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TITLE: PARK2, a Large Common Fragile Site Gene, is Part of a Stress Response Network in Normal Cells that is Disrupted During the Development of Ovarian Cancer

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PARK2, a Large Common Fragile Site Gene, is Part of a Stress Response Network in Normal Cells that is Disrupted During the Development of Ovarian Cancer

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13. ABSTRACT (Maximum 200 Words)

PARK2 (Parkin) is an extremely large gene that spans greater than 1.3 megabases of genomic sequence within chromosomal band 6q26. This gene is also derived from within the middle of the highly unstable FRA6E common fragile site (CFS). CFSs are large chromosomal regions that are highly unstable and prone to deletions and other alterations, especially in developing cancer cells. The central two questions that we want to address with this work are what role does the inactivation of Parkin play in the development of ovarian cancer and whether this gene functions as part of a stress response network. In order to address these two questions, we have analyzed the effect of reintroducing Parkin into ovarian cancer cell lines that do not express it. We then demonstrate that the re-introduction of Parkin is associated with greater sensitivity to the induction of apoptosis. This is consistent with our hypothesis that inactivation of this gene contributes to ovarian cancer development. In order to study whether Parkin is part of a stress response network, we have begun to characterize other proteins that Parkin interacts with, including another large CFS gene WWOX. WWOX functions as part of a stress response network and is specifically phosphorylated in stressed cells and then it binds to p53, translocates to the mitochondria and induces apoptosis. We demonstrate specific interactions between Parkin and WWOX, thus Parkin may also be part of a stress response network in normal cells that is disrupted during the development of ovarian cancer.
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Introduction
PARK2 is a gene spanning an extremely large chromosomal region of 1.36 Mbs. This large gene spans the most unstable region within the highly unstable FRA6E common fragile site (CFS), and our novel hypothesis that received Department of Defense Ovarian Cancer Research Program funding was that Parkin (and possibly other large CFS genes, including FHIT and WWOX) is part of a stress response system that is disrupted during the development of ovarian cancer. We had three Specific Aims in our proposal. The first goal was to determine if inactivation of Parkin does contribute to the development of ovarian cancer. Our second aim was to inactivate the expression of Parkin, FHIT and WWOX, singly and in combination, in normal ovarian epithelial cells and determine if this had any phenotypic effects on those cells. Our final specific aim was to directly determine if Parkin functions as part of a stress-response network within cells. We proposed to accomplish this by treating VOSE cells with a variety of genotoxic and cellular stresses and then observe the effect on the expression of the Parkin protein. We finally proposed to examine and stress VOSE cells that had FHIT and/or WWOX inactivated to determine if this altered Parkin expression.

Body
We would like to thank the Department of Defense for their support of our work. The study and characterization of common fragile sites has been a difficult field because instability in these regions extends for multiple megabases and the genes in these regions, especially the large genes, have not turned out to be traditional mutational targets in cancer. As a result, there is a notion that CFS regions are merely highly unstable chromosomal positions and that genes in these regions are merely passengers to the instability surrounding them, rather than important players in cancer development. Our laboratory has been working on the cloning and characterization of these regions for many years and it is only with the recent availability of complete genome sequences for humans, mice and rats that important insights about the CFS regions and the genes contained within them have come to light. The CFS regions, in spite of being among the most unstable chromosomal regions, are highly evolutionarily conserved as are the large genes contained within them. The co-conservation of the large genes and the highly unstable regions suggests that the two function together somehow. Complete genomic resources have greatly facilitated the characterization of the CFS regions and we have now localized over 1/3 of the 90 described CFSs. Many of these regions contain genes similar in size to Parkin; thus observations that we make about Parkin, FHIT and WWOX may be applicable to many of large CFS genes. We believe that the entire group of large CFS genes function to respond to different types of stress. In addition, the large CFS genes are highly susceptible to alterations in developing cancer cells and as a group these genes may make significant contributions to the development of ovarian cancer. The current paradigm to explain the development of most epithelial tumors is that alterations in as few as 5-7 tumor suppressors and oncogenes transform normal epithelium into invasive cancer. We believe that these initial alterations trigger increased genomic instability and this then causes alterations in multiple genes, including many of the large CFS genes. These cumulatively cause dramatic changes in the phenotype of the resulting cells, and are also responsible for the tremendous heterogeneity observed in different cancers. We hope to develop sufficient preliminary results from Department of Defense
support so that we can submit more competitive proposals to the National Cancer Institute to further characterize these important chromosomal regions.

In the past year we have made progress on each of the three Specific Aims. We will describe in detail the work that was completed for each Specific Aim.

**Specific Aim 1: To analyze the effect of reintroducing PARK2 into ovarian cancer cell lines without endogenous Park2 expression.**

We took two ovarian cancer cell lines, OV202 and SKOV3, that had no endogenous Parkin expression and established stable Parkin-expressing cell lines. In addition, we found that Parkin expression was completely abrogated in all of the hepatocellular carcinoma cell lines that we examined, so we also introduced Parkin into two HCC cell lines. After establishing stable Parkin-expressing cancer cell lines, we performed colony formation assays and cell proliferation assays to detect whether Parkin expression would modulate cell growth. The colony formation assays revealed that there was no significant difference between Parkin-expressing clones and vector-only clones; please see the figure below. However, cell proliferation assays showed significant growth retardation in Parkin-expressing clones compared with vector-only clones. These data indicate that Parkin expression can modulate cancer cell growth. The Figures below show the cell proliferation assays comparing Parkin-expressing clones to vector-only clones.

**Colony formation assay**

![Colony formation assay graph](image-url)
We then analyzed the effect of Parkin expression on cellular response to different stress treatments. We tested ovarian cancer cell lines for the effects of various stress treatments, including DNA damage, chemotherapeutic drugs, and mitochondria-dependent cell death. We observed that Parkin expression had no effect on DNA damage-induced apoptosis, including UV radiation, MMS and MMC-related DNA damage. However, Parkin expression protected cells from mitochondria-dependent apoptosis (ceramide induced apoptosis). The Figure below shows these results.

An aspect of this work was included in a paper that we published last year in *Genes, Chromosomes and Cancer*. The title of the paper was "Parkin gene alterations in Hepatocellular Carcinoma" by Fang Wang, Stacy Denison, Jin-Ping Lai, Leslie A. Phillips, Damien Montoya, Norman Kock, Birgitt Schule, Christine Klein, Viji Shridhar,
Lewis R. Roberts and David I Smith. This paper is included as Appendix 1 of this report. The results in Figure 6 of this paper shows the sensitivity of two HCC cell lines, Hep3B and PLC5, to apoptotic inducers depending upon whether they have a stably transfected Parkin construct or the empty vector. We obtained results identical to this with the two ovarian cancer cell lines. In our original application we also proposed using expression profiling to determine which genes were affected by the re-expression of Parkin. However, we also proposed a similar expression profiling experiment which looked at inactivation of Parkin expression using RNAi strategies. We did the inactivation experiment this year and this will be summarized under the next specific aim.

Specific Aim #2: To use RNAi strategies to inactivate the expression of the three large CFS genes (FHIT, WWOX and Parkin) in normal ovarian epithelial cells.

There were several goals of this Specific Aim. The first goal was to determine if inactivation of multiple large CFS genes produced discernible phenotypic changes to cells. We therefore proposed strategies to inactivate the genes FHIT, Parkin and WWOX, singly and in combination. Our second goal was to use expression profiling to determine other genes affected by the inactivation of these large genes. In the past year we focused our efforts on the second goal. We established stable Parkin-expressing PLC5 (with homozygous Parkin deletion) and then used the U133A Affymetrix chips to compare mRNA levels of approximately 22,000 human genes in cell lines with and without Parkin expression. Parkin expression has been shown to be involved in cell and matrix interaction, so we sought to identify the difference of gene expression when cells were cultured on matrigel (matrigel). As we expected, Parkin expression had high effects on gene expression when cultured on matrigel, indicating possible linkage between Parkin and cellular matrix interaction.

Table 1: Differential gene expressions in Parkin expressing and non-expressing PLC5 cells. The table lists the number of genes for which we found varying levels (up 5-fold) of differential expression in Parkin expressing PLC5 cells relative to wild-type PLC5 cells (no Parkin expression) under different growth conditions.

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Specific Aim 3: To determine if Parkin functions as part of a stress response network.

The overall hypothesis that we would like to test is that the entire group of very large CFS genes function together as some type of stress-response network. We originally proposed to just examine the three known large CFS genes at the time (FHIT, Parkin and WWOX). We proposed to treat VOSE cells with various genotoxic and cellular stresses and then observe the effect of these on Parkin expression. We also proposed to observe VOSE cells with WWOX or FHIT inactivation to see if this had any effect on Parkin expression.

In the past year there has been a number of other very large CFS genes discovered. The first one was the 1.46 Mb GRID2 gene at 4q22.3. Michelle Debatisse’s group demonstrated that this gene maps to a CFS in both mice and humans. In addition, spontaneous deletions of this gene in mice lead to the mouse mutant Lurcher, which lose 100% of their Purkinje cells postnatally. Interestingly, a spontaneous deletion which removed the Parkin gene and its neighbor PACRG results in another mouse neurological mutant called Quaker (viable).

Since there are a number of extremely large CFS genes, we contacted the University of California, Santa Cruz Human Genome Database to obtain lists of the largest known human, mouse and rat genes. We found that there were far more extremely large genes than anticipated. There are a total of 40 human genes that span greater than 1.0 Mb of genome sequence and another 200 that span between 500 Kb and 1.0 Mb. Interestingly, many of the largest human genes map to chromosomal bands known to contact a CFS. In the past year we have examined several other very large genes, including the 2.3 Mb CNTNAP2 gene, the 2.0 Mb DMD gene, and the 1.5 Mb DAB1 gene. We have discovered that each of these are large CFS genes. Thus far we have found that 6 of the 10 largest human genes are CFS genes. This is extremely important, as anything that we find out with Parkin, FHIT or WWOX may be generalized to all the large CFS genes.

We hypothesized that Parkin might be interacting with other large CFS genes. To test our hypothesis, we tested for possible functional interactions between WWOX and Parkin. The yeast two hybrid experiments revealed that Parkin interacts with wild type WOX1 but not the mutant WOX1 (Y33R). Co-immunoprecipitation confirmed the interaction between WOX1 and Parkin as shown in Figure A. We also observed that Parkin expression specifically decreased wild type WOX1 protein level as shown in Figure B, but did not decrease the expression of mutant WOX1. Moreover, Parkin expression blocked WOX1-induced apoptosis, which was consistent with our previous Specific Aim 1 observation that WOX1-induced apoptosis is mitochondria-dependent and is involved in the p53 and JNK pathway. In addition, Parkin expression protected mitochondrial swelling and blocked WOX1-induced cell death. We also found that Parkin expression markedly reduced the cytosolic protein level of WOX1 (Figure C), and this reduction was abrogated by proteasome inhibitors. This suggests that Parkin, a ubiquitin E3 ligase, may degrade cytosolic WOX1. This is currently under investigation.
Figure A. Parkin interacts with wild type human WOX1 and mouse WOX1 in vitro. To identify the possible interaction of Parkin and WOX1, Parkin-myc and WOX1-GFP plasmids were transfected into COS1 cells, and then total cellular protein was collected and subjected to co-immunoprecipitation. By use of anti-GFP and anti-myc antibody, respectively, we found that Parkin specifically precipitated with human WOX1 and mouse WOX1, not the GFP tag, indicating that Parkin interacts with WOX1 in vivo.
Figure B. Parkin expression decreased WOX1 protein level. COS1 cells were transiently transfected with different expression plasmids as indicated in the figure. Cytosol protein was collected and subjected to immunoblotting to detect the expression of GFP-WOX1 varieties in the presence or absence of Parkin. Actin was used as an internal control. Parkin expression reduced the protein level of hWOX1 and mWOX1, but not dominant negative (DN) WOX1 and Y33R mutant WOX1.
Parkin expression decreased the cytosolic level of WOX1 (Figure C). WOX1 is a proapoptotic protein capable of interacting with p53. A large portion of endogenous WOX1 protein is located in the mitochondria, and the mitochondrion-targeting sequence has been mapped within the ADH domain. This is consistent with Parkin’s location, as we found a proportion of Parkin was present in mitochondria. During apoptosis or stress responses, there is an increased synthesis of cytosolic WOX1, followed by translocation to the mitochondria. Most conclusively, by electron microscopy we have demonstrated an increased presence of WOX1 in the damaged mitochondria and condensed nuclei of retinal degenerating photoreceptors in rats. Induction of mitochondrial permeability transition by TNF, staurosporine, and atracyloside results in WOX1 release from the mitochondria and subsequent nuclear translocation.

Thus, we assume that the decrease in the cytosolic level of WOX1 by Parkin would block most of the pre-apoptotic function of WOX1, and meanwhile interfere with the translocation of WOX1. We transfected WOX1 and Parkin together into HEK293 cells and detected the cytosolic WOX1 level in the presence or absence of Parkin expression under the treatment of Staurosporine. As shown in Figure C below, without Parkin expression, the cytosolic WOX1 level was abundant. With the staurosporine treatment, cytosolic WOX1 was decreased due to translocation. However, with Parkin expression, we observed decreased cytosolic levels of WOX1 and blockage of translocation of WOX1 under staurosporine treatment. These data represent part of the mechanism by which Parkin blocks WOX1-induced apoptosis.

Figure C: mWOX1 was co-transfected with pcDNA3.1 or Parkin into HEK293 cells. 48hr after transfection, cells were incubated with staurosporine (5 uM) for indicated times, and cytosol was collected by use of NucBuster extraction kit (Novagen). mWOX1 expression was detected by use of anti-GFP Abs, Actin was detected as an internal control.
Key Research Accomplishments
We had three Specific Aims to determine the role that Parkin plays in the development of ovarian cancer. These aims were:

Specific Aim 1: To analyze the effect of reintroducing PARK2 into ovarian cancer cell lines without endogenous Park2 expression.

Specific Aim #2: To use RNAi strategies to inactivate the expression of the three large CFS genes (FHIT, WWOX and Parkin) in normal ovarian epithelial cells.

Specific Aim 3: To determine if Parkin functions as part of a stress response network.

Our key research accomplishments are:
(1) We have demonstrated that the reintroduction of Parkin into ovarian cancer cell lines (as well as cell lines from other cancers) results in cells that are more sensitive to inducers of apoptosis. This is consistent with our hypothesis that the inactivation of Parkin during the development of ovarian cancer contributes to the cancer phenotype.

(2) We have begun to demonstrate how Parkin interacts with another very large common fragile site gene, WWOX. Since WWOX has been shown to be functioning as a stress-response gene (this gene is specifically phosphorylated in response to stress and then binds to p53 to induce apoptosis), this provides further evidence that Parkin is also involved in cellular responses to stress.

(3) We now have evidence that many of the largest human genes (not just FHIT, Parkin and WWOX) are localized within common fragile site regions. The findings that we make with FHIT, Parkin and WWOX may be representative of what is occurring in all of the large CFS genes.

Conclusions
We are continuing to study the very exciting Parkin gene to determine its function in the normal cell and understand how loss of Parkin expression contributes to the development of ovarian cancer. We now know that loss of expression of this gene is associated with greater resistance to induction of apoptosis and that this gene interacts with another large CFS gene, WWOX. In the next year we will be examining how this gene functions to respond to stress. In addition, we will look for more functional connections between Parkin and other large CFS genes involved in stress response.

Appendix
Parkin Gene Alterations in Hepatocellular Carcinoma

Fang Wang,1 Stacy Denison,1 Jin-Ping Lai,2 Leslie A. Phillips,1 Damien Montoya,2 Norman Kock,3 Birgitt Schüle,4 Christine Klein,1 Viji Shridhar,1 Lewis R. Roberts,2 and David I. Smith1*

1Division of Experimental Pathology, Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, Minnesota
2Division of Gastroenterology and Hepatology, Department of Internal Medicine, Mayo Clinic College of Medicine, Rochester, Minnesota
3Departments of Neurology and Human Genetics, University of Lübeck, Lübeck, Germany
4Department of Genetics, Stanford University School of Medicine, Stanford, California

The Parkin gene is an extremely large gene (1.5 Mb) within the highly unstable FRA6E common fragile site (CFS) region, which is frequently altered in ovarian, breast, and hepatocellular carcinomas. Because Parkin/FRA6E has genomic similarities to FHIT/FRA3B and WWOX/FRA16D, two other large tumor-suppressor genes that are within CFS regions, we were interested in characterizing Parkin gene alterations and their possible association with cancer. After analyzing 50 cancer-derived cell lines including 11 hepatocellular carcinoma (HCC) cell lines, we found that one HCC cell line, PLC/PRF/5, had a detectable homozygous deletion encompassing exon 3. Using quantitative duplex PCR and fluorescence in situ hybridization analysis to characterize the copy number changes of Parkin exons in HCC cell lines, we found that 4 of 11 HCC cell lines had heterozygous deletions of Parkin exons and one, Hep3B, had an exon duplication. Parkin protein expression was significantly decreased or absent in all 11 HCC cell lines. Furthermore, more than 50% of HCC primary tumors had decreased Parkin expression compared to that in normal liver tissue. Parkin gene–transfected PLC5 and Hep3B cells grew more slowly than vector-only transfectants and also showed increased sensitivity to apoptosis induced by cell-cycle inhibitors. Therefore, we suggest that Parkin may be involved in tumor suppression and that the loss of Parkin contributes to the development of hepatocarcinoma.

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INTRODUCTION

The Parkin gene was first identified as a mutational (point mutation and exonic deletion) target in a proportion of patients with autosomal recessive juvenile parkinsonism (ARJP) (Hattori, 1998; Kitada et al., 1998; Periquet et al., 2001). The Parkin gene contains 12 small exons and encodes an E3 ligase, which is comprised of 465 amino acids with a molecular mass of approximately 52,000 Da. The gene itself is extremely large and spans 1.5 Mb of genomic DNA in chromosomal band 6q26. Various deletions and point mutations in Parkin have been discovered in patients with ARJP (Hedrich et al., 2002; Rawal et al., 2003; Tan, 2003). However, large genome rearrangements resulting in the deletion or duplication of Parkin exons account for approximately 50% of the alterations observed in patients with ARJP.

The location of Parkin is in a chromosomal region that is frequently deleted in multiple tumor types, including hepatocellular carcinoma (HCC), ovarian cancer, and breast cancer. The Parkin gene is within FRA6E, the third most active common fragile site (Kakhkonen, 1988; Denison et al., 2003a). Common fragile sites (CFSs) are chromosomal loci that are susceptible to forming gaps and/or breaks when cells are cultured in the presence of specific inhibitors of DNA replication, such as aphidicolin (Rao et al., 1988). The two most active CFSs are FRA3B, at 3p14.2 (Becker et al., 2002), and FRA16D, at 16q23.2 (Mangelsdorf et al., 2000). These CFSs (FRA3B, FRA16D, and FRA6E) also occur in chromosomal regions that are frequently deleted in HCC and many other tumor types (Yuan et al., 2000; Yakiciier et al., 2001; Denison et al., 2003a; Huebner et al., 2003). Instability within FRA3B and FRA16D extends for 4.0 and 2.0 Mb, respectively, and spanning the most unstable regions within these CFSs are two extremely large genes, FHIT (fragile histidine triad), which is 1.4 Mb (Ohta et al., 1996), and WWOX (WW do-
main–containing oxidoreductase), which is 1.0 Mb (Bednarek et al., 2000). Alterations in these two genes are frequently observed in multiple tumor types, and the proteins encoded by these genes are absent or decreased in many different cancers (Lee et al., 2001; Driouch et al., 2002; Yendamuri et al., 2003). However, FHIT and WWOX do not appear to be traditional mutational targets in cancer because point mutations are rarely found within them, although many tumors have larger heterozygous deletions within the CFS regions that presumably result in inactivation of gene expression.

We recently characterized the FRA6E region and found that instability within this region extended for 3.5 Mb (Denison et al., 2003a). A number of small genes are encoded within the centromeric half of FRA6E, but the distal half, which also contains its most unstable region, is spanned by the 1.5-Mb Parkin gene. We found that Parkin expression was decreased in 60% of primary ovarian tumors, and we also detected tumor-specific alternative transcripts of Parkin in primary ovarian tumors and in ovarian cancer cell lines (Denison et al., 2003b).

Cesari et al. (2003) performed a loss of heterozygosity (LOH) analysis with 40 malignant breast and ovarian tumors and identified a common minimal deleted region, which includes the markers D6S305 and D6S1599. These markers are within the large Parkin gene. In addition, they identified a homozygous deletion of exon 2 of Parkin in two lung adenocarcinoma cell lines, Calu-3 and H-1573. They also detected absent or diminished Parkin expression in breast and ovarian cancer cell lines and in primary tumors.

These three CFS regions and the large genes contained within them, therefore, have a number of similarities. A more detailed analysis of FHIT revealed that this gene is inactivated in many tumor types and that the inactivation of this gene can be detected early in cancer development, even in premalignant lesions (Birrer et al., 1999; Hao et al., 2000). The reintroduction of FHIT into some, but not all, cancer-derived cell lines can inhibit cell growth and, in some instances, can induce apoptosis (Ji et al., 1999; Sard et al., 1999). Similar alterations have been detected in WWOX in multiple tumor types, and cellular stress causes WWOX to interact with P53 to induce apoptosis (Chang et al., 2002, 2003). Hence, these large CFS genes are frequently inactivated in tumors, and their loss can play an important role in tumor development.

We report here on a study in which we examined 11 hepatocellular carcinoma cell lines in a search for Parkin gene alteration. We also observed decreased or absent Parkin protein expression in HCC cell lines and primary tumors. Stable expression of the Parkin gene in PLC5 and Hep3B HCC cells resulted in enhanced sensitivity to apoptosis induced by cell-cycle inhibitors.

MATERIAL AND METHODS

Reagents

Parkin antibodies were obtained from CellSignaling (Beverly, MA) and Chemicon (Temecula, CA). Parkin expression constructs were kindly provided by Dr. Paul J. Lockhart (Mayo Clinic, Jacksonville, FL). Plasmid pCDNA3.1 (+)/myc-his A was obtained from Invitrogen (Carlsbad, CA) and was used as a transfection vector–only control. Normal liver tissues and HCC primary tumors were obtained from surgical sections performed at the Mayo Clinic (Rochester, MN). Staurosporine, Trichostatin A (TSA), sodium butyrate, and DAPI were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Western Blotting Analysis of Parkin Expression

The HCC cell lines HepG2, Hep3B, PLC/PRF/5 (PLC5), Huh7, SNU182, SNU387, SNU398, SNU423, SK-Hep1, SNU449, and SNU 475 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to ATCC’s protocols. Western blotting was performed according to standard methods. Signals were detected using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

Screening for Homozygous Exonic Deletions in Cancer-Derived Cell Lines

Primers unique for each of Parkin’s 12 exons were synthesized according to Kitada (Kitada et al., 1998) at the Mayo Clinic Molecular Core Facility (Rochester, MN) and tested on a DNA panel consisting of genomic DNA isolated from 50 cancer-derived cell lines. Briefly, genomic DNA was isolated from cells by standard phenol–chloroform methods. The PCR mix (20 μl reaction volume) contained 20 ng of genomic DNA, 1× PCR buffer, 2 μl of dNTP (10 mM), 0.5 μM primers, and 1 U Taq polymerase (Promega, Madison, WI). The conditions for amplification were: 94°C for 3 min; then 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec; with a final extension of 72°C for 10 min.
Quantitative Duplex PCR for Parkin Gene Dosage Studies in Hepatocellular Cells

To analyze the HCC cell lines for heterozygous deletion (hD), homozygous deletion (HD), or exon duplications, we used quantitative duplex PCR based on the fluorescence resonance energy transfer technique on a LightCycler (Roche Diagnostics, Indianapolis, IN) according to Hedrich et al. (2001). Eleven HCC cell lines and two normal liver tissue samples (positive controls) were used as shown in Table 1. The genomic DNA was isolated using standard phenol–chloroform methods. The PCR samples (20.0 μl total volume) contained 50 ng of genomic DNA, 1× PCR buffer (supplied with enzyme), 4 mM MgCl₂, 0.2 mM dNTP, 0.3× Sybr green (Molecular Probes, Inc., Eugene, OR), 0.05% BSA, 0.5 μM concentration of forward and reverse primers for the specific exons, and 0.1 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA). The conditions for amplification were as previously described. The gene dosage ratio of Parkin exons was compared to the control gene β-actin (HBB) in order to identify deletions and/or duplications of each exon of Parkin. A standard curve was generated by use of human genomic DNA (Roche Diagnostics, Indianapolis, IN) in concentrations of 5, 1.25, and 0.3125 ng/μl. All standards (and samples) were amplified in duplicate, followed by calculation of a regression curve, based on which sample concentrations were inferred and accepted only when within the range of the standard templates.

Fluorescence In Situ Hybridization

Metaphase/interphase preparations were obtained from each of the HCC cell lines. Cell harvest and metaphase preparations were performed by routine cytogenetic techniques. Three different bacterial artificial chromosomes (BACs) were used to determine (1) whether any HCC cell line exhibited aneuploidy for chromosome 6 and (2) the Parkin dosage level for each cell line. The three BACs were a centromere probe specific to chromosome 6, RP-1 179P19 (which contains a portion of exons 3 and 4 of Parkin), and CTD-264317 (which contains a portion of HBB). For clones RP-1 179P19 and CTD-264317, 1 μg of purified DNA was labeled with biotin-16-dUTP (Boehringer/Roche, Indianapolis, IN) by nick translation, precipitated, and hybridized to the interphase chromosomes according to the protocol described by Verma and Babu (1995). Photomicroscopy was performed with a Zeiss Axioplan 2 fluorescence microscope and IPLab Spectrum P software (Scanalytics Inc., Fairfax, VA). A minimum of 50 interphase cells were analyzed for each individual HCC cell line.

Sequencing of Parkin Transcripts of PLC5 Cells

The total RNA from PLC5 cells was isolated with Trizol reagent (Gibco BRL, Rockville, MD) according to the manufacturer’s instructions. To amplify the complete coding region of the Parkin gene, 5 μg of total RNA was subjected to RT-PCR using 3 sets of primers, according to Kuroda (2001). Exons 1–6: forward, 5'-GACCATGATAGTGTTT-3' (nt 98–114); and reverse, 5'-GATGTCTCCGACTATTGTTTGGCATCAGT-3' (nt 781–809); exons 2–9: forward, 5'-CTGAGATGCTTCGACTGACTG-3' (nt 249–267); reverse, 5'-GACTTCTGTGGACCATACT-3' (nt 1051–1069); and exons 9–12: forward, 5'-GTCAACCCGTACAGCA GTATG-3' (nt 1034–1056); reverse, 5'-CTAACAGCGTCCAGACAGTGGTC-3' (nt 1479–1499). The PCR reactions were performed as above, and the amplified products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced by the Molecular Biology Core Facility at the Mayo Clinic, Rochester.

Construction of Stably Parkin-Expressing PLC5 and Hep3B Cell Lines

PLC5 and Hep3B cells were maintained in MEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. The Parkin expression vector was pcDNA3.1/Parkin, and the vector-only control was pcDNA3.1. Before transfection, the exponentially growing cells were washed with serum-free medium and transfected with 5 μg of plasmids and 20 μl of Lipofectamin 2000 (Invitrogen) according to the manufacturer’s instructions. After a 5-h incubation, complete growth medium was added. After 48 h, transfected cells were selected in 400 μg/ml Geneticin for 2–3 weeks. Subsequently, cloning cylinders were used to clone individual colonies. For each cell line, eight clones were selected and detected by Western blotting against the Parkin antibody. For each Parkin-expressing cell line, we chose the three most abundant expressing clones for further study.

Immunocytochemistry Detecting Parkin Localization

Parkin-expressing PLC5 and Hep3B cells as well as empty vector transfectants were plated in 4-well chambers in complete growth medium. After 24 hr, cells were washed three times with PBS and fixed in methanol (−20°C) for 10 min, followed by per-
<table>
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<th>Cell Line</th>
<th>Ratio</th>
<th>Normal</th>
<th>Het. Del.</th>
<th>Het. Dup.</th>
<th>Ex1</th>
<th>Ex2</th>
<th>Ex3</th>
<th>Ex4</th>
<th>Ex5</th>
<th>Ex6</th>
<th>Ex7</th>
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The Parkin gene dosage ratio was compared to that of the control gene HBB (β-actin) and adjusted by FISH. Only one cell line, PLCS, showed an HD of exon 3 and an HD of exon 4; SK-Hep 1 had an HD of exon 3; SNU398 had an HD of exons 4 and 8; Hep3B had a duplication of exon 5 and an HD of exon 9.

*The ratio of hybridization signals observed on interphase nuclei for a BAC (RP1-179P19) containing exons 3 and 4 of the Parkin gene compared to that of a BAC (264317) containing a portion of HBB.

*Normal range adjusted for differences in copy number.

*Homozygous deletion range based on copy number.

*Homozygous duplication range based on copy number.

*Because in PLCS the Parkin gene contains a homozygous deletion for exon 3, a BAC (94P15) from another part of the Parkin gene was hybridized, and the ratios were calculated.
meabilization with 0.1% Triton X-100 for 5 min. Fixed cells were incubated in 5% normal goat serum for 1 hr and then incubated with anti-Parkin primary antibody at 4°C overnight. Primary antibody was detected after incubation in FITC-conjugated IgG, and the nuclei area was stained with DAPI. Images were viewed with an Axiovert 35 epifluorescence microscope (Carl Zeiss, Thornwood, NY).

Proliferation Assay

Parkin-expressing PLC5 and Hep3B cells and the corresponding vector-only transfected cells were plated at 5,000 cells/well in triplicate in 24-well plates with complete medium. For each cell line, three independent clones were subjected to a proliferation assay. The total cell number was measured every 2 days for a total of 10 days with the MTS assay (Chemicon, Temecula, CA) according to the manufacturer’s instructions.

Analysis of Apoptosis

Hep3B and PLC5 cells (Parkin-expressing or vector-transfected only) were plated in 6-well plates at a density of 200,000 cells/well. Before treatment, cells were washed with PBS and changed to the serum-free medium. Staurosporine, TSA, and sodium butyrate were added to the serum-free medium at the indicated concentrations and incubated for 5 hr (staurosporine) or 24 hr (TSA and sodium butyrate). Apoptosis was measured by assessing the number of cells containing nuclear changes (chromatin condensation and nuclear fragmentation) after staining with DAPI (final concentration of 5 μg/ml). After incubation in the dark with DAPI for 20 min, cells were examined by fluorescence microscopy (Nikon Eclipse TE200; Nikon Corp., Tokyo, Japan). The experiments were performed in triplicate, and at least 300 cells were counted in each individual treatment.

RESULTS

Screening of 50 Cancer-Derived Cell Lines for Homozygous Deletion of Parkin Exons

We screened each Parkin exon for homozygous deletion in a panel of 50 cancer-derived cell lines by using genome DNA amplification (data not shown). Only a single cell line, PLC5, had a detectable homozygous deletion of a Parkin exon. This cell line exhibited no amplification of exon 3 (Fig. 1A). Sequence analysis of the PCR-amplified cDNA of Parkin in the PLC5 cells using three sets of primers specific to exons 1–6, 2–9, and 9–12 confirmed the deletion of exon 3, as the 3' end of exon 2 was directly connected to the 5' end of exon 4 (Fig. 1B).

Quantitative Duplex PCR and FISH for Parkin Exon Dosage Studies in HCC Cell Lines

We used quantitative duplex PCR to map possible homozygous (HD) and heterozygous (hD) deletions of Parkin gene exons according to Hedrich et al. (2001). This technique was applied to the analysis of the 11 HCC cell lines (including PLC5) for heterozygous deletions and/or duplications of Parkin exons. The gene dosage ratio of the Parkin gene was compared to the control gene β-actin (HBB) in order to identify deletions and/or duplications of whole exons of Parkin. The normal gene dosage for any Parkin exon as compared to HBB was determined to a range of 0.8–1.2. Dosage values less than 0.4, from 0.4 to 0.6, and from 1.4 to 1.6 were identified as representing an HD, hD, and heterozygous duplications, respectively, for that Parkin exon. However, these ratios specifically reflected the gene dosage levels for karyotypically normal individual cells.

Karyotypic analysis of primary tumors, as well as of tumor-derived cell lines, has demonstrated that most cancers are aneuploid, with variation in both chromosome number and structure (Knudson, 2001). Hence, we utilized a fluorescence in situ hybridization (FISH)-based technique to quantify the copy number of 6q sequences surrounding Parkin in the HCC cell lines (Fig. 2). We then modified the gene dosage ratios in order to accurately reflect the changes in the gene dosage ratio of Parkin exon to HBB that could calculate the exon variation observed among the cell lines. We analyzed a total of 50 interphase cells for each of the 11 cell lines in order to determine the ratio of observed hybridization signals of Parkin to HBB (Table 1). Each HCC cell line had a unique, relatively “normal” Parkin gene dosage ratio. For example, for SNU182 cells, the normal ratio is between 1.3 and 1.9, whereas for Hep3B cells the normal ratio is 1.4–2.1.

Quantitative duplex PCR analysis of PLC5 confirmed the HD mapping data and identified PLC5 as having a homozygous deletion of exon 3 (Parkin/HBB ratio of 0.03; Table 1), as well as a heterozygous deletion of exon 4 (Parkin/HBB ratio of 0.33; Table 1). SNU398 had a heterozygous deletion of exons 4 and 8, SK-Hep1 had a heterozygous deletion of exon 3, and Hep3B had both a heterozygous duplication of exon 5 and a heterozygous deletion of exon 9 (Table 1).
**Figure 1.** Homozygous deletion of exon 3 of Parkin gene in PLC5. (A) Genomic DNA PCR of 12 exons of Parkin gene in PLC5 cells showed the absence of exon 3 (B) Quantitative duplex PCR identified that confirmed the exon 3 and exon 4 was deleted in PLC5 cells. The ratio was determined by comparison with the PCR of the control, the HBB gene. Exon 3 represents the homozygous deletion and exon 4 was identified as heterozygous deletion.

**Figure 2.** FISH analyses of HCC cell lines to determine the ratio of Parkin exons to HBB. Probes for Parkin (yellow), HBB (red), and the centromere of chromosome 6 (aqua) were hybridized to interphase chromosomes obtained from each cell line. Chromosomal interphases were counterstained with DAPI. All three possible ratios are represented in the figure: (A) 1:1 ratio of Parkin to HBB in SNU398; (B) <1:1 ratio of Parkin to HBB in SNU475; (C) >1:1 ratio of Parkin to HBB in SNU182.

**Parkin Protein Expression in HCC Cell Lines and Primary Tumors**

Using Western blotting, we found that Parkin protein was completely absent or diminished in all 11 HCC cell lines tested compared with its level in normal liver tissue. As shown in Figure 3A, only SNU423, HepG2, SNU387, and SNU475 had Parkin protein expression. These data were confirmed using two different Parkin antibodies, which were raised against amino acids 295-311 of human Parkin and full-length Parkin, respectively. We also performed immunoblotting on extracts from HCC primary tumors. We found that more than 50% of primary tumors had diminished or absent Parkin protein expression compared to the benign pair (Fig. 3B).
A.

<table>
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<tr>
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<th>snu423</th>
<th>snu449</th>
<th>Hep3B</th>
<th>PLC5</th>
<th>HepG2</th>
<th>snu387</th>
<th>snu475</th>
<th>Huh7</th>
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![Parkin and actin blot](image)

B.

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![Parkin and actin blot](image)

Figure 3. Western blotting of Parkin expression in hepatocellular carcinoma cell lines and primary tumors. (A) Parkin antibody recognizes full length of human Parkin protein (NL: normal liver). All 11 HCC cell lines show decreased or absent Parkin expression compared to normal liver. (B) Parkin protein expression in hepatocellular carcinoma primary tumors. 10 of 12 HCC primary tumors show decreased or absent Parkin expression.

**Growth Retardation and Increased Sensitivity of Apoptosis of Parkin-Expressing PLC5 and Hep3B Cells**

We selected the PLC5 and Hep3B cell lines into which to reintroduce the Parkin gene and to detect potential physiological differences before and after Parkin gene expression. We picked up 8 individual clones after transfection and detected Parkin protein expression by using Western blotting (Fig. 4A). We chose the three most abundant Parkin-expressing clones for use in the proliferation and apoptosis assays. Meanwhile, using immunocytochemistry, we showed that in both the PLC5 and Hep3B transfected cells, Parkin protein was mostly localized in the cytoplasm and that there was no colocalization with DAPI (nuclear) (Fig. 4B). Results were confirmed in PLC5 and Hep3B, two independent Parkin-expressing cell lines. This was consistent with other reports that Parkin is a cytoplasmic protein.

To determine whether Parkin expression affects cell growth, we performed proliferation assays on vector-only PLC5 and Hep3B cells and on three independent Parkin-expressing clones of each cell line, PLC5, and Hep3B. In each well of 24-well plates, 5,000 cells were plated, and the MTS assay was used to measure the cell number every 2 days over a 10-day period (Fig. 5). Parkin-expressing clones grew more slowly than empty vector transfectants in both the PLC5 and Hep3B cell lines.

To examine the effects of Parkin expression on apoptosis, Parkin-expressing clones and vector-
transfected clones were exposed to different treatments. Three apoptosis-inducing drugs were used in the apoptosis induction assay: staurosporine, TSA, and sodium butyrate. Staurosporine is a widely used proapoptotic stimulus that acts as a broad-spectrum kinase inhibitor. Both TSA and sodium butyrate are potent and specific inhibitors of mammalian histone deacetylase, which induce cell apoptosis by regulating the eukaryotic cell cycle. For staurosporine treatment, cells were incubated at concentration ranging from 0 to 5 μM for 5 h and then stained with DAPI to detect, by fluorescence microscopy, nuclear condensation and fragments, which are evidence of apoptotic cells (Fig. 6). In the other two treatments, the concentrations of TSA ranged from 0 to 1 μM and of sodium butyrate from 0 to 10 mM. Parkin expression had no effect on staurosporine-induced apoptosis. In contrast, Parkin-expressing clones were more sensitive to either TSA- or sodium butyrate-induced apoptosis than were vector-transfected clones. The effect was significant at low drug concentrations (TSA at 0.25 and 0.5 μM, sodium butyrate at 1 and 2 mM). Similar results were obtained in all three PLC5 and all three Hep3B Parkin-transfected clones. These results showed that Parkin enhanced the sensitivity of TSA- and butyrate-induced apoptosis (Fig. 6A–C).

**DISCUSSION**

Common fragile sites are highly unstable chromosomal regions that predispose chromosomes to breakage and rearrangement (Michels, 1985; Smith et al., 1998). They are present in all individuals, but some are more highly expressed than others. More highly expressed CFSs include FRA3B (3p14.2), FRA16D (16q23.2), and FRA6E (6q26). Interestingly, all three regions were found to be consistently deleted in HCC (Kitay-Cohen et al., 2001; Yakiciier et al., 2001; Zhao et al., 2003) as well as in ovarian, breast, and prostate cancers. FRA3B, the most active CFS, spans 4.5 Mb within chromosome segment 3p14.2, and the 1.4-Mb FHIT gene is in the center of FRA3B (Ohta et al., 1996). FHIT is a diadenosine triphosphate hydrolase whose substrates are produced in cells in response to stress (Brenner et al., 2002). The reintroduction of FHIT into specific cancer-derived cell lines results in the induction of apoptosis (Ji et al., 1999; Sard et al., 1999). FRA16D spans 2 Mb and contains the
PARKIN ALTERATION IN HEPATOCELLULAR CARCINOMA

PLC5/vector region extends for 4.0, 2.0, and 3.5 Mb, respectively. Spanning the unstable region within each CFS are extremely large genes comprising relatively small exons, and aberrant transcripts of these genes are frequently observed in tumors. In addition, an absence of expression of all three genes has been observed in HCC and many other tumors.

Polymorphic markers within the large Parkin gene showed the highest LOH of any 6q26 markers tested against a panel of primary ovarian tumors (Denison et al., 2003b). Semiquantitative PCR revealed that Parkin mRNA was decreased or absent in many ovarian cancer cell lines and primary tumors (Denison et al., 2003b). Recently, Cesari et al. (2003) also identified an altered Parkin gene and decreased protein in breast, ovarian, and lung cancer cell lines, and they proposed that Parkin was a candidate tumor-suppressor gene.

Parkin was originally identified as a mutational target in patients with autosomal recessive juvenile parkinsonism, a rare form of parkinsonism associated with an early age of onset (Hattori, 1998; Kitada et al., 1998; Periquet et al., 2001). Mutations in Parkin have been demonstrated to be responsible for the pathogenesis of ARJP. Patients with ARJP have large deletions or duplications of Parkin exons more frequently than point mutations, although 1- or 2-bp, even 40-bp, small-fragment deletions have also been observed in patients (Hattori, 1998; Kitada et al., 1998; Periquet et al., 2001; Hedrich et al., 2002; Rawal et al., 2003; Tan, 2003). These mutations lead to amino-acid substitutions or to truncation of the Parkin protein. Mutations have been documented throughout the entire Parkin gene, but the majority localize between exons 2 and 8 of Parkin, which correspond to the most unstable region within FRA6E.

We tested a panel of 50 cancer-derived cell lines searching for homozygous deletions of Parkin exons. One HCC cell line, PLC5, had a detectable homozygous deletion encompassing exon 3 of Parkin. We then used the quantitative duplex PCR techniques of Hedrich et al. (2001) to characterize homozygous and heterozygous deletions and duplications of Parkin exons in 11 HCC cell lines. This technique was developed to characterize Parkin alteration in ARJP patients, who are generally diploid for 6q26 sequences. Because many cancer-derived cell lines are aneuploid, we utilized FISH analysis to determine copy numbers of the 6q26 sequences in the 11 HCC cell lines. We then could use quantitative duplex PCR to standardize the values obtained in order to determine accurately whether there were any deletions or duplications of

1.0-Mb WWOX gene, which also has been found to be frequently altered in multiple tumor types (Bednarek et al., 2000; Yakicier et al., 2001). Chang et al. (2002, 2003) showed that cellular stress induces phosphorylation of WWOX and that this phosphorylated protein functions to induce apoptosis by binding to P53 protein. Hence, both CFS genes appear to play an important role within the cells, and loss of expression of one or both is commonly observed in multiple tumor types, including HCC.

Parkin is the third very large gene that has been mapped within a frequently deleted CFS region (Denison et al., 2003a, 2003b). There are many similarities between Parkin/FRA6E, FHIT/FRA3B, and WWOX/FRA16D. FRA3B, FRA16D, and FRA6E are the three most active CFSs. Instability within each region extends for 4.0, 2.0, and 3.5 Mb, respectively.
Figure 6. Parkin expression enhanced the sensitivity of Hep3B and PLC5 cells to apoptosis induced by TSA and sodium butyrate treatments. Parkin-expressing cells and vector-only cells were incubated at the indicated concentrations of TSA. After 24 h of incubation, cells were stained with DAPI (5 μg/ml) for 20 min. Apoptosis percentage was measured by counting the number of cells containing nuclear changes (condensation and fragments). The non-apoptotic cells remain intact. At least three hundred cells were counted, and each experiment was performed in triplicate. (A) Effect of Parkin-expression on staurosporine-induced apoptosis. Three independent clones of Hep3B/Parkin, PLC5/vector, PLC5/Parkin1, PLC5/Parkin2, and PLC5/Parkin3 were subjected to staurosporine treatment (0, 0.5, 1, 2, and 5 μM) for 5 h. Parkin 1, 2, and 3 refer to three different Parkin-expressing clones. (B) Effect of Parkin expression on TSA-induced apoptosis. Cells were treated with TSA (0, 0.25, 0.5, 1 μM) for 24 h. (C) Effect of Parkin expression on sodium butyrate-induced apoptosis. Cells were incubated with sodium butyrate (0, 1, 2.5, 5, 10 mM) for 24 h. The apoptosis percentage in three treatments was measured by fluorescence microscopy nuclear counting after DAPI staining.
Parkin exons. The deletions and duplications we found to occur within the "center," which is the most unstable region, of the FRA6E CFS. Most importantly, we found that most of the HCC cell lines and primary tumors had undetectable or decreased Parkin protein. However, the molecular mechanism responsible for diminished or absent Parkin expression is unknown because only two of the cell lines showed significant changes in the Parkin gene. One HCC cell line, PLC5, had a homozygous deletion and the other, Hep3B, an exon duplication. However, it was presumed that instability within the highly unstable, large CFS region may result in multiple deletions and point mutations in the large Parkin gene. Aberrant regulation of gene expression may also play a role in the absence of Parkin protein.

To determine whether the loss of Parkin could play a role in the development of HCC tumors, we generated stable Parkin-expressing PLC5 and Hep3B cells. After reintroducing the Parkin gene, we measured cell growth and also treated the Parkin-expressing cells with apoptosis-inducing drugs. We found slight growth retardation of the two independent PLC5/Parkin-expressing and Hep3B/Parkin-expressing cells compared to in vector-only transfectants, but no gross growth retardation or cell apoptosis was observed under normal cell culture conditions. We then observed the effects of Parkin protein expression on sensitivity to apoptosis after treatments with three drugs. As mentioned above, TSA and sodium butyrate are cell-cycle inhibitors, and staurosporine is a potent kinase inhibitor. We chose to examine the effect of cell-cycle inhibitors because that Parkin protein has been shown to bind directly to microtubules and to enhance tubulin degradation (Ren et al., 2003). Parkin protein is also recruited to the centrosome in response to the inhibition of proteasomes (Zhao et al., 2003). Centrosome duplication, DNA replication, and cell-cycle regulation are tightly linked, and the balance of microtubule stabilization and destabilization plays an important role in cancer development. We hypothesized that Parkin might be involved in cell-cycle regulation, delaying cell-cycle progression under certain cellular stresses. As we expected, Parkin expression enhanced the sensitivity of cells to TSA and butyrate treatment, resulting in cell-cycle arrest and increased cell death. In contrast, Parkin-expressing cells showed no difference in sensitivity to treatment with staurosporine, as non-Parkin-expressing cells and Parkin-expressing cells that received staurosporine treatment showed a similar percentage of apoptotic cells.

Two aspects of these results require particular emphasis. First, Parkin protein expression alone did not lead to an increased number of apoptotic cells compared to that in vector-transfected clones. Instead, Parkin protein expression enhanced the sensitivity of cells to pro-apoptotic drugs. Second, Parkin protein overexpression did not lead to increased sensitivity to all pro-apoptotic induction. This is illustrated by the lack of apoptotic change following staurosporine treatment, in contrast to the enhanced sensitivity to cell-cycle disruption caused by TSA and butyrate. This raises the possibility that Parkin is not as broad a tumor-suppressor gene as the p53 and retinoblastoma genes are. Instead, Parkin may show specificity for a certain type of cellular stress.

In this study, we found that Parkin is a mutational target in hepatocellular carcinoma, results similar to those previously reported in ovarian and breast cancers. We also found that Parkin protein expression was decreased significantly in HCC cell lines as well as in primary tumors. This decreased expression was possibly a result of chromosomal instability and the regulation of gene expression. Further, we showed that Parkin increased the sensitivity of cells to apoptosis induced by cell-cycle inhibitors, suggesting that Parkin is involved in cell-cycle regulation, possibly through stabilization of microtubules. Because of the striking similarity of Parkin, FHIT, and WWOX, we suggest that Parkin could be another tumor-suppressor gene.

REFERENCES


