MK2 may lead to drug-resistance in MM (Figure 1C). The distinction of MM patients with MK2 is clinically relevant and patients with high-MK2 expression have 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 EFS and OS survival (Figure 1E-F). These findings from two independent cohorts suggest that increased MK2 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.

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 shRNA
transfection. We first detected the protein expression levels of MK2 in MM cells by Western blot and found that all the 9 MM cell lines, XG1, CAG, ARP1, U266, OMP2, H929, MM.1S, 8226 and OCI-MY5, 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). 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 clonogenecity 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 in vivo study and injected both ARP1\textsuperscript{KD} and ARP1\textsuperscript{Ctrl} cells subcutaneously into the opposite side flanks of each NSG mouse (n = 4). Tumor diameters were measured and recorded twice per week to examine the growth rate of the tumor cells. After 4 weeks, the tumors produced by ARP1\textsuperscript{KD} cells were visibly smaller than their corresponding ARP1\textsuperscript{Ctrl} counterparts. The average weight of ARP1\textsuperscript{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\textsuperscript{KD} tumors volume significantly fell behind the ARP1\textsuperscript{Ctrl} control tumors (Figure 2F). These results indicate that genetic knockdown of MK2 delayed myeloma growth in vivo.

Inversely to the knockdown assay, we transfected MM 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 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 cell 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.

To seek how MK2 mediates MM progression, Co-IP assay was performed to detect the down-stream target of MK2. We found that AKT can 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 AKT signal (Figure 3D) in both ARP1 and OCI-MY5 cells. Both two assays proved that MK2 directly binds AKT in MM cells. Due to that MK2 is a Ser/Thr protein kinase, we tried to know 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 phosphorylates AKT(Figure 3E). This interpretation was served by the specific AKT phosphorylation inhibitor, LY490002, which overcame the MK2 activation induced MM cellular drug-resistance and profoundly suppressed clonogenicity in ARP1 and OCI-MY5 OE cells (Figure 3F). Now it is plausible to conclude that MK2 promotes MM progression through directly activating AKT. In addition, we also validated that MK2 inhibitor IV, a selective MK2 inhibitor had potential inhibition effect on MM cells both in vitro and in 5TGM1 MM mouse model (data not shown).
In summary, we first evaluate the MK2 expression in MM cells relative to normal control cells, and correlate MK2 with MM patient outcomes in relapsed MM patients. We also show that MK2 mediates MM cellular growth and drug-resistance. Finally, we disclose that MK2 regulates MM progression through activating AKT signaling. Our findings indicate MK2 acting 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Reference


Figure legends

Figure 1. MK2 is a poor prognostic marker or a high-risk gene in MM.
(A) MK2 expression in TT2 high and low group based 70-gene model. (B) A box-plot exhibited the average MK2 expression in 8 MM subgroups of 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 & F) Kaplan-Meier curve on relapsed MM patients’ event free survival (E) and overall survival (F) in TT2 cohort divided by MK2 expression.

Figure 2. Decreased MK2 expression induces MM 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-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 48h. (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 MM progression.
(A) Western blot assay on MK2 expression in ARP1 and OCI-MY5 WT and 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.
Figure 3

Panel A: Western blot analysis showing the expression of MK2 and β-Actin in ARP1 and OCI-MY5 cells under WT and OE conditions.

Panel B: Immunoblot images for control, Bortezomib, and Doxorubicin treatments in WT and OE conditions.

Panel C: Immunoblot analysis of AKT, MK2, and β-Actin in ARP1 and OCI-MY5 cells under IP, IgG, Input, and Bait conditions.

Panel D: Immunofluorescence imaging of MK2 and AKT in ARP1 and OCI-MY5 cells.

Panel E: Western blot analysis of MK2 and pAKT in ARP1 and OCI-MY5 cells under WT and OE conditions.

Panel F: Immunoblot images of ARP1 and OCI-MY5 cells under OE, OE & LY492002 conditions.