Parkin regulates paclitaxel sensitivity in breast cancer via a microtubule-dependent mechanism

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Abstract

Parkin is an E3 ubiquitin ligase encoded by the Parkin gene (also called PARK2, located at 6q25.2-q27) and is involved in the pathogenesis of Parkinson’s disease and the development of cancer. Recently, Parkin has been demonstrated to interact with the microtubule cytoskeleton. However, the biological implication of the Parkin–microtubule axis has been poorly explored. In this study, we report for the first time that Parkin modulates sensitivity of the chemotherapeutic agent paclitaxel in breast cancer, via a microtubule-dependent mechanism. Our data reveal that Parkin binds to the outer surface of microtubules and increases paclitaxel–microtubule interaction, resulting in enhanced paclitaxel-induced microtubule assembly and stabilization. Our data further show that Parkin promotes the activity of paclitaxel to trigger multinucleation and apoptosis, rendering breast cancer cells more sensitive to this drug. Moreover, Parkin expression correlates with the pathological response of tumours to preoperative paclitaxel-containing chemotherapy. In addition, expression of Parkin also correlates with the sensitivity of paclitaxel in primary cultures of breast cancer cells. Our results identify Parkin as a novel mediator of paclitaxel sensitivity in breast cancer. In addition, our study suggests that patients harbouring tumours with high Parkin level would be more likely to benefit from paclitaxel-containing regimens.

Keywords: breast cancer; Parkin; paclitaxel; microtubule; sensitivity

Introduction

The Parkin gene (also called PARK2) is located at 6q25.2-q27 in a common fragile site FRA6E and in a candidate imprinting domain [1,2]. This gene encodes an E3 ubiquitin ligase that catalyses protein ubiquitination, leading to protein degradation by the proteasome [3–6]. Compelling evidence demonstrates that Parkin gene mutation is one of the most frequent causes of Parkinson’s disease [7]. In addition, reduced expression and inactivation of Parkin are frequently observed in human cancers [8–11]. It is also reported that Parkin expression can be epigenetically regulated; its expression can be reduced due to abnormal DNA methylation [12]. These studies suggest that Parkin may play a tumour suppressor role. Although the precise mechanisms of how Parkin is involved in the development of Parkinson’s disease and cancer remain elusive, alterations in its ubiquitin ligase activity are evident in a significant proportion of patients [7–11].

In addition to its function as a ubiquitin ligase, Parkin has recently been identified as a molecule capable of interacting with microtubules [13,14]. Parkin partly co-localizes with microtubules in cells and is present in purified microtubule preparations. The association of Parkin with microtubules appears to be strong and endures high concentrations of salt and urea [14]. In addition, the association is not affected by mutations that abolish the ubiquitin ligase activity of Parkin [13,14]. It has been suggested that microtubules may serve to anchor Parkin in the cytoplasm and thereby regulate its enzymatic activity [15]. However, considering that microtubules function in many cellular activities and act as a validated cancer chemotherapeutic target, it is possible that the association of Parkin with microtubules may have broader biological implications. In the present study, we provide evidence that the Parkin–microtubule axis has a remarkable impact on the sensitivity of breast cancer cells to the microtubule-targeting drug paclitaxel.
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Materials and methods

Materials

Paclitaxel, vinblastine and doxorubicin were from Sigma-Aldrich. Dimethylastron (Calbiochem), docetaxel (Aventis Pharmaceuticals), epothilone-B (Novartis), Oregon green fluorescent paclitaxel (Molecular Probes) and 3H-paclitaxel (Moravek Biochemicals) were from the indicated sources. Antibodies against α-tubulin, acetylated α-tubulin and β-actin were from Sigma-Aldrich. Antibodies against MBP (New England BioLabs), Parkin, cleaved caspase-3 and cleaved caspase-9 (Cell Signaling), horseradish peroxidase-conjugated and biotinylated secondary antibodies (Amersham Biosciences), and rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) were from the indicated sources.

Cell lines, adenoviruses and tumour specimens

Breast cancer cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). Adenoviruses were prepared and amplified in human embryonic kidney 293 cells, as described previously [16]. Breast cancer specimens were obtained from patients who underwent surgical resection for clinical treatment of breast cancer during 2006–2008 in the Cancer Hospital of Tianjin Medical University, Tianjin, China. For cancer response analysis, breast cancer specimens were obtained from 82 patients who received neoadjuvant chemotherapy containing paclitaxel (paclitaxel/doxorubicin, 12 cases; paclitaxel/doxorubicin/cyclophosphamide, eight cases; paclitaxel/epirubicin, 35 cases; paclitaxel/epirubicin/cyclophosphamide, 27 cases). To examine paclitaxel sensitivity in primary cultures of breast cancer cells, two sets of breast cancer specimens were obtained from 37 patients who underwent surgical resection without preoperative chemotherapy; one set of the specimens was used to obtain primary culture and examine paclitaxel sensitivity, and the other set was used to examine Parkin expression by immunohistochemistry. Use of human tissues in this study was approved by the Ethics Committee of the Cancer Hospital of Tianjin Medical University.

Evaluation of pathological response to chemotherapy

Pathological response to chemotherapy was classified into four groups, based on pathomorphological alterations: grade 0, no reaction; grade I, appearance of degenerated cancer cells; grade II, formation of granuloma; grade III, proliferation of fibrous tissues and formation of scars. Grades II and III were regarded as having a good response to chemotherapy. Grades I and 0 were regarded as having a poor response.

Measurement of paclitaxel sensitivity in primary cultures

Tumour tissues were minced and suspended in Hank’s balanced salt solution. The cells were dispersed by incubating tissues in 0.1% EZ (Nitta Gelatin, Japan) and filtered through nylon mesh. The cells were suspended in PCM-1 medium (Nitta Gelatin) and incubated in a collagen gel-coated flask in a CO2 incubator at 37 °C. To examine paclitaxel sensitivity, the collagen–cell mixture was placed in six-well plates on ice and allowed to gel at 37 °C (the final concentration was 3 × 103 cells/collagen gel droplet). Culture medium was then overlaid on each well and the cells were cultured overnight. Paclitaxel was added at a concentration of 10 μg/ml and incubated for 24 h. After removal of the medium containing paclitaxel, each well was overlaid with PCM-2 medium (Nitta Gelatin), and the cells were incubated for 7 days. At the end of the incubation, neutral red was added to each well and colonies in the collagen gel droplets were stained for 2 h. Each collagen droplet was fixed with 10% neutral formalin buffer, air-dried and quantified by image analysis, as described previously [17]. Paclitaxel sensitivity was expressed as the T:C ratio, where T was the reading of the treated group and C was the reading of the control group; a T:C ratio of 50% or less was regarded as being sensitive.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated with xylene and graded alcohols. Antigen retrieval was carried out in 5 mm citrate buffer. After inactivation of endogenous peroxidase with 3% H2O2, the sections were blocked with goat serum and incubated with anti-Parkin antibody. The sections were then incubated with biotinylated secondary antibody and streptavidin–biotin–peroxidase. Diaminobenzidine was used as a chromogen substrate. Finally, the sections were counterstained with haematoxylin. Parkin level was classified into four groups, using a modified scoring method [18] based on the intensity of staining (0 = negative; 1 = low; 2 = medium; 3 = high) and the percentage of stained cells (0 = 0% stained; 1 = 1–25% stained; 2 = 26–50% stained; 3 = 51–100% stained). A multiplied score (intensity score × percentage score) <2 was considered as negative staining (−), 2–3 as low staining (+), 4–6 as medium staining (++) and >6 as high staining (+++).

Statistics

The correlation between Parkin expression measured by immunohistochemistry of tumour tissues and the pathological response of tumours was examined by two-sample Wilcoxon rank sum test. The correlation between Parkin expression measured by immunohistochemistry of tumour tissues and paclitaxel sensitivity in primary cultures of cancer cells was examined by Spearman rank correlation test.
Examination of paclitaxel–microtubule interaction

Purified MBP or MBP–Parkin was incubated with purified tubulin for 30 min, and \(^{3}H\)-paclitaxel was then added and incubated for another 30 min. Microtubules were pelleted by centrifugation through a sucrose layer, and the radioactivity present in the pellet was measured. To study intracellular accumulation of paclitaxel, the cells were exposed to fluorescent paclitaxel for 30 min and then examined by flow cytometry.

Tubulin polymerization assay

Spectrophotometer cuvettes held a solution consisting of tubulin polymerization buffer with or without paclitaxel. The cuvettes were kept at room temperature before the addition of purified tubulin, with or without MBP or MBP–Parkin, and shifted to 37°C in a temperature-controlled spectrophotometer. Tubulin polymerization was monitored by measuring the optical absorbance at 350 nm.

Cytotoxicity assay

Cells grown in 96-well plates were treated for 48 h with gradient concentrations of drugs (0.01, 0.1, 1, 10, 100 and 1000 nM for microtubule-targeting drugs; 0.01, 0.1, 1, 10, 100 and 1000 μM for other drugs). The cells were then fixed with 50% trichloroacetic acid and stained with 0.4% sulphorhodamine B. Cells were washed with 1% acetic acid and the protein-bound dye was extracted with 10 mM Tris base to determine the optical density at 562 nm. The percentage of cell survival as a function of drug concentration was plotted to determine the drug concentration needed for 50% cell kill (IC\(_{50}\)).

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with methanol or paraformaldehyde. Cells were then blocked with bovine serum albumin and incubated in succession with primary and secondary antibodies, followed by staining with 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted with 90% glycerol and examined with an Olympus fluorescence microscope.

Results

Parkin binds to microtubule outer surface and increases paclitaxel–microtubule interaction

The building blocks of microtubules are \( \alpha \) and \( \beta \)-tubulin heterodimers. The C-termini of \( \alpha \)- and \( \beta \)-tubulin are exposed to the outer surface of the microtubule wall and mediate microtubule association with a number of proteins [19]. The nature of Parkin as an enzyme suggests that it may also bind to the microtubule outer surface to allow convenient access to various substrates. To test this possibility, pre-assembled microtubules were treated with subtilisin, which resulted in removal of the C-termini of \( \alpha \)- and \( \beta \)-tubulin, as evidenced by protein mobility shift in the gel (Figure 1A, upper panel). The Parkin–microtubule interaction was examined by microtubule co-sedimentation assay. We found that while the majority of MBP–Parkin resided in the pellet of control microtubules, it was not detectable in the pellet of subtilisin-digested microtubules (Figure 1A, lower panel). These data indicate that Parkin associates with microtubule outer surface.

There is evidence that certain microtubule-binding proteins can, through structural competition/stimulation or allosteric effect, influence the ability of microtubule-targeting drugs to interact with microtubules [20–22]. To investigate whether Parkin affects paclitaxel–microtubule interaction, \(^{3}H\)-labelled paclitaxel was incubated with microtubules assembled in the presence of MBP–Parkin. The radioactivity in the microtubule pellet was then measured. We found that MBP–Parkin increased paclitaxel binding to microtubules in a dose-dependent manner, whereas MBP alone did not affect paclitaxel binding (Figure 1B).

We then asked whether Parkin could modulate paclitaxel–microtubule interaction in cells. We examined by western blotting the expression of Parkin in eight breast cancer cell lines (MCF-7, T47D, MDA-MB-231, MDA-MB-453, SW527, BT549, ZR-75-1 and HCC1143). In agreement with the previous finding [8], Parkin expression was not detectable by western blotting in these cell lines (data not shown). Thus, for further studies we chose the means of over-expression to alter cellular Parkin level. MCF7, MDA-MB-231 and T47D cell lines were used because their genetic background, morphology and growth conditions are well characterized.

MCF7 cells were treated with adenoviruses, exposed to fluorescent paclitaxel, and then examined by flow cytometry. We found that cells treated with Parkin adenoviruses accumulated more fluorescent paclitaxel than those untreated or treated with \( \beta \)-galactosidase adenoviruses (Figure 1C). Similar results were obtained in MDA-MB-231 and T47D cells (data not shown). Given that intracellular paclitaxel binds mostly to microtubules [23], these data indicate that Parkin increases paclitaxel–microtubule interaction in cells.

Parkin enhances the activity of paclitaxel to promote microtubule assembly and stability

The remarkable effect of Parkin on paclitaxel–microtubule interaction suggests that it may increase the ability of paclitaxel to modulate microtubule properties. To test this possibility, MCF7 cells transfected with GFP or GFP–Parkin were treated with paclitaxel and examined by immunofluorescence microscopy.
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Figure 1. Parkin binds to the outer surface of microtubules and increases paclitaxel–microtubule interaction. (A) Upper panel, Coomassie-stained gel showing the shift of α- and β-tubulin mobility of subtilisin (Subt.)-digested microtubules compared with that of control (Con.) microtubules. Lower panel, purified MBP–Parkin fusion protein was incubated with control or subtilisin-digested microtubules. Microtubules were then pelleted by centrifugation and MBP–Parkin in pellet and supernatant fractions was detected by western blotting. (B) Purified MBP (20 µM) or MBP–Parkin (5, 10 or 20 µM) was incubated for 30 min with purified tubulin in tubulin polymerization buffer and 3H-paclitaxel (0.1 Ci/m) was then added and incubated for another 30 min. Microtubules were pelleted by centrifugation and the radioactivity present in the pellet was measured. (C) MCF7 cells untreated (mock) or treated with β-galactosidase or Parkin adenoviruses were exposed to Oregon green fluorescent paclitaxel (100 nM) for 30 min and then examined by flow cytometry. The M1 and M2 gates indicate fluorescence-negative and -positive populations, respectively.

Consistent with previous studies [14,23], overexpression of GFP or GFP–Parkin or treatment with sub-nanomolar concentrations of paclitaxel did not obviously affect the morphology of cellular microtubules (Figure 2A). Strikingly, in cells overexpressing GFP–Parkin, upon paclitaxel treatment 55% of cells exhibited microtubule bundles (Figure 2A, B), indicating that Parkin increases the ability of paclitaxel to promote microtubule assembly. We also found that Parkin enhanced the ability of paclitaxel to promote microtubule assembly in the purified system (Figure 2C).

Next, we investigated the effect of Parkin on paclitaxel-induced microtubule stabilization. Cells transfected with GFP or GFP–Parkin were treated with paclitaxel and then placed on ice to depolymerize microtubules. We found that Parkin significantly enhanced the activity of paclitaxel to stabilize microtubules (Figure 3A, B). In cells over-expressing GFP–Parkin, upon paclitaxel treatment 35% of cells contained microtubules (Figure 3B). In contrast, in cells over-expressing GFP alone, upon paclitaxel treatment only 5% of cells contained microtubules (Figure 3B). Similarly, by examining the level of tubulin acetylation, a marker of microtubule stability [24], we found that Parkin enhanced the activity of paclitaxel to stabilize microtubules (Figure 3C).

Parkin increases the ability of paclitaxel to trigger multinucleation and apoptosis and renders breast cancer cells more sensitive to this drug

We then examined the effect of Parkin on downstream cellular events triggered by paclitaxel. Immunofluorescence microscopy and flow cytometric analysis of cellular DNA content revealed that Parkin did not significantly affect paclitaxel-induced mitotic arrest (data not shown). Instead, the ability of paclitaxel to trigger multinucleation was remarkably increased by Parkin (Figure 4A, B). In addition, Parkin enhanced the ability of paclitaxel to cause the cleavage of caspase-3 and caspase-9 (Figure 4C) and accumulation of annexin V-positive cells (Figure 4D), indicating an enhanced apoptosis. Importantly, a Parkin mutant lacking the microtubule-binding domain failed to increase the ability of paclitaxel to trigger apoptosis (Figure 4E), demonstrating the importance of Parkin–microtubule interaction in mediating the sensitizing effect of Parkin.

We then investigated whether Parkin could modulate paclitaxel sensitivity in breast cancer cells by measuring the IC50 value of paclitaxel. We found that GFP–Parkin adenoviruses significantly decreased paclitaxel IC50 in MCF7 cells (Figure 5A), indicating that Parkin renders cells more sensitive to paclitaxel.

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Figure 2. Parkin enhances the activity of paclitaxel to promote microtubule assembly. (A) MCF7 cells transfected with GFP or GFP–Parkin were treated with 2 nM paclitaxel or equal volume of vehicle, and microtubule (MT) morphology (red) and GFP signal (green) were then examined by immunofluorescence microscopy. (B) Experiments were performed as in (A) and the percentage of cells with microtubule bundles was quantified by immunofluorescence microscopic analysis of microtubule morphology. The microtubule bundle refers to a strand of microtubules bound together instead of as separate microtubule fibres; 300 cells were counted in total. (C) Effect of Parkin on paclitaxel-induced tubulin polymerization in vitro, examined by measuring the optical absorbance at 350 nm. #1, tubulin alone; #2, tubulin + paclitaxel (10 µM); #3, tubulin + paclitaxel + MBP (20 µM); #4, tubulin + paclitaxel + MBP–Parkin (5 µM); #5, tubulin + paclitaxel + MBP–Parkin (5 µM); #6, tubulin + paclitaxel + MBP–Parkin (20 µM).

paclitaxel. Similar results were achieved in MDA-MB-231 and T47D breast cancer cells (data not shown). We also found that Parkin could increase the sensitivity of cells to other microtubule-targeting drugs, including docetaxel, epothilone-B and vinblastine (Figure 5A). In contrast, cell sensitivity to doxorubicin, a DNA-damaging drug, or dimethylenastron, a drug targeting the mitotic kinesin Eg5 [25], was not altered by Parkin (Figure 5A). A large proportion of cells treated with paclitaxel in the presence of GFP–Parkin adenoviruses exhibited round, abnormal morphology (Figure 5B), consistent with enhanced induction of apoptosis. Moreover, using the SH-SY5Y neuroblastoma cell line, which is known to express Parkin [26], we found that siRNA-mediated knockdown of Parkin rendered cells resistant to paclitaxel (Figure 5C, D).

Parkin expression predicts breast cancer response to paclitaxel treatment

Our finding that Parkin modulates the sensitivity of breast cancer cells to paclitaxel prompted us to study whether Parkin is associated with paclitaxel sensitivity in the clinical setting. Immunohistochemistry was performed to examine Parkin expression in breast
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Figure 3. Parkin strengthens paclitaxel-induced microtubule stabilization. (A) MCF7 cells transfected with GFP or GFP–Parkin were treated with 2 nM paclitaxel or equal volumes of vehicle and then placed on ice for 30 min. Microtubule (MT) morphology (red) and GFP signal (green) were examined by immunofluorescence microscopy. (B) Experiments were performed as in (A) and the percentage of cells containing microtubules was quantified; 300 cells were counted in total. (C) Cells were treated with paclitaxel or vehicle in the absence or presence of GFP or GFP–Parkin adenoviruses. Acetylated α-tubulin, total α-tubulin, and Parkin were examined by western blotting. Tubulin acetylation (acetyl.) level was determined by dividing the intensity of acetylated α-tubulin with the intensity of total α-tubulin on the blot.

cancer tissues from patients who received neoadjuvant chemotherapy containing paclitaxel. Pathological response to chemotherapy was classified into four groups, based on pathomorphological alterations: grade 0, no reaction; grade I, appearance of degenerated cancer cells; grade II, formation of granuloma; and grade III, proliferation of fibrous tissues and formation of scars. Grades II and III were regarded as having a good response to chemotherapy. Grades I and 0 were regarded as having a poor response. In agreement with the previous study [8], there was a significant variability in Parkin expression among tumours from different patients; of a total of 82 cases, 17 were Parkin-negative (−), 34 had low expression (++), 23 had medium expression (+++) and eight had high expression (++++) (Figure 6A, B). Strikingly, Parkin expression level in breast cancer tissues correlated with the pathological response to paclitaxel-containing regimen. In contrast, no obvious correlation was observed between Parkin expression and pathological response to the control CEF (cyclophosphamide, epirubicin and 5-fluorouracil) regimen (data not shown).

We also investigated paclitaxel sensitivity in primary cultures of breast cancer cells. Two sets of breast cancer specimens were obtained from 37 patients who underwent surgical resection without preoperative chemotherapy; one set of the specimens was...
Figure 4. Parkin increases paclitaxel-induced multinucleation and apoptosis. MCF7 cells were treated for 48 h with 2 nM paclitaxel or equal volumes of vehicle in the absence or presence of GFP or GFP–Parkin adenoviruses. Nuclear morphology (blue) and GFP signal (green) were then examined by fluorescence microscopy (A) and the percentage of multinucleated cells was quantified (B). The levels of cleaved caspase-3 (casp-3), cleaved caspase-9 (casp-9), Parkin and β-actin were examined by western blotting (C), and the percentage of apoptosis was determined by annexin V staining (D). (E) Cells transfected with GFP (control), GFP–Parkin or GFP–ParkinΔMB (a Parkin mutant that lacks the microtubule-binding domain) were treated with paclitaxel or vehicle and the percentage of apoptotic cells was then quantified.

Discussion

Parkin is an E3 ubiquitin ligase regulating the ubiquitination and proteasome-dependent degradation of diverse proteins [3–6]. Parkin gene mutations have been implicated in the pathogenesis of Parkinson’s disease and cancer [7–11]. In addition, break at the Parkin gene/common fragile site FRA6E is associated with poor outcome in breast cancer [27]. In this study, our results provide evidence to support a novel function for Parkin in regulating the sensitivity of breast cancer cells to paclitaxel: (a) Parkin increases the ability of paclitaxel to trigger multinucleation and
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Figure 5. Over-expression of Parkin renders breast cancer cells more sensitive to paclitaxel. (A) IC₅₀ values of paclitaxel, docetaxel, epothilone-B (EpoB), vinblastine, dimethylenastron (DIMEN) and doxorubicin in MCF7 cells untreated (mock) or treated with GFP or GFP–Parkin adenoviruses. **p < 0.01; *p < 0.05. (B) Visualization of cells treated for 48 h with 2 nM paclitaxel or equal volumes of vehicle in the absence or presence of GFP or GFP–Parkin adenoviruses. (C) Western blot analysis of the expression of Parkin and β-actin in SH-SY5Y cells transfected with control siRNA or two different Parkin siRNAs. (D) IC₅₀ values of paclitaxel in SH-SY5Y cells transfected with control (Con) siRNA or two different Parkin siRNAs (P#1 and P#2)

Our study also provides mechanistic insight into how Parkin might modulate cancer cell sensitivity to paclitaxel. We find that Parkin increases paclitaxel–microtubule interaction and enhances the activity of paclitaxel to promote microtubule assembly and stability, two hallmark events that underlie the cytotoxicity of paclitaxel [28]. Our data reveal that Parkin also increases cancer cell sensitivity to several other microtubule-targeting agents, but does not influence cell sensitivity to agents that target Eg5 or DNA. In addition, we find that the effect of Parkin on paclitaxel sensitivity is abolished by deletion of the microtubule-binding domain. Given that Parkin binds to microtubules with high affinity [14], these findings suggest that the activity of Parkin towards paclitaxel sensitivity is mediated specifically by a microtubule-dependent mechanism.

Microtubule-binding proteins are known to have the potential to alter the ability of microtubule-targeting drugs to interact with microtubules and to modulate microtubule properties [20–22]. This capacity of microtubule-binding proteins has been proposed to contribute to the variable sensitivity of cancer cells to microtubule-targeting drugs, thereby providing a unique avenue to predict or manipulate the clinical outcome [29]. In this study, consistent with the activity of Parkin to sensitize breast cancer cells to paclitaxel, gene expression analysis of breast cancer
Figure 6. Parkin expression predicts breast cancer response to paclitaxel treatment. (A) Immunohistochemical analysis of Parkin expression in breast cancer tissues. (B) Parkin expression level in breast cancer tissues correlates with the pathological response to preoperative paclitaxel-containing chemotherapy ($r = 0.454$, $p < 0.01$). (C) Parkin expression level correlates with paclitaxel sensitivity in primary cultures of breast cancer cells ($r = 0.371$, $p < 0.05$).

tissues reveals a striking correlation of Parkin level with pathological response of tumours to paclitaxel-containing regimen, but not to the control CEF regimen. These data thus suggest a potential for Parkin expression as a diagnostic marker to predict pathological response to paclitaxel therapy. In addition, these results implicate that Parkin might be exploited as a therapeutic target to improve paclitaxel sensitivity.

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