Parkin Pathway Activation Mitigates Glioma Cell Proliferation and Predicts Patient Survival


Abstract

Mutations in the parkin gene, which encodes a ubiquitin ligase, are a major genetic cause of parkinsonism. Interestingly, parkin also plays a role in cancer as a putative tumor suppressor, and the gene is frequently targeted by deletion and inactivation in human malignant tumors. Here, we investigated a potential tumor suppressor role for parkin in gliomas. We found that parkin expression was dramatically reduced in glioma cells. Restoration of parkin expression promoted G1 phase cell-cycle arrest and mitigated the proliferation rate of glioma cells in vitro and in vivo. Notably, parkin-expressing glioma cells showed a reduction in levels of cyclin D1, but not cyclin E, and a selective downregulation of Akt serine-473 phosphorylation and VEGF receptor levels. In accordance, cells derived from a parkin-null mouse model exhibited increased levels of cyclin D1, VEGF receptor, and Akt phosphorylation, and divided significantly faster when compared with wild-type cells, with suppression of these changes following parkin reintroduction. Clinically, analysis of parkin pathway activation was predictive for the survival outcome of patients with glioma. Taken together, our study provides mechanistic insight into the tumor suppressor function of parkin in brain tumors and suggests that measurement of parkin pathway activation may be used clinically as a prognostic tool in patients with brain tumor. Cancer Res; 72(10); 2543-53. ©2012 AACR.

Introduction

Parkinson disease and cancer are 2 important but obviously disparate human disorders. Yet, many of the Parkinson disease–linked genes identified to date, including α-synuclein, parkin, DJ-1, and PINK1, are also associated with cancer (1). In particular, DJ-1 was originally isolated as a putative oncogene (2). The overlapping of genes involved in Parkinson disease and cancer would imply a shared pathogenic pathway. However, the mechanism underlying the opposite cellular fates of the 2 diseases remains unclear, the elucidation of which could help conceive novel therapeutic approaches to both groups of disorders.

Mutations in the parkin gene, which encodes a ubiquitin ligase, were originally identified as a genetic contributor of autosomal recessive parkinsonism (3). Accordingly, much of the interest in characterizing the function of the parkin gene has been directed toward understanding its role in neurodegeneration. However, in recent years, the role of parkin in cancer has also gained much attention. It is now clear that parkin gene alterations are not restricted to familial forms of Parkinson disease but also occur frequently in a wide variety of malignancies (4–7), which include glioblastoma (GBM) and lung cancer, where somatic loss-of-function parkin mutations were found (8). Unlike the oncogenic DJ-1, parkin appears to act as a tumor suppressor. Supporting this, we and others have shown that restoration of parkin expression in a wide spectrum of parkin-deficient cancer cells results in a marked decrease in their proliferation rate (8, 9). Furthermore, at least one line of parkin-null mice exhibits a tendency to develop carcinoma (10).

Notably, parkin functions as a ubiquitin ligase (E3) associated with the ubiquitin-proteasome system, and one of its several putative substrates identified to date is cyclin E (11), a cell-cycle–related (G1) cyclin, whose accumulation is associated with cancer development. It is therefore attractive to suggest that altered cyclin E proteolysis in parkin-deficient cancer and neuronal cells may underlie their respective susceptibility to undergo cell division and degeneration. Indeed, cyclin E accumulation is associated with parkin deficiency in several cancer cell lines as well as in cultured postmitotic neurons (8, 11, 12), although this phenomenon is not universally observed (9, 13). Interestingly, parkin also exhibits an

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apparent E3-independent function in the control of gene transcription. Among the genes regulated by parkin is TPS3, a well-established tumor suppressor, whose expression is repressed by functional parkin (14). This is rather surprising, as the elevation of p53 levels in parkin-deficient cells should in theory retard (instead of promote) cell division. Two other genes whose expression is also regulated by parkin are follistatin (10) and cyclin-dependent kinase (CDK)6 (9). Whereas the level of follistatin increases in parkin-deficient cells, the reverse is true for CDK6 (9, 10). Both events are, however, thought to promote carcinogenesis (9, 10). Here, we provide evidence that parkin dysfunction is relevant to gliomagenesis and that restoration of functional parkin expression in glioma cells mitigates their growth via a mechanism that likely involves parkin-mediated downregulation of the Akt signaling pathway. Importantly, we further found that parkin pathway activation is predictive of survival prognosis of patients with glioma.

Materials and Methods

Antibodies and reagents

The following antibodies were used: monoclonal anti-parkin clone PRK8 (Covance), anti-phospho-Akt (S473 and T308), anti-Akt, anti-cyclin D1 and E, anti-VEGFR2, anti-phospho-PDK1, anti-PDK1, anti-mTOR, anti-Rictor, anti-Sin1, anti-phosphoFoxO3a, anti-FoxO3a, anti-phosphoGSK3β, anti-GSK3β (all from Cell Signaling), anti-Flag peroxidase (Sigma), monoclonal anti-β-actin (Sigma), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). All the cell lines used in this study were purchased from American Type Culture Collection. Primary mouse embryonic fibroblasts (MEF) from wild-type and parkin-null mice (exon 7; kind gifts from Dr. T.M. Dawson, Johns Hopkins Medicine, Baltimore, MD) were generated according to published protocol (15).

Analysis of parkin expression in glioma cells

Total RNA was isolated from various cancer cells described with the RNeasy Mini Kit (Qiagen). Subsequently, the isolated RNA was reverse transcribed with the Superscript First-Strand Synthesis System (Invitrogen). Real-time PCR (RT-PCR) was carried out in a LightCycler (Roche) with the FastStart DNA Synthesis System (Invitrogen). Real-time PCR (RT-PCR) was conducted as previously described (9).

Generation of stable cell lines, proliferation assays, and cell-cycle analysis

U87MG cells stably expressing wild-type or mutant parkin, or otherwise containing vector alone were generated by means of a previously described procedure (9). All positive cell lines used for the experiments described hereafter were maintained in serum-containing Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 200 μg/mL Geneticin (Invitrogen) to prevent extrusion of integrated constructs. A simple population growth assay was conducted by seeding cells to be counted in duplicates at a concentration of 2 × 10^3 cells in 6-well plates and subsequently quantifying their number each day for a period of 5 days by means of a hemocytometer. Bromodeoxyuridine (BrdUrd)-based proliferation assay (Roche) was carried out according to the manufacturer’s instructions. For soft agar colony formation, 0.3% agar overlaid on precast 0.5% bottom agar and allowed to solidify before incubating at 37°C in a 5% CO₂ incubator for a period of 21 days. Colonies formed on the soft agar were visualized under light microscopy before and after MTT staining. For cell-cycle analysis, cells were seeded at a concentration of 5 × 10^4 cells in 10 mL of culture medium in 10-cm tissue culture plates and incubated at 37°C in a 5% CO₂ incubator. After 24 hours, the culture medium was aspirated and the cells were rinsed 3 times with serum-free culture medium replaced with 10 mL of serum-free culture medium for 24 hours at 37°C in a 5% CO₂ incubator to synchronize them at G₀ resting phase. Cells were stained with the BrdU/7-AAD Flow Kit (Pharmingen) and then resuspended in 1 mL of PBS containing 5 mmol/L of EDTA before analysis by fluorescence-activated cell sorting (FACS) at 20,000 events with quadrant plot analysis on a flow cytometer (FACScalibur; Becton, Dickinson and Company).

In vivo NOD-SCID/J intracranial tumor model

All procedures involving animals were approved by and conformed to the guidelines of our Institutional Animal Care and Use Committee. Six-week-old nonobese diabetic-severe combined immunodeficient (NOD-SCID)/J mice were stereotactically implanted with U87-vector or U87-Parkin stable cells was overlaid on precast 0.5% bottom agar and allowed to solidify before incubating at 37°C in a 5% CO₂ incubator for a period of 21 days. Colonies formed on the soft agar were visualized under light microscopy before and after MTT staining. For cell-cycle analysis, cells were seeded at a concentration of 5 × 10^4 cells in 10 mL of culture medium in 10-cm tissue culture plates and incubated at 37°C in a 5% CO₂ incubator. After 24 hours, the culture medium was aspirated and the cells were rinsed 3 times with serum-free culture medium replaced with 10 mL of serum-free culture medium for 24 hours at 37°C in a 5% CO₂ incubator to synchronize them at G₀ resting phase. Cells were stained with the BrdU/7-AAD Flow Kit (Pharmingen) and then resuspended in 1 mL of PBS containing 5 mmol/L of EDTA before analysis by fluorescence-activated cell sorting (FACS) at 20,000 events with quadrant plot analysis on a flow cytometer (FACScalibur; Becton, Dickinson and Company).

Results

Parkin deficiency is a characteristic of glioma cells and restoration of its expression mitigates glioma growth both in vitro and in vivo

In view of the association between parkin gene alteration and cancer, we were interested to examine whether the expression of parkin is compromised in glioma cells. For this purpose, we analyzed the levels of parkin transcripts quantitatively by means of RT-PCR in a spectrum of glioma cells that included U87MG, U373MG, U251MG, and T98G. As controls, we also examined parkin expression in HEK293 cells, a non-tumor-derived cell line, and MCF7, a parkin-deficient breast cancer cell line (4, 9). Our analysis revealed an apparent reduction in the level of parkin mRNA in all the glioma cell lines examined, which decreases dramatically by an average of 16-fold, relative to HEK293 cells (Supplementary Fig. S1A). Notably, this reduced level of parkin mRNA expression is...
comparable with that in MCF7 cells (Supplementary Fig. S1A) and correlates well with the amount of parkin protein in extracts derived from the various glioma cell lines examined (Supplementary Fig. S1B). To examine the effects of restoring parkin expression in parkin-deficient glioma cells, we generated clonal populations of U87MG glioma cells stably expressing either FLAG-tagged parkin (U87-Parkin) or containing vector alone as a control (U87-vector). We have chosen U87MG cells for this purpose because it is a popular cell model for glioma studies and is also one that is highly deficient in parkin expression (Supplementary Fig. S1A). All the parkin-positive clones express parkin at a significantly higher level than vector control or parental cells (Fig. 1A) but are otherwise similar morphologically (not shown). We then measured the proliferation rate of U87-Parkin and U87-vector by means of a variety of assays. A simple population growth assay revealed that the proliferation rate of parkin-expressing U87MG cells is significantly reduced compared with control cells (Fig. 1A), suggesting that ectopic parkin expression in these cells mitigates their growth. Supporting this, the incorporation of BrdUrd, a thymidine analogue, in parkin-expressing U87MG cells also occurs significantly less frequently compared with control cells (Fig. 1C). Similar observation was made with the MTT-based proliferation assay (not shown). Because cancer cells are anchorage-independent and have the ability to form colonies in soft agar, we also examined whether ectopic parkin expression in
U87MG cells compromise their ability to generate colonies in soft agar. We found that the number of soft agar colonies formed by parkin-expressing U87MG cells is dramatically reduced and the size of these colonies also tends to be smaller than those generated by control U87MG cells (Fig. 1D, not shown for U87-Parkin #19 and #25). Thus, elevated expression of parkin in U87MG cells, apparently, could downregulate their rate of proliferation. This effect of parkin is however dependent on its catalytic activity, as the stable expression of a catalytically null parkin mutant (i.e., T415N) in U87MG cells failed to exert any negative influence on their proliferation rate (Fig. 1B).

Alongside our in vitro assays, we also examined the effects of parkin overexpression on the ability of U87MG cells to generate solid tumor in vivo. An intracranial glioma model (n = 5 for each group), which involved the injection of parkin-expressing (clone #7) or control U87MG cells into the cerebral cortex of NOD-SCID mice, was used for this purpose. At 4 weeks after injection, we observed the appearance of macroscopic tumors in 4 of 5 dissected brains of mice injected with control U87MG cells, but none in those injected with U87-Parkin cells (Fig. 1E) or vehicle alone (not shown). Furthermore, histologic examination of brain sections derived from these mice revealed that NOD-SCID mice injected with U87-vector control cells via the intracranial route developed visibly larger tumors in the brain at the end of the experimental period than those injected with U87-Parkin cells (Fig. 1E). Consistent with this, the average weight of brains harvested from all 5 mice injected with parkin-expressing U87MG cells is significantly reduced compared with those harvested from vector control mice (Fig. 1F). Taken together, our results strongly support a negative role for parkin in glioma cell proliferation.

As an extension of our in vivo study, we repeated the above experiment but allowed injected mice to come from different groups (i.e., U87-vector, U87-Parkin #7 and #19; n = 10 for each group) to live beyond 4 weeks to examine their respective survival profile. We found that whereas NOD-SCID mice harboring glioma generated from U87-vector control cells readily succumb to the tumor, those injected with U87-Parkin #7 exhibit significantly better survival (Supplementary Fig. S2A). Curiously, the U87-parkin #19 group showed a mortality curve that is between vector and U87-Parkin #7 groups, but with a profile tending toward and not significantly different from the vector group (Supplementary Fig. S2A). At postmortem analysis, we found that tumor derived from U87-Parkin #19 had lost much of its parkin expression over time, which likely accounted for its survival profile, whereas those derived from U87-Parkin #7 exhibit robust parkin expression even after prolonged periods in vivo (Supplementary Fig. S2B). Thus, parkin expression appears to correlate inversely with cancer mortality.

Parkin-expressing glioma cells exhibit delayed entry into mitosis and reduced cyclin D1 levels

Conceivably, the reduced proliferation rate of parkin-expressing U87MG cells may be due to alterations of the cell-cycle program. To investigate this, we analyzed the cell-cycle profile of control U87-vector and U87-Parkin cells via flow cytometry. In asynchronous cells, we observed a tendency for parkin-expressing U87MG cells to accumulate at the G1 phase relative to control cells (Supplementary Fig. S3A), suggesting that parkin expression may delay the entry of cells into the mitotic phase. In agreement with this, we recorded a corresponding reduction in the percentage of parkin-expressing cells at the S- and G2–M phases (Supplementary Fig. S3A). This trend is similarly observed in synchronized U87-Parkin cells following serum release but not in those that express parkin T415N mutant (which tend to show a reverse trend; Fig. 2A). We next examined whether enhanced cyclin E proteolysis may account for the cell-cycle profile in U87-Parkin cells. Surprisingly, we did not detect an apparent difference in the steady-state level of cyclin E between parkin-expressing and control U87MG cells (Fig. 2B). Instead, we found that the expression of cyclin D1 (which, like cyclin E, is also involved in G1–S transition) is dramatically repressed in parkin-expressing U87MG cells (Fig. 2B). In contrast, cyclin D1 levels remain largely unaltered in U87MG cells stably expressing the T415N mutant (Fig. 2C). Together, our results suggest that defective cyclin D1 expression, rather than cyclin E, possibly underlies the ability of parkin to influence the cell-cycle program of glioma cells in this case.

Akt Ser-473 phosphorylation and VEGFR-2 expression are significantly reduced in parkin-expressing glioma cells

Notably, a significant percentage of high-grade gliomas harbor mutations in PTEN, a tumor suppressor, whose loss of function is known to promote abnormal cellular growth via overactivation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (17). Consistent with this, Pt-en-deficient U87MG, U373MG, and U251MG cells exhibit elevated levels of phosphorylated Akt compared with other PTEN-expressing glioma cells (not shown). To investigate whether exogenous parkin expression in U87MG cells exerts any effect on Akt signaling, we immunoblotted lysates prepared from U87-Parkin and U87-vector cells with phosphorylation-specific Akt antibodies and found that the levels of Akt phosphorylated at Ser-473 is significantly reduced in parkin-expressing U87MG cells compared with control cells (Fig. 3A). This phenomenon is not due to changes in expression in the presence of parkin (Fig. 3A and B) and is not observed when we repeated the experiment with U87MG cells stably expressing parkin T415N mutant (Fig. 3A), suggesting that the catalytic activity of parkin is required for its effect on Akt Ser-473 phosphorylation. In contrast, phosphorylation of Akt in U87MG cells at another site, Thr-308, appears to be unaffected by parkin overexpression (Fig. 3B). Consistent with this, the levels of activated phosphoinositide-dependent kinase (PDK) 1, an upstream effector of Akt Thr-308 phosphorylation, are not appreciably altered in wild-type or mutant parkin–expressing U87MG cells (Fig. 3C and Supplementary Fig. S3B). Instead, the levels of TORC2 components (thought to be responsible for Akt Ser-473 phosphorylation) including mTOR, Rictor, and Sin1 as well as their associated substrates, such as FoxO3a and GSK3β, all appear to be reduced in U87-Parkin cells (Fig. 3D), suggesting that parkin-mediated downregulation of Akt Ser-473 phosphorylation may be due to its ability (directly or indirectly) to influence the expression of TORC2 complex. This reduction of...
To extend these findings, we also looked at the extent of Akt Ser-473 phosphorylation in the presence or absence of growth factor stimulation. Under serum-starved condition, the basal level of Ser-473 phosphorylated Akt in parkin-expressing U87MG cells is similarly reduced compared with control cells (Fig. 3E). Upon EGF stimulation, a marked increase in Akt phosphorylation is observed in both control and parkin-expressing U87MG cells, although the steady-state levels of Ser-473 phosphorylated Akt in U87-Parkin cells remain low relative to U87-vector control cells (Fig. 3D). Collectively, these results suggest that parkin-mediated suppression of glioma cell growth is in part contributed by its ability to regulate growth factor–dependent or –independent PI3K/Akt signaling pathway via the downregulation of Akt Ser-473 phosphorylation. In further support of this, we found that overexpression of Akt in U87-Parkin stable cells can overcome the suppressive effects of parkin to result in a significantly enhanced proliferation rate (Fig. 4A).

As cancer development is a multistep process involving a wide spectrum of factors, we were also interested to examine the global gene expression changes in glioma cells triggered by exogenously introduced parkin. We subjected parkin-expressing and vector control U87MG cells to microarray analyses using Affymetrix chips. Notably, the expression of cyclin D1 transcript was reduced by about 2-fold in the presence of parkin overexpression (not shown). On the basis of the changes in gene expression observed between control and parkin-expressing U87MG cells (Supplementary Fig. S4), we were able to derive a parkin gene signature (see section below). Among these, one gene that caught our attention is VEGF receptor (VEGFR)-2 (also known as KDR), whose expression is reduced by nearly 4-fold in U87-Parkin compared with vector control (Supplementary Fig. S4). Aberrant VEGFR-2 expression is associated with a wide variety of cancers, including gliomas. Consistent with our microarray analyses result, anti-VEGFR-2 immunoblotting revealed a significant reduction in parkin-expressing U87MG cells but not in those that expresses the T415N mutant, as compared with control cells (Fig. 4B). Thus, VEGFR-2, along with cyclin D1 and phospho-Akt (S473), all of which are known to enhance cellular proliferation, exhibits significantly reduced expression in glioma cells expressing exogenous parkin.

Figure 2. Parkin overexpression in U87MG cells reduces the level of cyclin D1 and delays entry into mitotic phase. A, bar graph showing the percentage of U87-vector and U87 wild-type parkin-expressing cells (left) or U87-vector and U87-mutant parkin T415N cells (TN5, 7, and 8; right) at different stages of the cell cycle, as measured by flow cytometry. *, P < 0.05, Student t test. B, immunoblots showing the levels of cyclin E and cyclin D1 in lysates prepared from native U87MG cells (U87), U87-vector (vector), and FLAG-parkin–expressing stable clones (PK7, 19, and 25). The anti-cyclin D1 immunoblots from 3 independent experiments were used to derive the relative densitometric units of cyclin D1 (normalized to respective actin level), which is presented as a histogram on the right. *, P < 0.05, Student t test. C, as in B except PK7, 19, and 25 are substituted by TN5, 7, and 8.
Parkin-null fibroblasts exhibit enhanced proliferation rate that is accompanied by increased levels of cyclin D1, phospho-Akt, and VEGFR-2

To further support the role of parkin as a suppressor of cellular proliferation, we analyzed the growth characteristics of MEFs prepared from wild-type and parkin-null mice that we have reported previously (18). As expected, parkin-null fibroblasts exhibit significantly enhanced rate of growth over time (Fig. 5A). Moreover, parkin−/- MEFs also show elevated levels of cyclin D1, phospho-Akt (S473), and VEGFR-2 (Fig. 5B). These observations were duplicated in MEFs derived from a separate line of parkin-null mice (generated by the means of a targeted deletion of parkin exon 3; ref. 10; Supplementary Fig. S3C).

Importantly, restoration of parkin expression in parkin−/- MEFs results in a significant reduction in the expression of cyclin D1, phospho-Akt (S473), and VEGFR-2, which is accompanied by a substantially slower growth rate (Fig. 5C and D). Taken together, our results support an inverse association between parkin function and cellular proliferation and identify cyclin D1, phospho-Akt (S473), and VEGFR-2 as key common denominators of this functional relationship between parkin and cellular growth that is relevant to gliomagenesis.

Parkin gene signature predicts survival outcome of human glioma patients

Given the tumor-suppressing function of parkin and its apparent ability to reduce mortality in the glioma mouse model, we sought to determine whether parkin mutations or deletions are present in clinical specimens. The PARK2 gene is located within a known chromosomal fragile site (FRA6E) and consequently, rearrangements of PARK2 are expected to be frequent in cancer cells (19–21). Furthermore, Clark and colleagues mate-paired sequenced the genome of the U87MG cell line and found an intrachromosomal translocation within the PARK2 gene (22). This, combined with the loss of expression we observed in several glioma cell lines, suggests a rearrangement leading to loss of PARK2. Veeriah and colleagues also found somatic mutations of PARK2 in 7 of 75 GBM samples and copy number loss in 53 of 216 samples (8). To substantiate our hypothesis, we explored one of the largest public glioma databases, REMBRANDT (23). We showed not only approximately 12% of samples with incomplete copy number loss (<1.7) but also a ‘mixed profile’ with one group of copy number identifiers located in the PARK2 gene showing slight gain, whereas another
group showed slight loss (Supplementary Fig. S5A). This is suggestive of a widespread translocation of \textit{PARK2}. Importantly, the segmentation analysis (24) found a significant deletion across the transcription start site of \textit{PARK2} (Supplementary Fig. S5B). Collectively, these data provide evidence for the presence of \textit{PARK2} mutations/deletions in the model systems presented.

Recent literature implicates that gene expression drives glioma disease progression (25–27). As the presence of \textit{PARK2} rearrangements does not definitively indicate whether parkin-activated signaling occurs, we verify that \textit{PARK2} gene expression correlates with glioma disease progression; derive a differentially regulated gene signature from \textit{in vitro} parkin-overexpressing and vector-only U87MG cells, our initial model; and interrogate the strength of gene signature association with individual patient gene expression profiles in REMBRANDT (23) and "Freije" (28), two well-known, public glioma databases. We show that \textit{PARK2} expression portends favorable prognosis and correlates inversely with tumor grade (Supplementary Fig. S6). As parkin pathway activation would be better represented by a collective set of genes rather than a single gene, we adapted the Connectivity Map, a rank-based pattern-matching approach that was recently successfully applied to determine the degree of oncogenic pathway activation in gastric cancer (29, 30). Patients with strong resemblance to the parkin gene signature (Supplementary Fig. S4 and Table S1; 50 genes, parkin-overexpressing vs. vector U87MG cells) would thus be expected to fare better. Indeed, the list of differentially regulated genes (Fig. 6A, right, REMBRANDT and B, right, Freije; Supplementary Table S2; false discovery rate \( \log_2 \) fold change \( \geq 1.2 \) consistently stratified patient survival in both databases (Fig. 6A and B, left); furthermore, a multivariate analysis indicated that the gene signature prognosticated survival better than current clinical indicators, age and histology (Table 1). This suggests that parkin pathway activation contributes to the molecular heterogeneity of gliomas. Patients exhibiting strong concordance with the parkin signature (parkin\(^+\)) correlated with lower tumor grade (Fig. 6A and B, middle; Supplementary Table S3). In addition, using an independent molecular classifier developed by Phillips and colleagues (26), we observed parkin\(^-\) patients falling mainly into the proneural subtype, which typifies patients with better prognosis. Conversely, parkin\(^-\) patients correlated with higher tumor grade (GBM) and the mesenchymal subtype, which typifies highly aggressive and recurrent gliomas. Interestingly, the mesenchymal subtype mainly correlated with \textit{PTEN} loss and \textit{EGFR} amplification with activated Akt signaling, thus providing clinical evidence and consistency with our \textit{in vitro} U87MG-based model. We also observed elevated \textit{AKT1}, \textit{AKT2}, and \textit{KDR} expression in parkin\(^-\) patient gene expression profiles, similar to our \textit{in vitro} findings (Supplementary Fig. S7A, REMBRANDT and S7B, Freije).
To conclusively map our gene expression–based parkin activation pathway as a consequence of varying PARK2 genomic levels, we used the copy number information in REMBRANDT. We found that both PARK2 gene expression levels (Supplementary Fig. S8A) and PARK2 activation signature (Supplementary Fig. S8B) are lower in this PARK2 DNA–lost group than in the group with diploid PARK2. Taken together, we provide strong evidence that parkin function correlates inversely with glioma mortality and that its pathway activation is predictive of survival outcome.

Discussion

The main finding of our current study is that loss of parkin function enhances cyclin D1 expression and Akt-related growth-promoting signaling and concomitantly promotes glioma cell proliferation. Importantly, our study also identified a signature pathway of parkin that is predictive of the survival outcome of patients with glioma and is therefore of potential prognostic value.

It is becoming increasingly clear that parkin dysfunction not only plays a role in neurodegeneration but also underlies the development of several types of cancers (4). Notably, Cesari and colleagues have originally shown that the chromosome 6q–located, 1.4-Mb parkin gene is frequently targeted by hemizygous deletion and inactivation in both malignant tumors and tumor-derived cell lines (4). Following this discovery, several other groups including ours have reported parkin gene alterations and expression variability in a wide variety of tumor types (4–7, 9), including GBMs (8). Collectively, these studies strongly implicate a role for parkin as a tumor suppressor. Furthermore, supporting this, we showed here that parkin level is dramatically reduced in several glioma cell lines and that restoration of functional (but not a catalytically null mutant) parkin expression in otherwise parkin-deficient glioma cells mitigates their growth both in vitro and in vivo. Our study also revealed that parkin negatively influences the cell-cycle program of U87MG cells but through the downregulation of cyclin D1 instead of cyclin E expression. We do not know whether this is a cell-specific phenomenon, although it is noteworthy to point out that the suggested role of parkin in cyclin E degradation is currently controversial (9, 31).

Precisely, how functional parkin regulates the expression of cyclin D1 is unclear to us, although its apparent ability to

Figure 5. Expression of cyclin D1, phospho-AKT, and VEGFR-2 are upregulated in parkin-null fibroblasts. A, graph showing the growth rate of primary embryonic fibroblasts derived from wild-type (parkin+/+) or parkin-null mice (parkin−/−). B, immunoblots showing the levels of cyclin D1 (top), phospho-Akt (S473; middle), and VEGFR2 (bottom) in parkin+/+ and −/− MEFs. These experiments were repeated at least 3 times and the relative densitometric units of cyclin D1/actin, phospho-AKT/AKT, and VEGFR2/actin in parkin+/+ and parkin−/− MEFs are presented as histogram alongside their respective immunoblots. C and D, as in A and B, respectively, except that data for parkin+/+ MEFs is replaced by parkin-null MEFs ectopically expressing FLAG-parkin. (Inset in both graphs shows anti-parkin immunoblot). *, P < 0.05; **, P < 0.001, Student t test.
Figure 6. Parkin gene signature is predictive of survival outcome of patients with glioma. The 50-gene signature stratified patient survival in 2 public glioma databases: REMBRANDT (A) and Freije (left; B). Survival data are plotted as Kaplan–Meier curves. +, concordance of parkin overexpression gene signature with patients’ gene expression and consequently better survival; −, inverse relation of parkin overexpression with clinical database gene expression and consequently poorer survival. Activation scores denote ranking of gene signature likeness to clinical database gene expression, and patient survival is linked to this ranking (middle). A positive score represents patients with similarity to parkin overexpression, whereas a negative score denotes patients with inverse relation, that is, similarity to vector-only cells. The tumor grade and molecular classification scheme were scored for individual patients and illustrated below the activation score graphs. Heatmaps illustrate the expression patterns of genes in individual patients (right). The corresponding parkin signature probe sets in the Affymetrix platforms can be found in Supplementary Table S2 and the list of patients in parkin+/− and parkin−/− in each data set can be found in Supplementary Table S3.
downregulate Akt phosphorylation may play a part here. The Akt pathway is known to enhance G1–S cell-cycle progression through the increase of cyclin D1 level (32). Maximum activation of Akt requires both T308 phosphorylation by PDK1 and S473 phosphorylation by the TORC2 complex (33). Interestingly, wild-type parkin expression leads to a specific reduction in phospho-Akt (S473; but not T308) phosphorylation, suggesting that parkin acts on TORC2 instead of PDK1. Consistent with this, we found that the levels of TORC2 components and associated substrates are reduced in parkin-expressing glioma cells whereas the expression of PDK1 remains unaltered. As overactivation of Akt signaling is a frequent feature of tumors including gliomas, our results thus offer a mechanism underlying parkin-mediated tumor suppressor function in gliomagenesis. Interestingly, the presence of parkin also apparently influences the expression of VEGFR-2, one of the 2 high-affinity tyrosine kinase receptors involved in tumor neoangiogenesis. Although predominantly found on endothelial cells, VEGFR has also been detected on cancer cells including gliomas, suggesting a possible autocrine effect on their growth (34, 35). Importantly, autocrine regulation of GBM cell-cycle progression and viability through VEGF–VEGFR2 interplay involves coactivation of the PI3K/Akt pathway (35), which parkin appears to regulate. Taken together, our results suggest that parkin-mediated suppression of glioma cell proliferation involves the regulation of VEGFR2/Akt/cyclin D1 pathway.

Finally, we provide clinical evidence for our U87MG-based model. We implicate frequent chromosomal rearrangements with loss of gene expression in the PARK2 locus. We showed that PARK2 pathway activation correlates inversely with disease progression and patient survival. Our findings are important for the following reasons: The parkin gene signature defines molecular heterogeneity in gliomas that cannot be accounted for by histology alone. This is impactful as histology-based diagnoses currently determine the treatment regimens. Our findings indicate that the parkin signature can potentially stratify patients and predict cohorts likely to receive treatment benefit from PI3K/Akt inhibitor therapies. Thus, reexamination of the 50-gene list including PARK2 could conceivably provide useful prognostic indicators to monitor disease progression. Our finding that a PARK2 deletion occurs at the transcription start site in all patients with glioma may indicate a driver role for PARK2 deletion in gliomagenesis, as a passenger mutational status would otherwise be captured in only certain subgroups. Collectively, these data strongly establish a role for parkin in gliomagenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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References
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