HHS Public Access

Author manuscript

Mol Cancer Ther. Author manuscript; available in PMC 2017 July 01.

Published in final edited form as:

Mol Cancer Ther. 2016 July ; 15(7): 1656–1668. doi:10.1158/1535-7163.MCT-15-0857.

MSK1-mediated β-catenin phosphorylation confers resistance to PI3K/mTOR inhibitors in glioblastoma

Shaofang Wu[1], **Shuzhen Wang**[1], **Siyuan Zheng**[2], **Roel Verhaak**[2], **Dimpy Koul**[1,*], and **W. K. Alfred Yung**[1,*]

[1]Brain Tumor Center, Department of Neuro-Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[2]Brain Tumor Center, Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

Abstract

Glioblastoma (GBM) represents a compelling disease for kinase inhibitor therapy because most of these tumors harbor genetic alterations that result in aberrant activation of growth factor signaling pathways. The PI3K/mammalian target of rapamycin (mTOR) pathway is dysregulated in over 50% of human GBM but remains a challenging clinical target. Inhibitors against PI3K/mTOR mediators have limited clinical efficacy as single agents. We investigated potential bypass mechanisms to PI3K/mTOR inhibition using gene expression profiling before and after PI3K inhibitor treatment by Affymetrix microarrays. Mitogen- and stress-activated protein kinase 1 (MSK1) was markedly induced after PI3K/mTOR inhibitor treatment and disruption of MSK1 by specific shRNAs attenuated resistance to PI3K/mTOR inhibitors in glioma initiating cells (GICs). Further investigation showed that MSK1 phosphorylates β-catenin and regulates its nuclear translocation and transcriptional activity. The depletion of β-catenin potentiated PI3K/mTOR inhibitor-induced cytotoxicity and the inhibition of MSK1 synergized with PI3K/mTOR inhibitors to extend survival in an intracranial animal model and decreased phosphorylation of β-catenin at Ser^{552}. These observations suggest that MSK1/β-catenin signaling serves as an escape survival signal upon PI3K/mTOR inhibition and provides a strong rationale for the combined use of PI3K/mTOR and MSK1/β-catenin inhibition to induce lethal growth inhibition in human GBM.

Keywords

PI3K/mTOR resistance; MSK1; β-catenin; glioblastoma; drug resistance

*Correspondence: W. K. Alfred Yung, Department of Neuro-Oncology, Unit 431, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030; tel: 713-794-1285; fax: 713-794-4999; wyung@mdanderson.org., Dimpy Koul, Department of Neuro-Oncology, Unit 1003, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030; tel: 713-834-6202; fax: 713-834-6230; dkoul@mdanderson.org.

Conflict of interest: The authors (W.K. Alfred Yung) disclose conflict of interest as a consultant with DNATrix. Rest all authors have no conflict of Interest to disclose.

Introduction

Glioblastoma multiforme (GBM), an aggressive brain tumor, is resistant to traditional chemotherapy and radiation therapy. Over 50% of GBM have *PI3KR1/PI3KCA/PTEN* mutations (1). As PI3K pathway activation and PTEN inactivation are associated with a poor prognostic outcome, the PI3K pathway represents an attractive therapeutic target. The downstream effector mammalian target of rapamycin (mTOR) links growth factor signaling through PI3K to energy and nutrient status, protein translation, autophagy, and tumor cell metabolism (2,3). Thus, mTOR is a critical integrator that regulates tumor growth, survival, and potentially, cancer drug resistance.

PI3K/mTOR inhibitors produce a partial response, but complete responses are rare. In preclinical experimental models, about half the responders who benefit from PI3K/mTOR inhibition treatment eventually develop drug resistance after a transient response. Therefore, an understanding of the molecular mechanisms that affect cancer cell sensitivity and resistance to PI3K/mTOR inhibitors is greatly needed. Recently, a number of clinical and preclinical studies indicated that ERK signaling is activated upon PI3K inhibition, and ERK signaling might serve as a compensatory pathway to escape PI3K inhibition (4-6).

Mitogen- and stress-activated protein kinase 1 (MSK1), also known as RPS6KA5, is a serine/threonine kinase that belongs to RSK (Ribosomal Protein-S6 Kinase) family and is ubiquitously expressed in various tissues and predominantly expressed in the brain, heart, placenta, and skeletal muscles (7). MSK1 is activated by extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase pathways in response to growth factor and cellular stress stimuli (7). Activated MSK1 phosphorylates multiple transcription factors and nuclear proteins, increasing their stability or activity. MSK1 phosphorylates CREB at Ser133 and is linked to the regulation of immediate early genes, including *c-Fos*, *Jun B*, *c-Jun*, and *Nurr77* (8,9). MSK1 has also been shown to mediate NF-κB-dependent transcription through phosphorylation of p65 on Ser276 (10). In addition, stress- and mitogen-induced phosphorylation of histone H3 and HMG-14 was found to be completely inhibited in primary embryonic fibroblasts from MSK1/MSK2-knockout animals, suggesting that MSK1 is a prominent kinase involved in the nucleosomal response (11). MSK1 plays a crucial role in integrating different extracellular signals to functionally regulate cell growth and cell death in response to growth factor and cellular stress stimuli (8,12,13). More importantly, MSK1 is required for Ciras-3 cells to maintain malignant phenotype (8) and for hormone-dependent breast cancer growth.(13), suggesting that MSK1 plays an important role in tumor progression. However, its function in response to PI3K/mTOR pathway inhibition is unknown.

Wnt/β-catenin signaling is important for glioma tumor cell proliferation and tumor progression. Although β-catenin mutations have not been found in glioma tissues and cell lines (14), β-catenin mRNA and protein levels are increased in GBM and are correlated with malignancy; therefore, they have been proposed as prognostic markers in GBM (15,16). In addition, an increased nuclear fraction of β-catenin and the elevated expression of β-catenin target genes such as cyclin D1 and c-Myc have also been observed in high-grade

astrocytomas and GBM (15-17). These results suggest that increased β-catenin activity is crucial for glioma progression.

In the current study, we determined the response to PI3K/mTOR inhibition in nude mice and in a panel of glioma-initiating cells (GIC), which retain the relevant molecular features of GBMs and serve as preclinical models for studies of tumor biology and therapeutics. We found that, as a result of selective pressure by PI3K/mTOR inhibition, MSK1 was up-regulated and phosphorylated by ERK signaling. In addition, MSK1 phosphorylated and promoted the transcriptional activity of β-catenin, the activity of which is crucial for glioma progression. The loss of function of MSK1 blocked PI3K/mTOR-inhibition induced phosphorylation of β-catenin and attenuated GICs resistance to PI3K/mTOR inhibition. Thus, our results show that MSK1 and β-catenin signaling acted as compensatory survival signaling pathways to escape from anti-PI3K/mTOR drug therapy and provides a strong rationale to combine PI3K/mTOR and MSK1 or β-catenin inhibition in targeted therapy for malignant glioma.

Materials and Methods

Cell lines and reagents

The GIC lines were established by isolating neurosphere-forming cells from fresh surgical specimens of human GBM tissue between the years of 2005 through 2008, as described previously (18). Cells were authenticated by testing short tandem repeats (STR) using the Applied Biosystems AmpFISTR Identifier kit (Foster City, CA). The last authentication testing was done in March 2014. This study was approved by the institutional review board of The University of Texas MD Anderson Cancer Center (Houston, Texas). These GBM neurospheres were cultured in DMEM/F12 medium containing B27 supplement (Invitrogen, Carlsbad, CA), basic fibroblast growth factor, and epidermal growth factor (20 ng/ml each). The PI3K/mTOR dual inhibitor BEZ235 was from Selleck (Houston, Texas), DS-7423 was provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan), and PD-0325901 was from Selleck (Houston, Texas). For in vitro use, all inhibitors were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO).

Cell proliferation assay

Cells were treated in triplicate with serial doses of BEZ235 and DS7423 for 72 h, and cell proliferation was tested using the CellTiter-Blue (Promega, Madison, WI) viability assay. The IC_{50} value was calculated as the mean drug concentration required to inhibit cell proliferation by 50% compared with vehicle-treated controls.

Gene arrays, reverse-phase protein arrays, and bioinformatics

For gene expression arrays, cells were treated with BEZ235, DS7423, or dimethyl sulfoxide for 24 hours; RNA was extracted and hybridized to Affymetrix U133 GeneChips. The array data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE79316. For reverse-phase protein arrays (RPPA), cells were incubated with inhibitors with duplicate treatments for 24 hours. Cell lysates were arrayed on nitrocellulose-coated slides and probed for a panel of 217 antibodies that targeted

major important signaling molecules and pathways to establish expression level and activation status.

Western blot analysis

Cells were harvested in lysis buffer, as described previously (18), and subjected to Western blotting. Anti-MSK1, anti-pMSK1, anti--β-catenin, anti-pS552-β-catenin, anti-ERK, anti-pERK, anti-S6, anti-pS6, anti-AKT and anti-pS473-AKT antibodies were from Cell Signaling (Boston, MA). Anti-β-actin antibody was purchased from Sigma (St. Louis, MO) and used as a loading control.

Plasmids and transfection

Myc-DDK-MSK1 plasmid was purchased from Origene (Rockville, MD) and was used for transient transfection. For stable transfection, MSK1 was subcloned into pMy-puro-IRES retrovirus vector. Flag-β-catenin, TOP-flash, and FOP-flash were gifts from Dr. Zhimin Lu (19). Lentivirus pLOC-TurboRFP-β-catenin was from GE Healthcare Dharmacon (Pittsburgh, PA). β-catenin S552A mutant was generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). Lentiviral vector pLKO.1-mediated expression of shRNAs for MSK1 and pGIPZ-mediated expression of shRNA of β-catenin were purchased from GE Healthcare Dharmacon (Pittsburgh, PA). The shRNA hairpin sequences are listed in Supplementary table 1. Lentiviral particles expressing targeting or control scramble shRNA (SCR) were produced in HEK293FT cells with the mixed set of packing plasmids, and the viruses were concentrated and titered as previously described (20). Cells were infected with lentivirus for 2 days and selected with 1 μg/μl puromycin.

Luciferase reporter assay

Cells (50,000/well) were plated in 24-well plates and transfected with a mixture containing TCF/LEF-1 reporter (TOP-FLASH) or control vector (FOP-FLASH) and 200 ng β-catenin, with or without 200 ng or 400 ng of MSK1. We analyzed 10 μl of 100-μl lysates for luciferase activity.

Immunoprecipitation

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 1 mM phenylmethylsulfonyl fluoride). Cell lysate (200 to 1000 μg of protein) was immunoprecipitated with anti-flag M2 beads (Sigma) for 18 h at 4°C.

In vitro kinase assay

The kinase reactions were performed by mixing purified His-catenin, with or without purified active MSK1, in kinase assay buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM $MnCl_2$, 1 mM dithiothreitol, and 20 μM ATP for 20 min at 30°C. Samples were separated by 10% SDS-PAGE and analyzed by western blot or by liquid chromatography-coupled ion trap mass spectrometry (LC-MS).

Chromatin IP (ChIP)

ChIP was performed using the SimpleChIP enzymatic chromatin IP kit (Cell Signaling Technology, Boston, MA), according to the manufacturer's instructions. The lysate was incubated with anti-β-catenin antibody (Santa Cruz), and the human MYC promoter was amplified by PCR with primers 5′-CAGCCCGAGACTGTTGC-3 (forward) and 5′-CAGAGCGTGGGATGTTAG-3′(reverse).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from GIC with the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Real-time quantitative PCR was performed with the SuperScript III One-Step RT-PCR System and sybgreen DNA polymerase (Invitrogen, Grand Island, NY), according to the manufacturer's instructions. Primers for quantitative PCR are listed in Supplemental table 2.

Animal studies

All animal studies were conducted in the veterinary facilities of MD Anderson in accordance with institutional rules. To create the intracranial disease model, we engrafted GSC20-SCR and GSC20-MSK1-sh cells (5×10^5) into the caudate nucleus of nude (nu/nu) 6- to 8-week-old mice using a previously described guide-screw system (21). Animals were randomly divided into 2 groups of 10 mice each. Starting on day 4 after the tumor cell implantation, mice were treated with 6 mg/kg DS7423 in 200 μL of 0.5% methylcellulose solution, and mice in the control group were given an equal volume of vehicle by oral gavage. The treatment frequency was once a day for 5 days, with 2 days off between treatments, for a total duration of 6 weeks. Mice were monitored daily and euthanized when they became moribund. Their whole brains were extracted, rapidly frozen in liquid nitrogen, and stored at −70°C.

Immunohistochemical staining

Sections (5-μm thick) of formalin-fixed, paraffin-embedded whole brains from animals were stained with anti-ki67 (BD Biosciences), anti-MSK1, anti-p-Ser473-AKT, anti-p-Ser235/236S6-S6, and anti-p-Ser552-β-catenin (Cell Signaling Technology, Boston, MA). The sections were visualized using a diaminobenzidine substrate kit, and the slides were examined under a bright-field microscope.

Statistical analysis

The statistical analysis was performed using Student's t–test. The results are presented as the mean of at least 3 independent experiments. A survival analysis was performed using the log-rank analysis module in SPSS software version 10.0 (SPSS, Inc., Armonk, NY). Differences were considered significant at $P < 0.05$ for all comparisons.

Results

PI3K/mTOR dual inhibitors increased MSK1 expression

To identify molecular targets and signatures that contribute to resistance to PI3K/mTOR inhibitors, we performed gene expression profiling, before and after PI3K/mTOR inhibitor treatment, and analyzed the results by Affymetrix microarrays; the top 10 up-regulated genes were identified (Figure 1A). We used two PI3K/mTOR inhibitors, BEZ235 (22) and DS7423 (Supplemental Figure S1 for structure), to exclude the possibility of non-specific drug toxicity. Both BEZ235 and DS7423 are in clinical trials in patients with advanced solid tumors, and a previous study from our laboratory showed that DS7423 is blood–tumor barrier permeable (23). We report that MSK1 was significantly up-regulated after BEZ235 and DS7423 treatment in all cell lines tested. Because of the important role of MSK1 in tumor progression, we focused on the potential function of MSK1 as an escape kinase for PI3K/mTOR inhibition.

First, up-regulation of MSK1 by PI3K/mTOR inhibitors was confirmed in several cell lines by quantitative PCR (Supplemental Figure S2). The MSK1 protein level was also markedly increased after BEZ235 and DS7423 treatment (Figure 1B). Both BEZ235 and DS7423 increased MSK1 expression in a dose-dependent manner (Figure 1C) and time course-dependent manner (Figure 1D).

To further confirm the response of MSK1 to PI3K/mTOR inhibition, we treated the cells with 100 nM BEZ235 or DS7423 for 24 hours, washed them for another 24 hours, and repeated 3 cycles (Figure 1E). Interestingly, MSK1 expression at both the mRNA level (Figure 1F) and protein level (Figure 1G) was up-regulated after PI3K/mTOR inhibitor treatment and decreased after the inhibitors had been washed out, suggesting that MSK1 expression is induced by PI3K/mTOR inhibition.

In addition to regulating MSK1 expression, PI3K/mTOR inhibition induced MSK1 phosphorylation (Supplemental Figure S3A). As MSK1 is reported to be phosphorylated by ERK and p38 under extracellular stimuli, we determined whether ERK or p38 phosphorylated MSK1 in response to PI3K/mTOR inhibition. The RPPA data showed that only ERK, not p38, was activated by PI3K/mTOR inhibitors (Supplemental Figure S3B). Phosphorylation of MSK1 and ERK was further confirmed by time-course treatment with BEZ235 (Supplemental Figure S3C). Suppression of ERK activation by the chemical inhibitor PD-0325901 (24) blocked MSK1 phosphorylation induced by BEZ235 (Supplemental Figure S3D). These results suggest that ERK phosphorylated MSK1 upon PI3K/mTOR inhibition.

Depletion of MSK1 decreased cell resistance to PI3K/mTOR inhibition

To determine the role of MSK1 in response to PI3K/mTOR inhibition, we knocked down MSK1 expression by shRNAs in GSC20 (Figure 2A). Our results showed that MSK1-depleted cells (MSK1-sh6 and MSK1-sh10) were more sensitive to BEZ235 treatment and DS7423 treatment than were SCR cells (Figure 2B-D). In parallel, we showed that after BEZ235 and DS7423 treatment, MSK1 knockdown cells formed smaller and fewer neuro-spheres than did SCR cells (Figure 2E). After BEZ235 and DS7423 treatment, cell

proliferation was also decreased by MSK1 knockdown (Figure 2F). Consistent with this finding, the Ki-67 index of the MSK1-knockdown cells was lower than that of SCR cells after treatment with BEZ235 or DS7423 (Figure 2G and H). In addition, MSK1-knockdown also increased PI3K/mTOR inhibition induced-apoptosis as indicated by TUNEL staining (Figure 2I and J). Notably, MSK1- knockdown alone had little effect on neuro-sphere formation (Figure 2E), cell viability (Figure 2F), cell proliferation index (Figure 2G-H) and cell growth (Supplemental Figure S4). These results indicate that knockdown of MSK expression did not affect cell growth, but attenuated resistance to PI3K/mTOR inhibition.

Overexpression of MSK1 increased cell resistance to PI3K/mTOR inhibition

To confirm the function of MSK1 in PI3K/mTOR response, we used GSC11, which is sensitive to PI3K/mTOR inhibition and with low endogenous expression of MSK1, to establish an MSK1-overexpressing cell line (Figure 3A). A cell viability assay showed that, compared with vector-infected cells, GSC11-MSK1 were more resistant to BEZ235 and DS7423 treatment (Figure 3B and C). The IC_{50} of BEZ235 was increased 8.5-fold as a result of MSK1 overexpression (86 nM for vector cells and 724 nM for MSK1-overexpressed cells); similarly, the IC_{50} to DS7423 increased 3-fold as a result of MSK1 overexpression (from 105 nM to 399 nM) (Figure 3D). After BEZ235 and DS7423 treatment, the neurospheres derived from MSK1-overexpressing cells were larger and more numerous than those from vector cells (Figure 3E). Although MSK1- overexpression alone did not affect cell viability, it increased cell viability after BEZ235 or DS7423 treatment (Figure 3F). Consistently, overexpression of MSK1 markedly increased the proliferative potential of GSC11 in response to BEZ234 and DS7423, as shown by the increased positivity of Ki-67 staining in treated cells (Figure 3G and H). In addition, MSK1-overexpression also suppressed PI3K/mTOR inhibition-induced apoptosis as indicated by TUNEL staining (Figure 3I and 3J). Taken together, these data suggest that MSK1 overexpression confers resistance to PI3K/mTOR inhibition.

MSK1 phosphorylated β-catenin at Ser^{552}

To explore the molecular mechanism by which MSK1 regulates cell proliferation, we analyzed the gene expression profile of GSC20-SCR and GSC20-MSK1-sh and found a panel of β-catenin signaling-targeted genes including MYC (25), MMP2 (26), MMP7(27), WISP1 (28), CYR61 (29), MET (30) *etc.* were down-regulated by MSK1 shRNA (Supplemental Figure S5). This result suggested that MSK1 regulates β-catenin signaling; therefore, we studied the link between β-catenin and MSK1. First, we determined whether MSK1 interacted with β-catenin by coimmunoprecipitation assay. β-catenin was co-immunoprecipitated with Flag-tagged MSK1 by anti-Flag M2 agarose (Figure 4A); conversely, MSK1 was also co-immunoprecipitated with Flag-tagged β-catenin (Figure 4B), suggesting a physical interaction between MSK1 and β-catenin.

Since MSK1 is a serine/threonine kinase, we determined whether it phosphorylates β-catenin. We performed *in vitro* kinase assays with purified active GST-tagged MSK1 mixed with purified His-β-catenin and analyzed a trypsin digest of β-catenin by mass spectrometry. Ser^{552} was identified as a phosphorylation site of β-catenin by liquid chromatography-coupled ion trap mass spectrometry (LC-MS/MS) (Figure 4C). The phosphorylation of

ser^{552}-β-catenin by MSK1 was further confirmed by immunoblotting with a commercial specific antibody against p-ser^{552}-β-catenin (Figure 4D). These results indicate that MSK1 directly phosphorylated β-catenin at Ser^{552} *in vitro*.

To determine whether MSK1 phosphorylates β-catenin in intact cells, we overexpressed MSK1 in GSC11 and detected endogenous β-catenin by immuno-blotting. MSK1 overexpression greatly increased phospho-ser^{552}-β-catenin but had little effect on the total protein level of β-catenin (Figure 4E). To confirm this result, we overexpressed MSK1 in another cell line GSC42. Similarly, overexpression of MSK1 in GSC42 also induced phospho-ser^{552}-β-catenin without affecting total β-catenin protein level (Figure 4F). Collectively, these results indicate that MSK1 directly phosphorylates β-catenin *in vitro* and *in vivo*.

PI3K/mTOR inhibitors induced β-catenin phosphorylation through MSK1

As previously reported, AKT phosphorylates β-catenin at Ser^{552}, and inhibition of AKT inhibits β-catenin phosphorylation at Ser^{552} (19). However, interestingly, in GSC, we found that treatment with the PI3K/mTOR inhibitors BEZ235 and DS7423 for 24 hours increased β-catenin phosphorylation at Ser^{552} in spite of the inhibition of AKT activity and S6 activity (Figure 4G), suggesting that BEZ235 or DS7423 treatment induced β-catenin phosphorylation independently of AKT activity.

We determined whether PI3K/mTOR inhibitor-induced β-catenin phosphorylation is mediated by MSK1 by knocking down MSK1 expression with shRNAs. In SCR cells, DS7423 increased Ser^{552}-β-catenin phosphorylation, but when MSK1 was depleted, Ser^{552}-β-catenin phosphorylation was decreased and was not further induced by DS7423 treatment (Figure 4H). These results indicate that PI3K/mTOR inhibitors increased MSK1 expression, which in turn phosphorylated β-catenin at Ser^{552}.

MSK1 regulated β-catenin localization and transcriptional activity

Since β-catenin localizes in different cellular compartments to execute differential cellular functions, we determined whether β-catenin phosphorylation by MSK1 affects its subcellular distribution. For that, wild type (wt) β-catenin or S552A mutant β-catenin was transfected with or without MSK1. Co-expression of MKS1 led to a partial translocation of β-catenin from the membrane to the nucleus (Figure 5A). S552A mutant β-catenin was primarily localized in the cell membrane and did not enter the nucleus even when co-expressed with MSK1.

As Ser^{552} phosphorylation is important for the transcriptional activity of β-catenin (31), we co-transfected TCF/LEF-1 luciferase reporter TOP-FLASH or a control vector, FOP-FLASH, with β-catenin and an increased dose of MSK1. Co-transfection of MSK1 increased β-catenin transcriptional activity in a dose-dependent manner (Figure 5B). To investigate the mechanisms underlying MSK1-regulated β-catenin transcriptional activity, we performed a ChIP analysis. As shown in Figure 5C, MSK1 increased β-catenin binding to the c-Myc promoter. The expression of β-catenin target genes, including c-Myc, cyclin D1, CYR61, and MMP 9, were up-regulated by MSK1 overexpression (Figure 5D and E). In contrast, the expression of these genes was down-regulated by MSK1 shRNA (Figure 5F).

β-catenin was essential for MSK1-mediated resistance to PI3K/mTOR inhibitors

To determine whether β-catenin confers resistance to PI3K/mTOR inhibitors, we knocked down β-catenin expression by shRNAs in a resistant cell line GSC20 (Supplemental Fig. S6A). Depletion of β-catenin expression sensitized cells to BEZ235 and DS7423 treatment (Supplemental Fig. S6B and S6C). To determine if β-catenin acts as a downstream target of MSK1 to mediate resistance to PI3K/mTOR inhibition, we depleted β-catenin expression in GSC11 MSK1-overexpressing cells (Figure 6A). Consistent with the results above, MSK1 overexpression led to resistance to DS7423 treatment as indicated by the increased IC_{50} of DS7423 (Figure 6B and C); this effect was reversed by depletion of β-catenin, as expression of β-catenin shRNAs in MSK1- overexpression cells resensitized cells to DS7423 as indicated by the decreased IC_{50} of DS7423 (Figure 6B and C). To study how the MSK1-overxepression and β-catenin depletion regulate cell response, we analyzed c-Myc and cyclin D1 expression by QPCR. C-Myc and cyclin D1 expression was increased by MSK1 overexpression, which was blocked by β-catenin depletion (Figure 6D). Taken together our results implied that β-catenin acts as a downstream of MSK1 upon PI3K/mTOR inhibition, and MSK1-regulated β-catenin phosphorylation and transcriptional activity is responsible for the resistance to PI3K/mTOR inhibition.

MSK1 inhibition synergized with PI3K/mTOR inhibitors to extend survival in an intracranial animal model

We determined whether inhibition of MSK1 potentiated PI3K/mTOR inhibition in a xenograft intracranial tumor model of glioma. Immunodeficient mice were injected with GSC20 that had been stably transfected with MSK1-shRNA (MSK1-sh) or SCR as a control. DS7423 (6 mg/kg) was started 4 days after injection for 6 weeks. DS7423 treatment very slightly extended the survival of SCR cells compared with vehicle treatment. MSK1 deletion also slightly extended the average survival duration; DS7423 treatment greatly extended survival in the MSK1 deletion group but had less effect in the SCR group (Figure 7A), suggesting that in vivo inhibition of both PI3K/mTOR signaling and MSK1 is more effective than either alone for blocking tumor progression.

An immunohistochemical analysis of the tumor tissue further proved that MSK1 inhibition synergized with PI3K/mTOR inhibitors to suppress tumor growth, as indicated by Ki-67 staining (Figure 7B). S6 ribosomal protein phosphorylation and AKT phosphorylation were reduced by DS7423 treatment, confirming the efficacy of DS7423 at inhibiting PI3K/mTOR signaling in vivo. Consistent with the *in vitro* analysis, immunohistochemical staining showed that DS7423 treatment increased MSK1 expression and β-catenin phosphorylation at Ser^{552} *in vivo*, which was blocked by MSK1 deletion (Figure 7B).

Discussion

PI3K/mTOR inhibitors are being developed as promising anti-cancer agents (32). However, the results of pre-clinical studies indicate that patients who benefit from PI3K/mTOR treatment eventually develop drug resistance after a transient response (33,34). To understand the mechanisms underlying resistance to the PI3K/mTOR inhibitor, the current study analyzed the gene expression profiles of pre- and post-PI3K/mTOR inhibitor

treatments in a panel of GIC. Here, we identified MSK1-upregulation as an adaptive resistance response to PI3K/mTOR inhibition. MSK1 was one of the top 10 up-regulated PI3K/mTOR inhibitor genes and is an attractive target for combination therapy for several reasons: first, it is a kinase; thus, it is potentially targetable. Second, it regulates diverse cellular processes, including cell proliferation and apoptosis, and is linked to tumorigenesis (8,13,35). Moreover, it is implicated in resistance to several agents, including As_2O_3, TGF-β, and paclitaxel (12,36,37).

In the present study, using quantitative RT-PCR and Western blot analysis, we demonstrated that MSK1 mRNA and protein were up-regulated after PI3K/mTOR inhibitor treatment. However, regulation of MSK1 expression remains largely unknown. Fujita et al showed that MSK1 was a direct target of MiR-148a in PC3 cells and that overexpression of MiR-148a led to decreased MSK1 expression (37). However, how PI3K/mTOR inhibition leads to up-regulation of MSK1 expression and whether MiR-148a is involved remain to be further elucidated. Nevertheless, we found that MSK1 was activated by ERK in response to PI3K/mTOR inhibition. This result is consistent with those of previous reports that ERK signaling was activated by PI3K/mTOR inhibition (38,39) and MSK1 was phosphorylated by ERK in various stimuli (9,11). Notably, our RPPA data (Supplementary Figure S3B) showed that EKR signaling was activated upon BEZ235 or DS7423 treatment for 24 hours, but other MAPKs, p38 and JNK, were not or slightly affected. As expected, the mTOR signaling was inhibited as indicated by p70S6K and S6 phosphorylation. However, pS473-AKT was not significantly inhibited by PI3K/mTOR inhibition. Time-course treatment with BEZ235 revealed that pS473-AKT was inhibited at beginning but then reactivated at 24 hours (Supplementary Figure S3C), which might be due to negative feedback by mTORC1-S6K(40), or reactivation by ERK (41).

The ectopic expression of MSK1 in cells with low endogenous expression and sensitive GIC potentiated resistance to PI3K/mTOR inhibition, while knockdown of MSK1 in cells with high endogenous expression and resistant GIC attenuated resistance both *in vitro* and *in vivo*. These results suggest that deregulation of MSK1 contributes to PI3K/mTOR resistance. Interestingly, Violeta Serra et al (42) used an ORF library and identified RSK3/RSK4, 2 other RSK family members that mediate resistance to PI3K pathway inhibition in breast cancer. Here, we report that MSK1 mediated resistance to PI3K/mTOR inhibition in GBM. These findings suggest that RSK families play a crucial role in resistance to PI3K/mTOR inhibition in addition to their well-known role in tumorigenesis, although different RSKs may be involved in different cancers.

Our *in vitro* kinase assay showed that MSK1 can directly phosphorylate β-catenin at Ser^{552}. This site was reported to be phosphorylated by several kinases, including AKT. In this study, the results that AKT activity was inhibited upon PI3K/mTOR inhibition while β-catenin was phosphorylated at Ser^{552} excluded the possibility that AKT phosphorylates β-catenin. The observation of parallel up-regulation of MSK1 expression and β-catenin phosphorylation suggests that MSK1 mediates PI3K/mTOR inhibitor-induced β-catenin phosphorylation. Consistently, β-catenin phosphorylation induced by PI3K/mTOR inhibitors was blocked by MSK1 depletion *in vitro* and *in vivo*, suggesting that MSK1 is the main kinase that phosphorylates β-catenin under the stress of PI3K/mTOR inhibition. Interestingly, MSK1

and AKT shared the same phosphorylation motif (43,44) that is present in Ser^{552} β-catenin. These results suggest that MSK1 serves as a supplemental kinase to AKT to phosphorylate β-catenin and provides a pathway to escape from PI3K/mTOR inhibition.

Phosphorylation of Ser^{552} is important for β-catenin nuclear localization and transcriptional activity (31,45). Here, we demonstrated that MSK1 phosphorylated β-catenin at Ser^{552} and promoted the nuclear translocation of wt-β-catenin but not S552A-mutated β-catenin. MSK1 also regulated the expression of β-catenin target genes, including c-Myc and cyclin D1, which are involved in cell proliferation. Depletion of β-catenin reversed the MSK1 overexpression-induced expression of c-Myc and cyclin D1 and overcame the resistance phenotype of MSK1-overexpressing cells, suggesting that β-catenin plays a crucial role in MSK1-mediated resistance to PI3K/mTOR inhibition. The function of β-catenin in PI3K/mTOR resistance was also supported by the results of a previous report that β-catenin confers resistance to PI3K/AKT inhibition in colon cancer (46).

Notably, MSK1 depletion not only down-regulated p-S552-β-catenin but also decreased total β-catenin (Figure 4H,) while interestingly, induction of p-S552-β-catenin by MSK1 overexpression (Figure 4E and F) or by DS/BEZ treatment (Figure 4G) exerted little effect on the total β-catenin. These results suggested that phosphorylation at Ser^{552} is important, and possibly a necessary but not a sufficient condition for the protein stability. Consistent with this speculation, Jar-Yi Ho et al reported that ovatodiolide decreased β-catenin protein stability by inhibiting phosphorylation of Ser^{552} (47). However, overexpression of S552D mutant β-catenin, a mimic of pS552-β-catenin, showed no difference in half-life compared with wt-β-catenin (19). These reports together with our results suggested that phosphorylation at Ser^{552} is critical but not sufficient for protein stability.

The BBB is a significant obstacle for delivering small molecules to brain tumor (48) and penetration of molecules across BBB is critical to achieve optimal effects. In current study, the IHC results showed that pAKT and pS6 was significantly decreased after DS7423 treatment. These results, combining our previous pharmacodynamics study (23), suggested DS7423 can cross BBB to achieve functional concentration to inhibit PI3K/mTOR signaling. While BEZ235 can also cross the BBB in orthotopic glioblastoma models (49), the potential *in vivo* synergy of BEZ235 and MSK1 knockdown or inhibitor was not tested in current study. Future study of identifying MSK1 specific inhibitor and combining the inhibitor with BEZ235 or DS7423 is needed for successful clinical combination therapy.

Collectively, our data support the model shown in Figure 7C: PI3K/mTOR inhibitors inhibit the PI3K/mTOR pathway but also activate ERK signaling and up-regulate MSK1 expression. MSK1 is phosphorylated by ERK and in turn, phosphorylates β-catenin to regulate the expression of c-Myc and cyclin D1 to promote cell proliferation; therefore, cells are resistant to PI3K/mTOR inhibition. MSK1 depletion by shRNA blocks PI3K/mTOR inhibitor-induced β-catenin activation and sensitizes cells to PI3K/mTOR inhibition. Our findings indicate that MSK1/β-catenin signaling serves as an escape survival signal upon PI3K inhibition and provides a strong rationale for the combined use of PI3K and MSK1/β-catenin inhibition to induce lethal growth inhibition in human GBM cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Verlene Henry and Lindsay Holmes for performing the animal studies. We thank Ann Sutton (Department of Scientific Publications, The University of Texas MD Anderson Cancer Center) for editing the manuscript, and M.D. Anderson Cancer Center, NIH High-End Instrumentation program grant# 1S10OD012304-01, and CPRIT Core Facility Grant# RP130397 for mass spectroscopy services.

Funding source: This study was funded by a CPRIT grant (RP120256 to W. K. A. Yung), a SPORE grant (P50 CA127001 to F. F. Lang), and a Cancer Center Support Grant (CA016672 to R. A. DePinho).

References

1. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008; 321:1807–12. [PubMed: 18772396]
2. Soliman GA. The integral role of mTOR in lipid metabolism. Cell cycle. 2011; 10:861–2. [PubMed: 21325894]
3. Laplante M, Sabatini DM. mTOR signaling at a glance. Journal of cell science. 2009; 122:3589–94. [PubMed: 19812304]
4. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nature medicine. 2008; 14:1351–6.
5. Migliardi G, Sassi F, Torti D, Galimi F, Zanella ER, Buscarino M, et al. Inhibition of MEK and PI3K/mTOR suppresses tumor growth but does not cause tumor regression in patient-derived xenografts of RAS-mutant colorectal carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012; 18:2515–25. [PubMed: 22392911]
6. Halilovic E, She QB, Ye Q, Pagliarini R, Sellers WR, Solit DB, et al. PIK3CA mutation uncouples tumor growth and cyclin D1 regulation from MEK/ERK and mutant KRAS signaling. Cancer research. 2010; 70:6804–14. [PubMed: 20699365]
7. Deak M, Clifton AD, Lucocq LM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 1998; 17:4426–41. [PubMed: 9687510]
8. Perez-Cadahia B, Drobic B, Espino PS, He S, Mandal S, Healy S, et al. Role of MSK1 in the malignant phenotype of Ras-transformed mouse fibroblasts. The Journal of biological chemistry. 2011; 286:42–9. [PubMed: 21071437]
9. Davie JR. MSK1 and MSK2 mediate mitogen- and stress-induced phosphorylation of histone H3: a controversy resolved. Science's STKE : signal transduction knowledge environment. 2003; 2003:PE33.
10. Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G. Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). EMBO J. 2003; 22:1313–24. [PubMed: 12628924]
11. Soloaga A, Thomson S, Wiggin GR, Rampersaud N, Dyson MH, Hazzalin CA, et al. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. EMBO J. 2003; 22:2788–97. [PubMed: 12773393]
12. van der Heide LP, van Dinther M, Moustakas A, ten Dijke P. TGFbeta activates mitogen- and stress-activated protein kinase-1 (MSK1) to attenuate cell death. The Journal of biological chemistry. 2011; 286:5003–11. [PubMed: 21106525]
13. Reyes D, Ballare C, Castellano G, Soronellas D, Bago JR, Blanco J, et al. Activation of mitogen- and stress-activated kinase 1 is required for proliferation of breast cancer cells in response to estrogens or progestins. Oncogene. 2013
14. Polakis P. The oncogenic activation of beta-catenin. Current opinion in genetics & development. 1999; 9:15–21. [PubMed: 10072352]

15. Liu C, Tu Y, Sun X, Jiang J, Jin X, Bo X, et al. Wnt/beta-Catenin pathway in human glioma: expression pattern and clinical/prognostic correlations. Clinical and experimental medicine. 2011; 11:105–12. [PubMed: 20809334]
16. Sareddy GR, Panigrahi M, Challa S, Mahadevan A, Babu PP. Activation of Wnt/beta-catenin/Tcf signaling pathway in human astrocytomas. Neurochemistry international. 2009; 55:307–17. [PubMed: 19576519]
17. Yang W, Xia Y, Ji H, Zheng Y, Liang J, Huang W, et al. Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. Nature. 2011; 480:118–22. [PubMed: 22056988]
18. Saito N, Fu J, Zheng S, Yao J, Wang S, Liu DD, et al. A high Notch pathway activation predicts response to gamma secretase inhibitors in proneural subtype of glioma tumor-initiating cells. Stem Cells. 2014; 32:301–12. [PubMed: 24038660]
19. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, et al. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. The Journal of biological chemistry. 2007; 282:11221–9. [PubMed: 17287208]
20. Saito N, Fu J, Zheng S, Yao J, Wang S, Liu DD, et al. A high Notch pathway activation predicts response to gamma secretase inhibitors in proneural subtype of glioma tumor initiating cells. Stem Cells.
21. Koul D, Fu J, Shen R, LaFortune TA, Wang S, Tiao N, et al. Antitumor activity of NVP-BKM120--a selective pan class I PI3 kinase inhibitor showed differential forms of cell death based on p53 status of glioma cells. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012; 18:184–95. [PubMed: 22065080]
22. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Molecular cancer therapeutics. 2008; 7:1851–63. [PubMed: 18606717]
23. Koul D, Wang S, Saito N, Zheng S, Wu S, Setoguchi M, et al. Preclinical Therapeutic Efficacy of a Novel Blood-Brain Barrier-Penetrant Dual PI3K/mTOR Inhibitor with Preferential Response in PI3K/PTEN Mutant Glioma. In submission.
24. Wang D, Boerner SA, Winkler JD, LoRusso PM. Clinical experience of MEK inhibitors in cancer therapy. Biochimica et biophysica acta. 2007; 1773:1248–55. [PubMed: 17194493]
25. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science. 1998; 281:1509–12. [PubMed: 9727977]
26. Wu B, Crampton SP, Hughes CC. Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. Immunity. 2007; 26:227–39. [PubMed: 17306568]
27. Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. The American journal of pathology. 1999; 155:1033–8. [PubMed: 10514384]
28. Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. Genes & development. 2000; 14:585–95. [PubMed: 10716946]
29. Si W, Kang Q, Luu HH, Park JK, Luo Q, Song WX, et al. CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. Molecular and cellular biology. 2006; 26:2955–64. [PubMed: 16581771]
30. Boon EM, van der Neut R, van de Wetering M, Clevers H, Pals ST. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer research. 2002; 62:5126–8. [PubMed: 12234972]
31. Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO. Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase. The Journal of biological chemistry. 2006; 281:9971–6. [PubMed: 16476742]
32. Fan QW, Weiss WA. Targeting the RTK-PI3K-mTOR axis in malignant glioma: overcoming resistance. Current topics in microbiology and immunology. 2010; 347:279–96. [PubMed: 20535652]
33. Wen PY, Lee EQ, Reardon DA, Ligon KL, Alfred Yung WK. Current clinical development of PI3K pathway inhibitors in glioblastoma. Neuro-oncology. 2012; 14:819–29. [PubMed: 22619466]

34. Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. Nature reviews Clinical oncology. 2013; 10:143–53.
35. Abecassis L, Rogier E, Vazquez A, Atfi A, Bourgeade MF. Evidence for a role of MSK1 in transforming growth factor-beta-mediated responses through p38alpha and Smad signaling pathways. The Journal of biological chemistry. 2004; 279:30474–9. [PubMed: 15133024]
36. Kannan-Thulasiraman P, Katsoulidis E, Tallman MS, Arthur JS, Platanias LC. Activation of the mitogen- and stress-activated kinase 1 by arsenic trioxide. The Journal of biological chemistry. 2006; 281:22446–52. [PubMed: 16762916]
37. Fujita Y, Kojima K, Ohhashi R, Hamada N, Nozawa Y, Kitamoto A, et al. MiR-148a attenuates paclitaxel resistance of hormone-refractory, drug-resistant prostate cancer PC3 cells by regulating MSK1 expression. The Journal of biological chemistry. 2010; 285:19076–84. [PubMed: 20406806]
38. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. The Journal of clinical investigation. 2008; 118:3065–74. [PubMed: 18725988]
39. Ercan D, Xu C, Yanagita M, Monast CS, Pratilas CA, Montero J, et al. Reactivation of ERK signaling causes resistance to EGFR kinase inhibitors. Cancer discovery. 2012; 2:934–47. [PubMed: 22961667]
40. Efeyan A, Sabatini DM. mTOR and cancer: many loops in one pathway. Current opinion in cell biology. 2010; 22:169–76. [PubMed: 19945836]
41. Toulany M, Minjgee M, Saki M, Holler M, Meier F, Eicheler W, et al. ERK2-dependent reactivation of Akt mediates the limited response of tumor cells with constitutive K-RAS activity to PI3K inhibition. Cancer biology & therapy. 2014; 15:317–28. [PubMed: 24351425]
42. Serra V, Eichhorn PJ, Garcia-Garcia C, Ibrahim YH, Prudkin L, Sanchez G, et al. RSK3/4 mediate resistance to PI3K pathway inhibitors in breast cancer. The Journal of clinical investigation. 2013; 123:2551–63. [PubMed: 23635776]
43. Moritz A, Li Y, Guo A, Villen J, Wang Y, MacNeill J, et al. Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. Science signaling. 2010; 3:ra64. [PubMed: 20736484]
44. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 1996; 15:6541–51. [PubMed: 8978681]
45. He XC, Yin T, Grindley JC, Tian Q, Sato T, Tao WA, et al. PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nature genetics. 2007; 39:189–98. [PubMed: 17237784]
46. Tenbaum SP, Ordonez-Moran P, Puig I, Chicote I, Arques O, Landolfi S, et al. beta-catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer. Nature medicine. 2012; 18:892–901.
47. Ho JY, Hsu RJ, Wu CL, Chang WL, Cha TL, Yu DS, et al. Ovatodiolide Targets beta -Catenin Signaling in Suppressing Tumorigenesis and Overcoming Drug Resistance in Renal Cell Carcinoma. Evidence-based complementary and alternative medicine : eCAM. 2013; 2013:161628. [PubMed: 23781255]
48. Nathanson D, Mischel PS. Charting the course across the blood-brain barrier. The Journal of clinical investigation. 2011; 121:31–3. [PubMed: 21183784]
49. Gil del Alcazar CR, Hardebeck MC, Mukherjee B, Tomimatsu N, Gao X, Yan J, et al. Inhibition of DNA double-strand break repair by the dual PI3K/mTOR inhibitor NVP-BEZ235 as a strategy for radiosensitization of glioblastoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014; 20:1235–48. [PubMed: 24366691]

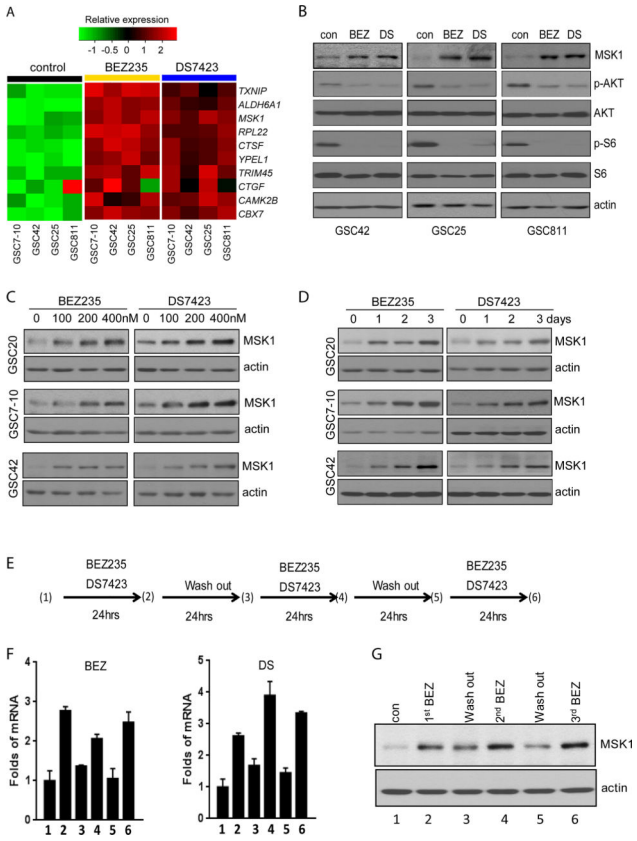

Figure 1. PI3K/mTOR dual inhibitors up-regulated MSK1 expression in GIC

A) GIC was treated with BEZ235 or DS7423, and gene expression was analyzed by microarray. The top 10 up-regulated genes are shown in the heat map. B) The indicated GIC were treated with 100 nM BEZ235 or 100 nM DS7423, and MSK1 protein was detected by Western blot analysis. C) GICs were treated with increasing doses of BEZ235 or DS7423 for 48 hours. MSK1 protein was detected by Western blot analysis. D) GSC20, GSC710, and GSC42 were treated with 100 nM BEZ235 or DS7423 for the indicated times, and MSK1 protein was detected. E-G) Pulse treatment of GSC42 with BEZ235 or DS7423 as indicated and mRNA and protein level of MSK1 was detected.

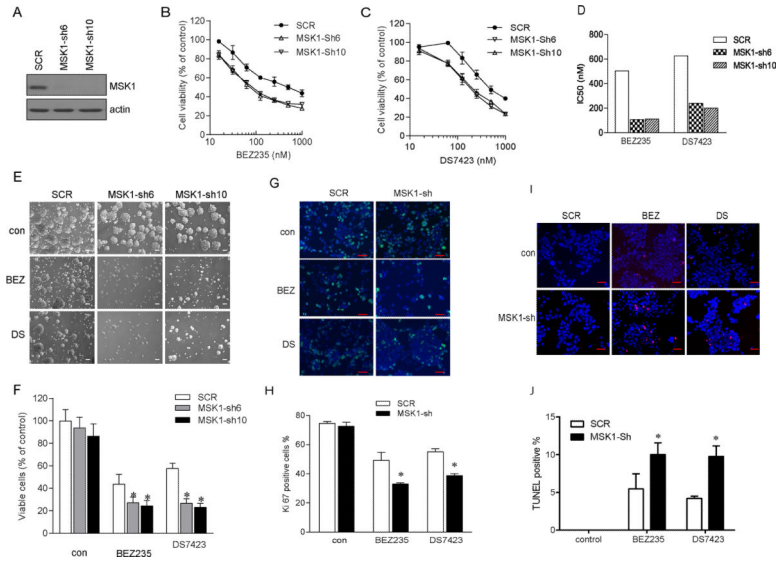

Figure 2. Depletion of MSK1 attenuated resistance to PI3K/mTOR inhibitors

A) MSK1 was knocked down by lentivirus shRNAs (MSK1-sh6 and MSK1-sh10), and this knockdown effect was confirmed by Western blot analysis. Scramble sequence shRNA (SCR) was used as the control. B-D) GSC20-SCR, GSC20-MSK1-sh6, and GSC20-MSK1-sh10 were treated with increasing doses of BEZ235 or DS7423 for 72 hours, and cell viability was assessed by the Cell Titer-Blue assay. The IC_{50} value was calculated and plotted. E) Micrographs of GSC20-SCR and GSC20-MSK1-sh treated with BEZ235 and DS7423 for 5 days. Scale bar, 100μM. F) GSC20-SCR and GSC20-MSK1-sh cells were treated with BEZ235 and DS7423 for 5 days, and the viable cells were counted. The number of viable untreated SCR cells was considered to be 100%. n = 4; ∗, $P < 0.001$. G) Immuno-staining of Ki-67 in GSC20-SCR and GSC20-MSK1-sh treated with BEZ235 or DS7423 for 72 hours. Scale bars, 50μM. H) The Ki-67-positive cell percentage was calculated. I) TUNEL-staining in GSC20-SCR and GSC20-MSK1-sh treated with BEZ235 or DS7423 for 72 hours. Scale bars, 50μM. J) Quantitation of percentage of TUNEL-positive cells. Experiments were carried out in triplicate. At least 300 cells were counted for each treatment.

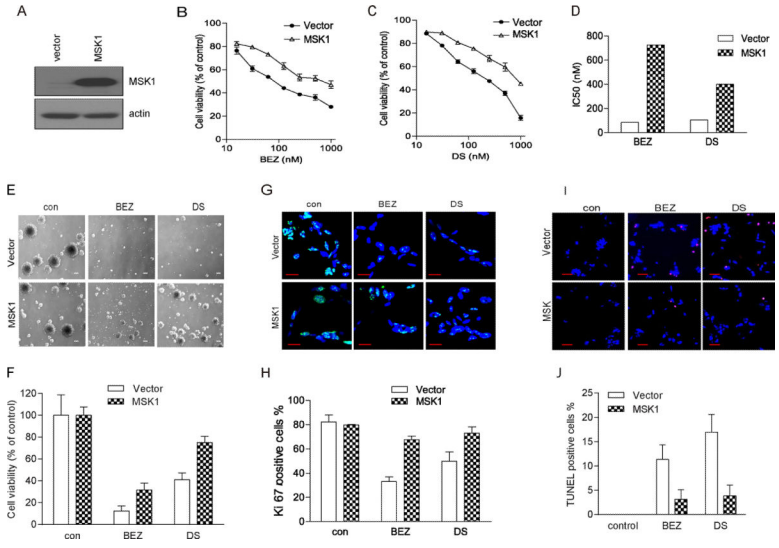

Figure 3. Overexpression of MSK1 potentiated the resistance of GIC to PI3K/mTOR inhibitors

A) A retrovirus containing empty vector or MSK1 was constitutively expressed in GSC11 and confirmed by Western blot analysis. B-D) GSC11-vector and GSC11-MSK1 were treated with BEZ235 or DS7423. Cell viability was tested, and the IC_{50} was calculated. E-F) GSC11-vector and GSC11-MSK1 were treated with BEZ235 or DS7423 for 5 days. The number of neuro-spheres was determined by microscopy, and viable cells were counted. Scale bars, 100μM. G-H) GSC11-vector and GSC11-MSK1 were treated with BEZ235 or DS7423 for 72 hours, and cell proliferation was examined by Ki-67 immunostaining. Scale bars, 50μM. I-J) GSC11-vector and GSC11-MSK1 were treated with BEZ235 or DS7423 for 72 hours, and cell apoptosis was examined by TUNEL staining. Scale bars, 50μM.

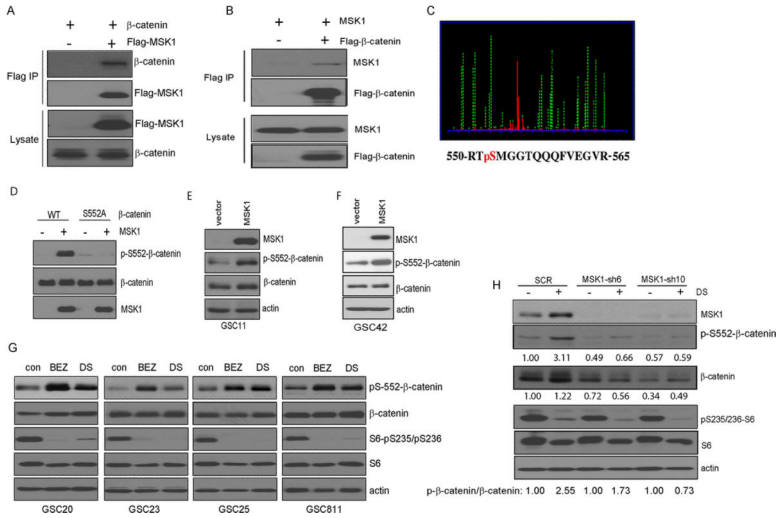

Figure 4. MSK1 phosphorylated β-catenin at Ser^{552}

A) β-catenin was transfected with or without Flag-tagged MSK1. Cell lysates were immunoprecipitated by anti-Flag M2 agarose and then analyzed by Western blot analysis. B) Flag-tagged β-catenin was immunoprecipitated with anti-Flag M2 agarose, and co-precipitated MSK1 was detected by Western blot analysis. C) An *in vitro* kinase assay was performed with purified active GST-MSK1 and purified bacterially expressed His-β-catenin. The reaction mixture was analyzed by mass spectrometry. A mass spectrometric analysis of the tryptic fragment Arg^{550}–Arg^{565} indicated that Ser^{552} was phosphorylated. D) An *in vitro* kinase assay was performed using purified active GST-MSK1 and purified bacterially expressed His-β-catenin or His-β-catenin-S552A. Phosphorylation of β-catenin at Ser^{552} was detected by Western blot analysis with specific antibody. E-F) Phosphorylation of β-catenin at Ser^{552} in GSC11-MSK1 and GSC42-MSK1 cells was detected by Western blot analysis. G) The indicated cells were treated with 100 nM BEZ235 or 100 nM DS7423 for 24 hours, and the cell lysates were analyzed by Western blot analysis. H) GSC20SCR, GSC20-MSK1-sh6, and GSC-MSK1-sh10 were treated with or without DS7423 for 24 hours, and the cell lysates were analyzed by Western blot analysis. p-β-catenin and total β-catenin expression was quantified by Image J, and p-β-catenin induction by DS treatment was shown at the bottom by the ratio of p-β-catenin/ β-catenin normalized to corresponding untreated samples.

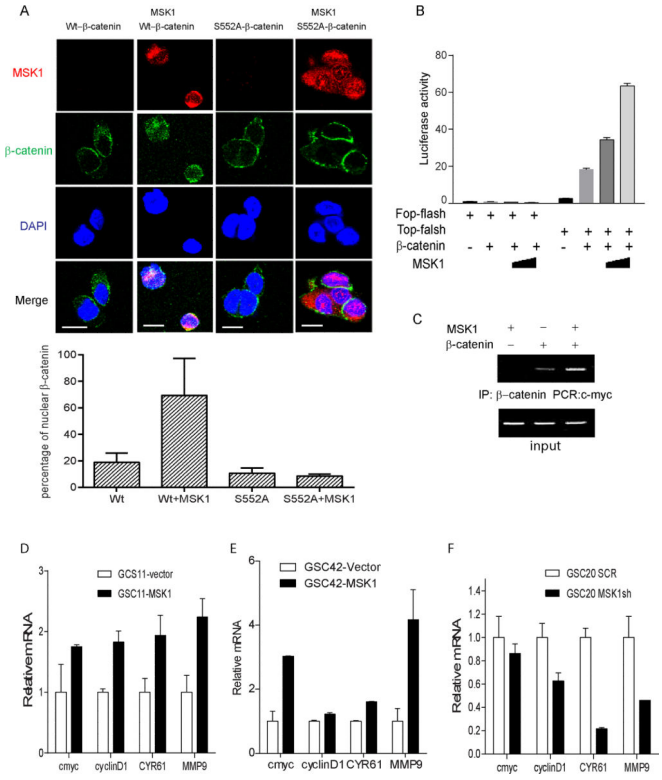

Figure 5. MSK1 regulated β-catenin localization and transcriptional activity

A) GSC11 that transiently expressed HA-β-catenin or HA-β-catenin-S552A, with or without Flag-tagged MSK1, were stained with anti-Flag antibody (red), anti-HA antibody (green), and DAPI for the nucleus. The distribution of β-catenin was quantified using Image J and percentage of nuclear β-catenin was shown at the bottom. B) TOP-FLASH or FOP-FLASH with β-catenin and MSK1 were transfected into U87 cells for 24 hours. Luciferase activity was determined and normalized with *Renilla* activity. C) MSK1 was transfected with or without β-catenin into U87 cells. A ChIP assay was performed with β-catenin antibody, and the c-Myc promoter was detected by PCR. β-catenin-targeted genes were detected by real-time PCR in GSC11 (D) and GSC42 (E) that overexpressed vector and MSK1. F) β-catenin target genes were detected by real-time PCR in GSC20 SCR and GSC20-MSK1-sh.

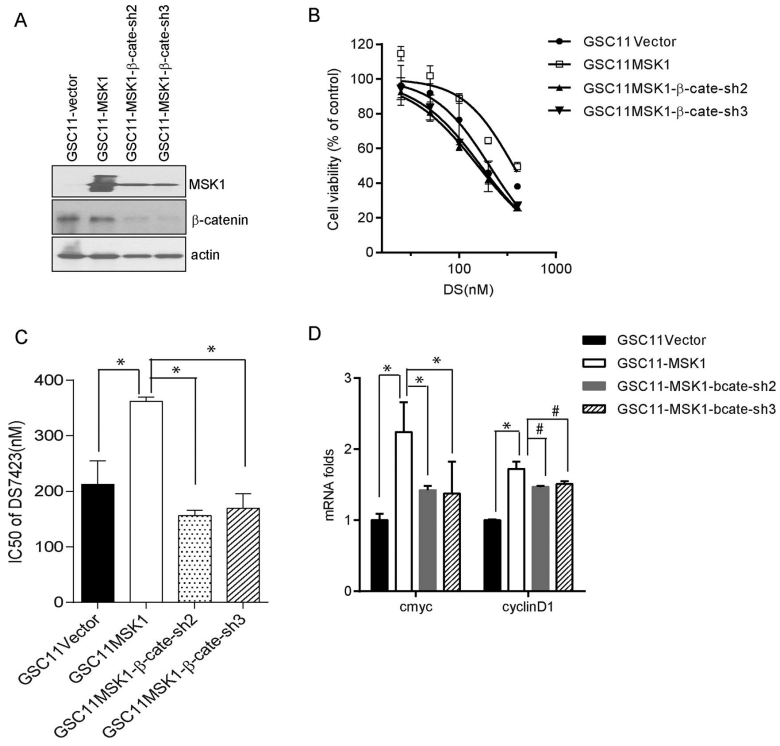

Figure 6. β-catenin acted downstream of MSK1 to potentiate resistance to PI3K/mTOR inhibition

A) β-catenin was knocked down by shRNAs in GSC11-MSK1. The expression of β-catenin and MSK1 was detected by Western blot analysis. B-C) Cell viability in response to DS7423 treatment was tested by CellTiter blue and the IC_{50} was calculated. D) c-Myc and cyclin D1 mRNA was analyzed by quantitative PCR. Symbol ∗ indicates $P < 0.001$ and # indicates $p < 0.05$.

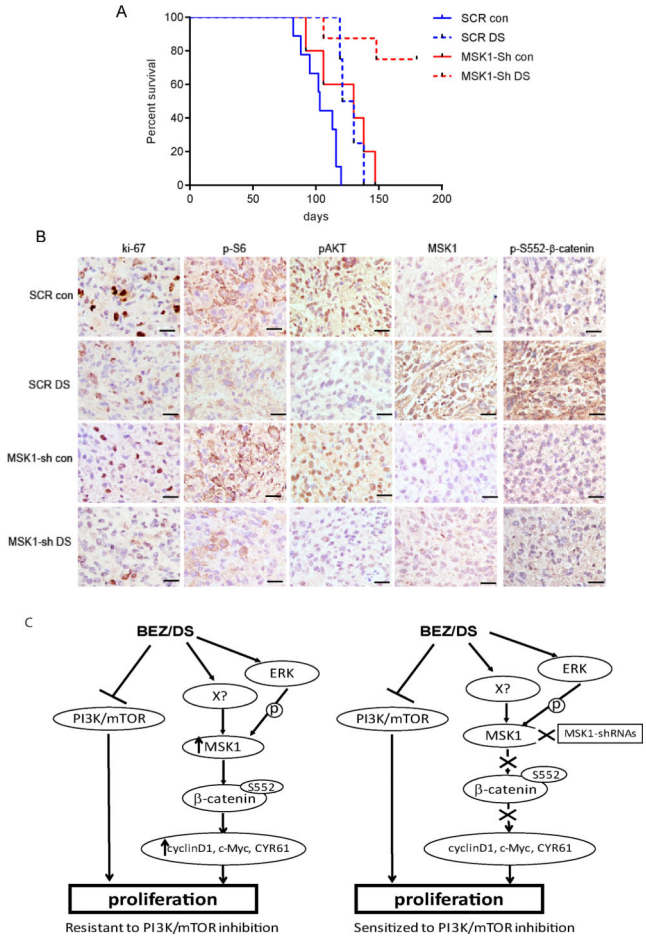

Figure 7. MSK1 depletion synergized with PI3K/mTOR inhibition to extend survival in an intracranial animal model

A) GSC20-SCR and GSC20-MSK1sh were implanted intracranially in nude mice (n=9 for SCR con, n=8 for SCR DS, n=5 for MSK1-sh con, n=8 for MSK1-sh DS); 4 days later, DS7423 (6 mg/kg) was administered orally 5 times a week for 6 weeks. Mice were killed at morbidity, and survival curves were compared using Kaplan-Meier survival plots. B) Immunostaining of the brain sections of animals that had been treated for 5 weeks. The tissue sections were incubated with antibodies against Ki-67, p-S6, pAKT, MSK1, and p-S552-β-catenin. Scale bars, 25 μM. C) Proposed Model for MSK1 mediated resistance to PI3K/mTOR inhibition: Left panel: BEZ235 and DS7423 inhibited the PI3K/mTOR pathway to affect proliferation. On the other hand, BEZ235/DS7423 treatment increased MSK1 expression through an unknown mechanism and activated ERK to phosphorylate MSK1. MSK1 further phosphorylated and activated β-catenin, which promotes cell proliferation, making cells resistant to PI3K/mTOR inhibition. Right panel: Upon MSK1 depletion by shRNA, β-catenin was not activated upon BEZ235/DS7423 treatment and no β-catenin transcriptional activation and no β-catenin targeted genes are activated therefore cells became sensitive to PI3K/mTOR inhibition.