Polo-like kinase 4 promotes tumorigenesis and induces resistance to radiotherapy in glioblastoma

JIA WANG[1,2], JIE ZUO[3], MAODE WANG[1,2], XUDONG MA[1], KE GAO[1],
XIAOBIN BAI[1], NING WANG[1], WANFU XIE[1] and HAO LIU[1]

[1]Department of Neurosurgery; [2]Center of Brain Science, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061; [3]The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710004, P.R. China

Received September 3, 2018; Accepted February 7, 2019

DOI: 10.3892/or.2019.7012

Abstract. Glioblastoma (GBM) is one of the most malignant tumors in adults, associated with severe outcomes (median survival, <2 years). Multiple mechanisms are known to be involved in tumor recurrence and treatment resistance in GBM, however, the key regulator for GBM tumorigenesis and therapy resistance remains unclear. To clarify a novel potential functional mechanism of GBM recurrence, a wide range of experiments including *in vitro* molecular biological experiments and *in vivo* intracranial xenograft tumor models were performed in the present study. With bioinformatics analysis, polo-like kinase 4 (PLK4) was initially identified as one of the most upregulated kinase encoding genes in GBM, which was functionally required for both *in vitro* cell proliferation and *in vivo* tumorigenesis in GBM. Clinically, an elevated PLK4 expression was observed in high grade glioma patients, which was associated with poor prognosis. In addition, PLK4 enhanced radioresistance in GBM, while PLK4 knockdown via lentivirus transfection significantly increased the radiosensitivity of GBM cells. Mechanically, PLK4 expression was markedly elevated by the exogenous overexpression of ATPase family AAA domain-containing protein 2 (ATAD2) in GBM cells. Collectively, the results suggested that the ATAD2-dependent transcriptional regulation of PLK4 promoted cell proliferation and tumorigenesis, as well as radioresistance in GBM, thus potentially inducing tumor recurrence. PLK4 could therefore serve as a potential therapeutic target for GBM treatment.

Introduction

Glioblastoma (GBM) is one of the most malignant tumors in adults which is associated with severe outcomes (median survival, <2 years) even with maximal therapy, including surgical resection followed by radiotherapy and adjuvant chemotherapy with temozolomide (1,2). The majority of GBM patients have been reported to suffer post-treatment recurrence, due to radiotherapy and chemotherapy resistance (3). Therefore, clarifying the mechanism for GBM treatment resistance may help to identify a novel therapeutic target for the treatment of GBM.

Polo-like kinase 4 (PLK4) is a centrosomal kinase which predominantly functions as a key regulator of centrosome duplication in human cells and serves an important role in chromosome instability (CIN) regulation, a unique genetic feature that is observed in human cancer cells (4-6). PLK4 auto-phosphorylation has been proven to be essential for centriole duplication and proteasomal degradation of PLK4 in the early G1 phase of cell cycles (7). In addition, a negative feedback loop for PLK4 kinase functions against the occurrence of centriole duplication, thus preventing multipolar spindle formation during the cell cycle (7-10). Furthermore, it has been reported that PLK4 kinase-dependent centrosome amplification promotes cell proliferation, motility, viability and treatment resistance, and therefore may be associated with poor prognosis in breast cancer (9,11,12). A previous study demonstrated that PLK4 expression is significantly elevated in gastric cancer, while enriched PLK4 results in the suppression of primary cilia formation (6). Through combined RNAi screening in human breast cancers, it was found that PLK4 may be a promising target for breast cancer, and a small molecule inhibitor, CFI-400945, was proven to be effective for breast cancer in xenograft models at well-tolerated doses (13-15). However, the physiological role and function of PLK4 in GBM remains unclear.

In the present study, PLK4 was identified as one of the most upregulated kinase encoding genes in GBM and was functionally required for both *in vitro* cell proliferation and *in vivo* tumorigenesis. Clinically, an elevated PLK4 was observed in high grade glioma patients and was associated with poor prognosis. In addition, PLK4 enhanced radiotherapy resistance in GBM, while PLK4 knockdown via lentivirus transfection significantly increased the radiosensitivity of GBM cells. Mechanically, PLK4 expression was markedly elevated by exogenous overexpression of ATPase family AAA domain-containing protein 2 (ATAD2) in GBM cells. Collectively, it was shown that the ATAD2-dependent

Correspondence to: Dr Hao Liu, Department of Neurosurgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: qilin75@qq.com

Key words: ATAD2, glioblastoma, PLK4, radioresistance, tumorigenesis

transcriptional regulation of PLK4 promotes cell proliferation and tumorigenesis, as well as radioresistance of GBM, thus potentially inducing tumor recurrence. PLK4 could therefore serve as a potential therapeutic target for GBM treatment.

Materials and methods

Ethics. The use of experimental animals was approved by the Ethics Committee of the School of Medicine, Xi'an Jiaotong University (Xi'an, China; approval no. 2016-085). The collection and use of the tumor samples and patient information was approved by the patients and the Scientific Ethics Committee of the First Affiliated Hospital of Xi'an (approval no. 2016-18). All usage of the human tissues was confirmed by the patients and all the necessary consent forms were signed.

Reagents and antibodies. The following reagents and antibodies were used in the present study: Dulbecco's modified Eagle's medium-nutrient mixture F12 (DMEM-F12; Thermo Fisher Scientific, Inc., Waltham, MA, USA), fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), accutase solution (Merck KGaA, Darmstadt, Germany), alamarBlue Cell Viability reagent (Thermo Fisher Scientific, Inc.), radioimmunoprecipitation assay (RIPA) lysis buffer (Merck KGaA), phosphatase inhibitor (Merck KGaA), protease inhibitor (Merck KGaA), Bradford solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA), bovine serum albumin (BSA) standard solution (New England BioLabs, Inc., Ipswich, MA, USA), PageRuler plus prestained protein ladder (Thermo Fisher Scientific, Inc.), iScript Reverse Transcription SuperMix (Bio-Rad Laboratories, Inc.), Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific, Inc.).

In vitro cell culture. GBM cell lines U138 and U251, as well as normal human astrocytes (NHAs), were provided by the Translational Medicine Center of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China) in 2013. The U87 cell line (GBM of unknown origin) was originally purchased from BeNa Culture Collection (Kunshan, China). GBM cells were cultured in DMEM-F12 containing 10% FBS at 37˚C with 5% CO_2. The medium was replaced every 3 days. Cells were dissociated with accutase and seeded into new medium with a density of 10^6 cells/10 ml. After 24 h culture at 37˚C with 5% CO_2, radiotherapy was performed *in vitro* using X-RAD 320 from Precision X-Ray at a dose of 12 Gy.

Lentivirus transduction. pGFP-shPLK4 lentivirus particles were purchased from OriGene Technologies, Inc. (cat. no. TL320644V; Beijing, China). pLenti-GIII-CMV ATAD2 lentivirus (cat. no. LVP082354) and pLenti-GIII-CMV PLK4 lentivirus were purchased from Applied Biological Materials, Inc. (Richmond, BC, Canada). U87 cells (2x10^5) were seeded in 6-well plates with 5 ml medium. Next, 10 μl lentivirus was added to the medium and incubated at 37˚C for 24 h. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were performed to confirm transfection efficiency.

RNA isolation and RT-qPCR. RNA isolation and RT-qPCR were performed as previously described (16). The following primers were used: PLK4 forward, CCTTCTGCAAATCTG GATGG and reverse, ACAGTGGTTTGGGAATCTGC; ATAD2 forward, AAGGAAGTTGAAACCTACCACCG and reverse, GCAAGTTGCTCCGTTATTTCCA; 18S forward, GGCCCTGTAATTGGAATGAGTC and reverse, CCAAGA TCCAACTACGAGCTT reverse.

Western blotting. Western blotting was performed as previously described (16). An anti-PLK4 primary antibody was purchased from Abcam (Cambridge, UK; cat. no. ab137398; 1:1,000; rabbit). Anti-rabbit IgG (cat. no. ab171870; 1:1,000; Abcam) was used as a negative control. Horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. ab97051; 1:2,000; Abcam) and goat anti-mouse IgG (cat. no. ab205719; 1:2,000; Abcam) were used as secondary antibodies.

Luciferase assays. PLK4 3' untranslated region (UTR) Lenti-reporter-Luciferase virus was purchased from Applied Biological Materials, Inc. (cat. no. MV-m16562). U87 cells were infected with 1 μg of either empty vector or PLK4 promoter luciferase reporter lentivirus and cultured for 3-5 days at 37˚C with 5% CO_2, and then infected with either control or ATAD2 overexpression lentivirus. Cells were cultured for 7 days at 37˚C with 5% CO_2. Luciferase assays were performed using the Bright-Glo™ Luciferase Assay system (Promega Corporation, Madison, WI, USA) on the Victor3 plate counter (PerkinElmer, Inc., Waltham, MA, USA). The luciferase activity of each sample was normalized to *Renilla* luciferase activity.

Flow cytometry. Flow cytometry was performed as previously described (16). The Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific, Inc.; V13241) was used to measure U87 cell apoptosis according to the manufacturer's protocol.

Immunohistochemistry (IHC). IHC was performed as previously described (16). Glioma samples were collected from 41 patients (aged 22-68; 14 males and 27 females). These patients had undergone surgical resection from 2006 to 2015 at the Department of Neurosurgery (First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China). All patients had been pathologically diagnosed and had died due to tumor recurrence, which was confirmed by computed tomography or magnetic resonance imaging. Three normal human brain tissue samples collected from patients with epilepsy (n=3; aged 32-41; male) were used as the negative controls. An anti-PLK4 primary antibody (cat. no. ab137398; 1:200; Abcam; rabbit) was used for PLK4 staining, and nuclei were counterstained with hematoxylin or Hoechst, respectively. Anti-rabbit IgG (cat. no. ab171870; 1:200; Abcam) was used as a negative control. Goat anti-rabbit IgG (cat. no. ab97051; 1:5,000; Abcam) and goat anti-mouse IgG (cat. no. ab205719; 1:5,000; Abcam) were used as secondary German immunohistochemical scoring (GIS) was used to measure the expression of PLK4 (17), in which the final immunoreactive score = % positive cells x average staining intensity. The percentage of positive cells was graded as follows: 0, negative; 1, <10% positive; 2, 11-50%; 3, 51-80%; 4, >80%. Staining intensity was graded as: 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive. A combination of >3 was considered

positive. Additionally, survival data from the Rembrandt database (Affymetrix HG U133 v.20 plus) was extracted and analyzed with G-doc (gdoc.georgetown.edu/gdoc/workflows/index) to compare the outcomes and PLK4 expression in glioma patients.

In vivo intracranial xenograft tumor models. Female nude mice (6 weeks; ~15 g; n=5 in each group) aged 6 weeks were used for *in vivo* experiments. The nude mice were purchased from Laboratory Animal Center, Xi'an Jiaotong University (Xi'an, China). Briefly, 1×10^5 U87 cells in 5 μl PBS transduced with non-target or shPLK4 lentivirus were implanted into the brains of nude mice following anesthesia. Mice were monitored once a day until at least one of the symptoms associated with tumor growth appeared, including an arched back, unsteady gait, leg paralysis and weight loss of 15%; at which point, the mice were sacrificed and brains were harvested following a ketamine/xylazine anesthesia overdose.

Gene expression analysis. Expression data of 669 kinase-encoding genes was extracted from GSE67089 dataset (18), which included 30 primary glioma sphere cultures (Glioma group) and three human fetal brain-derived sphere cultures (Control group). Hierarchical biclustering was performed to compare the expression of those genes, using Cluster 3.0 (www.geo.vu.nl/~huik/cluster.htm). Euclidean distance and average linkage were used as similarity metric and clustering method, respectively. The expression comparison was presented as fold-changes.

Pearson r correlation analysis. Expression data was extracted from TCGA database (cancergenome.nih.gov/). All data were converted into Log_2 form and the Pearson correlation coefficient was calculated with the following formula:

$$r_{x,y} = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i-\bar{y})^2}}$$

In terms of the strength of relationship, the value of the r coefficient varies between 1 and -1. When the value of the r coefficient is close to 1 or -1, there is a strong positive or negative association between the 2 variables, respectively. The statistical significance was calculated using F-test.

Statistical analysis. Results are presented as the mean ± standard deviation of three replicates. A two tailed t-test was used to evaluate the statistical differences between two groups. One-way analysis of variance followed by Dunnett's post hoc test was used to evaluate the statistical differences among multiple groups. The statistical significance of Kaplan-Meier survival plots was analyzed by the log-rank test. Statistical analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PLK4 is overexpressed in GBM. It is well-known that patients with GBM have a considerably poor prognosis, even following maximal treatment (3). To identify the molecular mechanism for GBM tumor recurrence and treatment resistance, the expression of 669 kinase-encoding genes was first compared using DNA microarray data (GSE67089) (18) from 30 primary glioma sphere cultures and three human fetal brain-derived sphere cultures (control). Based on this analysis, 28 kinase-encoding genes were identified to be significantly enriched in GBM samples, compared with astrocytes. Next, a total of 669 kinase-encoding genes were picked from the transcriptome microarray data of the glioma sphere samples-the results demonstrated a wide range of upregulated genes following radiation at 12 Gy, compared to naïve GBM cells. In total, seven kinase-encoding genes were found to overlap in these two sets of comparisons, including NIMA-related kinase 2, BUB1 mitotic checkpoint serine/threonine kinase B, cell division cycle 7, BUB1, PLK4, CDKN3 and CHEK2 (Fig. 1A).

With PLK4 identified as a functional regulator of tumor proliferation and treatment resistance in a variety of human tumors, including gastric and breast cancer (6,11), the present study focused on the physiological function of PLK4 in GBM. Expression data from the TCGA database were analyzed and PLK4 was found to be upregulated in all four subtypes of GBM, including classical, mesenchymal, neural and proneural, as compared with the normal brain tissues (Fig. 1B). This expression profile by microarray was validated by RT-qPCR in three GBM cell lines (U87, U138 and U251) and a normal astrocyte cell line (Fig. 1C). Western blotting showed a higher PLK4 expression in GBM cells, compared with normal astrocyte cells (Fig. 1D). Taken together, these results demonstrated that PLK4 expression was significantly elevated in GBM.

Overexpression of PLK4 implies poor prognosis in GBM. To further verify the expression of PLK4 in GBM tumors, IHC staining was performed in glioma samples from 41 patients that had undergone surgical resection from 2006 to 2015 at the Department of Neurosurgery of the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China GIS was used to quantify the expression levels of PLK4. In sharp contrast to the low PLK4 expression in normal brain tissues and low-grade glioma samples, PLK4 expression was highly expressed in GBM (Fig. 2A and B). Furthermore, a longer overall survival was observed in samples with a lower PLK4 expression, compared to those with a higher expression (median survival, 26 vs. 13 months; Fig. 2C). Similarly, data from the Rembrandt database (Affymetrix HG U133 v.20 plus) demonstrated that enriched PLK4 expression could be associated with poorer survival, when compared to samples with an intermediate or low PLK4 expression (Fig. 2D). In combination, this demonstrated that increased PLK4 expression was associated with a poor prognosis in patients with GBM, and could potentially serve as a clinically relevant molecular marker for GBM.

PLK4 promotes GBM proliferation and tumorigenesis. To further investigate the physiological functions of PLK4 in GBM tumorigenesis, U87 GBM cells were used as an *in vitro* cell model and were infected with either non-targeting control (shNT) or pGFP-shPLK4 lentiviruses. The efficiency of the lentivirus infection was confirmed by both GFP fluorescence (Fig. 3A) and RT-qPCR (Fig. 3B). The results indicated that the mRNA expression of PLK4 was significantly reduced

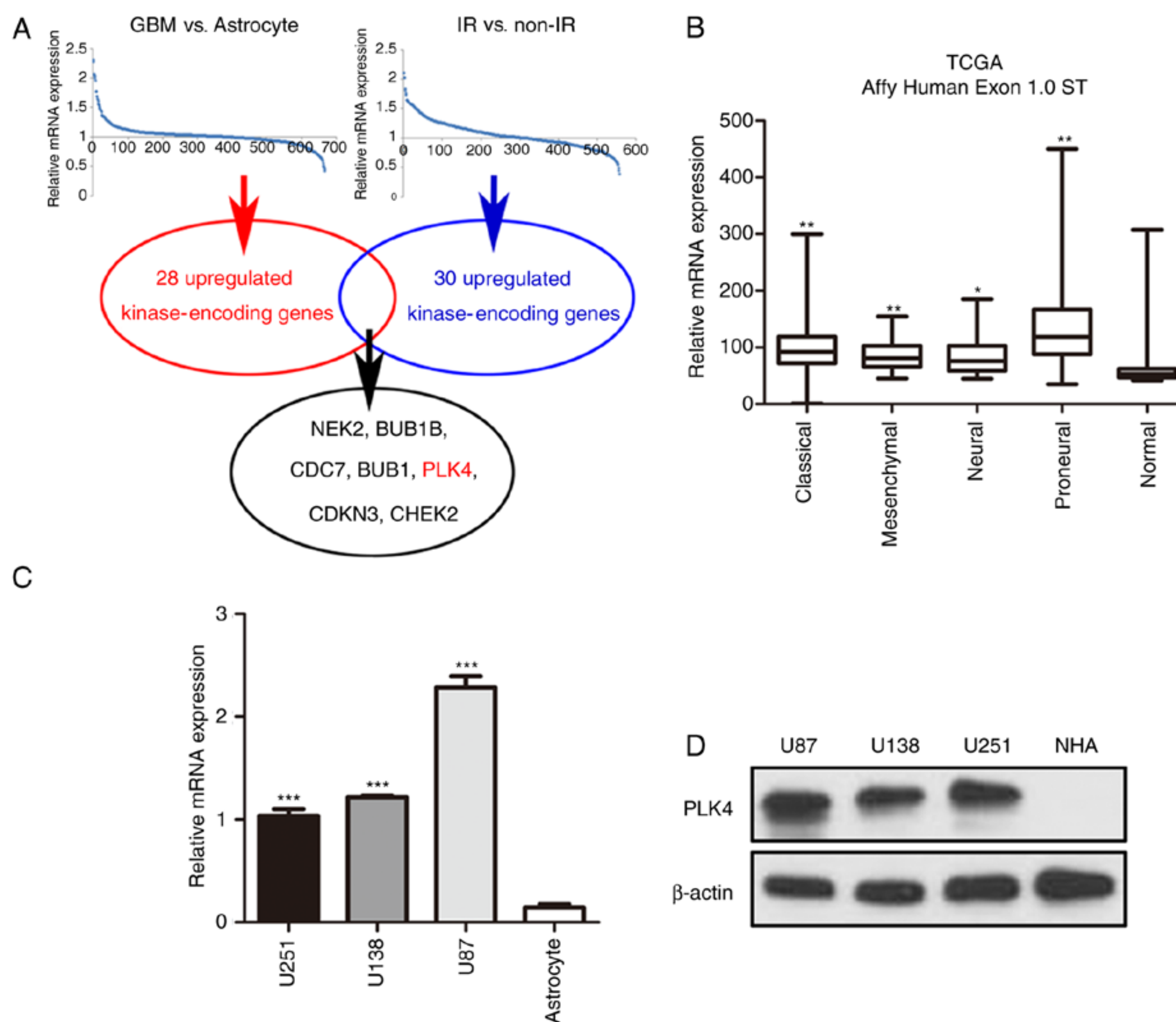

Figure 1. PLK4 is overexpressed in GBM. (A) Genome-wide transcriptome microarray analysis indicated that PLK4 was one of the seven overlapping gene candidates upregulated in GBM, as compared with normal astrocytes and in GBMs treated with or without radiotherapy. (B) Analysis of the TCGA database showed that PLK4 was highly expressed in all four subtypes of GBM. $^{*}P<0.05$ and $^{**}P<0.01$ vs. normal; one-way ANOVA followed by Dunnett's post hoc test. (C) RT-qPCR and (D) western blotting showed that PLK4 was overexpressed in the three GBM cell lines (U87, U138 and U251), compared with normal astrocytes ($^{***}P<0.001$, one-way ANOVA followed by Dunnett's post hoc test). β-actin served as the control. GBM, glioblastoma; ANOVA, analysis of variance; RT-qPCR, reverse transcription quantitative polymerase chain reaction; PLK4, polo-like kinase 4; IR, radiation.

in shPLK4 U87 cells. Western blotting yielded the same results (Fig. 3C). In addition, *in vitro* growth kinetics of shPLK4 lentivirus-infected U87 cells were inhibited proportionally to PLK4 reduction (Fig. 3D).

A mouse intracranial tumor model was used to investigate the functional role of PLK4 on GBM tumorigenesis *in vivo*. The results showed that shNT-transduced U87 cells formed GBM-like tumors within 30 days in mice (median survival, 27.2±4.21 days). However, a longer survival was observed in shPLK4-transduced U87 xenografted mice (45.6±10.04 days), highlighting a potential anti-tumorigenesis effect of PLK4 knockdown (Fig. 3E and F). In combination, these findings implied that PLK4 promoted GBM proliferation and tumorigenesis *in vitro* and *in vivo*.

PLK4 induces radioresistance in GBM. As PLK4 was found to be one of the most upregulated kinase-encoding genes for GBM following radiotherapy, it was assumed that PLK4-dependent radioresistance was essential for GBM cells. U87 cells were therefore treated with or without 12 Gy radiotherapy and analyzed by RT-qPCR. The results indicated that the PLK4 mRNA expression was significantly elevated following radiotherapy (Fig. 4A), while western blotting yielded the same results (Fig. 4B). To further clarify whether PLK4 induces radioresistance in GBM, PLK4 was knocked down via shPLK4 lentivirus infection, followed by 12 Gy radiotherapy. The RT-qPCR results showed that PLK4 mRNA expression was markedly increased following radiation and was partially eliminated by shPLK4 (Fig. 4C), as well as the *in vitro* cell proliferation (Fig. 4D). In addition, flow cytometry indicated that the percentage of U87 cells undergoing both early (AV^{+}; PI^{-}) and late (AV^{+}; PI^{+}) apoptosis were markedly increased following PLK4 knockdown followed by radiation, compared with radiotherapy alone (Fig. 4E). PLK4 was therefore essential for radioresistance, and the knockdown of PLK4 could increase radiosensitivity in GBM cells.

As it is well known that PLK4 predominantly functions as a mitosis regulating kinase (5), knockdown of PLK4 will increase cell cycle arrest in GBM. To eliminate the effects of PLK4 knock down on mitosis, PLK4 was overexpressed in

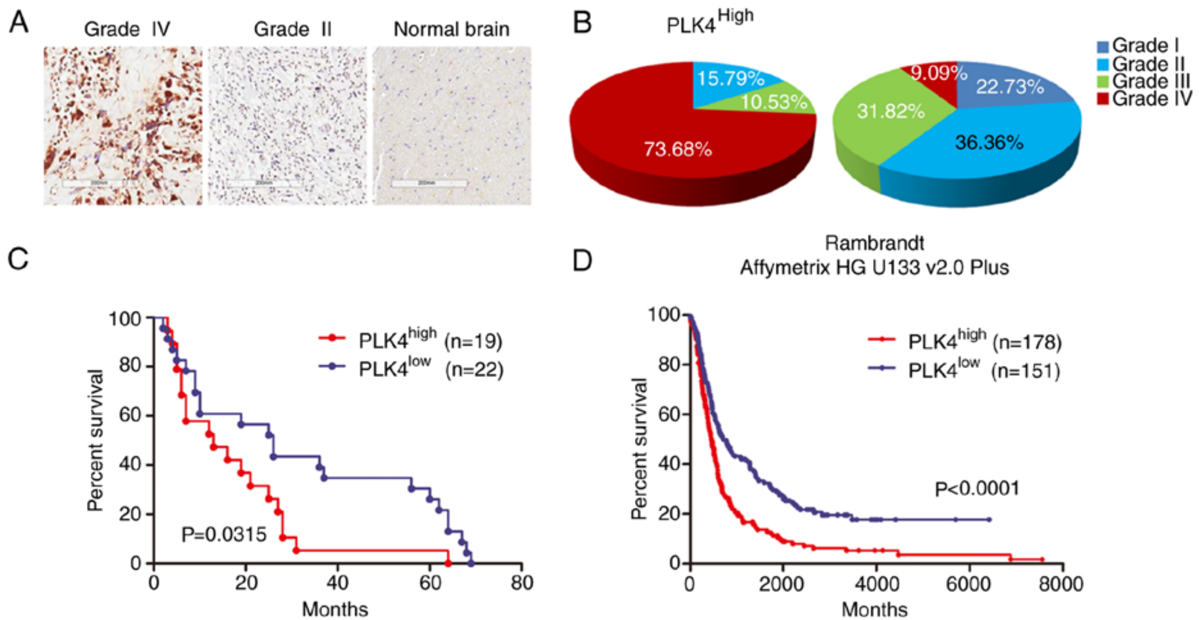

Figure 2. PLK4 overexpression implies poor prognosis in GBM. (A) Representative immunohistochemical images of PLK4 in GBM, low grade glioma and non-tumor brain tissue. (B) PLK4 was enriched in high grade glioma samples (WHO III-IV), compared with low grade glioma samples (WHO I-II). (C) Kaplan-Meier analysis exhibited a longer overall survival in samples with a lower PLK4 expression, compared with samples with a higher PLK4 expression among 41 glioma patients ($PLK4^{high}$ vs. $PLK4^{low}$ samples, P=0.0315; log-rank test). (D) Kaplan-Meier analysis of the Rembrandt database indicated an inverted correlation between PLK4 expression and the post-surgical survival of GBM patients ($PLK4^{high}$ vs. $PLK4^{low}$ samples; P<0.0001; log-rank test). GBM, glioblastoma; PLK4, polo-like kinase 4.

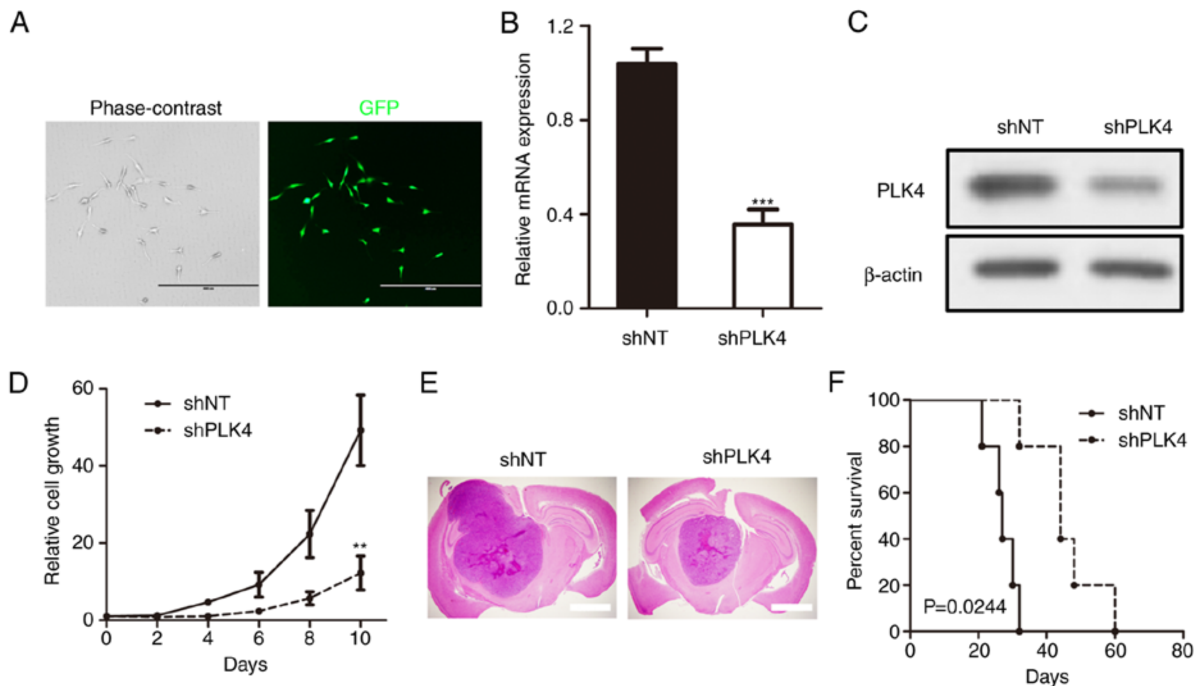

Figure 3. PLK4 promotes GBM proliferation and tumorigenesis. (A) Phase-contrast and fluorescence images showing that pGFP-shPLK4 lentivirus was successfully transduced into U87 cells. (B) RT-qPCR and (C) western blot analysis of U87 cells transduced with shPLK4 or shNT (***P<0.001 vs. shNT; t-test). β-actin served as the control. (D) *In vitro* cell proliferation assays showed that shPLK4 reduced U87 cell proliferation in cells (**P<0.01 vs. shNT; one-way ANOVA). (E) Representative images of hematoxylin and eosin-stained mouse brain sections implanted with U87 cells transduced with shPLK4 or shNT. (F) Kaplan-Meier analysis of nude mice intracranially implanted with transduced U87 cells (n=5; P=0.0244; log-rank test). GBM, glioblastoma; ANOVA, analysis of variance; RT-qPCR, reverse transcription quantitative polymerase chain reaction; PLK4, polo-like kinase 4; sh, small hairpin RNA; NT, non-targeting control.

U87 cells (Fig. 4F) then combined with radiation. The results indicated that exogenous overexpression markedly reduced cell apoptosis in U87 cells following radiotherapy (Fig. 4G), demonstrating that PLK4-induced radioresistance in GBM was independent, at least partially, from its functions in mitosis regulation.

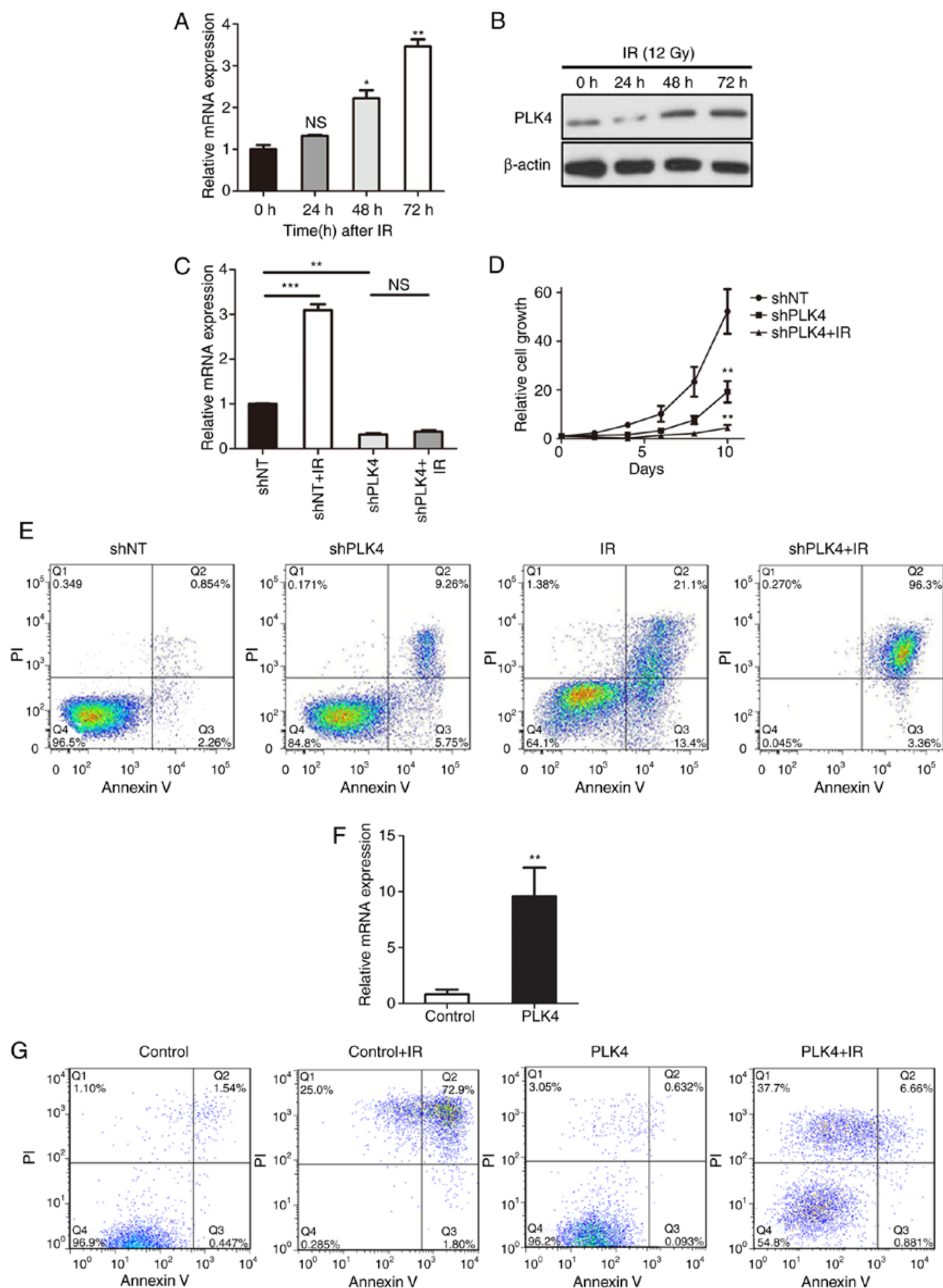

Figure 4. PLK4 induces radioresistance in GBM. (A) RT-qPCR and (B) western blot analysis for PLK4 expression in U87 cells treated with or without radiation (12 Gy; *P<0.05, **P<0.01 vs. 0 h; one-way ANOVA followed by Dunnett's post hoc test. β-actin served as the control. (C) RT-qPCR results showed that PLK4 mRNA expression was markedly increased following radiation (12 Gy), and could be partially eliminated by shPLK4. **P<0.01, ***P<0.001; one-way ANOVA followed by Dunnett's post hoc test. (D) *In vitro* cell proliferation assay for U87 cells transduced with either shNT or shPLK4 lentiviruses, followed (or not) by radiotherapy (12 Gy). **P<0.01 vs. shNT; one-way ANOVA followed by Dunnett's post hoc test. (E) Flow cytometry (Annexin V and propidium iodide) determined the apoptosis of U87 cells transduced with shNT or shPLK4, followed (or not) by radiotherapy (12 Gy). (F) RT-qPCR analysis of U87 cells transduced with PLK4 overexpression or control lentivirus. **P<0.01; t-test. (G) Flow cytometry determined the apoptosis of U87 cells transduced with PLK4 overexpression or control lentivirus, followed (or not) by radiotherapy (12 Gy). GBM, glioblastoma; ANOVA, analysis of variance; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ns, not significant; PLK4, polo-like kinase 4; sh, small hairpin RNA; IR, radiation.

PLK4 is transcriptionally regulated by ATAD2 in GBM. To further assess the regulatory mechanism of PLK4 expression in GBM, expression data from the TCGA dataset were analyzed. The results indicated that ATAD2 was one of the top genes

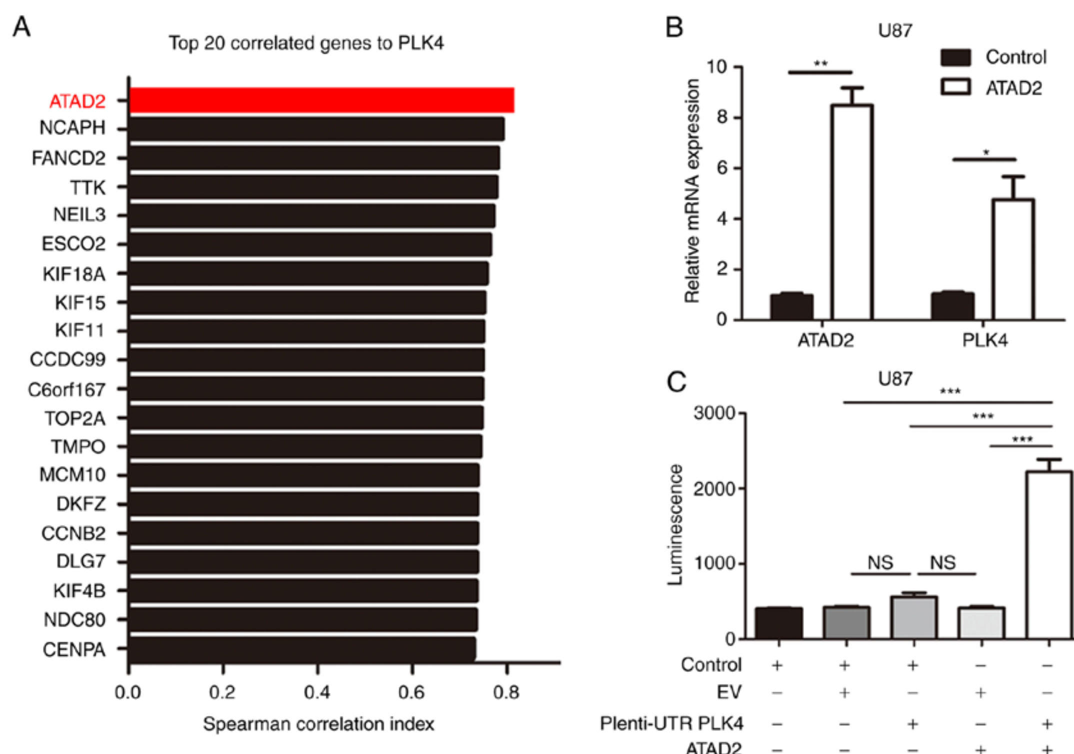

Figure 5. PLK4 is transcriptionally regulated by ATAD2 in GBM. (A) Top 20 most correlated genes to PLK4 expression in the TCGA database. Pearson's correlation analysis was performed. (B) RT-qPCR indicated that PLK4 expression was markedly increased following exogenous ATAD2 overexpression in U87 cells. *P<0.05, **P<0.01; t-test. (C) Luciferase assays showed that PLK4 promoter activity was increased following lentivirus-mediated overexpression of ATAD2 in U87 cells. ***P<0.001; one-way analysis of variance followed by Dunnett's post hoc test. ATAD2, ATPase family AAA domain-containing protein 2; GBM, glioblastoma; ANOVA, analysis of variance; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ns, not significant; PLK4, polo-like kinase 4; UTR, untranslated region; EV, empty vector.

correlated to PLK4 mRNA (Fig. 5A). Furthermore, the shRNA-mediated overexpression of ATAD2 increased the expression of PLK4 mRNA in U87 cells (Fig. 5B). To further study the mechanism of the ATAD2-dependent regulation of PLK4, luciferase reporter assays were performed on U87 cells transfected with human PLK4 promoter. According to the findings, the lentivirus-mediated overexpression of ATAD2 elevated the transcription activity of the PLK4 promoter in U87 cells, compared with the control cells (Fig. 5C). Collectively, these data suggest that ATAD2 may be a key regulator of PLK4 transcription in GBM cells.

Discussion

Accumulating data has demonstrated that kinase-dependent tumorigenesis and treatment resistance is essential for the recurrence of multiple types of tumors, and targeting kinase activity has been proven to be an effective way to reduce tumor growth (19-25). PLK4 is a serine/threonine centrosomal protein kinase, whose main function is to regulate the number of centrosomes in cells (26). Previous studies have indicated that PLK4 serves an essential role in cell proliferation, tumorigenesis, invasion and viability, as well as treatment resistance in a wide range of cancers, including gastric adenocarcinoma, colon, liver and breast cancer (6,9,12,27). However, the function of PLK4 in GBM remains ambiguous. In the present study, PLK4 was identified as one of the most enriched kinase-encoding genes in GBM, and was proven to be an essential regulator of GBM proliferation. PLK4 was shown to be functionally indispensable for both *in vitro* cell proliferation and *in vivo* tumorigenesis in GBM. PLK4 expression was highly enriched in GBM and implied poor prognosis.

Mechanically, PLK4 overexpression has been shown to regulate tumor proliferation and cell migration by inducing centrosome amplification and CIN, resulting in the suppression of cilia formation (6). Dzhindzhev *et al* (28) reported that PLK4 regulates centrosome duplication by interacting with centrosomal protein of 152 kDa (CEP152). These findings suggest that PLK4 may induce proliferation in tumor cells by inducing centrosome amplification and CIN. The inhibition of PLK4 in a lung cancer model induced apoptosis through a temperature-sensitive p53 mutant, while PLK4 overexpression diminished p53-dependent apoptosis (29). In addition, the Rho-GTPase signaling pathway could be disrupted by haploid expression of PLK4 during cytokinesis in liver cancer, leading to aneuploidy and tumorigenesis (12). Additional research has revealed that the artificial silencing of PLK4 inhibits stress-induced Akt activation, thus promoting apoptosis in lung cancer cells, and that the gradual activation of p53 downregulates PLK4 to promote apoptosis (30). In accordance with these findings, it was found in the present study that PLK4 induced tumorigenesis and radioresistance in GBM, with this mechanism potentially dependent on the induction of centrosome amplification and CIN. However, the mechanism and downstream target of PLK4 in GBM remains unclear and further study is required.

Since PLK4 was identified as an essential kinase for tumorigenesis and radioresistance in GBM, it was useful to investigate the detailed mechanism of PLK4 regulation in GBM. Spearman correlation analysis was performed between PLK4 and 14,731 genes using the TCGA dataset. Among these genes, ATAD2 was the gene most significantly correlated with PLK4 in GBM. ATAD2 was identified as a significantly conserved gene predominantly expressed in germ cells, but was remarkably elevated in a wide range of different subtypes of tumors, including thyroid, breast, cervical and gastric cancer (31-34). As a transcriptional coactivator of a wide subset of estradiol target genes, ATAD2 is associated with a variety of key regulatory mechanisms in human cancer cells, including the regulation of cell proliferation and tumor metastasis, via the transcriptional regulation of cyclin D1, c-myc and E2F (32,33). However, the potential downstream targets of ATAD2 in the DNA repair response of GBM remains unclear. In the present study, it was found that the PLK4 expression was increased by the exogenous overexpression of ATAD2, which suggested that the ATAD2-dependent transcriptional regulation of PLK4 was essential for tumor growth and treatment resistance in GBM cells. However, the transcription factor which directly binds to the PLK4 promoter area is unknown. Further experiments, including chromatin immunoprecipitation sequencing for PLK4, should be performed on co-immunoprecipitation of ATAD2-binding protein in order to clarify the interaction and exact pathway between ATAD2 and PLK4.

In the present study, bioinformatics analysis results identified PLK4 as one of the most upregulated kinase-encoding genes in GBM which was found to be functionally required for both *in vitro* cell proliferation and *in vivo* tumorigenesis. Clinically, elevated PLK4 expression was observed in high grade glioma patients and was linked to poor prognosis in GBM. In addition, PLK4 enhanced radioresistance in GBM cells, whereas PLK4 knockdown significantly increased the radiosensitivity of GBM cells. Mechanically, PLK4 expression was markedly elevated by the exogenous overexpression of ATAD2 in GBM cells. Collectively, the data showed that the ATAD2-dependent transcriptional regulation of PLK4 promoted cell proliferation and tumorigenesis, as well as GBM radioresistance, thus potentially inducing tumor recurrence. PLK4 could therefore serve as a potential therapeutic target for GBM treatment.

Acknowledgements

The authors would like to thank Dr Ruichun Li, Dr Ping Mao and all other members of the Department of Neurosurgery and Center of Brain Science (First Affiliated Hospital of Xi'an Jiaotong University) for their technical assistance.

Funding

Project was supported by National Natural Science Foundation of China (grant no. 81802502).

Availability of data and materials

The data used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW performed the experiments. JW, MW, XM and KG analyzed the data. DNA microarray database analysis was performed by JW, JZ and XB. JW, NW and JZ collected the patient samples and performed follow-up surveys. Bioinformatics analysis was performed by JW, MW and XM. Mice intracranial xenograft experiments were performed by JW, KG, XB and NW. Pearson's correlation analysis was performed by JW, WX and HL. The primers were designed by JW and HL. JW and JZ wrote the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The use of experimental animals in this study was approved by the Ethics Committee of the School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi Province, China (approval no. 2016-085). The collection and use of the tumor samples and patient information was approved by the patients and the Scientific Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China (approval no. 2016-18).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ho VK, Reijneveld JC, Enting RH, Bienfait HP, Robe P, Baumert BG and Visser O; Dutch Society for Neuro-Oncology (LWNO): Changing incidence and improved survival of gliomas. Eur J Cancer 50: 2309-2318, 2014.
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
3. Signorovitch J, Li N, Ohashi E, Dastani H, Shaw J and Orsini L: Overall survival (Os), quality of life (Qol), and neurocognitive function (Nf) in recurrent glioblastoma multiforme (Gbm): A systematic literature review. Value Health 18: A433, 2015.
4. A PLK4 inhibitor has single-agent activity in preclinical tumor models. Cancer Discov 4: OF11, 2014.
5. Korzeniewski N, Hohenfellner M and Duensing S: CAND1 promotes PLK4-mediated centriole overduplication and is frequently disrupted in prostate cancer. Neoplasia 14: 799-806, 2012.
6. Shinmura K, Kurabe N, Goto M, Yamada H, Natsume H, Konno H and Sugimura H: PLK4 overexpression and its effect on centrosome regulation and chromosome stability in human gastric cancer. Mol Biol Rep 41: 6635-6644, 2014.
7. Cunha-Ferreira I, Rodrigues-Martins A, Bento I, Riparbelli M, Zhang W, Laue E, Callaini G, Glover DM and Bettencourt-Dias M: The SCF/Slimb ubiquitin ligase limits centrosome amplification through degradation of SAK/PLK4. Curr Biol 19: 43-49, 2009.
8. Rogers GC, Rusan NM, Roberts DM, Peifer M and Rogers SL: The SCF Slimb ubiquitin ligase regulates Plk4/Sak levels to block centriole reduplication. J Cell Biol 184: 225-239, 2009.
9. Guderian G, Westendorf J, Uldschmid A and Nigg EA: Plk4 trans-autophosphorylation regulates centriole number by controlling betaTrCP-mediated degradation. J Cell Sci 123: 2163-2169, 2010.

10. Holland AJ, Lan W, Niessen S, Hoover H and Cleveland DW: Polo-like kinase 4 kinase activity limits centrosome overduplication by autoregulating its own stability. J Cell Biol 188: 191-198, 2010.
11. Marina M and Saavedra HI: Nek2 and Plk4: Prognostic markers, drivers of breast tumorigenesis and drug resistance. Front Biosci 19: 352-365, 2014.
12. Rosario CO, Ko MA, Haffani YZ, Gladdy RA, Paderova J, Pollett A, Squire JA, Dennis JW and Swallow CJ: Plk4 is required for cytokinesis and maintenance of chromosomal stability. Proc Natl Acad Sci USA 107: 6888-6893, 2010.
13. Mason JM, Lin DC, Wei X, Che Y, Yao Y, Kiarash R, Cescon DW, Fletcher GC, Awrey DE, Bray MR, *et al*: Functional characterization of CFI-400945, a Polo-like kinase 4 inhibitor, as a potential anticancer agent. Cancer Cell 26: 163-176, 2014.
14. Sampson PB, Liu Y, Forrest B, Cumming G, Li SW, Patel NK, Edwards L, Laufer R, Feher M, Ban F, *et al*: The discovery of Polo-like kinase 4 inhibitors: Identification of (1*R*,2S).2-(3-((*E*).4-(((*cis*).2,6-dimethylmorpholino)methyl)styryl) -1*H*.indazol-6-yl)-5'-methoxyspiro[cyclopropane-1,3'-indolin] -2'-one (CFI-400945) as a potent, orally active antitumor agent. J Med Chem 58: 147-169, 2015.
15. Yu B, Yu Z, Qi PP, Yu DQ and Liu HM: Discovery of orally active anticancer candidate CFI-400945 derived from biologically promising spirooxindoles: Success and challenges. Eur J Med Chem 95: 35-40, 2015.
16. Wang J, Cheng P, Pavlyukov MS, Yu H, Zhang Z, Kim SH, Minata M, Mohyeldin A, Xie W, Chen D, *et al*: Targeting NEK2 attenuates glioblastoma growth and radioresistance by destabilizing histone methyltransferase EZH2. J Clin Invest 127: 3075-3089, 2017.
17. van Diest PJ, van Dam P, Henzen-Logmans SC, Berns E, van der Burg ME, Green J and Vergote I: A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC-GCCG. European organization for research and treatment of cancer-gynaecological cancer cooperative group. J Clin Pathol 50: 801-804, 1997.
18. Mao P, Joshi K, Li J, Kim SH, Li P, Santana-Santos L, Luthra S, Chandran UR, Benos PV, Smith L, *et al*: Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc Natl Acad Sci USA 110: 8644-8649, 2013.
19. Chen C, Wang X, Xiong X, Liu Q, Huang Y, Xu Q, Hu J, Ge G and Ling K: Targeting type Igamma phosphatidylinositol phosphate kinase inhibits breast cancer metastasis. Oncogene 34: 4635-4646, 2015.
20. Hu J, Ahuja LG, Meharena HS, Kannan N, Kornev AP, Taylor SS and Shaw AS: Kinase regulation by hydrophobic spine assembly in cancer. Mol Cell Biol 35: 264-276, 2015.
21. Stransky N, Cerami E, Schalm S, Kim JL and Lengauer C: The landscape of kinase fusions in cancer. Nat Commun 5: 4846, 2014.
22. Kim SH, Joshi K, Ezhilarasan R, Myers TR, Siu J, Gu C, Nakano-Okuno M, Taylor D, Minata M, Sulman EP, *et al*: EZH2 protects glioma stem cells from radiation-induced cell death in a MELK/FOXM1-dependent manner. Stem Cell Reports 4: 226-238, 2015.
23. Minata M, Gu C, Joshi K, Nakano-Okuno M, Hong C, Nguyen CH, Kornblum HI, Molla A and Nakano I: Multi-kinase inhibitor C1 triggers mitotic catastrophe of glioma stem cells mainly through MELK kinase inhibition. PLoS One 9: e92546, 2014.
24. Joshi K, Banasavadi-Siddegowda Y, Mo X, Kim SH, Mao P, Kig C, Nardini D, Sobol RW, Chow LM, Kornblum HI, *et al*: MELK-dependent FOXM1 phosphorylation is essential for proliferation of glioma stem cells. Stem Cells 31: 1051-1063, 2013.
25. Gu C, Banasavadi-Siddegowda YK, Joshi K, Nakamura Y, Kurt H, Gupta S and Nakano I: Tumor-specific activation of the C-JUN/MELK pathway regulates glioma stem cell growth in a p53-dependent manner. Stem Cells 31: 870-881, 2013.
26. Habedanck R, Stierhof YD, Wilkinson CJ and Nigg EA: The Polo kinase Plk4 functions in centriole duplication. Nat Cell Biol 7: 1140-1146, 2005.
27. Macmillan JC, Hudson JW, Bull S, Dennis JW and Swallow CJ: Comparative expression of the mitotic regulators SAK and PLK in colorectal cancer. Ann Surg Oncol 8: 729-740, 2001.
28. Dzhindzhev NS, Yu QD, Weiskopf K, Tzolovsky G, Cunha-Ferreira I, Riparbelli M, Rodrigues-Martins A, Bettencourt-Dias M, Callaini G and Glover DM: Asterless is a scaffold for the onset of centriole assembly. Nature 467: 714-718, 2010.
29. Li J, Tan M, Li L, Pamarthy D, Lawrence TS and Sun Y: SAK, a new polo-like kinase, is transcriptionally repressed by p53 and induces apoptosis upon RNAi silencing. Neoplasia 7: 312-323, 2005.
30. Nakamura T, Saito H and Takekawa M: SAPK pathways and p53 cooperatively regulate PLK4 activity and centrosome integrity under stress. Nat Commun 4: 1775, 2013.
31. Sun W, Lan X, Zhang H, Wang Z, Dong W, He L, Zhang T, Zhang P, Liu J and Qin Y: NEAT1_2 functions as a competing endogenous RNA to regulate ATAD2 expression by sponging microRNA-106b-5p in papillary thyroid cancer. Cell Death Dis 9: 380, 2018.
32. Kalashnikova EV, Revenko AS, Gemo AT, Andrews NP, Tepper CG, Zou JX, Cardiff RD, Borowsky AD and Chen HW: ANCCA/ATAD2 overexpression identifies breast cancer patients with poor prognosis, acting to drive proliferation and survival of triple-negative cells through control of B-Myb and EZH2. Cancer Res 70: 9402-9412, 2010.
33. Zheng L, Li T, Zhang Y, Guo Y, Yao J, Dou L and Guo K: Oncogene ATAD2 promotes cell proliferation, invasion and migration in cervical cancer. Oncol Rep 33: 2337-2344, 2015.
34. Zhang M, Zhang C, Du W, Yang X and Chen Z: ATAD2 is overexpressed in gastric cancer and serves as an independent poor prognostic biomarker. Clin Transl Oncol 18: 776-781, 2016.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.