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 glioma, and low PARK2 expression is associated with poor survival. Restoration of PARK2 significantly inhibited glioma cell growth both in vitro and in vivo, whereas depletion of PARK2 promoted cell proliferation. PARK2 attenuated both Wnt- and EGF-stimulated pathways through downregulating the intracellular level of β-catenin and 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-/radioresistance 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 intermolecular 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 PARK2 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.

In this study, we describe that PARK2 shows frequent mutations, deletions, and downregulation in multiple types of human cancers, especially in glioma. Low-expression level of
Materials and Methods

**Cell culture**

HEK293T, HEK293, immortalized mouse embryonic fibroblast (MEF, kindly provided by Dr. Yoshiaki Ito, Cancer Science Institute of Singapore, Singapore), A172 (kindly provided by Dr. Koichi Okumura, Cancer Science Institute of Singapore, 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 DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin, at 37°C, 5% CO₂ in a humidified atmosphere. All the cell lines were authenticated in July 2014, by short tandem repeat analysis with the Geneprint 10 System Kit (Promega).

**Plasmids**

Both FLAG-tagged and HA-tagged PARK2 vectors were constructed on the basis of pcDNA3.1 plasmid and were further introduced into both pMSCV-PIC and pBABE-Puro vectors. Point mutations were introduced by site-directed mutagenesis. Lentiviral β-catenin overexpression constructs (including WT, ΔA5, and S33Y mutants) were generated on the basis of 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. Bopopathy 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 Xho 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**

Nontargeting control siRNA (siGENOME Nontargeting siRNA Pool #2) and PARK2-targeting siRNAs were purchased from Thermo Fisher Scientific, and were transfected using RNAi MAX (Invitrogen). The sequences of all siRNAs and shRNAs are listed in Supplementary Table S2. For lentiviral particle production, HEK293T cells were cotransfected using jetPRIME (Polyplus transfection) with shRNA constructs. SHC003-based overexpression constructs and MISSION packaging plasmid mix (Sigma). For retroviral particle production, pMSCV-PIC- or pBABE-Puro–based vectors were cotransfected 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 and 72 hours after transfection. For generation of stable lines, the cells were infected with viral particles in the presence of 8 μg/mL polybrene followed by puromycin selection.

**Coimmunoprecipitation**

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

**Western blotting**

Cells were lysed with M-Per Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with 1X protease inhibitor cocktail (Roche), 0.2 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride. BCA assay (Santa Cruz Biotechnology) was used for protein quantification. Cell lysates or immunoprecipitation eluates 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 horseradish peroxidase–conjugated antibodies (Millipore).

**Quantitative real-time and cDNA microarray analysis**

Total RNA was extracted using the 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 S3. GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) was performed according to the manufacturer’s instructions and analyzed with Partek Genomics Suite.

**Immunohistochemistry**

Tissue microarrays 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% to 25% (score = 1), 26% to 50% (score = 2), 51% to 75% (score = 3), and more than 76% (score = 4).

**Tumor formation assay in nude mice**

Three million of U251-control or U251-PARK2 cells were mixed with 80 μL of Matrigel solution (BD Biosciences) per injection, and the mixture was injected s.c. on the upper flanks of nude mice. Tumor volume was measured every week for 8 weeks. Mice experiments were approved by the Institutional Animal Care and Use Committee, Cedars-Sinai Medical Center.

**Bioinformatics and data analysis**

Copy-number data of human cancer cell lines were extracted from the Cancer Cell Line Encyclopedia database (http://www.broadinstitute.org/ccle/home). Copy-number data of low-grade glioma as well as GBM were collected from The Cancer Genome Atlas (TCGA: http://cancergenome.nih.gov/) via cBio Cancer Genomics Portal (http://www.cbioportal.org/) and analyzed with IGV software (http://www.broadinstitute.org/igv). High-amplitude deletion (gene dosage value < −1) in cell lines was defined on the basis of the assumptions that loss of both alleles occurred in more than 75% of total cells. High-amplitude
Results

PARK2 is somatically mutated, deleted, and underexpressed in human cancers

To determine comprehensively the genetic lesions affecting PARK2, a large number of datasets were interrogated (see Materials and Methods). Generally, PARK2 mutations were observed across different tumor types (Supplementary Table S1). Several recurrent mutations (Fig. 1A) and those shared by Parkinson 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 biologic relevance. Furthermore, high-amplitude PARK2 deletions (see Materials and Methods) were prevalent in primary tumor samples of various tissue origins, especially in GBM and ovarian cancer (Fig. 1E and Supplementary Fig. S1A). 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%; Fig. 1D and E), indicating that loss of PARK2 may contribute to the progression
of this disease. Analysis of TCGA dataset further supported the downregulation of PARK2 transcriptional levels in GBMs compared with 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 of six 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. S1B). Moreover, in additional independent datasets, lower PARK2 expression was strongly associated with a worse prognosis in patients with low-grade glioma (Supplementary Fig. S1C), high-grade glioma (Supplementary Fig. S1E), as well as GBM (Supplementary Fig. S1D), but not ovarian cancer (Supplementary Fig. S1F). 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 (Fig. 2A and B). 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'-untranslated region of PARK2 transcripts, and our ectopic PARK2 vector contained only coding DNA sequence. Importantly, reintroduction of ectopic PARK2 into PARK2-depleted GBM cells robustly expressing 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 s.c. 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 the Wnt pathway**

To characterize further the molecular mechanisms, cDNA microarray analysis was performed comparing U251-PARK2 with U251-control cells. Pathway enrichment analysis revealed that both Wnt and ErbB–EGFR signaling were among the top signaling pathways, significantly affected by PARK2 overexpression (Supplementary Fig. S2). We then examined how PARK2 was involved in these pathways.

In the Wnt–β-catenin pathway, the protein levels of canonical Wnt targets, including Cyclin D1, c-Myc, and TCF4, were markedly downregulated upon ectopic expression of PARK2, whereas p27kip1 (suppressed by active Wnt; ref. 29) was upregulated (Fig. 3A and B). In parallel, mRNA levels of CCND1, TCF4, cMyc, and LEF1 substantially decreased in U251-PARK2 cells (Fig. 3C). Notably, β-catenin protein level itself was decreased after restoration of PARK2 (Fig. 3A). Knockdown of endogenous PARK2 with pooled siRNAs increased β-catenin expression (Fig. 3D), which was verified independently by two individual siRNAs (Supplementary Fig. S3A). 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 the 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 (Fig. 3G and H), indicating a possible feedback regulation between PARK2 and Wnt–β-catenin pathways.

**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, the autophagy–lysosome pathway and the 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 downregulation (Fig. 4C). These results suggest that PARK2 downregulates 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 semiependymal coimmunoprecipitation (co-IP) assays were performed, which showed that PARK2 is physically bound to β-catenin irrespective of tissue origins (Fig. 4D–F and Supplementary Fig. S3B–S3D). To extend these findings, co-IP was performed using immortalized MEF cells and showed that endogenous murine PARK2 pulldown-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 degreon, as PARK2 interacted with both total and non–phospho-β-catenin (Fig. 4G). Moreover, both β-catenin S33Y and β-catenin Δ45Y mutants, which are known to be phosphorylation deficient in the canonical degreon, were coimmunoprecipitated with PARK2 antibody (Supplementary Fig. S3C). Interestingly, appreciable amount of low-mobility wild-type or mutant β-catenin were detected by both total and/or non–phospho-β-catenin antibodies (Supplementary Fig. S3B–S3E), 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, first, we found that EGFR itself was downregulated in glioma cells following PARK2 overexpression (Fig. 5A). Importantly, knockdown 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 (Fig. 5B, E, and F, Supplementary Fig. S4A). Next, the downstream 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 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 (Fig. 5C and D). In contrast, PARK2 depletion augmented these cellular responses in both glioma and HEK293 cells (Fig. 5E and 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 (Fig. 6C and D). Together, these results suggest that PARK2 possibly controls the protein level of EGFR through ubiquitination modification. In addition, depletion of PARK2 significantly upregulated the amount of EGFR mRNA (Supplementary Fig. S4B), 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.

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 depletion key mediators of these pathways, including β-catenin, AKT1, and EGFR, and found that knockdown of any one of them resulted in marked growth retardation in glioma cells (Supplementary Fig. S4C). 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. S4D) and overall survival (Supplementary Fig. S4E) in glioma patients. EGFR amplification and high expression also predicted poor survival in these cohorts of patients (Supplementary Fig. S4F and S4G). These data collectively support that Wnt–β-Catenin and EGFR–AKT pathways are two crucial drivers in this disease.

To explore further the biologic consequences of dual suppression of both Wnt–β-catenin and EGFR–AKT pathways by PARK2, rescue assays were performed. β-Catenin ΔΔ5 mutant (constitutively active form; refs. 30, 31) fully restored the cell growth in the presence of ectopic PARK2, whereas wild-type β-catenin either fully or partially rescued this phenotype (Fig. 6F and G). 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

On the basis of the above results, we tested targeting Wnt–β-catenin and/or EGFR–AKT pathways by small-molecule drugs in glioma cells. The antiglioma 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 (Supplementary Fig. S4C), these inhibitors potently suppressed glioma growth (Fig. 7A), and their on-target effects were confirmed (Supplementary Fig. S4H). As PARK2 showed robust antiproliferative 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, because they are well tolerated as shown by multiple studies and/or clinical trials (http://clinicaltrials.gov; refs. 32, 33). First, we determined the optimal combination ratio as 1:1 by checkerboard assay (see Materials and Methods). With this ratio, combinational application of ICG001 and MK2206 markedly reduced each IC50 and dramatically suppressed glioma cell proliferation at very low concentrations (Fig. 7A and B and Supplementary Fig. S5). 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 (Fig. 7D and E). 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 (RPPA) data further indicated a strong correlation between the expression of β-catenin and EGFR (Fig. 7G–J). Collectively, these results provide a novel potential strategy for glioma treatment by dual targeting Wnt–β-catenin and EGFR–AKT signaling pathways.

**Discussion**

In this study, we report the frequent genomic deletions and mutations, expression downregulation, prognostic value, and biologic 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. On the basis of our pan-cancer genomic analysis of PARK2 mutations, 15% of mutations lead to detrimental frameshift, mis-splicing or premature 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 and colleagues (20) showed that cancer-derived R42C, N254S, R275Q, and E344G mutants lost their ability to suppress tumor cell growth, 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 downregulation 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, Viotti and colleagues (34) recently reported that the protein level of PARK2 was inversely correlated with glioma grade, 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 biologic relevance of PARK2 in this malignancy.

Alterations in Wnt–β-catenin signaling have been implicated in gliomagenesis (6, 35–39). 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 (40). In this 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 the 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 (41, 42). 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 the Wnt–β-catenin pathway. Very interestingly, Wnt–β-catenin activation elevated PARK2

Figure 7.
Dual targeting of Wnt–β-catenin and EGFR–AKT pathways in glioma. A, the IC_{50} value 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} value, three to five independent biologic replicates with four technical controls were performed. Values represent mean ± SEM, * 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 μmol/L) for 24 hours and then harvested for Western blot analysis. D and E, Western blot analysis of glioma cells transduced with lentiviral particles encoding either nontargeting 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 + 0.25 SD) and β-catenin low (RPPA Z-score < mean + 0.25 SD) groups. K, a model of PARK2 functions in glioma and proposed therapeutic strategy targeting both Wnt-β-catenin and EGFR-AKT pathways.
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 and colleagues (19) showed that ectopic expression of PARK2 in U87 cells inhibited AKT phosphorylation. However, Fallon and colleagues (43) found that ectopic expression of PARK2 in U87 cells inhibited AKT signaling, 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 (Fig. 7D–I). In agreement with our findings, stimulation or activation of EGFR were reported to promote β-catenin transactivation (44, 45); and on the other hand, EGFR was shown to be a direct Wnt–β-catenin target gene in several other cancers (46–48). 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 because of either unresponsiveness, high toxicity, or acquired resistance (49–53). 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 antianglioma 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 (54). 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 under-expression 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 signaling. 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.

Disclosure of Potential Conflicts of Interest

M. Kahn has ownership interest (including patents) in Prion Pharma. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.-C. Lin, L. Xu, D. Yin
Development of methodology: L. Xu, Y. Chen, J.W. Said, D. Yin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Yan, J.W. Said, L.-W. Ding, S. Yu, D. Yin
Writing, review, and/or revision of the manuscript: D.-C. Lin, L. Xu, Y. Chen, J.W. Said, M. Kahn, D. Yin, H.P. Koehler
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Xu, Y. Chen, M. Kahn, D. Yin
Study supervision: H.P. Koehler

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