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REVIEW

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Genetics of parkin-linked disease

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Abstract Research into Parkinson's disease (PD), once considered the archetypical non-genetic neurodegenerative disorder, has been revolutionized by the identification of a number of genes, mutations of which underlie various familial forms of the disease. Whereas such mutations appear to exist in a relatively small number of individuals from a few families, the study of the function of these genes promises to reveal the fundamental disease pathogenesis, not only of familial forms of the disease, but also of the much more common sporadic PD. The observation that mutations in the second identified PD locus (parkin) are common in juvenile- and early-onset PD and increasing evidence supporting a direct role for parkin in late-onset disease make this gene a particularly compelling candidate for intensified investigation. The determination of the frequency and effect of parkin mutations in various subsets of PD will be crucial for understanding the way in which parkin is related to neurodegenerative mechanisms, and whether these subsets might be effectively identified and treated. In addition, many aspects of parkin-linked disease, originally thought to be well defined, have now been obscured both by genetic studies that preclude a simple model of disease transmission and by clinical and pathological studies that demonstrate broad variability in cases with parkin mutations. Future studies that address the issues in question should have a far-reaching impact in downstream biochemical studies and our understanding of parkin's role in PD.

Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disorder, with classical symptoms including bradykinesia, rigidity, resting tremor, and postural instability. Cases often present with heterogeneous symptoms, perhaps reflecting the underlying complex nature of its pathogenesis, which confounds the accurate diagnosis and treatment of PD (Rao et al. 2003). PD is confirmed pathologically by the loss of pigmented dopaminergic neurons in the substantia nigra pars compacta (SNpc); however, additional brain regions are affected prior to and subsequent to neurodegeneration in the midbrain (Braak et al. 2003). Eosinophilic inclusions containing alpha-synuclein and ubiquitin are also hallmarks of the disease and are commonly found in the SN and locus coeruleus, in both the perikarya (Lewy bodies) and processes (Lewy neurites) of the remaining neurons (Spillantini et al. 1997). Whereas treatment with L-dopa and dopaminergic agonists usually provides good symptomatic benefit, the therapy fails to alter disease progression and provokes undesirable side-effects (Melamed et al. 2000). As a result, PD is a major cause of morbidity and mortality (Tanner and Aston 2000). Although the majority of PD patients appear to present without a family history of disease, studies have demonstrated the importance of genetic susceptibility factors (Maher et al. 2002; Sveinbjornsdottir et al. 2000). The identification and isolation of genes that contribute to PD presents an opportunity to explore the molecular basis of the disease and thereby to provide a rational approach to therapeutic intervention.

The risk of developing PD increases with age, the disease being uncommon before the age of 30 (Langston 2002). A genetic locus for juvenile-onset PD, a rare condition usually confined to patients with age at disease onset <21 years (Langston and Tan 2000), has been mapped to chromosome 6q25.2-27 in consanguineous Japanese families (Matsumine et al. 1998), attributable in part to the serendipitous proximity of the manganese superoxide dismutase gene (Mizuno et al. 1999). Analysis of additional Japanese families with positive linkage to this region has fortuitously revealed a patient with a homozygous deletion of a microsatellite marker (D6S305), and an exon-trapping strategy has identified a nearby expressed-sequence tag corresponding to a novel gene, dubbed parkin (Kitada

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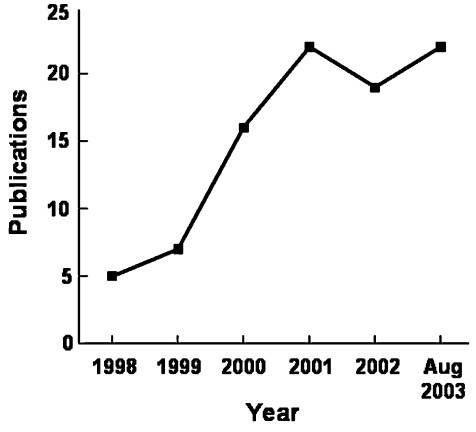

Fig. 1 Number of publications, indexed on Pubmed, in which the parkin gene was analyzed in case and/or control samples

et al. 1998). Homozygous exonic deletions have been detected in five Japanese juvenile-onset PD patients from four independent families; thus, alterations in the parkin gene have been hypothesized to be the predominant cause of recessively inherited juvenile-onset PD in the Japanese.

Despite the identification of PARK2 and the underlying gene having occurred in the scientific wake of the discovery of mutations in the alpha-synuclein gene, parkin has since established a unique role for itself in PD genetics. Mutations in other genes associated with PD, such as alpha-synuclein, UCH-L1, and DJ-1, are probably confined to a small number of individuals from a few families. Parkin mutations, on the other hand, are now known to be prevalent in PD, with mutations having been found in nearly every ethnicity and PD case population studied. Several excellent recent reviews have described advances in the genetic loci associated with PD (Hardy et al. 2003; Skipper and Farrer 2002), whereas other reviews have dealt more specifically with the biology of the parkin protein (Cookson 2003; Feany and Pallanck 2003). To our knowledge, a comprehensive survey of the more than 90 studies that are indexed on Pubmed and that explore the genetic relationship between the parkin gene and PD does not exist. Interest in this subject nevertheless appears to be greater than ever (Fig. 1); therefore, this review is dedicated toward dissecting parkin's genetic association with PD, the once quintessential "non-genetic" neurodegenerative disorder. Attention is also given to the clinical and pathological aspects of parkin-linked disease, transgenic models, and potential future directions for the application and analysis of parkin-related genetic research.

Parkin mutations in early-onset PD

Shortly after the discovery of the parkin gene, mutation analyses in index cases who were from families of diverse ethnic origins, and who were consistent with early onset (age of disease onset <45 years) recessively inherited PD, revealed that mutations in the parkin gene were not limited to homozygous exon deletions, juvenile-onset PD, or the Japanese population (Hattori et al. 1998; Leroy et al. 1998; Lucking et al. 1998). To ascertain the frequency of parkin mutations in early-onset PD patients in the European population, Abbas et al. (1999) sequenced each of the 12 exons of the parkin gene, and mutations were identified in 12 families (32%). In their study, point mutations were approximately twice as common as homozygous exonic deletions; however, in three families, a second mutation (either a homozygous exon deletion or point mutation) was not found. The authors acknowledged that the methodology used to screen for parkin mutations would be insufficient to detect heterozygous exon rearrangements or alterations outside of the open reading frame, and thus the true frequency of parkin mutations would be underestimated.

To overcome these limitations, a semi-quantitative polymerase chain reaction assay was developed to obtain a more accurate estimation of the frequency of parkin mutations in early-onset PD (Lucking et al. 2000). Cases from 73 families of predominantly European descent with a history of early-onset PD compatible with recessive inheritance (familial index cases), in addition to 100 early-onset PD patients with no known family history of PD (sporadic cases), were analyzed for parkin mutations. A wide variety of mutations were identified, including missense and nonsense mutations, intra-exonic deletions and insertions, and exon multiplications and deletions. In total, parkin mutations were found in 49% of familial index cases and 18% of sporadic cases. Further stratification of the sporadic cases revealed that 77% (10 out of 13 studied) with age of disease onset <20 years had parkin mutations, whereas mutations were found in 3% of cases with age of disease onset between 31 and 45 years (2 out of 64 cases studied). The reciprocal correlation between age of disease onset and frequency of parkin mutations was further analyzed in a larger case series, in which parkin mutations were found in 67% of cases with age of onset <20 years and in ~8% of cases with an age of onset between 30–45 years (Periquet et al. 2003).

Given the commonness and wide variety of parkin mutations, viz., from large deletions spanning multiple exons to single basepair deletions and insertions (and in various combinations with one another), it became clear that extensive genomic analyses requiring exon-dosage experiments in addition to sequencing were necessary. Hedrich et al. (2002) evaluated 50 index cases from a variety of ethnicities for parkin mutations, all with age of disease onset <50 years, and identified seven cases with compound heterozygous mutations and six cases with a single heterozygous mutation. As noted by the authors, the second parkin mutation in six of the seven cases with compound heterozygous mutations would have been missed had exon-dosage analysis not been performed. Yet six cases, despite dosage analysis of all parkin exons, remained heterozygous for a single parkin mutation. It was therefore hypothesized that loss of a single parkin allele is associated with early-onset PD.

Whereas the high prevalence of parkin mutations in early-onset PD has been reproduced in multiple studies, Kann et al. (2002) have argued that parkin mutation rates

in early-onset PD may have been overestimated because of referral bias in patient sample collection. To address this issue, the parkin gene was analyzed in 111 community-based German PD patients with age of disease onset <50 years, and an overall mutation rate of 9% was obtained, with 5.4% of cases being heterozygous for a single parkin mutation. If referral bias had indeed led to an overestimation of the frequency of parkin alterations in early-onset PD, then the potential for additional genetic modifiers being important in early-onset PD may also have been underestimated. To address this question, Scott et al. (2001) performed a 10-cM genome-wide screen in 18 Caucasian families, with at least one early-onset PD case. A peak LOD score of 5.47 was obtained for marker D6S305, with no other scores >1.5 being found in the analysis, thereby suggesting that the parkin gene (at least in the population studied) is unique in its relationship to the development of early-onset PD. A follow-up screening for parkin mutations was performed, and mutations were discovered in 18% of the early-onset cases; however, the true frequency may have been underestimated as exon-dosage experiments were not performed in these patients (Oliveira et al. 2003a).

Parkin mutations in late-onset PD

Given the prevalence of parkin mutations in familial and sporadic, and early-onset and juvenile PD, it seems reasonable to hypothesize that parkin might have a role in later-onset sporadic disease. Studies utilizing common single nucleotide polymorphisms (SNPs) in tests for association with late-onset PD have produced mixed results (Hu et al. 2000; Oliveri et al. 2001; Satoh and Kuroda 1999; Wang et al. 1999). Table 1 lists published polymorphisms in the parkin open reading frame, many of which have been analyzed in case/control association studies. SNP positions in this table correspond to the published parkin mRNA sequence (NCBI accession AB009973). In a study of 607 cases and 872 controls, Oliveiera et al. (2003b) failed to find an association with any of the previously reported parkin polymorphisms associated with disease. However, in lieu of a causative effect of a given polymorphism, it is unlikely that standard association analyses would be able to capture the genetic diversity in even a small portion of the parkin gene, because of its expanded introns and high recombinability rates (West et al. 2003). Perhaps suggestive of the unstable nature of the parkin gene as a whole, our group has found unexpectedly low rates of linkage disequilibrium (LD) among SNPs just a few hundred basepairs apart in the parkin promoter (West et al. 2002a); likewise, an unexpectedly low rate of LD among SNPs in the same parkin exon has also been observed (Oliveira et al. 2003b). The technical problems introduced by the apparent lack of LD blocks of appreciable size in association analyses are probably exponentially worsened by the massive size of the parkin gene. Thus, studies utilizing common polymorphisms have failed to define the role that parkin plays in the development of late-onset PD.

By using techniques originally reserved for early-onset cohorts, the parkin gene was analyzed in 118 late-onset (>45 years) cases, and no homozygous exonic deletions were detected (Oliveri et al. 2001). Whereas exon dosage analysis was not performed in this study, it seemed clear that mutations were not as common as in early-onset PD. Another study involving a larger population analyzed 363 affected subjects from 307 families and identified parkin mutations in 2% of all late-onset families screened, thereby directly implicating the parkin gene in late-onset PD (Oliveira et al. 2003a). However, exon-dosage screening was not performed; instead, the authors used a denaturing high-pressure liquid chromatography methodology with follow-up sequencing to identify parkin mutations; thus, the number of cases with parkin mutations was probably underestimated.

Foroud et al. (2003) screened the parkin gene using exon dosage and sequencing analysis within families that either tested positive for a genetic marker (D6S305) within parkin and had a disease inheritance compatible with a recessive transmission or that had at least one affected family member with an age of disease onset <50 years. Within their cohort of 278 families, parkin mutations were identified in 11.2% of cases (50/448) with PD onset >50 years of age. In addition, individuals with two identified parkin mutations (affecting both parkin alleles) were found to have a significantly earlier age at disease onset (41.3 years) compared with individuals with one identified parkin mutation (56.3 years). The authors suggested that genetic alterations that attenuate but do not ablate parkin activity, such as particular heterozygous point mutations, might also result in a less-severe phenotype with a later onset of disease.

Single parkin mutations and dominant inheritance

Early studies indicated that parkin-linked PD is recessively inherited, where a deleterious alteration is presumed on

Table 1 Polymorphisms in the open reading frame of parkin

SNP position	Amino acid change	Exon	Frequency (%)	Enzyme	Reference
300G→C	Gln100His	3	45	*Bpl*I	Chen et al. 2003
373C→T	Silent	3	50	*Tsp*RI	Chen et al. 2003
601G→A	Ser167Asn	4	1	*Alw*NI	Abbas et al. 1999
813A→T	Arg271Ser	7	14	*Bsm*AI	Chen et al. 2003
1015G→T	Ala339Ser	9	29	*Dpn*I	Chen et al. 2003
1239G→C	Val380Leu	10	16	–	Abbas et al. 1999
1281G→A	Asp394Asn	11	7	*Taq*I	Abbas et al. 1999

both alleles, affected individuals are located in a single generation, and heterozygous carriers are unaffected. Initial parkin mutation screening studies that identified affected individuals in early-onset families with only a single parkin mutation might have been explained by the lack of advanced genetic screening techniques (Abbas et al. 1999; Maruyama et al. 2000). However, as alluded to above, the implementation of exon-dosage analysis to detect heterozygous exon rearrangements in the parkin gene produced an unanticipated result: the prevalence of early-onset PD cases with single parkin mutations increased (Foroud et al. 2003; Hedrich et al. 2002; Kann et al. 2002; Lucking et al. 2000; Periquet et al. 2001; Rawal et al. 2003). In some studies, more than half of the total number of cases identified with a parkin mutation had only a single heterozygous mutation (Kann et al. 2002). Thus, the mode of parkin-linked disease transmission became obscured.

Rudimentary techniques for parkin mutation screening include sequencing the open reading frame from genomic DNA and/or screening for "common" parkin point mutations. Parkin exon-dosage experiments are now a requirement for satisfactory genetic screening, as parkin heterozygous exon rearrangments are known to be common in parkin-positive cases and may occur de novo (Periquet et al. 2001). Our group hypothesized that pathogenic mutations could extend outside of the parkin open reading frame into the introns and gene-regulatory regions. For this reason, we collected cases described in the literature (before January 2001) with a single parkin mutation and early-onset PD, comprehensively re-screened them for parkin mutations, and extended the analysis to include the promoter region and intron-exon junctions (West et al. 2002a). Out of 20 samples analyzed, we identified a second mutation in half of the samples (including intronic splice site mutations), whereas half the samples remained heterozygous for a single parkin mutation. Our study illustrated the technical difficulties in screening the parkin gene and that single parkin mutation frequencies are probably over-estimated.

Support for the idea that single parkin mutations might be sufficient to produce disease has come from descriptions of families with a single heterozygous parkin mutation associated with disease in multiple generations, thereby suggesting dominant inheritance. A heterozygous intra-exonic deletion was discovered in family Ph, and subsequent analyses failed to find additional parkin alterations, despite the evaluation of parkin at DNA, RNA, and protein levels (Farrer et al. 2001). Smaller families have also been described and, as a whole, illustrate the wide variety of single parkin mutations associated with disease (e.g., exon triplications, deletions, and point mutations). Additional susceptibility alleles were postulated to exist in cases with a single parkin mutation, and a genome-wide linkage analysis was performed on 23 families (Pankratz et al. 2003). Although a LOD score of 7.2 was obtained near the parkin gene, some additional evidence for linkage to a 29-cM region of chromosome 10 (LOD=2.3) was observed; however, no known genes associated with PD has been localized to this region. Thus, heterozygous single parkin mutations appear to be the primary cause of disease in some cases.

To account for the existence of bona fide single parkin mutation cases within the genetic framework of a phenotype that is usually recessively inherited, four hypotheses might be envisaged. (1) A second loss of function mutation in the parkin gene does indeed exist, outside of the regions usually analyzed for mutations, including the promoter, introns, and intron/exon junctions. We have previously described the extremely high sequence conservation between human and mouse parkin introns (West et al. 2003). Conservation might imply function, and thus, alterations within parkin's introns may deleteriously effect splicing and act to negate the generation of a full-length transcript but yet be undetectable in conventional parkin screening assays. Contrary to this hypothesis, some affected individuals with a single parkin mutation have been analyzed at the RNA and protein level with wild-type RNA and protein being identified, thereby suggesting a second mutation does not exist in these individuals (Farrer et al. 2001). (2) Dominant-negative mechanisms induced by particular mutations may cause disease with severity comparable to loss of function mutations. However, a review of the literature suggests that most parkin mutations have also been identified in at least one unaffected related carrier (age-matched or older). If a particular class of mutations has dominant-negative potential, it is not obvious what mutations these might be given the current literature that utilizes family-based genetics. (3) Cases described with a single parkin mutation may be incidental parkin mutation carriers, where disease is not directly related to the existence of a parkin mutation, particularly in cases with heterozygous exonic rearrangements. If single parkin mutation cases are truly overestimated in the literature, as we have previously found (West et al. 2002a), then it is important to ascertain the frequency of the single mutations in age-matched control populations. A review of the literature demonstrates that the frequencies of newly identified parkin point mutations are routinely analyzed in at least some type of control population; however, we were unable to find studies that estimated the frequency of heterozygous exon rearrangements in large control populations. Until the frequencies of parkin gene alterations in clinically and pathologically confirmed control populations are known, the proportion of incidental parkin mutation carriers will remain undefined. (4) Cases with a single parkin mutation may suffer from haploinsufficiency, where a loss of one parkin allele reduces normal expression and subsequent enzymatic activity, and therefore is a risk factor for disease. This model assumes that parkin expression is truly reduced in single parkin mutation cases, and that parkin is the rate limiting factor for a downstream biochemical event(s).

Whereas one or more of the above hypotheses may account for single parkin mutation cases, data converging from diverse experimental approaches seem to favor the haploinsufficiency model. First, (^{18}F)-Dopa PET scanning in a family with parkin mutations has revealed that carriers of parkin mutations (single parkin mutation cases) have a

significantly lower dopaminergic input to the striatum compared with controls and might be presumed to have preclinical PD (Hilker et al. 2001, 2002). These results have been replicated in an additional family with parkin mutations (Khan et al. 2002). Second, an SNP in the parkin promoter, associated with a low-expressing parkin allele, is slightly more common in PD cases than controls (West et al. 2002b). Third, over-expression of parkin in assays in vitro favors a neuroprotective role for parkin in dopaminergic neurons, and thus partial loss of expression might be detrimental (Darios et al. 2003; Petrucelli et al. 2002). However, it cannot be excluded that dominant-negative mutations might also account for some proportion of single parkin mutation cases. Additional biochemical studies and perhaps the generation of transgenic animals should help to determine the potential roles of different parkin mutations in pathogenesis.

The nature of parkin mutations

Although the existence of large homozygous deletions in PD cases greatly aided the discovery of the parkin gene and its association with PD, it was quickly recognized that the number and types of genetic alterations in the parkin gene extended beyond large deletions. New assays that were able to detect a deletion as small as one basepair to those extending more than a megabase across the parkin gene were developed and utilized in screening case samples. As a result, hundreds of cases with a wide variety of mutations have been described, a meta-analysis of which may provide insight into the mechanisms of the disease.

The protein encoded by the parkin gene is characterized by four motifs, including the ubiquitin-like domain (UBL) near the N-terminus, a really-interesting new gene (RING) finger domain, an in-between RING domain (IBR), and a second RING finger domain (Kitada et al. 1998). Figure 2A displays all published point mutations (as of August 2003) in the parkin gene that result in a pathogenic change in amino acid sequence aligned with the protein domains of parkin. Point mutations that result in the generation of a truncated protein (nonsense mutations) were excluded. The majority of missense mutations cluster into the functional domains of the parkin protein (Fig. 2A), and almost without exception, these mutations occur in residues evolutionarily conserved in mouse parkin protein. Whereas biochemical analysis of every point mutation identified is incomplete, to our knowledge, all studied mutant proteins have been shown to possess attenuated or negated enzyme activity in assays in vitro, thus contributing to the hypothesis that loss of function of parkin is the predominant cause of disease.

Large deletions that lie within the parkin gene and that span multiple exons are also common types of mutations found in PD cases; however, the mechanisms that govern such alterations are unknown. A schematic presentation of all published deletions in the parkin gene in cases is given in Fig. 2B together with an aligned diagram indicating the positions of the 12 exons of parkin. Clearly, a common deleted region does not exist among the cases in the literature, as deletions have been reported to span nearly every exon of parkin. However, any given deletion appears to be more likely to include the center of the gene (near exons 4 and 5) than the 5' or 3' ends of the gene.

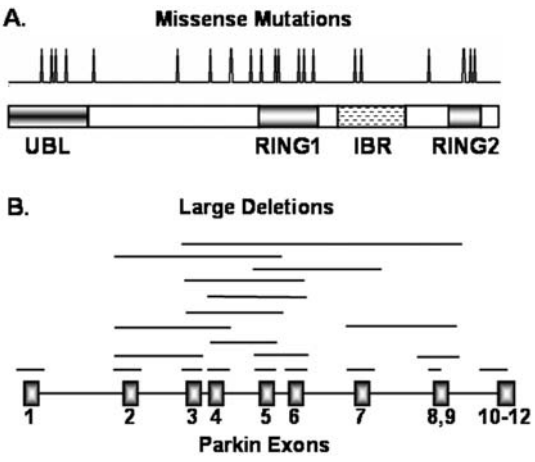

Fig. 2A, B Mutations in the parkin gene. **A** Missense mutations are represented in the histogram aligned with the amino acid sequence and protein domain structure of parkin. **B** Deletions spanning whole exons are represented as *black lines* above a diagram indicating the relative positions of parkin exons within the parkin gene. *Boxes* representing exons are not to scale

Perhaps some light is shed regarding the nature of these deletions in humans by the parkin gene being one of the largest genes in the genome (1.34 Mb) and spanning the third most common human fragile site, Fra6E (Denison et al. 2003; Smith et al. 1998). Common fragile sites are regions known to be unstable and hyper-recombinable and are frequently subject to extensive rearrangements in tumors (Richards 2001). Deletions within parkin, similar to other genes spanning common fragile sites, have been detected in both ovarian and breast cancer; however, the role of parkin in cancer is as yet undefined (Cesari et al. 2003; Denison et al. 2003). Given the high frequency of common fragile site rearrangements in cancer cells, coupled with the finding that parkin spans a particularly unstable common fragile site, it is an unnerving prospect to imagine that the parkin gene may be subject to rearrangements upon EBV exposure and thus to the establishment of a lymphoblastoma cell line. Investigators screening for parkin mutations may need to keep in mind the origin of the materials used.

Clinical and pathological findings in parkin-linked disease

The characterization of the clinical and pathological aspects of parkin-related disease has been of interest since the cloning of the parkin gene. Parkin-linked disease might have initially been presumed to be restricted to autosomal-recessive juvenile PD cases in the Japanese; however, this assumption was certainly premature as parkin mutations have been identified as the causative factor in both early-onset and late-onset, and sporadic and familial PD cases

of diverse ethnicities. If parkin-linked disease closely resembles the clinical and pathological phenotypes observed in the majority of PD cases, then a close association may be more likely to exist between parkin function and the pathogenic mechanisms occurring in PD. Unfortunately, PD represents a heterogeneous syndrome, and PD-associated symptoms are also known to occur in other neurological disorders. Therefore, detailed studies are required to determine the extent to which parkin-linked disease resembles "idiopathic" PD.

An early large-scale study clinically evaluating parkin-positive and -negative cases revealed that, in general, cases with parkin mutations had significantly higher frequencies of dystonia and symmetric symptoms, and a better and prolonged response to dopamine replacement therapy compared with cases without parkin mutations (Lucking et al. 2000). In general, however, most studies indicate a close overlap between symptoms in age-matched cases with and without parkin mutations, thereby limiting the accuracy of a clinical-evaluation based prediction of parkin mutations. Interestingly, a proportion of cases with parkin mutations have been described with symptoms atypical for cases without parkin mutations, including psychiatric manifestations, signs of cerebellar dysfunction and neuropathy (Khan et al. 2003; Lohmann et al. 2003). The significance of these findings is unknown, as more studies are required that utilize even larger patient populations in addition to appropriate control groups.

Imaging studies with (^{18}F)-Dopa PET have suggested that early-onset or juvenile-onset cases resemble that of idiopathic PD, although a worse clinical outcome might be predicted by PET in parkin mutation cases than is actually observed (Broussolle et al. 2000). In agreement, no correlation between PET data and clinical presentation in parkin mutation cases has been found, whereas cases without parkin mutations display a significant correlation (Thobois et al. 2003). In addition, Khan et al. (2002) has performed PET over a number of years in a family with parkin mutations and observed a slower disease progression in parkin mutations cases versus cases without parkin mutations. Hilker et al. (2001, 2002) have included asymptomatic family controls in a study utilizing PET and found that carriers of a parkin mutation have significantly reduced dopamine input to the striatum compared with individuals with two normal parkin alleles. This result has partially been confirmed in another study, in which some, but not all, of the asymptomatic single parkin mutation carriers had significant PET changes (Khan et al. 2002). Taken together, studies that utilize PET scans in cases without parkin mutations typically show similar results to cases with parkin mutations, illustrating the inability of PET to accurately predict parkin mutations (Pal et al. 2002). However, similar to clinical findings, subtle differences between cases with and without parkin mutations may warrant further investigation and could reveal additional details of the pathogenic mechanisms associated with parkin mutations.

The first reports of neuropathological examinations of parkin-proven disease revealed a nearly complete loss of pigmented neurons in the SNpc, similar to advanced cases of sporadic PD (Ishikawa and Takahashi 1998; Mori et al. 1998). Autopsy of additional juvenile-onset Japanese patients demonstrated that neuronal loss and gliosis were restricted to the SN and locus ceruleus, without Lewy pathology (Hayashi et al. 2000). Perhaps suggesting that lack of Lewy pathology is specific to parkin-linked disease, Lewy pathology has been reported in juvenile-onset PD (Bernheimer et al. 1973; Yokochi et al. 1984); however, the parkin gene has not been screened in these cases, and therefore, mutations cannot be ruled out. In contrast, Farrer et al. (2001) identified a case with compound heterozygous parkin mutations, early-onset PD, and prominent Lewy pathology. A Japanese juvenile-onset PD case and a Dutch early-onset parkin mutation case with tau-positive neurofibrillary tangles have also been described (Mori et al. 1998; van de Warrenburg et al. 2001). Interestingly, four other early-onset PD cases were found to have no Lewy pathology but demonstrated neurofibrillary tangles, neuronal loss, and gliosis restiricted to the SN and locus ceruleus; the status of the parkin gene was unknown in these cases (Rajput et al. 1989). Clearly, it is presumptuous to formulate conclusions because of the limited number cases, with and without parkin mutations, that have come to autopsy. A screen for parkin mutations in the relevant cases pathologically described in the literature (before the parkin gene was discovered) would probably contribute essential information as to the nature of the relationship between the parkin gene and Lewy body and neurofibrillary tangle formation and the potential differential effects of various parkin mutations on pathological presentation.

Transgenic models of parkin-linked disease

The molecular scenario by which parkin mutations result in selective dopaminergic neuron degeneration is the subject of intense speculation. In an effort to provide animal models with which to address this question, several groups have created mutants in which the ortholog of the human parkin gene is disrupted (Table 2). The first such model to be described was created in the fruit fly (Greene et al. 2003). In addition to a shorter lifespan, parkin-null flies exhibit compromised locomotor activity, but this was apparently attributable to muscle rather than neuronal degeneration. Indeed, with the possible exception of a very subtle (and unquantified) shrinkage of some tyrosine-hydroxylase-positive cell bodies, the brains of these flies appeared normal. The utility of these flies as a model of PD pathology might therefore be questioned, but given the lack of information concerning the physiology of their neurons, this may be premature; indeed, the abnormal mitochondrial morphology and apoptosis found in the degenerating muscle may prove to be indicative of a general mechanism of toxicity pertinent to the neuronal degeneration of PD, as the authors speculate.

Two other reports describe the generation of parkin-knockout mice, both involving deletion of exon 3 of the

Table 2 Characteristics of parkin-deficient animals

Species	Key observations	Reference
Drosophila melanogaster	Spermatid individualization failure resulting in male sterility Reduced life span Age-associated locomotor defect attributable to apoptotic cell death in muscle subsets with mitochondrial abnormalities	Greene et al. 2003
Mus musculus	Reduced body weight and temperature Behavioral defects, including reduced exploratory behavior Reduced levels of DAT and VMAT2 in the striatum Reduced [3H]-dopamine uptake in fetal midbrain cultures Decrease in amphetamine induced motor activation and [3H]-dopamine release Defects in glutamate synaptic transmission	Itier et al. 2003
Mus musculus	Increased extracellular concentration of dopamine in the striatum Decreased synaptic excitability of striatal neurons Behavioral impairments, including an increased slips/step ratio in a beam traversal task and poorer performance in an adhesive removal test	Goldberg et al. 2003

parkin gene and resulting in the absence of measurable wild-type mRNA or parkin protein (Goldberg et al. 2003; Itier et al. 2003). In each case, similar to the fly model, gross brain morphology is normal and appears to retain the normal complement of tyrosine-hydroxylase-positive neurons. Moreover, there is no indication of aberrant dopaminergic cell morphology. However, both groups have described neurochemical findings suggesting the abnormal regulation of dopamine release or intraneuronal metabolism/compartmentalization. This is potentially important given that dopamine itself has previously been proposed as a possible mediator of dopaminergic neuron toxicity because of its potential for the generation of reactive oxygen species (Lotharius and Brundin 2002). The implication is that the aberrant handling of dopamine, either intra- or extracellularly, in the parkin-knockout mouse, could represent an early stage in the degenerative disease process, which, for species-specific reasons, fails to progress to cell death. Interestingly, indications of synaptic dysregulation in these mice do not appear to be restricted to dopamine neurons, since striatal and hippocampal neurons show signs of reduced excitability. Perhaps abnormal changes in pre- or post-synaptic components of glutamatergic transmission account for these observations.

One might be tempted to dismiss the parkin-knockout mouse as an irrelevant model of PD because of the lack of dopamine cell loss. However, subtle behavioral deficits, arguably consistent with dopaminergic neuronal dysfunction, have been observed in these mice. These are not overtly obvious deficits, such as have been described in animals with chemically introduced lesions and in which a profound loss of dopamine neurons occurs, but rather have been revealed by challenging the animals in stringent tests of motor coordination. At the risk of stating the obvious, neurons become sick before they die, and such compromised neurons probably also contribute to subtle early physical signs of the disease process prior to cell loss in the human condition. Put another way, by focusing on cell death, we are restricting our study to the terminal stages of the disease process. It may prove more profitable to direct our attention to earlier signs of functional discordance within and, for that matter, between neurons if we are to understand the underlying disease process. In this context, the parkin-knockout mouse becomes a more compelling model. However, it would certainly be comforting if dopamine neurons that lack parkin in these models prove to be more susceptible than their wild-type counterparts to insults inducing cellular death. On the other hand, an understanding of why mouse neurons are particularly resistant could also be of significant therapeutic consequence.

Future directions for the genetic analysis of parkin

Mutations in the parkin gene are a major cause of early-onset and familial PD, and gene alterations have been found in nearly every population studied. Although parkin mutations are commonly identified in the majority of cases with an age of onset <21 years, the frequency of parkin mutations in older-onset cases has proven more variable among different studies. However, given the technical heterogeneity used by different laboratories to identify parkin mutations, a meta-analysis that might estimate the frequency of mutations in PD would be unreliable. Whereas it is tempting to envision a smooth decline in the frequency of parkin mutations with increasing age of disease onset, a decline that might correlate with reduced but not ablated parkin activity (such as single heterozygous parkin mutations), this has not yet been clearly indicated or reproduced, perhaps illustrating the importance of additional susceptibility factors. Nevertheless, most studies find at least twice as many parkin mutations in familial PD cases versus affected individuals with no family history of disease, and parkin mutations appear to be more common in certain ethnicities, such as northern Europeans, than in other populations, although this may be attributable to differences in genetic screening techniques among different centers.

To determine a truer estimate of the frequency of parkin mutations in familial and sporadic PD, a standard genetic screening methodology and uniform inclusion criteria for cases and controls should be applied, regardless of the size of the population to be analyzed. Exon-dosage analysis, usually performed at least in triplicate for each exon of the parkin gene, in addition to the sequencing of each exon, has been shown to identify most parkin mutations, and studies utilizing these methods usually produce higher frequencies of identified parkin mutations in any given population. However, imposing this screening requirement may present unreasonable financial and technical demands on an individual laboratory; thus, the formation of a consortium may be warranted, perhaps involving genome-sequencing centers with high throughput capabilities. Alternatively, advanced screening technologies involving array-based methods may also be developed and thereby contribute significantly to the advancement of PD genetics. Perhaps with detailed knowledge of the relationship between the various parkin mutations and disease, genetic counselors and physicians will be better informed when making decisions, and therefore the accurate identification of parkin mutations in patients will be paramount. Interestingly, a screen for mutations in the parkin gene is now commercially available for use by physicians; however, the utility of information derived from such a screen would certainly be questionable in the majority of cases. As outlined in this review, many basic questions remain to be addressed and a fundamental working knowledge of the relationship between parkin mutations and PD has not yet been attained. Therefore, the use of a parkin mutation screen for non-research purposes would appear to be premature and should be monitored closely by regulatory committees.

The existence of parkin mutation cases with a single parkin mutation remains an enigma but may reflect the nature of parkin's relationship to the development of PD. To help clarify this issue, mutation screening in these individuals should be extended to include the analysis of parkin mRNA and, when available, parkin protein. Our group has routinely been able to amplify parkin from RNA derived from cell lines or blood samples; however, parkin is poorly expressed in these tissues (methods described in Farrer et al. 2001). If wild-type parkin mRNA cannot be isolated in these cases, a second mutation that nullifies the expression and/or activity of parkin can be assumed, and further analyses might identify a novel class of mutations, such as intra-intronic rearrangements or promoter alterations. Analysis of parkin protein expression might also reflect the status of both parkin alleles; however, in our experience, parkin is expressed so poorly in the human periphery as to be undetectable by standard Western analysis.

Alterations in the parkin gene clearly cause a disease that closely overlaps clinically with sporadic PD, the major exceptions being average age of disease onset and a sustained positive response to dopamine replacement therapy. Heterogeneity in the clinical subtleties that are associated with parkin mutation cases might prevent the prediction of parkin mutations without molecular analysis; likewise, PET does not necessarily distinguish between cases with and without parkin mutations. The effects of parkin mutations are least known on the pathologic level, in the few cases that have come to autopsy. Therefore, parkin's relationship to Lewy body and neurofibrillary tangles is unclear. If a direct relationship between the parkin gene and Lewy body formation is hypothetically assumed, the complete knockout of the parkin gene might be predicted to prevent cytoplasmic inclusions from forming via parkin-related deficiencies in the ubiquitin proteasome system, whereas the loss of one parkin allele, partial reduction in parkin expression, or the presence of particular dominant negative mutants might still allow the formation of some Lewy bodies. If the formation of Lewy bodies is assumed to be a neuroprotective measure undertaken by a cell to sequester toxic cellular components, then perhaps the loss of function of a protein required for that event would result in premature cell death and therefore an earlier disease onset**.** Likewise, if partial function of parkin is retained, disease onset would be delayed, with some neurons being able to survive by forming Lewy inclusions. Although it may be many years before adequate numbers of human parkin mutation cases are pathologically evaluated to test these hypotheses, transgenic parkin-knockout animals are likely to provide insight and testable models for deciphering early events in disease pathogenesis.

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