

Kdm6a and Kdm6b: Altered expression in malignant pleural mesothelioma

SIAN CREGAN¹⁻³, MAEVE BRESLIN¹, GERARD ROCHE¹, SIGRID WENNSTEDT¹,
LAUREN MacDONAGH¹, CINARIA ALBADRI¹, YUN GAO^{1,4}, KENNETH J. O'BYRNE^{1,5},
SINEAD CUFFE⁶, STEPHEN P. FINN⁷ and STEVEN G. GRAY^{1,8,9}

¹Thoracic Oncology Research Group, Institute of Molecular Medicine, St. James's Hospital, Dublin 8;

²Department of Surgery, Trinity College Dublin, Dublin; ³MSc in Translational Oncology Program, Trinity College, Dublin, Ireland; ⁴Department of Oncology, Aerospace Central Clinical Medical College of Peking University, Beijing, P.R. China;

⁵Cancer and Ageing Research Program, Queensland University of Technology, Brisbane, QLD, Australia; ⁶HOPE Directorate, St. James's Hospital, Dublin 8; ⁷Department of Histopathology and Morbid Anatomy, Trinity College Dublin, Dublin;

⁸Department of Clinical Medicine, Trinity College Dublin, Dublin 8; ⁹Labmed Directorate, St. James's Hospital, Dublin 8, Ireland

Received November 9, 2016; Accepted January 16, 2017

DOI: 10.3892/ijo.2017.3870

Abstract. Malignant pleural mesothelioma (MPM) is a rare aggressive cancer of the pleura primarily associated with prior exposure to asbestos. The current standard of care for patients suffering from MPM is a combination of cisplatin and pemetrexed (or alternatively cisplatin and raltitrexed). Most patients, however, die within 24 months of diagnosis. New therapies are therefore urgently required for this disease. Inflammation is thought to be a key element in the pathogenesis of MPM, and recently Kdm6 family members (Kdm6a and Kdm6b) have been identified as playing important roles in inflammatory processes. As such these genes could potentially represent novel candidate targets for intervention in MPM. Using RT-PCR we examined the expression of Kdm6aA and Kdm6b in a panel of MPM cell lines and in a cohort of snap-frozen patient samples isolated at surgery comprising benign, epithelial, biphasic and sarcomatoid histologies. Both Kdm6a and Kdm6b were found to be significantly overexpressed in MPM at the mRNA level. However, tests examining if targeting therapeutically Kdm6a/b using a specific small molecule inhibitor (GSK-J4) was potentially useful for treating MPM, revealed that anti-proliferative activity was higher at lower drug concentrations in cell lines derived from normal mesothelial cells compared to those derived from malignant

cells. Treatments with GSK-J4 were found to be associated with the induction of apoptosis and increased expression of pro-inflammatory cytokines. As such our results demonstrate that whilst members of the Kdm6 family are overexpressed in MPM they may not be suitable candidates for therapy and may elicit a cytokine storm.

Introduction

Associated with prior exposure to asbestos (1), malignant pleural mesothelioma (MPM) is an aggressive inflammatory cancer arising from mesothelial cells lining the pleural, peritoneal and pericardial cavities. It has a long latency period, and because of this, the vast majority of patients present at an advanced stage resulting in a poor prognosis with a median survival time of 6-12 months. Current estimates suggest that 43,000 people die from MPM each year with a mortality rate in the order of 6.2 per million population (2,3). Many countries have banned the use of asbestos, and data are emerging to suggest that the incidence of mesothelioma in these countries may be falling (4-6), although there is some dispute regarding these projections (7). Furthermore, the long-term risk to younger people exposed to asbestos still present in many buildings is not currently known but could be substantial (8). Nevertheless, it is generally accepted that MPM mortality rates will continue to increase by 5-10% per year in most industrialized countries for the next 2-3 decades, despite asbestos ablation efforts (7). In addition, asbestos consumption continues to increase in emerging countries such as such as Brazil, and a new wave of MPM is predicted in such countries (9).

The current standard of care is a combination of pemetrexed (or raltitrexed) and cisplatin chemotherapy (10). Unfortunately, this treatment only has a patient response rate of between 23-40% and is non-curative (11,12). Consequently, there is an urgent need to identify novel therapeutic avenues in this disease to improve patient outcomes.

Correspondence to: Dr Steven G. Gray, Thoracic Oncology Research Group, Trinity Centre for Health Sciences, St. James's Hospital, Room 2.09, Dublin D08 W9RT, Ireland
E-mail: sgray@stjames.ie

Key words: KDM6, ubiquitously transcribed tetratricopeptide repeat gene on X chromosome, Jumonji domain-containing protein 3, malignant pleural mesothelioma, GSK-J4, therapy

Dysregulation of epigenetic transcriptional control, particularly in the areas of promoter DNA methylation and histone post-translational modifications, is a well-established feature of human malignancies, including mesothelioma (13-15). The pharmaceutical sector has devoted significant efforts to develop agents capable of targeting the epigenetic machinery. The potential to target the epigenetic machinery in MPM initially came from data in a phase I trial of vorinostat a histone deacetylase (HDAC) inhibitor, in which 4 of 13 patients with MPM (30%) who received vorinostat had stable disease lasting more than 4 months with two unconfirmed partial responses (16). Unfortunately, when vorinostat was given as a second-line or third-line therapy in the phase III trial (VANTAGE 014) of 660 pretreated advanced MPM patients it did not improve overall survival, and the recommendation was therefore that it was unsuitable as a therapy in MPM patients (17).

Despite this, evidence continues to emerge suggesting that targeting the epigenetic machinery may be a viable therapeutic option in MPM. For example, we recently demonstrated that KAT5 (a lysine acetyltransferase) was overexpressed in MPM and a suitable potential candidate for therapeutic intervention (18). Furthermore, the potential importance of targeting the epigenetic machinery in MPM has been highlighted by data emerging regarding MPM patients who have mutated BRCA1-associated protein 1 (BAP1). BAP1 plays critical roles in chromatin remodeling, and loss of BAP1 in MPM cells has now been associated with altered sensitivity to HDAC inhibitors through regulation of a specific HDAC, HDAC2 (19).

In a recent analysis of MPM, a subset of genes was found to be silenced by histone H3 lysine 27 trimethylation (H3K27me3), a mark most often found at or near the promoters of silent genes (20,21). Polycomb repressive complex 2 (PRC2) catalyses tri-methylation of Histone H3 at lysine 27 (H3K27me2/3) (22), and contains the lysine methyltransferase EZH2. Targeting this complex with the methyltransferase inhibitor DZNep has been shown to be a potential therapeutic option in MPM (23), and in particular sensitivity to DZNep has been linked to a subset of MPM patients that contain (BAP1) mutation (24), and a phase II clinical trial of the EZH2 inhibitor tazmetostat (EPZ-6438) in mesothelioma is currently in progress (clinicaltrials.gov NCT02860286).

The proteins which demethylate H3K27me3 have been collectively identified as the Kdm6 family (20) and include Kdm6a (also known as Utx) and Kdm6b (also known as JMJD3) both of which have been shown to catalyze the demethylation of H3K27me3 (25,26). The roles of these demethylases in cancer are less well defined. There are many instances where loss of expression of these demethylases are found in cancer through mutation (27). Likewise, there are also many studies which have shown overexpression of these proteins in cancer (27-29), and specific inhibitors (GSK-J1/GSK-J4) for the Kdm6 family have now been developed (30).

Given the pro-inflammatory nature of MPM, and the therapeutic potential to target the PRC2 complex, it may also be possible that the Kdm6 family is a potential candidate therapeutic avenue. In the present study, we assessed the expression of Kdm6 family members in MPM and the effects of Kdm6 inhibition on MPM cellular health. Our results indicate that while the Kdm6 family is overexpressed in MPM, selective

inhibition of this family may not be suitable as a therapeutic option in MPM.

Materials and methods

Primary tumor samples. Surgical specimens were obtained as discarded tumor samples from patients who had undergone extended pleuro-pneumectomy at Glenfield Hospital (Leicester, UK). Benign specimens were acquired from patients never diagnosed with MPM. Informed consent was obtained from each patient, and the study was conducted after formal approval from the relevant Hospital Ethics Committee (Leicestershire REC references 6742 and 6948). Samples consisted of the following: 5 benign lesions and 17 MPM samples (epithelioid, n=7; sarcomatoid, n=4; biphasic, n=6), and details are provided in Table I.

All research on these samples was conducted at St. James's Hospital under the SJH/AMNCH research ethics committee approval (041017/8704).

Cell culture. All MPM cell lines were maintained in a humidified atmosphere containing 5% v/v CO₂ in appropriate media supplemented with 10% v/v fetal bovine serum (FBS) and penicillin streptomycin (500 U/ml). Cell culture reagents were purchased from Lonza (Walkersville, MD, USA). A panel of normal mesothelial cell lines (LP9 and Met5A) and malignant MPM cell lines (NCI-H2596, MMP, MMB, NCI-H2052, NCI-H28, Ju77, One58, RS-5, DM-3, ACC-MESO-1, ACC-MESO-4, Y-MESO-8D, Y-MESO-9, Y-MESO-12, Y-MESO-14, NCI-H226 and REN) were used in the present study.

ACC-MESO-1, ACC-MESO-4, Y-MESO-8D, Y-MESO-9, Y-MESO-12 and Y-MESO-14 were generously provided by Yoshitaka Sekido (Aichi Cancer Center Research Institute, Nagoya, Japan).

NCI-H2052, One-58 and JU77 cells were provided by Duncan Stewart (University of Leicester, Leicester, UK). The REN and NCI-H226 cell lines were provided by Dean Fennell (Queen's University, Belfast, Northern Ireland). NCI-H28 and the immortalized non-tumorigenic mesothelial cell line, Met-5A were purchased from the ATCC (LGC Promochem, Teddington, UK). DM-3 and RS-5 were purchased from the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

The NCI-H226 cell line was authenticated by STR-profiling (Source BioScience, Nottingham, UK).

Total RNA isolation and RT-PCR amplification. Total RNA was extracted using TRI reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH, USA). cDNA was synthesized as follows: 250 ng (primary tumors) or 1000 ng (cell lines) 250 ng - 1 µg of total RNA was first pre-treated by digestion with RQ1 DNase (Promega, Madison, WI, USA). Following inactivation the RQ1 DNase treated mRNA was converted to cDNA using RevertAid (Thermo Fisher Scientific) and random hexamers (Roche) according to the manufacturer's instructions. cDNA was then stored at -20°C until use.

Expression of Kdm6a, Kdm6b and 18S rRNA was subsequently examined by RT-PCR, using the primers outlined in

Table I. Details of pleura/mesothelioma samples used in the present study.

Patients	Pathology (benign, epithelial, biphasic, sarcomatoid)	Age (years)	Gender
1	Benign-pleural plaque (Benign fibrous plaque and focal chronic inflammation)	55	Male
2	Benign-pleural plaque (Benign hyaline plaques with chronic inflammation + reactive changes)	55	Male
3	Benign-pneumothorax (Chronic inflammatory infiltrate, mesothelial proliferation)	30	Male
4	Benign empyema (Acute and chronic inflammation + fibrosis)	68	Male
5	Benign-pleural plaque (Inflammatory + talc granuloma)	55	Male
6	Epithelial	62	Male
7	Epithelial	73	Male
8	Epithelial	66	Male
9	Epithelial	56	Female
10	Epithelial	52	Male
11	Epithelial	56	Male
12	Epithelial	54	Male
13	Biphasic	54	Male
14	Biphasic	54	Female
15	Biphasic	41	Male
16	Biphasic	58	Male
17	Biphasic	N/A	Male
18	Biphasic	60	Female
19	Sarcomatoid	74	Male
20	Sarcomatoid	64	Male
21	Sarcomatoid	59	Male
22	Sarcomatoid (desmoplastic)	64	Male

N/A, not available.

Table II. PCR primers used in the present study.

Primer	Sequence	Expected size (bp)
Kdm6a Forward	5'-GGCACTGTTCATTGGGTTTCAGG-3'	305
Kdm6a Reverse	5'-TTTGTCCGCCCATGCCATAT-3'	
Kdm6b Forward	5'-AGCAAACGGGATGCCTTCTCAC-3'	179
Kdm6b Reverse	5'-TGCACCTGGGTGCGAACTTC-3'	
CXCL1 Forward	5'-ATGGCCCGCGCTGCTCTCTC-3'	324
CXCL1 Reverse	5'-TCAGTTGGATTGTCACTGTTC-3'	
CXCL2 Forward	5'-ATGGCCCGCGCCACGCTCTC-3'	324
CXCL2 Reverse	5'-TCAGTTGGATTGCCATTTTTCAGC-3'	
CXCL8 Forward	5'-ATGACTTCCAAGCTGGCCGTG-3'	297
CXCL8 Reverse	5'-TGAATTCTCAGCCCTCTTCA-3'	
18S rRNA Forward	5'-GATGGGCGGCGGAAAATAG-3'	166
18S rRNA Reverse	5'-GGCGTGATTCTGCATAATGG-3'	
β-actin Forward	5'-TGTTTGAGACCTTCAACACCC-3'	529
β-actin Reverse	5'-AGCACTGTGTTGGCGTACAG-3'	

Table II. PCR cycling conditions consisted of 95°C for 5 min followed by 35 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C with a final extension at 72°C for 10 min. Products were

electrophoresed on a 2% agarose gel. Product quantification was performed using TINA 2.09c (Raytest, Isotopenmeßgeräte GmbH, Straubenhardt, Germany) densitometry software. The

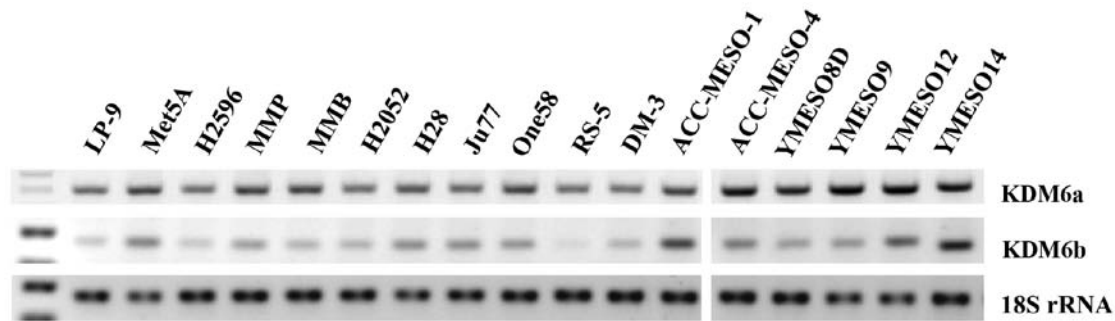


Figure 1. Kdm6 family mRNA expression in a panel of mesothelioma cell lines. Kdm6a and Kdm6b mRNA expression was examined in a panel of MPM cell lines including the normal pleural/peritoneal mesothelial cell lines (Met5A and LP9) by RT-PCR. 18S rRNA was included as a loading control.

mRNA expression was normalized to either 18S rRNA or β -actin controls, and was expressed as a ratio of experimental gene expression: control gene expression.

Drug treatment and cellular viability assays. GSK-J4 was purchased from Selleck (St. Louis, MO, USA; cat. no. O7753), and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 1 M. Cells were serum starved (0.5% v/v FBS) for 24 h prior to addition of either drug or vehicle, and incubated for a further 48 h. Cellular viability was assessed using either a resazurin reduction assay as previously described (31), or by a BrdU ELISA (Roche Diagnostics, Ltd., Sussex, UK), according to the manufacturer's instructions.

Cellular apoptosis (FACS). NCI-H226 cells were plated in 6-well plates (1×10^5 cells/well) and allowed to adhere overnight. Complete media was then removed and the cells washed with 100 ml phosphate-buffered saline (PBS). At this point, serum depleted media (0.5% FBS) was then added and the cells incubated for a further 24 h. Following this cells were subsequently treated with various concentrations of GSK-J4, diluted in serum depleted media, and incubated in the presence of drug for a further 48 h. Where appropriate, control cells were treated with either vehicle or left untreated with media only. Following treatment, the culture media was transferred to labeled FACS tubes and placed on ice. Remaining adherent cells were trypsinized and transferred to the same corresponding FACS tubes. Cells were then centrifuged (1300 rpm for 3 min) and all supernatants were discarded. The pellet of cells was resuspended and washed in 1 ml of 1X binding buffer (BB) diluted in ice cold PBS, centrifuged and subsequently resuspended in 100 μ l BB. A total of 2 μ l Annexin V (IQ Products BV, Groningen, the Netherlands) was added to each tube, with the exception of the negative control and media only samples, and incubated at 4°C for 20 min, protected from light. The cells were washed in 1 ml 1X binding buffer and supernatant removed. Prior to flow cytometric analysis the pellet of cells was resuspended in 400 μ l BB containing 0.5 μ g/ml PI (Invitrogen, Paisley, UK), except the negative control and FMO (fluorescence minus one) control for PI for which BB alone was used and analyzed by flow cytometry.

Cellular apoptosis (caspase-3/-7 activation). NCI-H226 cells were seeded at a density of 4×10^3 cells/well in Corning®

96 Well Flat Clear Bottom Black Polystyrene Tissue Culture-treated 96-well plates and allowed to adhere overnight. Subsequently, the media was removed and the cells washed with 100 ml PBS. The cells were then incubated in serum depleted media (0.5% FBS) for a further 24 h, at which point they were then treated with GSK-J4 at various concentrations for a further 48 h. Caspase-3/-7 activation was then measured using a FluoroFire caspase-3/-7 fluorescent assay kit according to the manufacturer's instructions (Molecutools, Dublin, Ireland).

In silico analysis. Data-mining of available mesothelioma datasets were conducted using Oncomine (www.oncomine.org) (32), or cBioPortal (www.cbioportal.org) (33) using the default settings. The results shown here are in whole or part based upon data generated by the The Cancer Genome Atlas Research Network (cancergenome.nih.gov).

Statistical analysis. Data are expressed as mean \pm standard error of multiple experiments ($n=3$). Statistical analysis was performed using either Mann-Whitney or unpaired Student's t-tests, or one-way ANOVA with Dunnett's post-test using the GraphPad Prism 5.01. Differences were considered to be statistically significant at $P<0.05$.

Results

Kdm6 family members are ubiquitously expressed in mesothelioma cell lines. Utilizing RT-PCR, the levels of expression of Kdm6a and Kdm6b mRNA were examined in a panel of cell lines including those derived from normal pleura (LP9 and Met5A) and mesotheliomas. Kdm6a and Kdm6b were readily detectable in all cell lines as shown in Fig. 1.

Kdm6 family members are overexpressed in MPM. To assess the expression of Kdm6 family members in primary patient material, RT-PCR was performed on a panel of benign pleura and MPM tumor samples isolated at surgery from patients (Fig. 2A). Densitometric analysis of the gels revealed a significant increase in the expression of Kdm6a mRNA ($P=0.0036$) and Kdm6b mRNA ($P=0.0122$) in MPM tumor samples compared with normal pleura (Fig. 2B). When stratified by histology, significant overexpression of both Kdm6a mRNA was observed across all histological subtypes, whereas only Kdm6b mRNA was significantly altered in the biphasic subtype

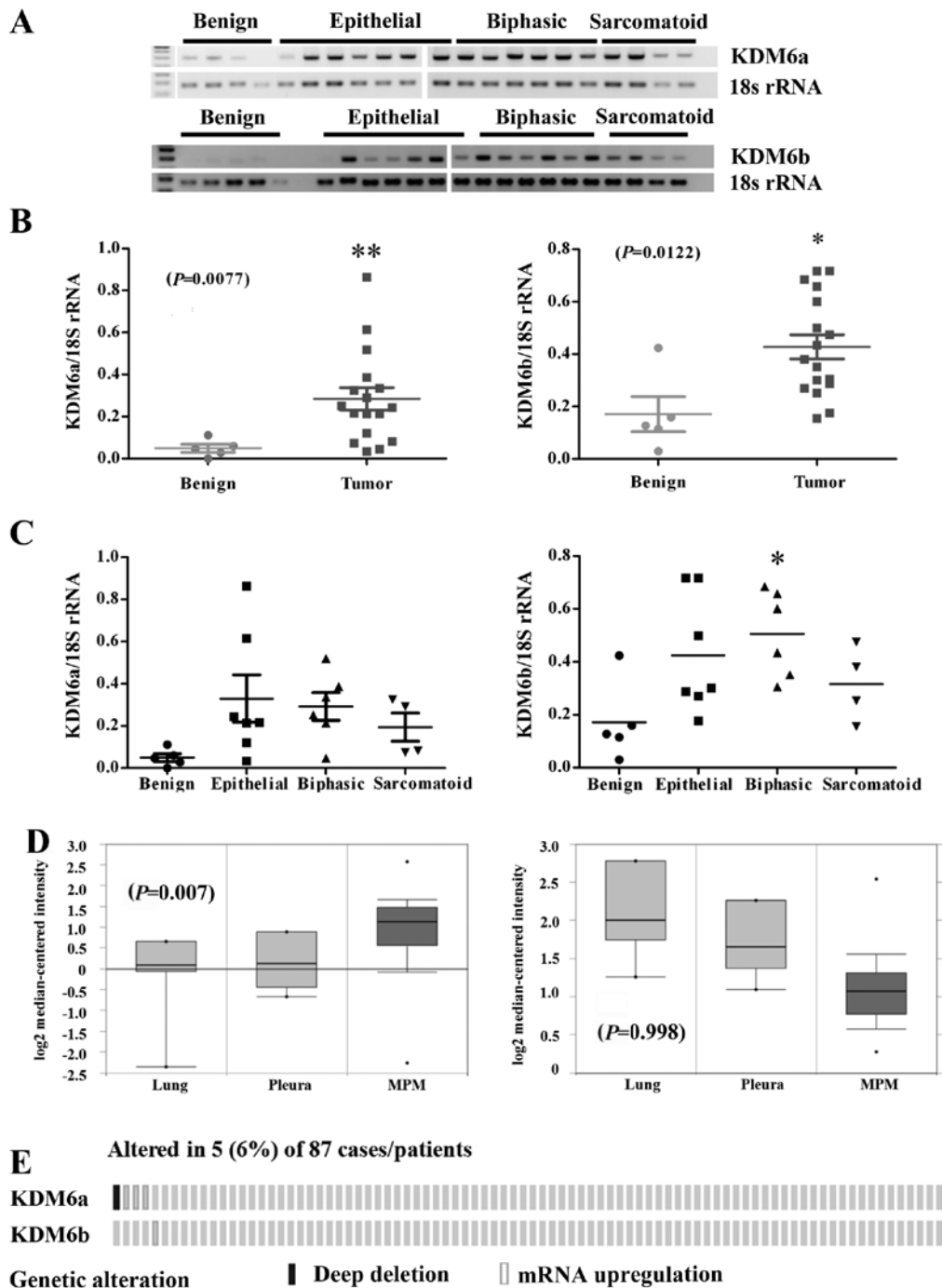


Figure 2. Kdm6 family members mRNA expression are significantly overexpressed in primary mesothelioma tumors. (A) Kdm6a and Kdm6b mRNA expression was examined by RT-PCR in a series of primary MPM (n=17) vs. benign pleura (n=4/5). Loading order is as per sample order in Table I. (B) Semi-quantitative densitometric analysis of the results determined that Kdm6a and Kdm6b mRNA were significantly elevated in the tumors compared to benign pleura. (C) When separated on the basis of histology Kdm6a was shown to be significantly elevated across all histological subtypes, whereas for Kdm6b, only the biphasic samples were shown to have significantly altered expression. (D) *In Silico* Oncomine analysis demonstrating significant overexpression of Kdm6a but not Kdm6b in a separate cohort of mesothelioma samples. (E) *In Silico* analysis using cBioPortal of an existing TCGA NGS mesothelioma dataset, indicating that overexpression of Kdm6a and Kdm6b occurs in a small subset of patients. Statistical significance was measured using either the Mann-Whitney two-tailed t-test, or a one-way ANOVA with Dunnett's post-test (*P<0.05; **P<0.01; ***P<0.001).

(Fig. 2C). Using Oncomine (32), we queried the expression of both Kdm6 family members in the Gordon *et al* (34) dataset. In this dataset only Kdm6a was shown to be significantly overexpressed in the MPM specimens compared to benign pleura or lung (P=0.007), whereas Kdm6b levels were not significantly altered (Fig. 2D). Using cBioPortal (33), we also examined an available mesothelioma TCGA NGS dataset comprising

(n=87) samples (Fig. 2E). Overexpression of Kdm6a and Kdm6b were observed in a small cohort of patients (Fig. 2E). Overall, the results suggest that the Kdm6 family could be a potential therapeutic target in MPM.

Inhibition of the Kdm6 family results in decreased MPM proliferation and altered expression of pro-inflammatory

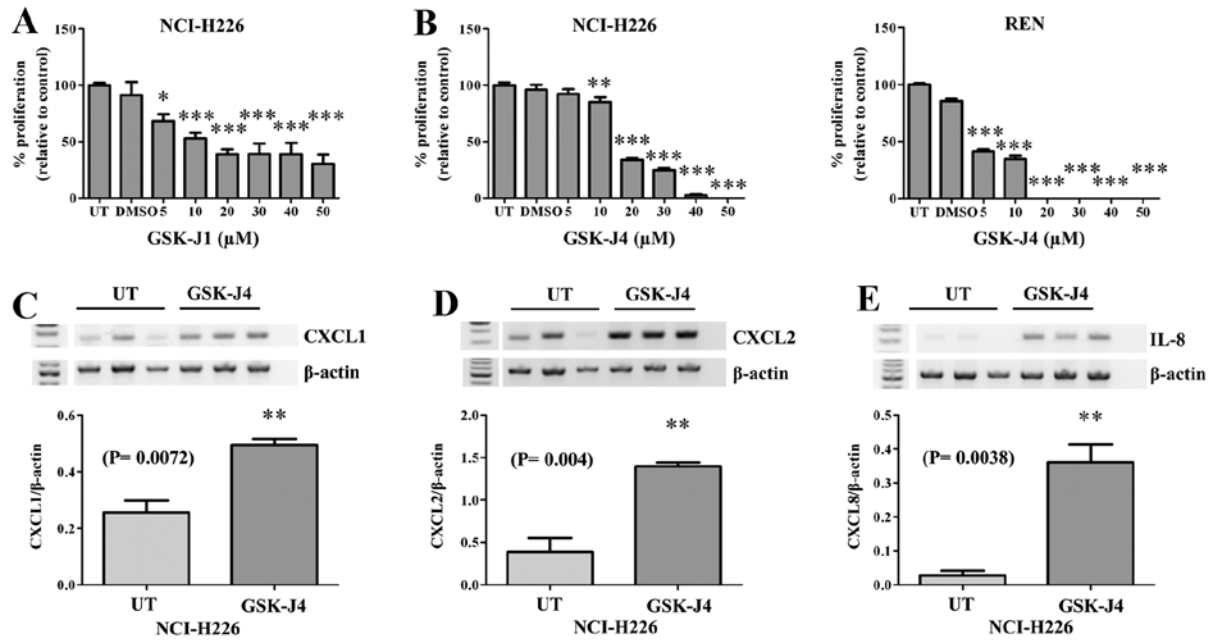


Figure 3. Effects of Kdm6 inhibitors on MPM cellular proliferation and cytokine expression. (A) NCI-H226 MPM cells were exposed to GSK-J1 at various concentrations for 48 h and effects on cellular proliferation were measured using a BrdU incorporation assay (n=3). (B) NCI-H226 and REN MPM cells were exposed to GSK-J4 at various concentrations for 48 h and effects on cellular proliferation were measured using a BrdU incorporation assay (n=3). RT-PCR analysis examining the effects of GSK-J4 on (C) CXCL1, (D) CXCL2 and (E) CXCL8/IL-8 in NCI-H226 cells treated for 24 h with GSK-J4 at a final concentration of 15 μ M (n=3). Densitometric analysis following electrophoresis demonstrates significant induction of these pro-inflammatory cytokines/chemokines. Statistical significance was measured using either an unpaired Student's two-tailed t-test, or a one-way ANOVA with Dunnett's post-test (*P<0.05; **P<0.01; ***P<0.001).

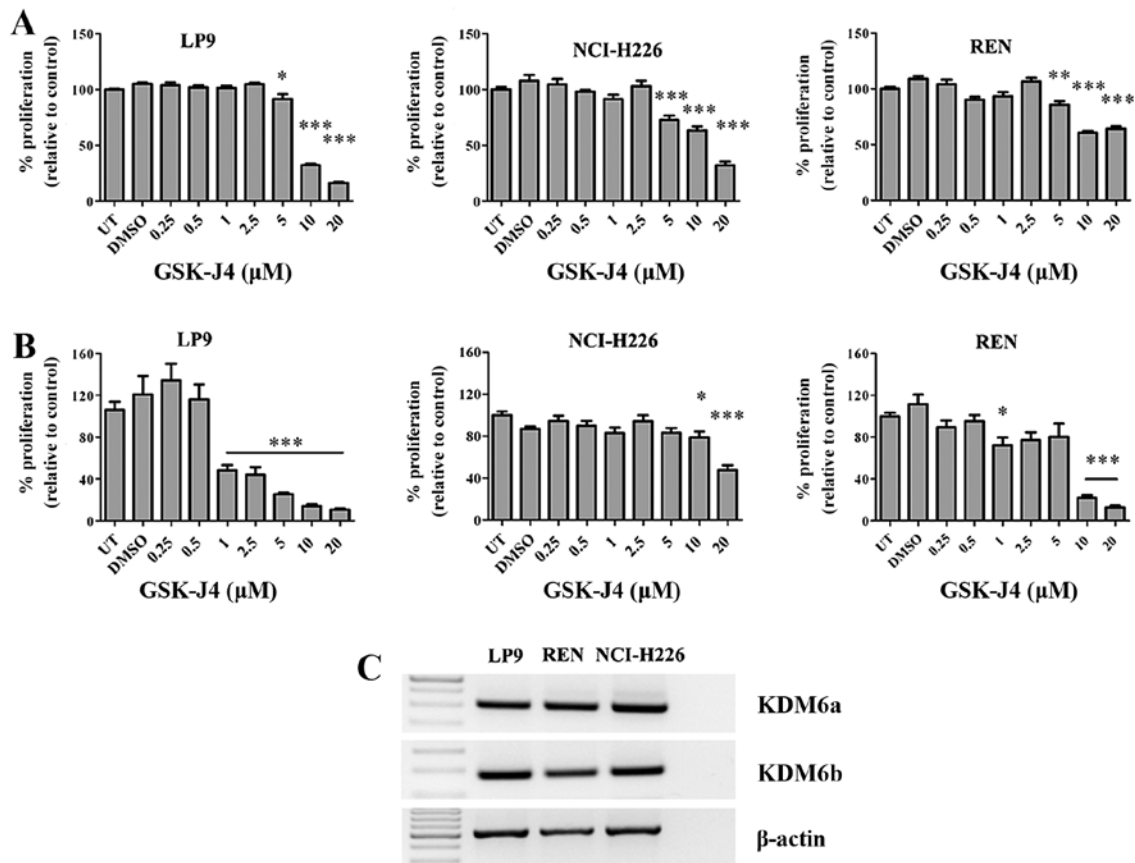


Figure 4. Cellular proliferation of normal pleural cells are more sensitive to Kdm6 inhibitors than MPM cells. LP9, NCI-H226 and REN MPM cells were exposed to GSK-J4 at various concentrations for 48 h (n=3) and effects on cellular proliferation were measured using (A) a resazurin based assay and (B) a BrdU incorporation assay. (C) The expression of Kdm6a and Kdm6b was confirmed by RT-PCR in all cell lines. Statistical significance was assessed using a one-way ANOVA with Dunnett's post-test (*P<0.05; **P<0.01; ***P<0.001).

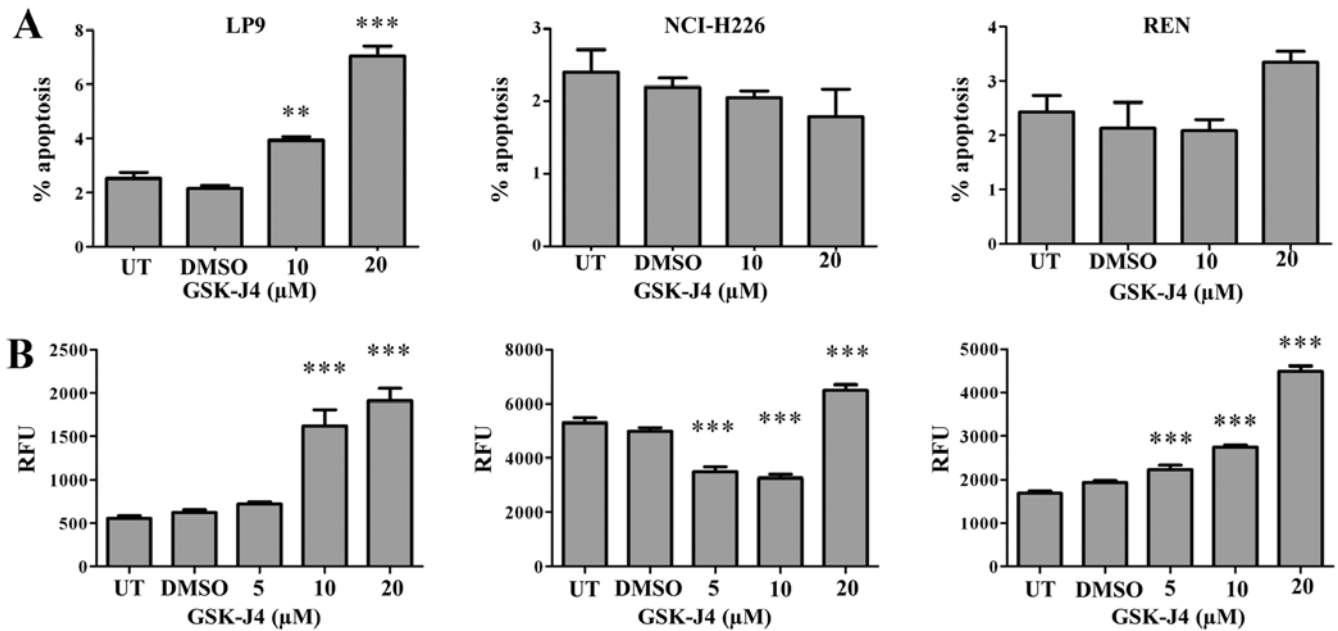


Figure 5. GSK-J4 induces apoptosis in normal pleural cells and MPM cells. LP-9, NCI-H226 and REN cells were exposed to different concentrations of GSK-J4 for 48 h and then apoptosis was assayed using either: (A) Annexin V/PI FACS analysis (n=3), or (B) caspase-3/-7 activation assay (n=3). Statistical significance was assessed using a one-way ANOVA with Dunnett's post-test (* $P<0.01$; *** $P<0.001$).

cytokines. The only current selective inhibitor targeting Kdm6a and Kdm6b is GSK-J1, with a corresponding cell-active pro-drug form GSK-J4 (30). We initially tested the effect of GSK-J1 on the proliferative capacity of NCI-H226 cells (Fig. 3A), the results of which indicated that this compound could indeed inhibit MPM cellular proliferation. We subsequently tested the ability of the pro-drug to inhibit cellular proliferation and confirmed that GSK-J4 inhibited MPM cellular proliferation in two MPM cell lines tested (Fig. 3B). Given the known role of Kdm6a as a key regulator in inflammation (27,30,35), and the ability of GSK-J4 to block pro-inflammatory cytokine expression (30), and the pro-inflammatory nature of MPM, we examined the effect of GSK-J4 on the expression of a panel of pro-inflammatory cytokines. However, treatment of MPM cells with GSK-J4 resulted in significant upregulation of CXCL1 ($P=0.0072$; Fig. 3C), CXCL2 ($P=0.004$; Fig. 3D) and CXCL8/IL-8 ($P=0.0038$; Fig. 3E).

Non-malignant pleural cells are more sensitive to GSK-J4 than MPM cells. Whilst GSK-J4 has significant anti-proliferative effects on MPM cell lines, given that expression of Kdm6a and Kdm6b was found to be almost ubiquitous in our panel of cell lines (Fig. 1), we subsequently examined the sensitivity of normal mesothelial cells (LP-9) in parallel with two malignant MPM cell lines (NCI-H226 and REN). Using either a Resazurin based assay (Fig. 4A), or BrdU incorporation (Fig. 4B), we demonstrated that the normal pleural cell line is more sensitive to GSK-J4. We re-screened the cell lines for expression of Kdm6a and Kdm6b mRNA and confirmed that both cells were expressed (Fig. 4C). The increased sensitivity of the normal pleural cell line to Kdm6 inhibition was reflected in increased levels of apoptosis in comparison with the MPM cell lines (Fig. 5).

Discussion

MPM is an aggressive cancer with very limited treatment options. Currently, the established first-line therapy is a combination of cisplatin and an anti-folate. Most MPM patients do not respond to this or other therapies, and the duration of response is short with a rapid development of resistance (36). There is no current defined second-line therapy, and as a consequence there is an urgent need to identify new therapeutic options for the treatment of this cancer. The recent demonstration that MPMs are in fact polyclonal tumors formed by the coalescence of different independent subclones highlights the need for new therapeutic approaches, as each clone may carry its own distinct set of molecular alterations, and the intra-tumoral heterogeneity arising may allow for the emergence of drug-resistant subpopulations, and as such multi-targeted approaches to therapy may be required to overcome the issue of clonality (37).

EZH2 is associated with the H3K27me3 histone post-translational modification mark common to silenced chromatin or bivalent 'poised' promoters (20,38). The lysine demethylases that catalyze the removal of this mark have been identified as Kdm6a (Utx) and Kdm6b (Jmjd3). Given the potential importance of this mark in BAP1 mutated MPM, in this study we therefore sought to examine the expression of these lysine demethylases in MPM. An initial screen showed that the mRNA for both Kdm6a and Kdm6b was commonly expressed in all cell lines derived from both normal mesothelial and malignant MPM cells (Fig. 1).

However, when examined in patient tumors we demonstrate that both Kdm6a and Kdm6b show significantly elevated mRNA for these lysine demethylases in the tumors compared to benign pleura (Fig. 2A). When separated according to histological subtype we observed no significant overexpression

of Kdm6a across all histological subtypes (Fig. 2C), whereas elevated Kdm6b mRNA was only significant in the biphasic subtype (Fig. 2C). In this regard the current sample size is clearly insufficient to make any clear comparisons across histological subtype, and that a larger sample size will be required to address this. *In silico* analysis confirmed that altered expression of these Kdms does occur in MPM, but significantly may only be restricted to Kdm6a (Fig. 2D and E).

It could also be construed that normal pleural tissues from patients with resected lung cancer would be a more suitable control than the inflammatory benign pleural samples used in the present study. In our opinion that moving forward, this may be a useful additional control to compare non-inflammatory pleura to inflammatory pleura. Nevertheless, given the fact that MPM is a pro-inflammatory cancer, and that Kdm6 family members have been shown to play key roles in the regulation of pro-inflammatory responses (30), including the potential role of Kdm6b as a key element in 'inflammaging' that can contribute to tumor progression (27), our data demonstrates that overexpression of these demethylases occurs in malignant tissue (Fig. 2) compared to the benign pleural plaques which contain a significant inflammatory profile (Table I). In this regard, our data remove a potential confounding issue; that inflammation itself may be driving the upregulation of Kdm6a and Kdm6b in malignant tissue.

Inhibitors specific to members of the Kdm6 family (GSK-J1 and GSK-J4) have been developed (30), and given both the pro-inflammatory nature of MPM, and the elevated expression of Kdm6a and Kdm6b in our patient samples, we subsequently examined the effects of these inhibitors on MPM cellular health and pro-inflammatory cytokine expression. Both drugs were shown to have significant anti-proliferative activity on MPM cell lines with the cell-active ethyl ester pro-drug GSK-J4 showing greater anti-proliferative effects at lower concentrations than GSK-J1 (Fig. 3A and B).

GSK-J4 was originally demonstrated to prevent LPS-mediated induction of pro-inflammatory cytokines in human primary macrophages (30). However, treatment of an MPM cell line with GSK-J4 resulted in significant upregulation of several pro-inflammatory cytokines including IL-8 (Fig. 3C). In this regard, induction of IL-8 by anticancer regimens has previously been observed in an experimental model of mesothelioma (39). Furthermore, elevated levels of IL-8 have also been found to be increased in mesothelioma patients undergoing therapy following pleurectomy or extra-pleural pneumonectomy (EPP) (40), or in MPM patients treated tumor necrosis factor- α (TNF- α) (41). It has been noted that elevated levels of IL-8 may not be a reliable marker for predicting response to therapy (42), and the observation that GSK-J4 is capable of elevating the expression of several pro-inflammatory cytokines suggests that inhibition of Kdm6 family members may have the potential to induce cytokine response syndrome (CRS) (43), as sometimes seen in MPM (44).

The increased production of pro-inflammatory cytokines in MPM cells was accompanied by decreased cellular proliferation (Fig. 4) and an associated induction of apoptosis (Fig. 5) and necrosis (Lauren MacDonagh, unpublished). Moreover, we were able to show that cells derived from normal pleura were more sensitive to GSK-J4 than those derived from MPM.

These results suggest that whilst the expression of Kdm6 family members are significantly elevated in MPM, targeting the same may not be sparing of patients normal pleura. However, our results also indicate that in primary tissues levels of the Kdm6 family are much higher in patient tumors than in normal pleura (Fig. 2), and this discrepancy remains to be resolved.

In conclusion, our results suggest that the Kdm6 family may represent a novel therapeutic target for the treatment of MPM, but given the potential for damage to the normal pleura, or the potential for inducing a cytokine storm, further studies are warranted. We believe that more studies will be required to further determine whether or not targeting the Kdm6 family is possible in MPM, or indeed even in a subset of MPM and to determine if a multi-targeting approach combining epigenetic therapies (including GSK-J4) is possible.

Acknowledgements

The authors are grateful to Dr Warren Thomas, Dr Yoshitaka Sekido, Dr Dean Fennell and Dr Hannu Norppa for their generosity in providing access to various mesothelioma cell lines. The present study was supported in part by funding for consumables from the Masters in Translational Oncology program (TCD) for Sian Cregan.

References

1. Wagner JC, Sleggs CA and Marchand P: Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. *Br J Ind Med* 17: 260-271, 1960.
2. Delgermaa V, Takahashi K, Park EK, Le GV, Hara T and Sorahan T: Global mesothelioma deaths reported to the World Health Organization between 1994 and 2008. *Bull World Health Organ* 89: 716-724, 724A-724C, 2011.
3. Ramazzini C: The global health dimensions of asbestos and asbestos-related diseases. *J Occup Health* 58: 220-223, 2016.
4. Järholm B and Burdorf A: Emerging evidence that the ban on asbestos use is reducing the occurrence of pleural mesothelioma in Sweden. *Scand J Public Health* 43: 875-881, 2015.
5. Price B and Ware A: Time trend of mesothelioma incidence in the United States and projection of future cases: An update based on SEER data for 1973 through 2005. *Crit Rev Toxicol* 39: 576-588, 2009.
6. Tan E, Warren N, Darnton AJ and Hodgson JT: Projection of mesothelioma mortality in Britain using Bayesian methods. *Br J Cancer* 103: 430-436, 2010.
7. Carbone M, Ly BH, Dodson RF, Pagano I, Morris PT, Dogan UA, Gazdar AF, Pass HI and Yang H: Malignant mesothelioma: Facts, myths, and hypotheses. *J Cell Physiol* 227: 44-58, 2012.
8. Gilham C, Rake C, Burdett G, Nicholson AG, Davison L, Franchini A, Carpenter J, Hodgson J, Darnton A and Peto J: Pleural mesothelioma and lung cancer risks in relation to occupational history and asbestos lung burden. *Occup Environ Med* 73: 290-299, 2016.
9. Algranti E, Saito CA, Carneiro AP, Moreira B, Mendonça EM and Bussacos MA: The next mesothelioma wave: Mortality trends and forecast to 2030 in Brazil. *Cancer Epidemiol* 39: 687-692, 2015.
10. Baas P, Fennell D, Kerr KM, Van Schil PE, Haas RL and Peters S; ESMO Guidelines Committee: Malignant pleural mesothelioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 26 (Suppl 5): v31-v39, 2015.
11. van Meerbeeck JP, Gaafar R, Manegold C, Van Klaveren RJ, Van Marck EA, Vincent M, Legrand C, Bottomley A, Debruyne C and Giaccone G; European Organisation for Research and Treatment of Cancer Lung Cancer Group; National Cancer Institute of Canada: Randomized phase III study of cisplatin with or without raltitrexed in patients with malignant pleural mesothelioma: An intergroup study of the European Organisation for Research and Treatment of Cancer Lung Cancer Group and the National Cancer Institute of Canada. *J Clin Oncol* 23: 6881-6889, 2005.

12. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, Gatzemeier U, Boyer M, Emri S, Manegold C, *et al*: Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol* 21: 2636-2644, 2003.
13. Baird A, Richard D, O'Byrne KJ and Gray SG: Epigenetic Therapy in Lung Cancer and Mesothelioma. In: *Epigenetic Cancer Therapy*. 1st edition. Gray SG (ed). Academic Press, ppl89-213, 2015.
14. Baylin SB and Jones PA: Epigenetic determinants of cancer. *Cold Spring Harb Perspect Biol* 8: a019505, 2016.
15. Zhang X, Tang N, Rishi AK, Pass HI and Wali A: Methylation profile landscape in mesothelioma: Possible implications in early detection, disease progression, and therapeutic options. *Methods Mol Biol* 1238: 235-247, 2015.
16. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, MacGregore-Cortelli B, Tong W, Secrist JP, Schwartz L, *et al*: Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J Clin Oncol* 23: 3923-3931, 2005.
17. Krug LM, Kindler HL, Calvert H, Manegold C, Tsao AS, Fennell D, Ohman R, Plummer R, Eberhardt WE, Fukuoka K, *et al*: Vorinostat in patients with advanced malignant pleural mesothelioma who have progressed on previous chemotherapy (VANTAGE-014): A phase 3, double-blind, randomised, placebo-controlled trial. *Lancet Oncol* 16: 447-456, 2015.
18. Cregan S, MacDonagh L, Gao Y, Barr MP, O'Byrne KJ, Finn SP, Cuffe S and Gray SG: KAT5 (Tip60) is a potential therapeutic target in malignant pleural mesothelioma. *Int J Oncol* 48: 1290-1296, 2016.
19. Sacco JJ, Kenyani J, Butt Z, Carter R, Chew HY, Cheeseman LP, Darling S, Denny M, Urbé S, Clague MJ, *et al*: Loss of the deubiquitylase BAP1 alters class I histone deacetylase expression and sensitivity of mesothelioma cells to HDAC inhibitors. *Oncotarget* 6: 13757-13771, 2015.
20. Bosselut R: Pleiotropic functions of H3K27Me3 demethylases in immune cell differentiation. *Trends Immunol* 37: 102-113, 2016.
21. Goto Y, Shinjo K, Kondo Y, Shen L, Toyota M, Suzuki H, Gao W, An B, Fujii M, Murakami H, *et al*: Epigenetic profiles distinguish malignant pleural mesothelioma from lung adenocarcinoma. *Cancer Res* 69: 9073-9082, 2009.
22. Conway E, Healy E and Bracken AP: PRC2 mediated H3K27 methylations in cellular identity and cancer. *Curr Opin Cell Biol* 37: 42-48, 2015.
23. Kemp CD, Rao M, Xi S, Inchauste S, Mani H, Fetsch P, Filie A, Zhang M, Hong JA, Walker RL, *et al*: Polycomb repressor complex-2 is a novel target for mesothelioma therapy. *Clin Cancer Res* 18: 77-90, 2012.
24. LaFave LM, Béguelin W, Koche R, Teater M, Spitzer B, Chramiec A, Papalexi E, Keller MD, Hricik T, Konstantinoff K, *et al*: Loss of BAP1 function leads to EZH2-dependent transformation. *Nat Med* 21: 1344-1349, 2015.
25. Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappasilber J, Issaeva I, Canaani E, Salcini AE and Helin K: UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* 449: 731-734, 2007.
26. Hong S, Cho YW, Yu LR, Yu H, Veenstra TD and Ge K: Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. *Proc Natl Acad Sci USA* 104: 18439-18444, 2007.
27. Perriguet PM, Najbauer J and Barciszewski J: Histone demethylase JMJD3 at the intersection of cellular senescence and cancer. *Biochim Biophys Acta* 1865: 237-244, 2016.
28. Paolicchi E, Crea F, Farrar WL, Green JE and Danesi R: Histone lysine demethylases in breast cancer. *Crit Rev Oncol Hematol* 86: 97-103, 2013.
29. Shen Y, Guo X, Wang Y, Qiu W, Chang Y, Zhang A and Duan X: Expression and significance of histone H3K27 demethylases in renal cell carcinoma. *BMC Cancer* 12: 470, 2012.
30. Kruidenier L, Chung CW, Cheng Z, Liddle J, Che K, Joberty G, Bantscheff M, Bountra C, Bridges A, Diallo H, *et al*: A selective jumoni H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature* 488: 404-408, 2012.
31. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ and Minor L: Cell viability assays. In: *Assay Guidance Manual* (Internet). Sittampalam GS, Coussens NP, Nelson H, Arkin M, Auld D, Austin C, Bejcek B, Glicksman M, Ingles J, Iversen PW, *et al* (eds). Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, MD, 2004.
32. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, Barrette TR, Anstet MJ, Kincaid-Beal C, Kulkarni P, *et al*: Oncomine 3.0: Genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia* 9: 166-180, 2007.
33. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, *et al*: The cBio cancer genomics portal: An open platform for exploring multi-dimensional cancer genomics data. *Cancer Discov* 2: 401-404, 2012.
34. Gordon GJ, Rockwell GN, Jensen RV, Rheinwald JG, Glickman JN, Aronson JP, Pottorf BJ, Nitz MD, Richards WG, Sugarbaker DJ, *et al*: Identification of novel candidate oncogenes and tumor suppressors in malignant pleural mesothelioma using large-scale transcriptional profiling. *Am J Pathol* 166: 1827-1840, 2005.
35. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G and Natoli G: The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell* 130: 1083-1094, 2007.
36. Bononi A, Napolitano A, Pass HI, Yang H and Carbone M: Latest developments in our understanding of the pathogenesis of mesothelioma and the design of targeted therapies. *Expert Rev Respir Med* 9: 633-654, 2015.
37. Comertpay S, Pastorino S, Tanji M, Mezzapelle R, Strianese O, Napolitano A, Baumann F, Weigel T, Friedberg J, Sugarbaker P, *et al*: Evaluation of clonal origin of malignant mesothelioma. *J Transl Med* 12: 301, 2014.
38. Arcipowski KM, Martinez CA and Ntziachristos P: Histone demethylases in physiology and cancer: A tale of two enzymes, JMJD3 and UTX. *Curr Opin Genet Dev* 36: 59-67, 2016.
39. Lansley SM, Varano Della Vergiliana JF, Cleaver AL, Ren SH, Segal A, Xu MY and Lee YC: A commercially available preparation of *Staphylococcus aureus* bio-products potentially inhibits tumour growth in a murine model of mesothelioma. *Respirology* 19: 1025-1033, 2014.
40. Yom SS, Busch TM, Friedberg JS, Wileyto EP, Smith D, Glatstein E and Hahn SM: Elevated serum cytokine levels in mesothelioma patients who have undergone pleurectomy or extrapleural pneumonectomy and adjuvant intraoperative photodynamic therapy. *Photochem Photobiol* 78: 75-81, 2003.
41. Stam TC, Swaak AJ, Kruit WH, Stoter G and Eggermont AM: Intrapleural administration of tumour necrosis factor-alpha (TNFalpha) in patients with mesothelioma: Cytokine patterns and acute-phase protein response. *Eur J Clin Invest* 30: 336-343, 2000.
42. Nowak AK, Millward MJ, Creaney J, Francis RJ, Dick IM, Hasani A, van der Schaaf A, Segal A, Musk AW and Byrne MJ: A phase II study of intermittent sunitinib malate as second-line therapy in progressive malignant pleural mesothelioma. *J Thorac Oncol* 7: 1449-1456, 2012.
43. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, Grupp SA and Mackall CL: Current concepts in the diagnosis and management of cytokine release syndrome. *Blood* 124: 188-195, 2014.
44. Nowak AK, Cook AM, McDonnell AM, Millward MJ, Creaney J, Francis RJ, Hasani A, Segal A, Musk AW, Turlach BA, *et al*: A phase Ib clinical trial of the CD40-activating antibody CP-870,893 in combination with cisplatin and pemetrexed in malignant pleural mesothelioma. *Ann Oncol* 26: 2483-2490, 2015.