Oncogenic KRAS Sensitizes Lung Adenocarcinoma to GSK-J4–Induced Metabolic and Oxidative Stress

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Abstract

Genetic and epigenetic changes (e.g., histone methylation) contribute to cancer development and progression, but our understanding of whether and how specific mutations affect a cancer’s sensitivity to histone demethylase (KDM) inhibitors is limited. Here, we evaluated the effects of a panel of KDM inhibitors on lung adenocarcinomas (LuAC) with various mutations. Notably, LuAC lines harboring KRAS mutations showed hypersensitivity to the histone H3K27 demethylase inhibitor GSK-J4. Specifically, GSK-J4 treatment of KRAS mutant–containing LuAC downregulated cell-cycle progression genes with increased H3K27me3. In addition, GSK-J4 upregulated expression of genes involved in glutamine/glutamate transport and metabolism. In line with this, GSK-J4 reduced cellular levels of glutamate, a key source of the TCA cycle intermediate α-ketoglutarate (αKG) and of the antioxidant glutathione, leading to reduced cell viability. Supplementation with an αKG analogue or glutathione protected KRAS-mutant LuAC cells from GSK-J4–mediated reductions in viability, suggesting GSK-J4 exerts its anticancer effects by inducing metabolic and oxidative stress. Importantly, KRAS knockdown in mutant LuAC lines prevented GSK-J4–induced decrease in glutamate levels and reduced their susceptibility to GSK-J4, whereas overexpression of oncogenic KRAS in wild-type LuAC lines sensitized them to GSK-J4. Collectively, our study uncovers a novel association between a genetic mutation and KDM inhibitor sensitivity and identifies the underlying mechanisms. This suggests GSK-J4 as a potential treatment option for cancer patients with KRAS mutations.

Significance: This study not only provides a novel association between KRAS mutation and GSK-J4 sensitivity but also demonstrates the underlying mechanisms, suggesting a potential use of GSK-J4 in cancer patients with KRAS mutations.

Introduction

Lung adenocarcinoma (LuAC) is the most common subtype of non–small cell lung carcinoma (NSCLC; ref. 1). The 5-year survival rate for LuAC is about 18%, owing mainly to a lack of effective treatment options. Current treatment options include surgery, chemo- and radiotherapies, and targeted therapies against EGFR and ALK (anaplastic lymphoma kinase) mutant–containing LuAC (1, 2). Although several strategies to target LuAC with KRAS mutations, the most prevalent type of mutation in LuAC (3, 4), have been suggested (5–7), no effective treatments for this subtype are currently available, necessitating novel approaches.

In addition to genetic mutations, epigenetic deregulation also contributes to LuAC (8–10). Thus, epigenetic inhibitors have been explored as alternative anticancer drugs for LuAC. For example, inhibitors against DNA methyltransferases and histone deacetylases are used as therapeutic agents for several cancers (11). In the case of LuAC, therapeutic strategies combining these epigenetic inhibitors with EGFR inhibitors or other chemotherapeutic agents are currently in clinical trials (11).

Furthermore, inhibitors targeting histone lysine methyltransferases (KMT) and demethylases (KDM) are being tested as cancer therapeutics. For example, inhibitors against H3K27 demethylase EZH2 are under clinical trial for solid tumors (12). In lung cancer, EZH2 inhibition sensitizes BRG1 and EGFR mutant NSCLC to TopoII inhibitors in a pre-clinical model (13). In addition, inhibitors targeting H3K4 demethylase LSD1 and H3K79 methyltransferase DOT1L are being evaluated for leukemia (12, 14). Inhibitors targeting KDM5 and KDM6, demethylases for H3K4 and H3K27, respectively, have even been tested in some preclinical cancer models (15, 16).

The importance of targeting KMTs and KDMs in cancer is also supported by an interplay between these enzymes and...
metabolism (17). Cancer cells often undergo oncogene-induced metabolic rewiring, which then leads to changes in cellular levels of a number of metabolites (17, 18). Because KMTs and KDMs use metabolic intermediates as cofactors [e.g., SAM for EZH2 and α-ketoglutarate (αKG) for Jumonji C-domain-containing (JMID) enzymes], changes in cellular levels of these cofactors can alter the activities of KMTs and KDMs in cancer cells (17, 18). Reciprocally, these enzymes can regulate the expression of metabolic genes (19–21). For example, EZH2 reprograms metabolism in glioblastoma and leukemia by regulating metabolic genes (19, 20). In addition, KDM6B regulates genes involved in β-oxidation and anabolism in hepatocytes, although their roles in cancer metabolism are unknown (21).

Despite the recent development of KMT and KDM inhibitors, their effects and mechanisms of action remain unclear. This emphasizes the need for an in-depth investigation of the association between genetic mutations, their underlying mechanisms, and their susceptibility to epigenetic modification.

Here, we report a novel association between oncogenic KRAS and sensitivity to GSK-J4 in LuAC and the underlying mechanism.

**Materials and Methods**

**Cell culture**

The human LuAC and pancreatic ductal adenocarcinoma (PDAC) cell lines were cultured in RPMI1640 containing 10% FBS. WI38 and IMR90 were cultured in MEM containing 10% FBS. HCC827, H23, H2009, and SN1-324 were purchased from Korean cell line bank. H1975, A549, MIA-Paca-2, and Panc-1 were originally obtained from the ATCC. All cell lines were recently authenticated by DNA fingerprinting analysis and regularly tested for Mycoplasma contamination by e-Mycoplus Mycoplasma PCR Detection Kit. All cell lines were used within 15 passages.

**RNAi-mediated gene knockdown**

SiRNA pools were synthesized from Bioneer. ON-TARGETplus SMARTpool siRNA targeting for KRAS were purchased from Dharmacon. Cells were transfected with siRNAs using Lipofectamine 2000. siRNA sequences used in this study are summarized in Supplementary Materials and Methods.

**Cell viability assay**

Viabilities of LuAC cells treated with DMSO or GSK-J4 for 24 hours and stained with 30 μg/mL 7-AAD (Sigma Aldrich) or Annexin V – Alexa 488 (Life Technologies) and 7-AAD solution (BD) for 30 minutes. Flow cytometry was conducted using BD LSRFortessa (BD). Cell-cycle distribution and percent apoptotic cells were analyzed by using FlowJo software v10 (FlowJo, LLC).

**Cell-cycle and apoptosis analysis**

Cells were treated with 10 μmol/L GSK-J4 for 24 hours and stained with 30 μg/mL 7-AAD (Sigma Aldrich) or Annexin V – Alexa 488 (Life Technologies) and 7-AAD solution (BD) for 30 minutes. Flow cytometry was conducted using BD LSRFortessa (BD). Cell-cycle distribution and percent apoptotic cells were analyzed by using FlowJo software v10 (FlowJo, LLC).

**Genome-wide RNA sequencing analysis**

Total RNA was extracted from LuAC cells treated with DMSO or 10 μmol/L GSK-J4 for 24 hours or siKDM6B. Libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina) according to the manufacturer’s instructions. The resulting RNA sequencing (RNA-seq) libraries were then sequenced on an Illumina NextSeq 500 using the single-end protocol with a read length of 75 nt. For detailed information on analysis, see Supplementary Materials and Methods.

**Gene set enrichment analysis**

Gene set enrichment analysis (GSEA) was performed according to the instructions. Briefly, GSK-J4 UP and GSK-J4 DN were generated on the basis of the genes defined in Fig. 3A. For GSEA preranked analyses of KDM6B knockdown samples, log2 of the fold changes calculated from RNA-seq analysis were provided.

**Genome-wide ChIP sequencing analysis**

To align chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq) reads, raw reads from the fastq files were aligned to the human reference genome assembly hg19 using Bowtie with default settings except for allowing two-mismatches for unique alignments (i.e., `-v 2 -m 1`). To avoid clonal artifacts introduced in the library amplification steps, duplicated mapped reads were further removed using Samtools. BAM files were subjected to other analyses with the HOMER suite. Peaks were identified in the processed BAM files and ChIP-seq tags were calculated using the findPeaks tool set to find histone-enriched regions (i.e., “-style histone”). Detailed information is provided in Supplementary Materials and Methods.

**Gene ontology**

A total of 246 up- and 591 downregulated genes were subjected to Gene Ontology (GO) term enrichment with Metascape (http://metascape.org; ref. 22).

**Quantification of cellular glutamine, glutamate, and glutathione levels**

The cellular concentrations of glutamine/glutamate and glutathione from cells treated with DMSO or 5 μmol/L GSK-J4 for 12 hours were measured by using the Glutamine/ Glutamate-Glo and GSH-Glo Glutathione Assay Kit (Promega), respectively.

**Quantification of reactive oxygen species**

Cells were plated on 96-well plates and treated with 5 μmol/L GSK-J4 with or without 100 μmol/L VitE for 48 hours. Reactive oxygen species (ROS) levels were quantified using ROS-Glo H2O2 Assay Kit (Promega).
Statistical analysis
Results are reported as means ± SEM and statistical significance was determined by two-tailed unpaired Student t test, unless indicated otherwise. P values of < 0.05 were considered statistically significant.

Data availability
RNA-seq and ChIP-seq data are available in NCBI GEO: GSE133970.

Additional information is described in the Supplementary Materials and Methods.

Results
LuAC cell lines exhibit differential GSK-J4 sensitivity
To determine whether LuAC cell lines show differential sensitivity toward various KDM inhibitors, we analyzed the viability of a panel of LuAC cell lines following treatment with JIB-04 (a pan-JMJC inhibitor), SD70 (a KDM4C inhibitor), IOX1 [a 2-oxoglutarate (2OG)-dependent KDM inhibitor], PBIT (a KDM5 inhibitor), GSK-1SD1 (a LSD1 inhibitor), and GSK-J4 (a KDM5 and 6 subfamily inhibitor; refs. 23 and 6 subfamily inhibitor), GSK-LSD1 (a LSD1 inhibitor), and GSK-J4 (a pan-JMJC inhibitor), SD70 (a KDM4C inhibitor), IOX1 [a 2-oxoglutarate (2OG)-dependent KDM inhibitor], PBIT (a KDM5 inhibitor), GSK-1SD1 (a LSD1 inhibitor), and GSK-J4 (a KDM5 and 6 subfamily inhibitor; refs. 23–26). Notably, at concentrations with minimal effects on the normal lung fibroblast cell lines WI38 and IMR90, only GSK-J4 treatment segregated LuAC lines into two groups in cell viability and colony formation assays: “GSK-J4 sensitive” and “GSK-J4 resistant” lines (Fig. 1A–C; Supplementary Fig. S1A–S1C). These differences in GSK-J4 sensitivity were recapitulated by KDM6B but not KDM6A knockdown (Fig. 1D; Supplementary Fig. S1D and S1E), suggesting KDM6B as a major GSK-J4 target. Further supporting this, GSK-J4 attenuated tumor growth by the sensitive (H2303 and A549), but not by the resistant (PC9 and HCC827), lines (Fig. 1E and F; Supplementary Fig. S1F–S1H). Similar results were observed upon KDM6B knockdown (Supplementary Fig. S1I).

GSK-J4 attenuates cell-cycle progression of GSK-J4–sensitive LuAC
On the basis of differential GSK-J4 sensitivities observed in LuAC, we investigated the underlying cellular mechanisms. GSK-J4 and KDM6B knockdown induced cell-cycle arrest only in GSK-J4–sensitive lines (Fig. 2A; Supplementary Fig. S2A and S2B). GSK-J4 also increased apoptosis in the sensitive line H23 (Fig. 2B) without affecting resistant lines (Fig. 2C; Supplementary Fig. S2C and S2D). In addition, GSK-J4 did not induce apoptosis in the other sensitive lines even at a later time point (Supplementary Fig. S2E–S2G), which is consistent with H23 being the most sensitive to GSK-J4 (refer to Fig. 1A–C). Whether or not GSK-J4 induces apoptosis in the sensitive lines are probably dependent on differences in their intrinsic biological properties. Together, GSK-J4’s antitumor effects on the sensitive lines occur via cell-cycle arrest and/or induction of apoptosis.

GSK-J4 affects specific transcriptional programs in sensitive LuAC
Next, we investigated the molecular mechanisms underlying the differential GSK-J4 sensitivities of LuAC. Because KDM6B knockdown mimicked GSK-J4’s effect on cell viability, we first looked for a correlation between GSK-J4 sensitivity and basal levels of H3K27me3 and its modifiers EZH2, KDM6A, and KDM6B, but found none (Supplementary Fig. S3A and S3B). We hypothesized that GSK-J4 sensitivity may arise from GSK-J4–mediated modulation of specific transcriptional programs in sensitive LuAC lines. To test this, we performed RNA-seq experiment and selected genes exhibiting GSK-J4–induced expression changes (fold change > 1.5, P < 0.05) in two sensitive lines (H23 and A549) but not the resistant line (HCC827). We defined the resulting list of 246 downregulated and 591 upregulated genes as a “GSK-J4 signature” (Fig. 3A; Supplementary Data S1). GSEA then revealed GSK-J4 signature genes show significant overlap with KDM6B–regulated genes in GSK-J4–sensitive lines (Fig. 3B; Supplementary Fig. S3C), again supporting preferential targeting of KDM6B by GSK-J4.

Most of the genes downregulated by GSK-J4 and KDM6B knockdown in sensitive lines were cell-cycle progression genes (Fig. 3C; Supplementary Data S2; Supplementary Fig. S3D). Furthermore, promoters of downregulated genes exhibited an enrichment of binding sites for cell cycle–related transcription factors (Supplementary Fig. S4E; refs. 27–30). This suggests GSK-J4 attenuates the cell cycle in sensitive LuAC by downregulating cell cycle–related genes. On the other hand, a majority of GSK-J4 upregulated genes belong to the stress-related pathways that respond to external stimuli, endoplasmic reticulum (ER) stress, and ROS (Fig. 3D; Supplementary Table S1; Supplementary Fig. S3F; Supplementary Data S3). We also observed significant enrichment for genes that function in amino acid transport and metabolism (Fig. 3D; Supplementary Data S3), which will be further discussed below.

Next, we investigated whether GSK-J4–mediated gene expression changes are associated with changes in histone H3 methylations. Consistent with our Western blotting results, ChIP-seq analysis revealed that GSK-J4–induced enrichment of H3K27me3 in the sensitive A549 line without affecting expression of its target KDMs (Fig. 3E; Supplementary Fig. S4A–S4C). Moreover, about half of the chromatin regions exhibiting GSK-J4–induced H3K27me3 increase were common to KDM6B knockdown including cell-cycle genes like E2F8 region (Fig. 3F and G; Supplementary Fig. S4D and S4E). We also observed H3K4me3 increase in a subset of GSK-J4–upregulated genes (Supplementary Fig. S4F and S4G). Thus, our data support that GSK-J4 affects several transcriptional programs in sensitive LuAC and this is associated with changes in histone methylation.

GSK-J4 induces oxidative and metabolic stress in sensitive LuAC
As described in Fig. 3D, we found GSK-J4 treatment enhanced the expression of stress-related genes. Consistent with this, the promoters of most of these genes contained binding sites for activating transcription factor 4 (ATF4), an important effector in the integrative stress response (ISR; Supplementary Fig. S5A). ATF4 is induced by oxidative and metabolic stress (31, 32) and upregulates transcription of target genes (31, 32) that lead to cell-cycle arrest and/or apoptosis (32). GSK-J4 increased ATF4 expression at the mRNA and protein levels as well as eIF2α phosphorylation, which promotes ATF4 translation (Supplementary Fig. S5B and S5C; ref. 32). Increased ATF4 was accompanied by upregulation of ATF4 target genes (Supplementary Fig. S5D), suggesting GSK-J4 specifically activates the ATF4 pathway in sensitive LuAC lines.

On the basis of this, we hypothesized that GSK-J4–mediated ATF4 induction may contribute to an antitumor effect of GSK-J4 in sensitive LuAC. Contrary to our expectation, however, ATF4 knockdown had no effect on GSK-J4 sensitivity (Supplementary
Thus, rather than directly contributing to GSK-J4-mediated reductions in cell viability, ATF4 induction may simply reflect the activation of the ISR pathway in sensitive lines. On the basis of this, we searched for other pathway(s) that could contribute to GSK-J4 sensitivity. Interestingly, GSK-J4 changed the expression of genes associated with amino acid transport and metabolism and with responses to nutrient deprivation (refer to Fig. 3D; Supplementary Table S1). Further analysis of RNA-seq data revealed that GSK-J4 altered the expression of genes involved in glutamine (Gln)/glutamate (Glu) transport and metabolism only in the sensitive lines (Supplementary Fig. S6A). Basal levels of Gln and Glu showed no correlation with GSK-J4 sensitivity (Supplementary Fig. S6B). GSK-J4, however, led to accumulation and reduction of cellular Gln and Glu levels only in the sensitive lines, respectively (Fig. 4A and B). Similar effects were observed upon KDM6B knockdown (Supplementary Fig. S6C).

Glutamate is a major source for the cellular antioxidant glutathione as well as αKG, a major intermediate in the TCA cycle (33, 34). Thus, we hypothesized that GSK-J4-mediated reduction in glutamate may cause a subsequent decrease in glutathione and αKG, leading to increased oxidative and metabolic stress, respectively. Although we found no correlation between basal levels of ROS and glutathione and GSK-J4 sensitivity (Fig. 4C and D), GSK-J4 reduced cellular glutathione levels and increased ROS levels in the sensitive lines (Fig. 4D and E). In addition, changes in Glu/Gln and ROS levels in H23 were saturated at 5 μmol/L GSK-J4, while A549 cells showed further decrease at 10 μmol/L (refer to Fig. 1B and C). This supports a direct association between GSK-J4 sensitivity and GSK-J4-mediated induction of oxidative stress.

Figure 1.
GSK-J4 treatment segregates LuAC cell lines into GSK-J4-sensitive and resistant groups. A, Relative viability of the LuAC cell lines 48 hours post GSK-J4 treatment. Cell viability was normalized to DMSO-treated cells. Resistant lines: HCC827, PC9, H1975, and HCC4006. Sensitive lines: H2030, H23, A549, and H2009. The highest P values between the resistant and sensitive groups at the indicated concentration of GSK-J4 are shown [n = 7 (HCC827, H23, A549, H2030); n = 6 (PC9); n = 4 (H2009); n = 3 (H1975, HCC4006)]. B and C, Colony formation by indicated LuAC cell lines upon GSK-J4 treatment. Cells were treated with GSK-J4 for 3 days and grown for another 3 days in fresh media without GSK-J4. Quantification of relative colony formation is shown with same color codes used in A (n = 3). Representative images are shown (C), D, Comparison of cell viability 96 hours posttransfection with 50 nmol/L of siNT (nontargeting), siKDM6A, or siKDM6B (n = 6 except for siKDM6B #1, n = 4). E and F, Tumor growth rate of PC9 (E) and H2030 (F) upon DMSO or GSK-J4 treatment in xenograft assays. Cells were subcutaneously injected into the immunocompromised mice. When tumor sizes reached 50 to 70 mm3, mice were treated with DMSO or GSK-J4 (100 mg/kg) every 2 days for five times. D0, the initial day of the drug treatment. Arrows on the x-axis indicate the completion of drug treatment. Data, means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.0001. n.s., nonsignificant.
Furthermore, relieving oxidative stress by supplementation of glutathione and the ROS scavenger α-tocopherol (VitE) reduced the GSK-J4 susceptibility of the sensitive lines, albeit not completely (Fig. 4F–H). Finally, supplementation of D2OG, a cell-permeable αKG analogue, protected GSK-J4–sensitive lines from GSK-J4 and this was accompanied by a prevention of the GSK-J4–mediated induction of ATF4 (Fig. 5A–D; Supplementary Fig. S6F). This indicates that GSK-J4–induced glutamate deprivation accounts for the activation of the ISR pathway in sensitive LuAC. Collectively, our data indicate that GSK-J4–induced reductions in glutamate reduce cellular glutathione and αKG levels, which together contribute to the GSK-J4 susceptibility of sensitive LuAC cells.

An activating KRAS mutation sensitizes LuAC to GSK-J4

Although our study identified the mechanism underlying GSK-J4 sensitivity of LuAC, we were curious how mutations in oncogenes and/or tumor suppressors would affect GSK-J4 sensitivity. Interestingly, GSK-J4–sensitive and resistant lines harbored activating KRAS and EGFR mutations, respectively (Supplementary Table S2).

Neither overexpression of EGFR in GSK-J4–sensitive lines nor EGFR knockdown in the resistant line affected their GSK-J4 sensitivity (Supplementary Fig. S7A–S7C). However, KRAS knockdown in GSK-J4–sensitive lines significantly reduced their sensitivity to GSK-J4 (Fig. 6A; Supplementary Fig. S7D and S7E). As reported, the viability of KRAS mutant LuAC cells was sensitive to KRAS knockdown (35). Thus, we performed these experiments with a suboptimal KRAS knockdown that only caused a minimal reduction in cell viability. Overexpression of activating KRAS mutants (KRASG12V and KRASG12C) in the resistant H1975 line increased GSK-J4 susceptibility in vitro and in vivo (Fig. 6B and C; Supplementary Fig. S7F). Furthermore, KRAS knockdown in H1975 reduced GSK-J4 sensitivity at a higher concentration of GSK-J4 (Supplementary Fig. S7G), demonstrating that KRAS dependence of GSK-J4 sensitivity in isogenic cell lines. We noted that mutant KRAS-containing PDAC cell lines also showed GSK-J4–mediated increase in ROS and higher sensitivity to GSK-J4, compared with wild type–containing lines (Supplementary Fig. S7H and S7I). Furthermore, KRAS knockdown protected mutant KRAS-containing
Figure 3.
GSK-J4 affects specific transcriptional programs in sensitive LuAC. A, Venn diagram showing genes down- (left) and upregulated (right) upon GSK-J4 treatment in sensitive (H23, A549) and resistant (HCC827) lines according to an RNA-seq analysis. Genes subjected to further gene ontology analyses are indicated by asterisks. B, GSEA plots showing enrichment of GSK-J4 signature gene sets downregulated (left) or upregulated (right) upon KDM6B knockdown in A549. NES, normalized enrichment score. C and D, Gene Ontology (GO) analysis of 246 GSK-J4-DN (C) and 591 GSK-J4-UP (D) genes in sensitive lines. The top 20 enriched biological processes ranked by their \(-\log_{10}(P)\) values. E and F, Box plots showing genome-wide H3K27me3 enrichment upon GSK-J4 (E) or siKDM6B (F) treatment. \(P\) values were obtained from Welch \(t\) test. Data, means ± Max/Min. G, Venn diagram showing common H3K27me3 ChIP-seq signals upon GSK-J4 and KDM6B knockdown.
cell line from GSK-J4's effect on cell viability (Supplementary Fig. S7J and S7K). Thus, oncogenic KRAS renders LuAC and PDAC more susceptible to GSK-J4.

Because GSK-J4 reduces the viability of sensitive lines by inducing oxidative and metabolic stress, we asked whether oncogenic KRAS sensitizes LuAC to GSK-J4 via these mechanisms. KRAS knockdown in A549 impaired GSK-J4-mediated ROS induction (Fig. 6D) while H1975-KRASG12V showed increased Gln and decreased Glu levels upon GSK-J4 (Fig. 6G). Consistent with this, KRAS knockout in H1975 impaired GSK-J4-mediated increases in ROS and Gln (Supplementary Fig. S7L and S7M).

Furthermore, D2OG restored the viability of H1975-KRASG12V in the presence of GSK-J4 (Fig. 6H), which is similar to what we observed with the sensitive lines (refer to Fig. 5A). Finally, KRAS knockout in the sensitive line prevented GSK-J4-mediated induction of ATF4, while KRAS overexpression in the resistant

Figure 4.
GSK-J4 induces oxidative stress in sensitive LuAC cell lines. A and B, Comparison of glutamine (A) and glutamate (B) levels in GSK-J4-sensitive and -resistant lines upon GSK-J4 treatment (5 μmol/L, 12 hours; n = 6 (A549); n = 5 (H23); n = 4 (PC9, H2069); n = 3 (HCC4006, HCC827, H1975)). C, Comparison of basal ROS levels in GSK-J4-resistant and -sensitive lines in the absence of GSK-J4. The ROS level in H23 was set to 1 (n = 5 (H23, A549); n = 4 (HCC4006, HCC827, H2030); n = 3 (PC9, H975, H2009)). D, Basal levels (DMSO) and GSK-J4-mediated changes (GSK-J4, 5 μmol/L, 12 hours) in total glutathione levels in resistant and sensitive lines (n = 3 except for A549, n = 4). E, GSK-J4 induced fold changes in ROS levels in resistant and sensitive cell lines (5 μmol/L, 48 hours). ROS levels were normalized to DMSO-treated samples. No cells: ROS levels in GSK-J4-containing media (n = 3 except for H23 and A549, n = 4). F, Comparison of A549 cell viability upon GSK-J4 treatment (5 μmol/L, 48 hours), with or without Vit E (100 μmol/L; n = 4). Data, means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., nonsignificant.
line stimulated it (Fig. 6I). These results are consistent with GSK-J4–mediated activation of ISR in the sensitive lines. Together, our data indicate oncogenic KRAS sensitizes LuAC to GSK-J4 by inducing oxidative and metabolic stress.

**Discussion**

Deregulated histone methylation contributes to the progression of several types of cancers, suggesting KMT and KDM inhibitors as anticancer drug candidates. This promising concept, however, has not been as straightforward as it first appeared mainly because of the low specificity of these inhibitors and insufficient information on their mechanisms of action. More importantly, a lack of appropriate biomarkers often makes it difficult to identify the patient groups who will benefit most from epigenetic inhibitors.

Here, we demonstrated KRAS mutations render LuAC susceptible to GSK-J4 and identified the underlying mechanisms. To our knowledge, this is the first report of an association between a genetic mutation in a non-histone gene and a cancer’s susceptibility to GSK-J4. Our data indicate GSK-J4 confers its antitumor effect on LuAC mainly by inhibiting KDM6B activity. Consistent with our study, other reports have suggested KDM6B as a main target of the GSK-J4 in high-risk

![Figure 5.](image-url)
neuroblastoma, leukemia, and H3.3 K27M mutant–containing glioma (36–38). Although GSK-J4 seems to function in sensitive LuAC primarily via inhibition of KDM6B, it may also act via KDM5 family members.

Our study suggests that GSK-J4–mediated glutamate reduction as an important contributor to LuAC GSK-J4 sensitivity. This glutamate reduction then induces oxidative and metabolic stress by limiting cellular levels of the anti-oxidant glutathione and the TCA cycle intermediate αKG. Consistent with our results, recent studies reported glutaminase inhibitor shows antitumor effects in hepatocarcinoma, triple-negative breast cancer, IDH1 mutant–containing glioma, and KRAS/KEAP1/NRF2 mutant–containing LuAC (39–41).

In addition to being a TCA cycle intermediate, αKG is a cofactor for JMJC domain–containing KDM family members. Thus, changes in αKG can affect KDM activities. In BRAF-mutant melanoma, for example, tumor areas with low glutamate (and low αKG) showed increased H3K27me3 owing to reduced KDM6B

Figure 6. An activating KRAS mutation sensitizes LuAC to GSK-J4. A, Relative GSK-J4 response in A549 cells upon KRAS knockdown. A549 cells were transfected with 3 nmol/L siNT, siKRAS #1, or #2. Seventy-two hours posttransfection, cells were treated with GSK-J4 for 48 hours, and relative viability was analyzed (n = 5). B, Similar experiments to A except that H1975-expressing control vector (Vec) or oncogenic KRAS<sup>G12V</sup> or KRAS<sup>G12C</sup> were used (n = 4). C, Tumor growth rates of the indicated cell lines upon DMSO (D) or GSK-J4 (G) treatment in xenograft assays (n = 12 except for H1975-KRAS<sup>G12V</sup> DMSO, n = 11). D and E, Relative ROS levels in cells from A and B upon GSK-J4 treatment (5 μmol/L, 48 hours; n = 3 for D, 4 for E). F and G, Relative Gln and Glu levels in the indicated A549 (F) and H1975 (G) cell lines upon GSK-J4 treatment (5 μmol/L, 12 hours; n = 3). H, Comparison of GSK-J4 sensitivity in H1975 cells with or without D2OG. The indicated cells were pretreated with 2 mmol/L D2OG for 2 hours, followed by GSK-J4 treatment for 24 hours (n = 3). I, Changes in ATF4 protein levels in the same lines used in F and G upon GSK-J4 treatment (5 μmol/L, 12 hours). Representative images are shown (n = 3). D, DMSO; G, GSK-J4. Data, means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
activity (42). Thus, we speculate GSK-J4-mediated reduction of eGK may amplify the effects of GSK-J4 by further inhibition of KDM6B function. This may explain why GSK-J4’s antitumor effect in vivo lasts so long after drug removal, but this warrants further investigation.

The oxidative and metabolic stress caused by GSK-J4 activates the ISR in LuAC as demonstrated by ATF4 induction, but KDM6B function. This may explain why GSK-J4’s antitumor effect in vivo lasts so long after drug removal, but this warrants further investigation.

Importantly, we found GSK-J4-induced oxidative and metabolic stress depends on the presence of oncogenic KRAS. Previous studies showed that KRAS-mutant cancer cells are more sensitive to nutrient and oxidative stress (43, 44). Furthermore, another recent study showed in NSCLC that oncogenic KRAS alters expression of genes involved in amino acid transport and metabolism upon glutamine deprivation (45). Thus, our study links the role of oncogenic KRAS in the metabolic stress response to GSK-J4 sensitivity. In addition to having KRAS mutation, three of four GSK-J4-sensitive LuAC lines contained LKB1 comutations. Because KRAS/LKB1 comutations have been reported to confer hypersensitivity to a DNA methyltransferase inhibitor in LuAC and PDAC (46), we also examined the contribution of LKB1 mutation in GSK-J4 sensitivity, but found no association. This suggests that oncogenic KRAS is a major determinant of GSK-J4 sensitivity.

To data, association between histone H3.3 K27M mutations and GSK-J4 sensitivity in glioma has been validated (47). A potential association of GSK-J4 sensitivity with PTEN loss and CEBP/p300 mutations has been suggested, but experimental data was insufficient (48, 49). In addition, consistent with our results, a recent study reported A549 is more sensitive to GSK-J4 than the PC9 line (50), but this was not investigated further. Thus, our study is the first to experimentally validate an association between a nonhistone gene mutation and GSK-J4 sensitivity. Collectively, we have demonstrated an unprecedented synthetic lethal interaction between oncogenic KRAS and GSK-J4 and discovered its novel mechanism of action. Further studies on this synthetic lethality may expand the use of GSK-J4 in treating various cancers with KRAS mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: B.-J. Hong, W.-Y. Park, M.-Y. Kim

Development of methodology: W.-Y. Park, J.W. Moon, J.H. Park

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.-J. Hong, W.-Y. Park, H.-R. Kim, J.W. Moon, H.Y. Lee, J.H. Park, Y. Oh, J.-S. Roe, M.-Y. Kim

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