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Genomic and functional analysis of the E3 ligase PARK2 in glioma

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Abstract

PARK2 (PARKIN) is an E3 ubiquitin ligase whose dysfunction has been associated with the progression of Parkinsonism and human malignancies, and its role in cancer remains to be explored. In this study, we report that PARK2 is frequently deleted and underexpressed in human

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D-C. Lin and L. Xu share first authorship for this work.

Conflict of interest

M.K. is a consultant and equity holder in Prism Pharma Co. Ltd. The rest of the authors declare no conflict of interest related to this study.

Accession codes

Digital cDNA microarray files have been deposited to Gene Expression Omnibus (GSE61973).

TCGA, http://cancergenome.nih.gov/; Clinical trials database, http://clinicaltrials.gov/; CCLE, http://www.broadinstitute.org/ccle/home; Integrated Genomics Viewer (IGV), http://www.broadinstitute.org/igv; COSMIC, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/; cBio Cancer Genomics Portal, http://www.cbioportal.org/; Rembrandt database, http://rembrandt.nci.nih.gov; LOVD Database, http://grenada.lumc.nl/LOVD2/TPI/home.php.

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glioma, and low PARK2 expression is associated with poor survival. Restoration of PARK2 significantly inhibited glioma cell growth both in vitro and in vivo, while depletion of PARK2 promoted cell proliferation. PARK2 attenuated both Wnt- and EGF-stimulated pathways through down-regulating the intracellular level of β-catenin and EGFR. Notably, PARK2 physically interacted with both β-catenin and EGFR. We further found that PARK2 promoted the ubiquitination of these two proteins in an E3 ligase activity dependent manner. Finally, inspired by these newly identified tumor suppressive functions of PARK2, we tested and proved that combination of small-molecule inhibitors targeting both Wnt-β-catenin and EGFR-AKT pathways synergistically impaired glioma cell viability. Together, our findings uncover novel cancer-associated functions of PARK2 and provide a potential therapeutic approach to treat glioma.

Keywords

PARK2; Glioma; Wnt; EGFR

Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive human malignancies with median survival less than 15 months (1). To date, genetic and functional studies have identified important signaling transductions in GBM including receptor tyrosine kinase and PI3K pathways (2, 3). In addition, albeit not genetically altered, Wnt signaling appears to be important for maintenance and chemo-/radio-resistance of glioma cancer initiating cells (4–6). However, further studies are required to characterize more comprehensively the GBM-associated network and translate the findings into effective clinical management of GBM patients.

PARK2 (PARKIN) encodes a well-conserved RBR type E3 ubiquitin ligase. Many studies have revealed a complex regulation of PARK2 activity through multiple intra- and inter-molecular interactions (7–10), implying its critical function in human disease. Indeed, germline mutations of *PARK2* have been identified as a cause of dopaminergic neuron loss in recessive familial early onset Parkinsonism (11). Somatic alterations of *PARK2* are also prevalent among various human malignancies (12). However, the functional consequences of PARK2 inactivation during cancer initiation and progression remain uncertain. In our previous reports, we identified loss of *PARK2* in glioma, and summarized current knowledge of cancer-associated functions of PAKR2 and further discussed the potential involvement of PARK2 inactivation during cancer development (12, 13). Briefly, PARK2-deficient mice are more susceptible to colorectal adenoma (14), hepatocellular carcinoma (15) and γ-irradiation-induced tumorigenesis (16). Restoration of PARK2 expression suppresses the proliferation of PARK2-deficient cancer cells derived from brain, breast, colon, lung and pancreas (14, 17–21). Although PARK2 has been implicated in several cellular processes including cell cycle progression and mitochondrial function, its precise role in tumor cells still remains inconclusive and obscure (12). Thus, the molecular events and signaling underlying the functional relevance of PARK2 in cancer await further characterization.

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In this study, we describe that PARK2 shows frequent mutations, deletions, and down-regulation in multiple types of human cancers, especially in glioma. Low expression level of PARK2 is associated with poor prognosis of glioma patients in multiple cohorts. Further study reveals that PARK2 inhibited the growth of glioma through negatively regulating both Wnt-β-catenin and EGFR-AKT pathways. In addition, inferred from these results, we demonstrated synergism between small-molecule inhibitors targeting both Wnt-β-catenin and EGFR-AKT pathways, therefore providing a new strategy for glioma treatment.

Materials and Methods

Cell culture

HEK293T, HEK293, immortalized murine embryonic fibroblast (MEF, kindly provided by Dr. Yoshiaki Ito, Cancer Science Institute of Singapore), A172 (kindly provided by Dr. Koichi Okumura, Cancer Science Institute of Singapore), U87-MG, U138-MG, U251-MG and U343-MG (hereafter referred to as U87, U138, U251 and U343, respectively), and T98G were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin/streptomycin, at 37 °C, 5% CO_2 in a humidified atmosphere. All the cell lines were authenticated in July 2014 by short tandem repeat analysis with Geneprint 10 system kit (Promega).

Plasmids

Both FLAG-tagged and HA-tagged PARK2 vectors were constructed based on pcDNA3.1 plasmid and were further introduced into both pMSCV-PIG and pBABE-Puro vectors. Point mutations were introduced by site-directed mutagenesis. Lentiviral β-catenin overexpression constructs (including WT, 45 and S33Y mutants) were generated based on the backbone of SHC003 (Sigma) using *Nhe* I and *Fse* I sites from parental plasmids (a gift from Dr. Bert Vogelstein, Johns Hopkins University). EGFP-tagged EGFR vector was subcloned from pCMV6-EGFR-TurboGFP (kindly provided by Dr. Boopathy Gandhi Theerthagiri Kuppusamy, Institute of Molecular and Cell Biology, Singapore) to pEGFP-C2 vector. FLAG-tagged-Ubiquitin was cloned from a human UBB cDNA, and inserted into p3xFLAG-CMV vector using *EcoR* I and *Xba* I sites. Ubiquitin G76V mutant was constructed by site-directed mutagenesis. All the shRNA constructs were made with PLKO. 1 backbone using *Age* I and *EcoR* I sites.

Modulation of gene expression and generation of stable cell lines

Non-targeting control siRNA (siGENOME Non-Targeting siRNA Pool #2) and PARK2-targeting siRNAs were purchased from Thermo Scientific, and were transfected using RNAi MAX (Invitrogen). The sequences of all siRNAs and shRNAs are listed in Supplementary Table 2. For lentiviral particle production, HEK293T cells were co-transfected using jetPRIME (Polyplus-transfection) with shRNA constructs, SHC003-based overexpression constructs and MISSION packaging plasmid mix (Sigma). For retroviral particle production, pMSCV-PIG- or pBABE-Puro-based vectors were co-transfected together with Env and Gagpol plasmids into HEK293T. The culture medium was replaced with fresh medium after 6 h, and supernatants were harvested at 48 h and 72 h post transfection. For generation of

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stable lines, the cells were infected with viral particles in the presence of 8 μg/mL polybrene followed by puromycin selection.

Co-Immunoprecipitation (IP)

Cells were treated either with or without 10 μM MG132 for 6–8 h before lysis. Indicated antibody was added and incubated overnight with each cell lysate at 4°C. Protein A/G PLUS-Agarose beads (Santa Cruz) were added after washing for 3 times with lysis buffer. After 2-hour incubation, beads were washed four times, 5 minutes per wash in IP buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40).

Western blotting

Cells were lysed with M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific) supplemented with 1X protease inhibitor cocktail (Roche), 0.2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). BCA assay (Santa Cruz) was used for protein quantification. Cell lysates or IP elutes were subjected to SDS/PAGE followed by either conventional wet transfer or dry transfer using iBlot® Gel Transfer Device (Invitrogen). Membranes were incubated with antibodies as indicated and exposed to secondary HRP-conjugated antibodies (Millipore).

Quantitative real-time and cDNA microarray analysis

Total RNA was extracted using RNeasy Kit (Qiagen) and processed to cDNA with Superscript III (Invitrogen). Quantitative real-time PCR analysis was performed using Kapa SYBR Fast Master Mix (KAPA Biosystems) on a 7500 Real-time PCR System (Applied Biosystems). The sequences of PCR primers are listed in Supplementary Table 3. GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix) was performed according to manufacturer's instructions and analyzed with Partek Genomics Suite.

Immunohistochemistry

Tissue microarray (TMA) containing gliomas and corresponding normal tissues were prepared and incubated with anti-β-catenin antibody (Cell Signaling Technology). The staining results were evaluated and quantified by two independent pathologists by the percentage of β-catenin-positive cells: negative (score = 0), 1–25% (score = 1), 26–50% (score = 2), 51–75% (score = 3) and more than 76% (score = 4).

The rest of the Materials and Methods are shown in the Supplementary Information.

Results

PARK2 Is Somatically Mutated, Deleted and Under-expressed in Human Cancers

To determine comprehensively the genetic lesions affecting *PARK2*, a large number of datasets were interrogated (see Methods). Generally, *PARK2* mutations were observed across different tumor types (Supplementary Table 1). Several recurrent mutations (Fig. 1a) and those shared by Parkinson's disease and cancer (Fig. 1b) were identified; and of note, most cancer-associated recurrent mutations of *PARK2* occurred in the evolutionally conserved amino acid residues, suggesting their biological relevance. Furthermore, high-

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amplitude *PARK2* deletions (see Methods) were prevalent in primary tumor samples of various tissue origins, especially in GBM and ovarian cancer (Fig. 1e and Supplementary Fig. 1a). Copy number loss of *PARK2* was also observed in 10.3% (100/971) of human cancer cell lines (Fig. 1c).

Interestingly, the frequency of *PARK2* deletions increased from low grade glioma (4.83%) to GBM (19.3%) (Figs. 1d and 1e), indicating that loss of *PARK2* may contribute to the progression of this disease. Analysis of TCGA dataset further supported the down-regulation of PARK2 transcriptional levels in GBMs compared to normal brain tissues (Fig. 1f), which is consistent with our previous results (13). We further screened the protein level of PARK2 in a panel of glioma cell lines and found that three out of 6 cell lines showed undetectable PARK2 (Fig. 1g).

Next, the prognostic value of PARK2 expression in glioma was analyzed. Notably, low mRNA expression of PARK2 predicted poor survival in all gliomas based on NCI Rembrandt database (Supplementary Fig. 1b). Moreover in additional independent datasets, lower PARK2 expression was strongly associated with a worse prognosis in patients with low grade glioma (Supplementary Fig. 1c), high grade glioma (Supplementary Fig. 1e), as well as GBM (Supplementary Fig. 1d), but not ovarian cancer (Supplementary Fig. 1f). Collectively, these findings suggest that PARK2 is targeted by various genomic defects in human cancers, especially glioma. PARK2 loss might contribute to development and progression of this malignancy.

PARK2 Attenuates Glioma Cell Proliferation Both in Vitro and in Vivo

To explore the role of PARK2 in glioma, cell line models with ectopic PARK2 were established and investigated. Exogenous PARK2 expression resulted in significant reductions in cell proliferation in liquid culture and colony formation in soft agar (Figs. 2a and 2b). To evaluate the activity of cancer-associated *PARK2* mutations, three recurrent mutants, namely T173A, T240M, and P294S, were constructed and studied. All three mutants lost their growth suppressive activity in GBM cells (Fig. 2c). Furthermore, silencing of endogenous PARK2 by both siRNAs and shRNA resulted in significantly increased growth of colonies in soft agar (Fig. 2d). To confirm the specificity of the knockdown assay, rescue experiments were performed by taking advantage of the fact that the sh-PARK2-1 targeted the 3′-UTR (Untranslated Region) of PARK2 transcripts, and our ectopic PARK2 vector contained only CDS (Coding DNA Sequence). Importantly, reintroduction of ectopic PARK2 into PARK2-depeleted GBM cells robustly suppressed their growth (Fig. 2e). To assess whether PARK2 affects the tumorigenicity of glioma cells in vivo, U251 cells stably expressing either PARK2 or GFP (Control) were subcutaneously injected into nude mice, and tumor volumes were measured every week. PARK2 overexpression substantially delayed tumor progression and reduced the tumor burden (Fig. 2f). All together, these results strongly suggest that PARK2 is a tumor suppressor in glioma.

PARK2 Negatively Regulates Wnt Pathway

To characterize further the molecular mechanisms, cDNA microarray analysis was performed comparing U251-PARK2 with U251-Control cells. Pathway enrichment analysis

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revealed that both Wnt and ErbB/EGFR signaling pathways were among the top signaling pathways significantly affected by PARK2 overexpression (Supplementary Fig. 2). We then examined how PARK2 was involved in these pathways.

In Wnt-β-catenin pathway, the protein levels of canonical Wnt targets including Cyclin D1, c-Myc and TCF4, were markedly down-regulated upon ectopic expression of PARK2, while $p27^{KIP1}$ [suppressed by active Wnt (22)] was up-regulated (Figs. 3a and 3b). In parallel, mRNA levels of CCND1, TCF4, MYC and LEF1 substantially decreased in U251-PARK2 cells (Fig. 3c). Notably, β-catenin protein level itself was decreased after restoration of PARK2 (Fig. 3a). Knock-down of endogenous PARK2 with pooled siRNAs increased β-catenin expression (Fig. 3d), which was verified independently by two individual siRNAs (Supplementary Fig. 3a). We further asked whether PARK2 affected the intracellular distribution of β-catenin, and cell fractionation assay showed that the nuclear proportion of β-catenin was significantly reduced in PARK2-overexpressing cells (Fig. 3e).

To test directly the effect of PARK2 on Wnt response, glioma cells were stimulated with Wnt3a-conditioned medium, and the mRNA level of conventional Wnt target gene AXIN2 was measured. As expected, dramatic induction of AXIN2 transcription was detected in Control cells, whereas the response was attenuated by expressing PARK2 (Fig. 3f). Very interestingly, activation of Wnt-β-catenin signaling by either Wnt3a treatment or overexpression of active β-catenin (S33Y mutant) elevated the expression of endogenous PARK2 (Figs. 3g and 3h), indicating a possible feedback regulation between PARK2 and Wnt-β-catenin pathway.

PARK2 Physically Interacts with β-Catenin and Promotes Its Turnover

As β-catenin is the key mediator of Wnt signaling, we further investigated how PARK2 regulates β-catenin. First, the half-life of β-catenin decreased upon expression of PARK2 as indicated by the cycloheximide chase assay (Fig. 4a). Next, the major protein degradation machineries controlling β-catenin turnover, namely, autophagy-lysosome pathway and ubiquitin-proteasome pathway, were examined. Depletion of ATG5, the essential component of autophagy, could not restore the β-catenin protein level (Fig. 4b). However, proteasome blockade by MG132 abolished β-catenin down-regulation (Fig. 4c). These results suggest that PARK2 down-regulates β-catenin through the proteasome pathway.

As PARK2 has E3 ligase activity, we hypothesized that PARK2 might directly bind and promote β-catenin degradation through the proteasome pathway. To test this, a series of endogenous and semi-endogenous co-immunoprecipitation (CO-IP) assays were performed which showed that PARK2 physically bound to β-catenin irrespective of tissue origins (Figs. 4d–4f and Supplementary Figs. 3b–d). To extend these findings, CO-IP was performed using immortalized MEF cells and showed that endogenous murine Park2 pulled down β-catenin (Fig. 4g), suggesting that the interaction is conserved from mice to humans. Surprisingly, this binding was independent of the phosphorylation status of canonical β-catenin degron, as Park2 interacted with both total and non-phospho-β-catenin (Fig. 4g). Moreover, both β-catenin S33Y and β-catenin 45Y mutants, which are known to be phosphorylation deficient in the canonical degron, were co-immunoprecipitated with PARK2 antibody (Supplementary Fig. 3c). Interestingly, appreciatable amount of low-

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mobility wild-type or mutant β-catenin were detected by both total and/or non-phospho-β-catenin antibodies (Supplementary Figs. 3b–e), indicating that PARK2 interacts with active β-catenin and may modify it. Having identified and confirmed the physical interaction, we next examined whether PARK2 promotes the ubiquitination of β-catenin. Importantly, exogenous expression of PARK2 significantly increased the ubiquitination level of β-catenin (Fig. 4h), whereas silencing of endogenous PARK2 expression reduced its ubiquitination (Fig. 4i). Ubiquitination assays were next performed with either wild-type PARK2 or its inactive mutants, including loss-of-function mutants T173A, T240M, and P294S (Fig. 2c), as well as a known ligase-dead mutant C431S (10). Notably, compared with wild-type PARK2, all of the mutants showed markedly decreased activity in ubiquitinating β-catenin (Fig. 4j), suggesting that PARK2-dependent ubiquitination of β-catenin requires its ubiquitin ligase activity. Together, these data identify a novel role of PARK2, serving as an E3 ligase of β-catenin and promoting its degradation.

PARK2 Inhibits EGFR-AKT Signaling

Concerning the EGFR-AKT pathway, firstly we found that EGFR itself was down-regulated in glioma cells following PARK2 overexpression (Fig. 5a). Importantly, knock-down of endogenous PARK2 with independent shRNA or siRNAs resulted in the elevation of EGFR proteins in both glioma and HEK293 cells, indicating that the regulation of EGFR by PARK2 is not tissue specific (Figs. 5b, e, f, Supplementary Fig. 4a). Next, the down-stream signaling pathway of EGFR, in particular, AKT signaling was examined. PARK2 suppressed the phosphorylation of AKT at Ser473, as well as S6K at both Thr421 and Ser424 (Fig. 5a), demonstrating the decrease of AKT activity and its downstream signaling. To extend these findings, we examined the effect of PARK2 on acute epidermal growth factor (EGF) stimulation. PARK2 overexpression significantly mitigated the molecular events induced by EGF, as evidenced by reduced levels of phospho-EGFR, phospho-ERK1/2, phospho-AKT and phospho-GSK3β (Figs. 5c and 5d). In contrast, PARK2 depletion augmented these cellular responses in both glioma and HEK293 cells (Figs. 5e–f). In further support of these results, in silico analysis showed that both total EGFR protein and phospho-EGFR levels were negatively correlated with PARK2 mRNA expression in GBM primary samples (Fig. 6a). Together, these results suggest that PARK2 suppresses EGFR-AKT signaling.

The molecular mechanisms by which PARK2 regulates the expression of EGFR were further explored. CO-IP assays showed that PARK2 interacted with EGFR (Fig. 6b). Moreover, exogenous expression of wild-type PARK2, but not PARK2 loss-of-function mutants, promoted the ubiquitination of EGFR (Figs. 6c–d). Together, these results suggest that PARK2 possibly controls the protein level of EGFR through ubiquitination modification. In addition, depletion of PARK2 significantly up-regulated the amount of EGFR mRNA (Supplementary Fig. 4b), indicating that PARK2 also modulates the expression of EGFR through transcriptional regulation. However, as PARK2 is not a well-established transcriptional factor, this transcriptional regulation is possibly indirect and needs further characterization.

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PARK2 Inhibits Glioma Cell Growth through Regulating Both Wnt-β-Catenin and EGFR-AKT Signaling

To examine the involvement of Wnt-β-Catenin and EGFR-AKT pathways in glioma, we first examined the cell viability by depleting key mediators of these pathways, including β-catenin, AKT1 and EGFR, and found that knock-down of any one of them resulted in marked growth retardation in glioma cells (Supplementary Fig. 4c). Immunohistochemistry analysis found that β-catenin levels were significantly elevated in glioma samples and correlated positively with tumor grade (Fig. 6e). Moreover, high β-catenin transcriptional levels were associated with poor disease-free survival (Supplementary Fig. 4d) and overall survival (Supplementary Fig. 4e) in glioma patients. EGFR amplification and high expression also predicted poor survival in these cohorts of patients (Supplementary Figs. 4f–g). These data collectively support that Wnt-β-Catenin and EGFR-AKT pathways are two crucial drivers in this disease.

To explore further the biological consequences of dual suppression of both Wnt-β-catenin and EGFR-AKT pathways by PARK2, rescue assays were performed. β-catenin 45 mutant [constitutively active form (23, 24)] fully restored the cell growth in the presence of ectopic PARK2, while wild-type β-catenin either fully or partially rescued this phenotype (Figs. 6f and 6g). In parallel, culture medium supplemented with EGF partially restored the proliferation of PARK2-expressing GBM cells but showed no effect on control cells (Fig. 6h). Taken together, these data suggest that PARK2 regulates glioma cell growth through both Wnt-β-catenin and EGFR-AKT pathways.

Therapeutic Merit by Dual Targeting Wnt-β-catenin and EGFR-AKT Pathways in Glioma

Based on the above results, we tested targeting Wnt-β-catenin and/or EGFR-AKT pathways by small-molecule drugs in glioma cells. The anti-glioma activities of two Wnt-β-catenin pathway inhibitors (ICG001 and PKF-118-310) and a pan-AKT inhibitor (MK2206), were examined in six glioma cell lines. Similar to our results obtained by shRNA-mediated knockdown, these inhibitors potently suppressed glioma growth (Fig. 7a), and their on-target effects were confirmed (Supplementary Fig. 4h). As PARK2 showed robust anti-proliferative function through dual suppression of both Wnt-β-catenin and EGFR-AKT pathways, we hypothesized that combinational targeting of both pathways by small-molecule inhibitors might be synergistic. ICG001 and MK2206 were chosen as candidate molecules for combinational study, since they are well-tolerated as shown by multiple studies and/or clinical trials (25, 26) (see URLs). First, we determined the optimal combination ratio as 1:1 by checkerboard assay (see Methods). With this ratio, combinational application of ICG001 and MK2206 markedly reduced each IC_{50} and dramatically suppressed glioma cell proliferation at very low concentrations (Figs. 7a and 7b, Supplementary Fig. 5). The markedly enhanced inhibitory effect was also evident by measuring downstream molecules, such as phospho-S6K (Fig. 7c). Very interestingly, expression of EGFR and β-catenin was mutually dependent. Depletion of either protein resulted in the decrease of the other (Figs. 7d and 7e). Overexpression of both wild-type and constitutive active mutant (S33Y) β-catenin elevated the EGFR expression (Fig. 7f). Analysis of TCGA reverse phase protein array data further indicated a strong correlation between the expression of β-catenin and EGFR (Figs. 7g–7j). Collectively, these results

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provide a novel potential strategy for glioma treatment by dual targeting Wnt-β-catenin and EGFR-AKT signaling pathways (Fig. 7k).

Discussion

In this study, we report the frequent genomic deletions and mutations, expression down-regulation, prognostic value, and biological relevance of PARK2 in glioma. With extensive molecular studies, we uncovered the glioma-suppressive role of PARK2 through negatively regulating both Wnt-β-catenin and EGFR-AKT pathways.

Genome instability is one of the hallmarks of cancer, and somatic mutation and copy number loss are commonly observed in genes encoding tumor suppressors. Based on our pan-cancer genomic analysis of *PARK2* mutations, 15% of mutations lead to detrimental frameshift, mis-splicing or pre-mature truncation of PARK2 protein. In addition, several recurrent mutations were identified across different functionally important domains, and among which we functionally validated three that resulted in loss-of-function (T173A, T240M and P294S). Similar to our results, Veeriah *et al.* showed that cancer-derived R42C, N254S, R275Q, and E344G mutants lost their ability to suppress tumor cell growth (27), suggesting that somatic mutations compromise PARK2 function during tumorigenesis. Copy number analysis of 269 low grade glioma samples and 497 GBM samples showed frequent high-amplitude *PARK2* deletion. Together with the analysis from TCGA cDNA microarray (Fig. 1f), we further confirmed the down-regulation of PARK2 expression in gliomas. In an attempt to examine the protein expression of PARK2 in primary glioma tissues, two different PARK2 antibodies were tested; but unfortunately, no specific signals were observed by IHC staining. Alternatively, we confirmed the absence of PARK2 protein in half of the examined glioma cell lines by western blot analysis (Fig. 1g). With a similar approach, J Viotti *et al.* recently reported that the protein level of PARK2 was inversely correlated with glioma grade (28), which is in line with our observations that PARK2 expression is lost during glioma progression. For the first time, we report the prognostic value of PARK2 expression in both low grade and high grade gliomas (including GBM), again highlighting the biological relevance of PARK2 in this malignancy.

Alterations in Wnt-β-catenin signaling have been implicated in gliomagenesis (6, 29–33). Here, we noted that total β-catenin levels in primary glioma samples correlated both with the grade of tumor and the survival probability, and that β-catenin is required for glioma cell proliferation. Murine Park2 has been shown to reduce the steady state level of β-catenin in murine cell lines and protect murine dopaminergic neurons from excessive Wnt signaling (34). In the current study, a conserved physical interaction was shown between PARK2 and β-catenin across different species, and PARK2 was identified further as a novel E3 ligase promoting β-catenin ubiquitination and turnover through proteasome pathway. Of note, similar to SIAH1, another E3 ligase for β-catenin, PARK2-mediated β-catenin degradation is independent of the conventional β-catenin degron (35, 36). PARK2 strongly suppressed the expression of canonical Wnt targets as well as the response to Wnt stimulation. Constitutive Wnt activation through overexpression of either wild-type or mutant β-catenin abolished PARK2-induced growth inhibition, supporting the concept that PARK2 functions as an upstream modulator of Wnt-β-catenin pathway. Very interestingly, Wnt-β-catenin activation

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elevated PARK2 expression which in turn constrained both β-catenin protein levels and downstream signaling, suggesting a potential feedback regulation.

EGFR activation through mutation, amplification, alternative splicing and genomic rearrangement occurs in gliomas. We found that PARK2 enhanced EGFR ubiquitination, and that PARK2 potently suppressed both EGFR expression and its downstream signaling. In support of our data, Yeo *et al.* showed that ectopic expression of PARK2 in U87 cells inhibited AKT phosphorylation (19). However, Lara Fallon *et al.* found that PARK2 interacted with EGFR and promoted its endocytosis, thereby promoting the EGFR-AKT signaling (37), which is discordant with our discoveries. Together, these results suggest that the regulation between PARK2 and EGFR-AKT is intricate and context-dependent.

Very interestingly, we found that expression of EGFR and β-catenin was positively correlated with each other (Figs. 7d–7j). In agreement with our findings, stimulation or activation of EGFR were reported to promote β-catenin transactivation (38, 39); and on the other hand, EGFR was shown to be a direct Wnt-β-catenin target gene in several other cancers (40–42). Collectively, as PARK2 inhibits both EGFR and β-catenin, its glioma-suppressive function is likely to be further amplified through the crosstalk between EGFR and β-catenin.

Effective targeted therapies are in urgent need for clinical interventions of glioma patients. EGFR was considered as an attractive target in glioma; however, use of several EGFR inhibitors was hindered due to either unresponsiveness, high toxicity or acquired resistance (43–47). As AKT is the key downstream molecule mediating the action of activated EGFR, targeting AKT instead of EGFR itself may have additional benefits. Considering that PARK2 potently suppressed gliomas through inhibiting both Wnt-β-catenin and EGFR-AKT pathways, we combined ICG001 and MK2206, and showed a remarkable synergistic anti-glioma effect. Mechanistically, the synergisms may result from the aforementioned crosstalk between Wnt-β-catenin and EGFR-AKT pathways; and indeed, β-catenin was recently reported to confer resistance to AKT inhibition in colon cancers (48). Given that both MK2206 and ICG001 derivative have entered phase I/II clinical trials, our preclinical studies may offer translational benefit for treating glioma patients.

In summary, we show that somatic inactivations and underexpression of PARK2 contribute to gliomagenesis, and that PARK2 expression is a novel biomarker for glioma prognosis. By detailed mechanistic studies, we unmask PARK2 as an important modulator of both Wnt-β-catenin and EGFR-AKT signalings. Together with the results that dual inhibition of Wnt-β-catenin and EGFR-AKT pathways synergistically killed glioma cells, our findings elucidate a comprehensive network involving PARK2/Wnt-β-catenin/EGFR-AKT signaling, and provide a candidate therapeutic approach for this deadly disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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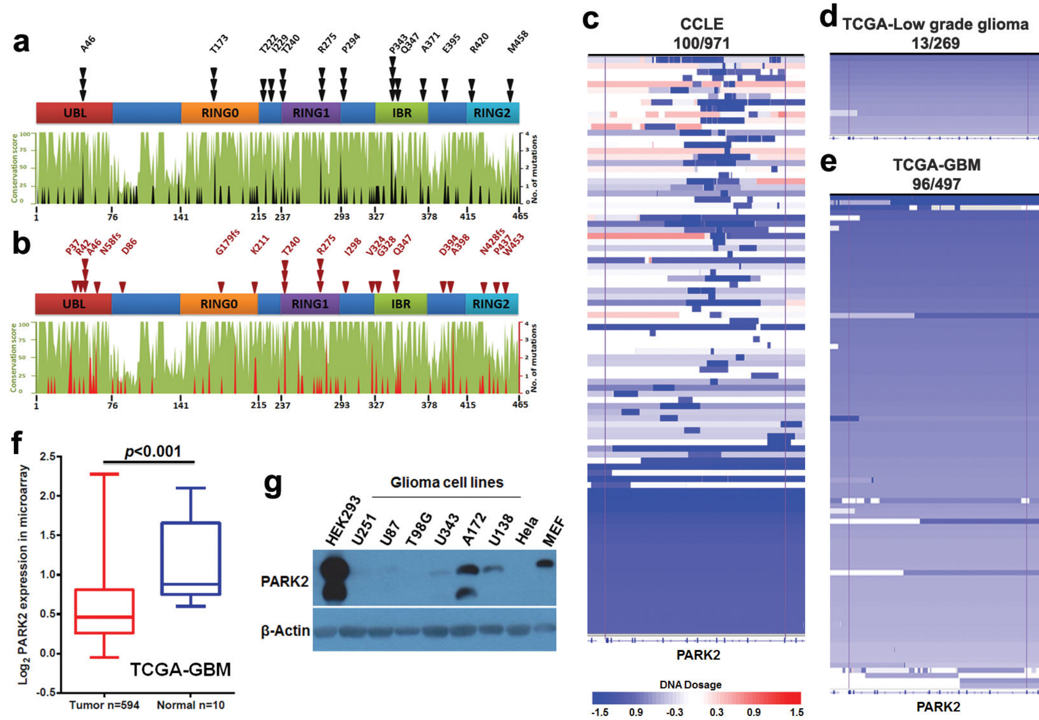

Figure 1. *PARK2* is frequently altered in cancers, and low PARK2 expression associates with poor prognosis in glioma

(a) Pan-cancer analysis of somatic *PARK2* mutations. Recurrent mutations are highlighted in the upper panel. Frequency of somatic mutations (black) and conservation score of each amino acid (green) are indicated in the lower panel. (b) Cancer-derived *PARK2* mutations which have been reported in PD are highlighted in the upper panel. Frequency of all PD-derived mutations (red) and conservation score of each amino acid (green) are indicated in the lower panel. (c) IGV plots showing high-amplitude *PARK2* deletion (see Methods) occurred in 100 out of 971 cancer cell lines (CCLE), (d) 13 out of 269 low grade glioma samples, and (e) 96 out of 497 GBM samples (TCGA). (f) Down-regulation of PARK2 transcription in GBM samples compared to normal brain tissues (TCGA). (g) PARK2 protein expression in glioma cell lines measured with western blot analysis.

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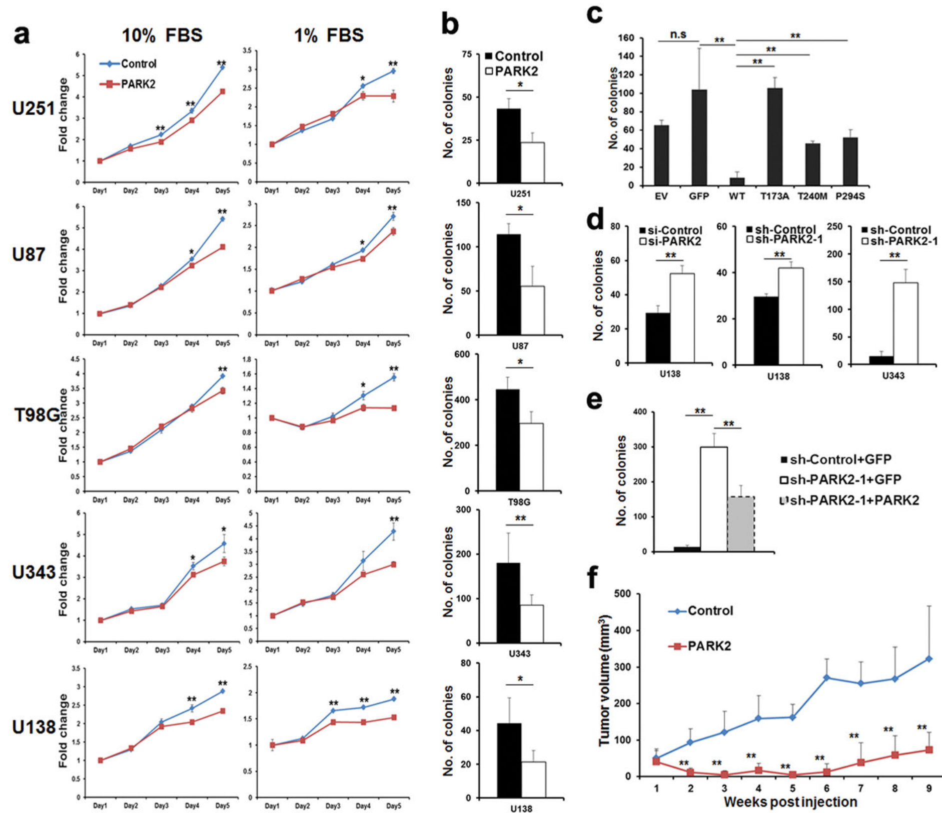

Figure 2. PARK2 suppresses glioma cell growth

(a) Short-term proliferation assay of glioma cell lines stably expressing either ectopic wild-type PARK2 or GFP (Control) under either 10% FBS or 1% FBS conditions. (b) Soft agar colony formation assay of glioma cell lines with either ectopic wild-type PARK2 or GFP (Control) protein expression. (c) Soft agar colony formation assay of U87 cells which stably expressed either wild-type PARK2 or its cancer-derived mutants. (d) Endogenous PARK2 in glioma cells was depleted by either siRNA or shRNA and then examined by soft agar colony formation assay. (e) U343 cells were transduced with lentiviral particles encoding shRNAs against either PARK2 (sh-PARK2-1) or control (sh-Control), and then exogenous PARK2 was introduced by retroviral particles. Subsequently, cells were subjected to soft agar colony formation assay. (f) U251 cells stably expressing either GFP (Control) or PARK2 were injected subcutaneously on the upper flanks of nude mice. Tumor volumes were measured every week. Data represent Mean ± SD. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$.

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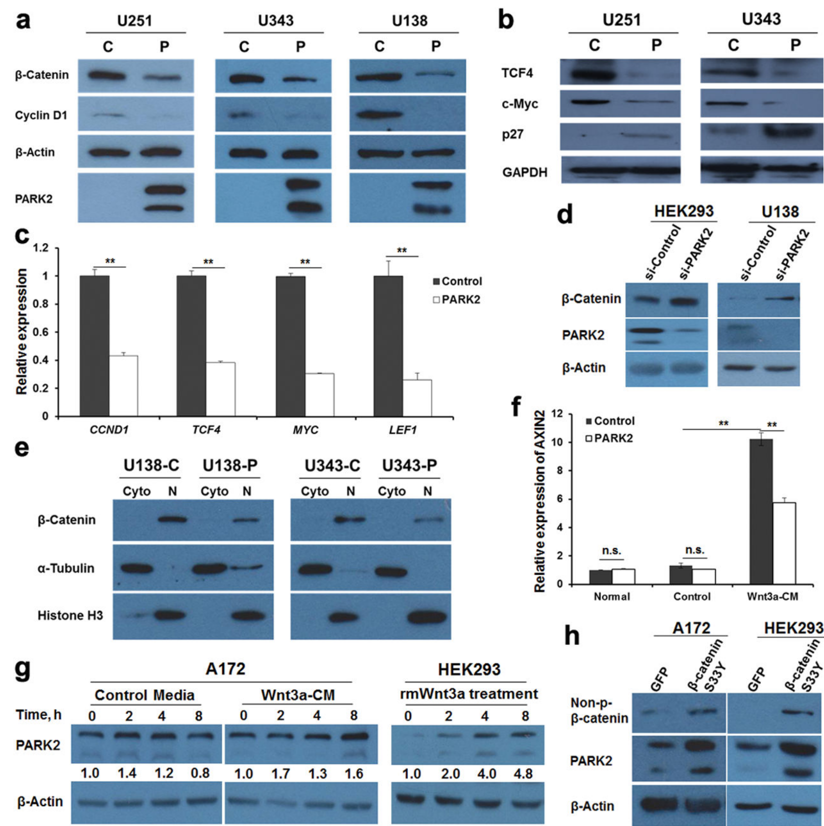

Figure 3. PARK2 inhibits Wnt signaling

(a, b) Western blot and (c) qRT-PCR analysis of gene expressions regulated by Wnt signaling in glioma cells stably expressing either GFP (C) or PARK2 (P). (d) Depletion of endogenous PARK2 with pooled siRNAs elevated the expression of β-catenin. (e) Cytosolic (Cyto) and nuclear (N) fractions of U138 and U343 cells stably expressing either GFP (C) or PARK2 (P) were subjected to western blot analysis, and probed with β-catenin antibody. α-Tubulin and Histone H3 were used as loading controls for cytosolic and nuclear proteins, respectively. (f) U251 cells stably expressing either GFP (Control) or PARK2 were treated with complete growth medium (Normal), L-cell control medium (Control) or Wnt3a-conditioned medium (Wnt3a-CM) for 4 hours. Subsequently, cells were harvested for qRT-PCR analysis of AXIN2 expression. (g) Western blot analysis of endogenous PARK2 expression in A172 (left panel) and HEK293 (right panel) after Wnt3a treatment. A172 cells were treated with either L-cell control medium (Control Medium) or Wnt3a-conditioned medium (Wnt3a-CM) for indicated time courses. HEK293 cells were stimulated with 10 ng/mL recombinant murine Wnt3a (rmWnt3a) for indicated durations. The level of PARK2 was quantified by ImageJ® and normalized to β-actin. (h) A172 and HEK293 cells stably expressing either GFP or β-catenin S33Y mutant were generated and subjected to western blot analysis using indicated antibodies. Data of (c, f) represent Mean ± SD. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$.

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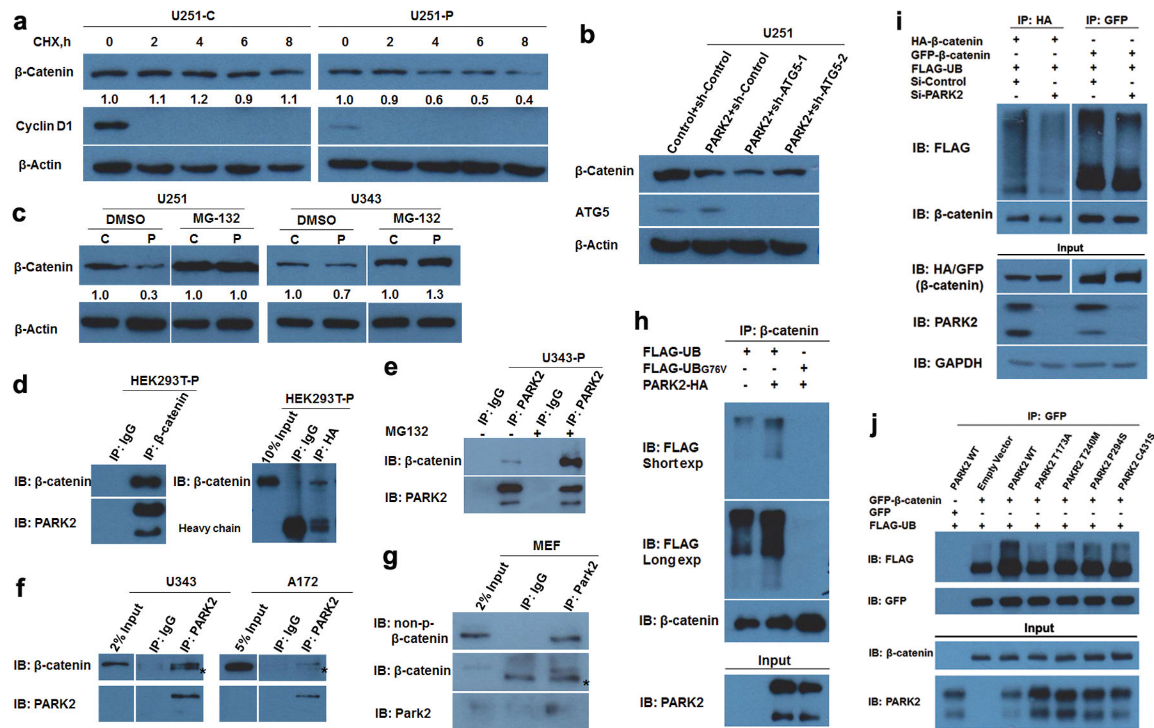

Figure 4. PARK2 interacts with β-catenin and promotes its ubiquitination

(a) Cycloheximide chase assay to measure β-catenin half-life in U251-C (expressing Control GFP) and U251-P (expressing ectopic PARK2) cells. Cyclin D1 protein was used as a positive control. The level of β-catenin was quantified by ImageJ® and normalized to β-actin. (b) U251 cells stably expressing either GFP (Control) or PARK2 proteins were transduced with lentiviral particles encoding shRNAs against either ATG5 or Non-targeting. Protein lysates were subjected to western blot analysis using indicated antibodies. (c) Western blot analysis of β-catenin levels in glioma cells stably expressing either GFP (C) or PARK2 (P) proteins in the presence of either DMSO or MG132 (24 hours). (d) In HEK293T cells stably expressing HA-tagged PARK2 (HEK293T-P), antibodies against β-catenin (left panel) and HA (right panel) were used to perform CO-IP. (e) Exogenously expressed PARK2 interacted with endogenous β-catenin in U343 cells (U343-P). (f) CO-IP with endogenous PARK2 and β-catenin in glioma cells and (g) in mouse embryonic fibroblast cells (MEF). * indicates a non-specific band. (h) PARK2 enhanced the ubiquitination of β-catenin. HEK293T cells were transfected with indicated vectors for 24 hours, and subjected to IP analysis with β-catenin antibody. Ubiquitin^{G76V} mutant (deficient in conjugation) was used as a negative control. (i) HEK293 cells were transfected with indicated siRNAs for 48 hours, and followed by co-transfection of indicated vectors for 24 hours. After MG132 treatment, cells were subjected to IP by either HA (lanes 1, 2) or GFP (lanes 3, 4) antibodies. (j) HEK293T cells were transfected with indicated vectors for 24 hours, and subjected to IP by GFP antibody. In (h, i, j), MG132 was added 4 hours before IP.

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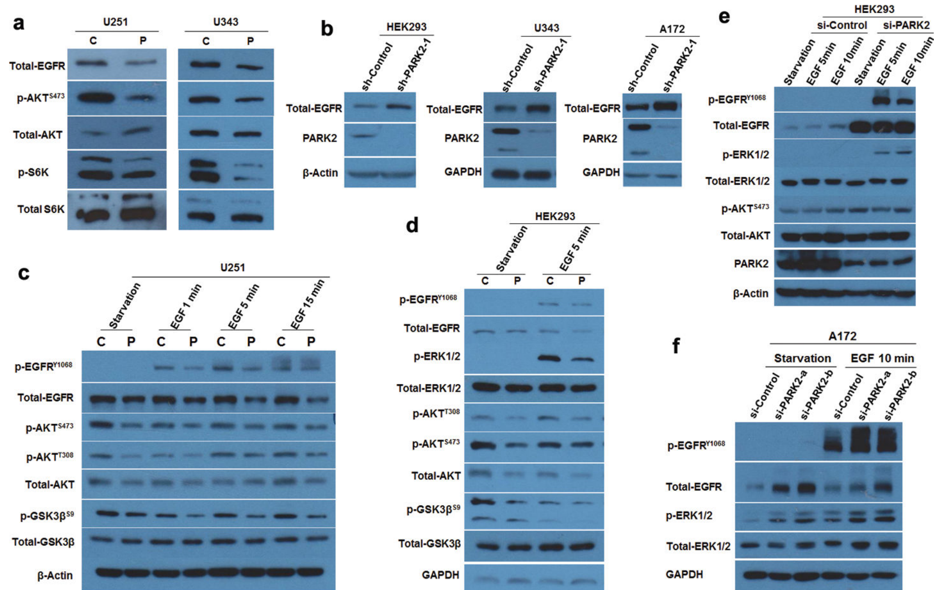

Figure 5. PARK2 suppresses EGFR-AKT pathway

(a) Western blot analysis of EGFR and its downstream targets in glioma cells stably expressing either GFP (C) or PARK2 (P) proteins. (b) Western blot analysis of EGFR expression in indicated cells transduced with lentiviral particles encoding shRNAs against either PARK2 (sh-PARK2-1) or Non-targeting control (sh-Control). (c–f) Western blot analysis of EGFR and its downstream targets in either serum-starved or EGF-stimulated conditions in indicated cells. All cells were serum-starved for at least 24 hours and subsequently stimulated with 10 ng/mL EGF for indicated durations.

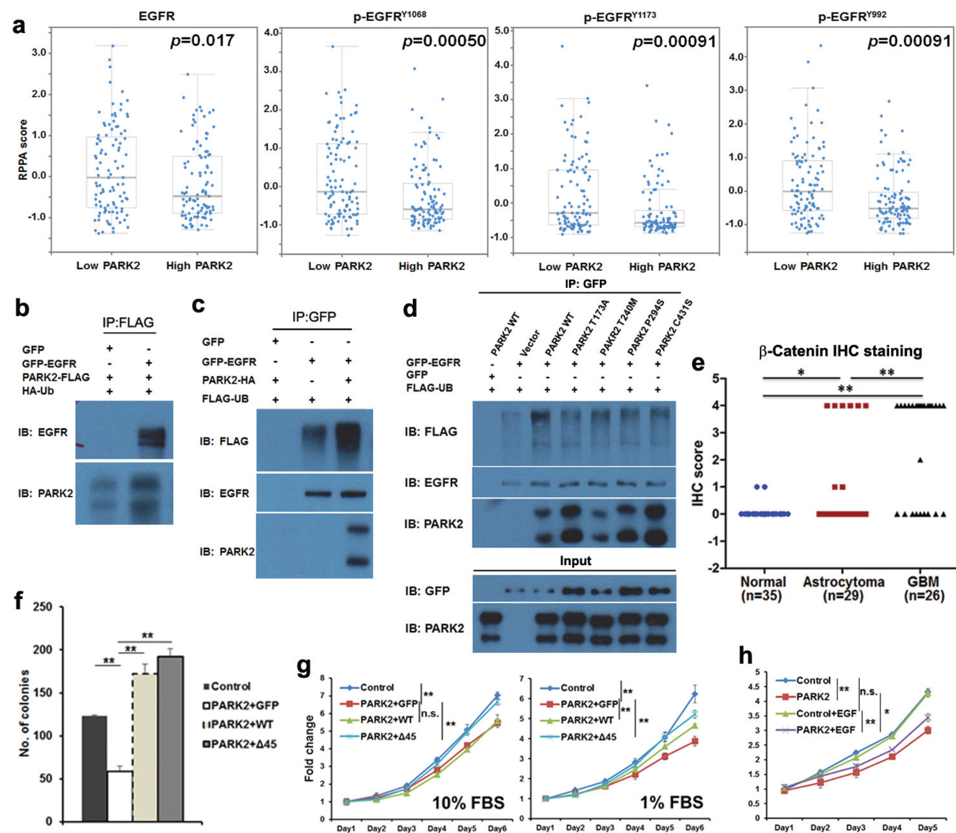

Figure 6. PARK2 inhibits glioma cell growth through Wnt-β-catenin and EGFR-AKT pathways

(a) Boxplots of reverse phase protein array (RPPA) data showing the positive correlation between the mRNA levels of PARK2 and protein levels of EGFR/p-EGFR. GBM patients (TCGA, n = 542) without pretreatment were divided into PARK2 high (mRNA/miRNA expression Z-score Mean + 0.35SD) and PARK2 low (mRNA/miRNA expression Z-score < Mean + 0.35SD) group. (b, c) CO-IP with exogenous PARK2 and EGFR in HEK293T cells. (d) HEK293T cells were transfected with either wild-type or mutant PARK2 for 24 hours, and subjected to IP by GFP antibody. In (b–d), MG132 was added 4 hours before IP. (e) Immunohistochemistry assay of β-catenin expression in either normal brain tissues or glioma samples. One-way ANOVA was employed for statistical analysis. (f, g) Stable U87 cells expressing either ectopic PARK2 or GFP (Control) were infected with lentiviral particles encoding either GFP, β-catenin (WT), or mutant β-catenin (45) and then subjected to (f) soft agar colony formation assay and (g) short-term proliferation assay. (h) Short-term proliferation assay of U251 cells stably expressing either ectopic PARK2 or GFP (Control) proteins under either normal or conditioned medium supplemented with 200 ng/mL recombinant EGF. Data of (e–h) represent Mean ± SD. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$.

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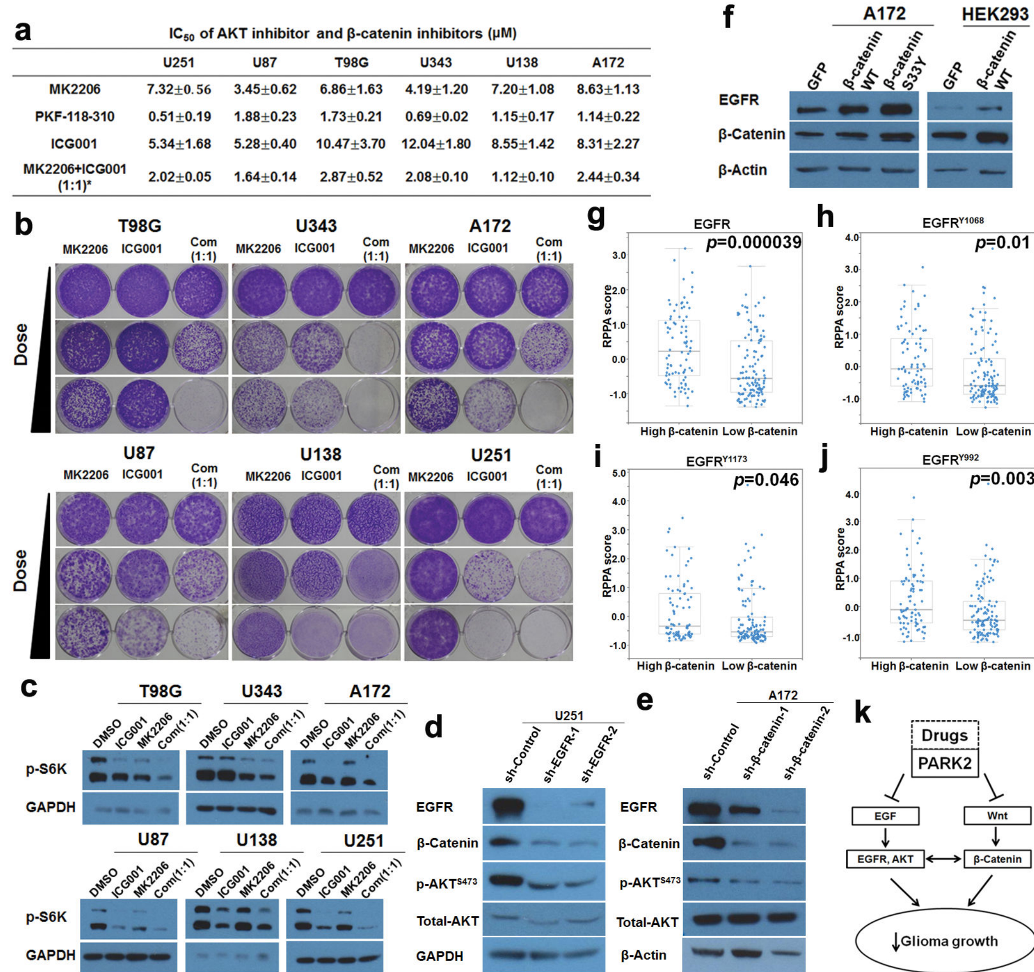

	U251	U87	T98G	U343	U138	A172
MK2206	7.32±0.56	3.45±0.62	6.86±1.63	4.19±1.20	7.20±1.08	8.63±1.13
PKF-118-310	0.51±0.19	1.88±0.23	1.73±0.21	0.69±0.02	1.15±0.17	1.14±0.22
ICG001	5.34±1.68	5.28±0.40	10.47±3.70	12.04±1.80	8.55±1.42	8.31±2.27
MK2206+ICG001 (1:1)*	2.02±0.05	1.64±0.14	2.87±0.52	2.08±0.10	1.12±0.10	2.44±0.34

Figure 7. Dual targeting of Wnt-β-catenin and EGFR-AKT pathways in glioma

(a) IC_{50} of AKT inhibitor (MK2206), β-catenin inhibitors (PKF-118-310 and ICG001) and combined drugs (MK2206 + ICG001) in glioma cell lines. For each IC_{50}, 3–5 independent biological replicates with 4 technical controls were performed. Values represent Mean ± SEM. * indicates the molecular ratio between MK2206 and ICG001. (b) Long-term proliferation assay of glioma cells under different drug treatments. (c) Cells were treated with indicated inhibitors (1 μM) for 24 hours and then harvested for western blot analysis. (d, e) Western blot analysis of glioma cells transduced with lentiviral particles encoding either Non-targeting shRNA (sh-Control) or shRNAs against EGFR (d) or β-catenin (e). (f) Western blot analysis of A172 glioma cells and HEK293 cells stably expressing GFP, wild-type (WT) or mutant (S33Y) β-catenin. (g–j) Boxplots of RPPA data showing the positive correlation between the protein levels of β-catenin and EGFR/p-EGFR. GBM patients (TCGA, n = 542) without pretreatment were divided into β-catenin high (RPPA Z-score Mean + 0.25SD) and β-catenin low (RPPA Z-score < Mean + 0.25SD) group. (k) A model of PARK2 functions in glioma and proposed therapeutic strategy targeting both Wnt-β-catenin and EGFR-AKT pathways.

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