Alterations of the Tumor Suppressor Gene Parkin in Non-Small Cell Lung Cancer

Maria Cristina Picchio, Eric Santos Martin, Rossano Cesari, George Adrian Calin, Sai Yendamuri, Tamotsu Kuroki, Francesca Pentimalli, Manuela Sarti, Kristine Yoder, Larry R. Kaiser, Richard Fishel, and Carlo Maria Croce

1Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania; 2Department of Surgery, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania; and 3Istituto Dermopatico dell’Immacolata, Rome, Italy

ABSTRACT

Purpose: Parkin, a gene mutated in autosomal recessive juvenile Parkinsonism and mapped to the common fragile site FRA6E on human chromosome 6q25-q27, is associated with a frequent loss of heterozygosity and altered expression in breast and ovarian carcinomas. In addition, homozygous deletions of exon 2 creating deleterious truncations of the Parkin transcript were observed in the lung adenocarcinoma cell lines Calu-3 and H-1573, suggesting that the loss of this locus and the resulting changes in its expression are involved in the development of these tumors.

Experimental Design: We examined 20 paired normal and non-small cell lung cancer samples for the presence of Parkin alterations in the coding sequence and changes in gene expression. We also restored gene expression in the Parkin-deficient lung carcinoma cell line H460 by use of a recombinant lentivirus containing the wild-type Parkin cDNA.

Results: Loss of heterozygosity analysis identified a common region of loss in the Parkin/FRA6E locus with the highest frequency for the intragenic marker D6S1599 (45%), and semi-quantitative reverse transcription-PCR revealed reduced expression in 3 of 9 (33%) lung tumors. Although we did not observe any in vitro changes in cell proliferation or cell cycle, ectopic Parkin expression had the ability to reduce in vivo tumorigenicity in nude mice.

Conclusion: These data suggest that Parkin is a tumor suppressor gene whose inactivation may play an important role in non-small cell lung cancer tumorigenesis.

INTRODUCTION

Several loss of heterozygosity (LOH) studies have indicated that the chromosome 6q25-q27 region is frequently altered in a variety of human cancers. Deletions within the long arm of chromosome 6 are associated with several solid tumors, including carcinoma of the ovary (1, 2), breast (3), kidney (4), and lung (5); melanoma (6); and hematological cancers such as acute lymphoblastic leukemia (7), Burkitt’s lymphoma (8), and non-Hodgkin’s B-cell lymphoma (9). These observations suggest that chromosome 6q25-q27 harbors a tumor suppressor gene (TSG) involved in a wide variety of human cancers.

Recently, Parkin, a gene implicated in autosomal recessive juvenile Parkinsonism (10), was found to be a target of LOH (4) at chromosome 6q25-q27 in breast and ovarian carcinomas (11, 12). Although various deletions and point mutations have been described in patients with early onset of Parkinsonism (13), mutation analysis failed to identify somatic point mutations in any of the breast or ovarian tumors with LOH at the Parkin/FRA6E locus examined (11). However, truncating deletions were found in 3 of 20 tumor samples, and homozygous deletions of exon 2 were identified in the lung adenocarcinoma cell lines Calu-3 and H-1573 (11). In addition, Parkin expression was down-regulated or absent in the majority of the breast and ovarian samples examined, suggesting that Parkin expression is targeted by the LOH observed at 6q25-q27 and may play a role in the development of these tumors.

In this report, we describe the LOH analysis of the Parkin/FRA6E locus at 6q25-q27 and the status of the Parkin coding sequence and its expression in 20 matched normal and non-small cell lung cancer (NSCLC) samples in gene sequence screening. The identification of a common region of deletion in combination with the absence of or down-regulated expression in these tumors indicates that Parkin is targeted by LOH in lung tumorigenesis. In addition, we show the in vivo reduction of tumorigenicity in the lung-tumor-derived cell line H460 lentivirally transduced with recombinant Parkin. Overall, these data suggest that Parkin is a TSG that may play an important role in the development of lung cancer.

MATERIALS AND METHODS

Tumor Specimens and Cell Lines. Tumor samples and corresponding noncancerous tissues were obtained from patients undergoing cancer surgery at the Hospital of University of Pennsylvania. Tumor (NSCLC) and normal tissue were snap-frozen. A portion of the tissue specimens was assessed for tumor content by histology, and only tissues with >60% tumor cells were used. All cell lines were purchased from the American Type Culture Collection. Cells were maintained in DMEM.
containing 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD), and each was supplemented with 100 μg/ml gentamicin (BioWhittaker).

**LOH Analysis.** LOH analysis was performed by a PCR approach using fluorescently end-labeled primers derived from the chromosome 6q25-q27 region as described previously (11). Primer sequences for each highly polymorphic (>60%) microsatellite markers used (Table 1) are available at the National Center for Biotechnology Information database.³ PCR and fragment analysis were performed as described previously (11). LOH was defined for those samples that had XLOH values <0.7 or an allelic loss of ≈ 40%.

**Semi quantitative reverse transcription-PCR (RT-PCR).** RNA was obtained for 12 samples (3 normal and 9 tumor). Total RNA was isolated from homogenized tumor material by use of the TRI Reagent (Molecular Research Center, Cincinnati, OH) and reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), each according to the manufacturer’s instructions. PCR conditions and primer sequences are available on request. Parkin and β-actin control PCR products were separated on a 1.0% agarose gel and quantified with the Personal Densitometer SI (Molecular Dynamics, Little Chalfont, Buckinghamshire, UK) and IMAGEQUANT software, Version 5 (Molecular Dynamics). The relative values of Parkin expression were determined by calculating the ratio of normalized expression levels with that of the corresponding normal tissue. The criterion for overexpression or reduced expression was a ratio >1.5 or <0.5, respectively.

**Mutation Analysis.** Mutation analysis was performed as described previously (14). PCR products were resolved on Tris-borate-EDTA 2% agarose gels and purified by use of the Qiagen Gel Extraction kit (Qiagen, Valencia, CA) followed by direct sequencing. The following primer pairs were used: ParkFw (5'-CGTAGGCTGGACATAAGGTGTTT-3')/ParkRw (5'-TGAAGGGTACGTCGACACTGGTTAT-3'); and ParkRIRw (5'-CGTGAGAAAGG-3')/ParkIRRw (5'-CTGCTGGTACCGGTTGTA3'). In addition, six sets of oligonucleotide primers were designed to amplify Parkin exons from genomic DNA isolated from patients undergoing cancer surgery by standard methods (15). PCR conditions and primer sequences are available on request. All sequence analyses and alignments were performed with the SEQUENCER program (Gene Codes, Ann Arbor, MI).

**Methylation-Specific PCR.** The methylation status of the Parkin promoter (16) was analyzed by amplification of a 541-bp PCR product including the first exon (followed by bisulfite sequencing as described; Ref. 17) in all samples used for RT-PCR. Primer sequences and conditions are available on request.

**Lentiviral Vector Production and in Vitro Transduction.** The full-length Parkin cDNA in frame with a carboxyterminal hemagglutinin epitope tag was generated by PCR and cloned into the BamHI site of the pNaldini.CMV.IRES.EGFP self-inactivating HIV-based provirus vector. Primers are available on request. Lentiviral vector production by transient transfection was performed as described previously (18–20). Transient transfections of pNaldini.CMV.IRES.EGFP or pNaldini.CMV.PARKINHA.IRES.EGFP and packaging vectors into 293FT cells (Invitrogen) were performed by the calcium phosphate precipitation method using the Profection Mammalian Transfection System (Promega, Madison, WI) according to the manufacturer’s instructions. Viruses were pseudotyped with the vesicular stomatitis virus glycoprotein (VSVG) with use of the pVSVG-G vector (Clontech, Palo Alto, CA). Viral supernatants were collected after 48 and 72 h, filtered, and snap-frozen in liquid nitrogen. Titers were determined by infecting 293FT cells with serial dilutions of virus supplemented with Polybrene (Sigma, St. Louis, MO) at a final concentration of 8.0 μg/ml. Infectivity was determined by green fluorescent protein (GFP) expression analysis of target cells by flow cytometry (FACS-Calibur; Becton Dickinson Immunocytometry Systems) 48 h after infection. Transduction units are expressed as a percentage of GFP-positive cells relative to the total number of cells ana-

---


---

**Table 1  Loss of heterozygosity frequency at the Parkin locus in non-small cell lung cancer**

<table>
<thead>
<tr>
<th>Samples</th>
<th>D6S1581</th>
<th>D6S1579</th>
<th>D6S305</th>
<th>D6S1599</th>
<th>D6S1008</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>49T</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>56T</td>
<td>NI</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>58T</td>
<td>HZ</td>
<td>LOH</td>
<td>HZ</td>
<td>HZ</td>
<td>LOH</td>
<td>Squamous</td>
</tr>
<tr>
<td>63T</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>64T</td>
<td>LOH</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>66T</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>71T</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>NI</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>75T</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>NI</td>
<td>HZ</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>82T</td>
<td>HZ</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>NI</td>
<td>Squamous</td>
</tr>
<tr>
<td>83T</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>NI</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>86T</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>LOH</td>
<td>LOH</td>
<td>Squamous</td>
</tr>
<tr>
<td>87T</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>LOH</td>
<td>LOH</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>90T</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>LOH</td>
<td>LOH</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>91T</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>NI</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>92T</td>
<td>HZ</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>94T</td>
<td>HZ</td>
<td>HZ</td>
<td>NI</td>
<td>HZ</td>
<td>HZ</td>
<td>Squamous</td>
</tr>
<tr>
<td>LOH, n (%)</td>
<td>1/10 (10)</td>
<td>1/11 (9)</td>
<td>3/15 (20)</td>
<td>4/9 (45)</td>
<td>4/13 (31)</td>
<td></td>
</tr>
</tbody>
</table>

⁴ NI, not informative; HZ, heterozygous; LOH, loss of heterozygosity; ND, not done.
lyzed. Typically, conditioned medium collected from transfections performed in T-175 flasks (Becton Dickinson) seeded with 5 \times 10^6 293FT cells yielded \sim 1 - 10 \times 10^7 transduction units/ml. Subsequently, infections of target cells were performed to achieve 90–100% GFP-positive cells.

**Cell Cycle and Cell Proliferation Analysis.** For cell cycle analysis, cells were harvested 24 and 48 h post-transduction, washed in PBS, and fixed in ethanol. After RNase treatment (Roche, Indianapolis, IN), cells were stained with 50 \mu g/ml propidium iodide (Molecular Probes). All samples were analyzed by flow cytometry (FACSCalibur) and the FlowJo Version 3.4 Software Package (Tree Star, Inc., San Carlos, CA). Cell proliferation was measured by the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer’s directions (Promega).

**Tumorigenicity in Nude Mice.** Cells transduced with and without Parkin cDNA were evaluated for tumorigenicity in nude mice. We gave 6-week-old female athymic mice nude (nu/nu) s.c. injections containing 1–10 \times 10^6 cells in 0.2 ml of PBS. Cells transduced with each construct were injected into three to four mice each and followed individually. Mice were examined two to three times per week for tumor formation at the sites of injection for 5 weeks. Tumors were measured with linear calipers, and tumor volumes were calculated (v = \frac{ab^2}{2}).

**RESULTS AND DISCUSSIONS**

Five polymorphic microsatellite markers from human chromosome 6q25–6q27 used to define the minimal region of loss in breast and ovarian samples (11) were tested for LOH in 16 lung normal-tumor DNA pairs. Overall, 7 of 16 (44%) samples showed LOH in at least one locus in the region examined spanning \sim 3 Mb. The percentage of LOH for each of the five markers ranged from 9% (D6S1579) to 45% (D6S1599). The intragenic marker D6S1599 is located in the 5’ end of the Parkin gene, between exons 2 and 3, and the minimal region of LOH is delineated by the markers D6S305 and D6S1599 (Fig. 1; Table 1). These data are in accordance with the minimal region of deletion observed in ovarian and breast cancers (11). A high frequency of LOH was also observed for the D6S1008 marker, suggesting that this region of frequent deletion may extend beyond the 5’ end of Parkin. Among the four tumors demonstrating LOH at more than one loci, three were adenocarcinomas, suggesting that deletion of the Parkin gene may have a unique association with a histological subtype (Table 1). A larger cohort of histologically characterized tumor specimens will be necessary to explore such a possibility.

Subsequently, we analyzed Parkin expression in a series of lung tumors by semiquantitative RT-PCR. Three of nine samples (33%) showed decreased or no expression of Parkin transcript relative to normal lung tissue, whereas five of nine (55%) showed nearly identical levels of expression, and one of nine (11%) cases demonstrated a reduction in Parkin gene expression, indicating that a mechanism other than deletion may account for the reduction in the levels of Parkin in some tumors.

RT-PCR followed by direct sequencing of the full-length Parkin cDNA was performed on available tumor samples used in both the expression and LOH analyses. No point mutations or truncations were found in any of the eight cases (data not shown). Although several mutations and deletions have been identified in patients with autosomal recessive juvenile Parkinsonism (13), to date only two intragenic deletions have been found in three patients with ovarian adenocarcinomas (11). A recent study showed that Parkin was down-regulated in 60% of

![Fig. 1 Summary of allelic loss observed spanning the Parkin/FRA6E locus in non-small cell lung cancer. Each vertical line represents an informative case; •, ○, and □ represent LOH, retention of heterozygosity, and noninformative results, respectively. Right, the genomic locus of the Parkin gene and the positions of the intragenic markers D6S305 and D6S1599 are shown according to Cesari et al. (11).](image)
the primary ovarian tumors analyzed (12). These data suggest that in human tumors the primary mechanism of Parkin inactivation is probably a combination of LOH coupled with down-regulation through an alternative means, possibly through an epigenetic mechanism such as aberrant promoter hypermethylation or promoter mutations (21). To date, we have not found any difference in the methylation pattern of the published Parkin promoter (16) between normal and tumor samples regardless of the expression status. However, the possibility remains that methylation of an alternative promoter or mutations in regulatory sequences could exist and could possibly explain such findings.

To study the in vitro and in vivo effects of restored Parkin gene expression, we used a self-inactivating lentiviral system of gene transfer to infect the human tumor-derived cell line H460, which lacks endogenous Parkin expression (11). Infection efficiencies >90% were observed in H460 cells by recombinant lentiviruses containing Parkin or EGFP alone. Typically, Parkin and EGFP gene expression levels became stable 48 h postinfection and continued for several weeks (>12 weeks). In each of these infections, there were no cytotoxic effects or changes in growth characteristics and cell cycle in H460 cells (Fig. 3). These data suggest that in vitro there are no direct consequences of ectopic Parkin expression on cell growth or cell cycle in these cells.

To study the in vivo behavior of restored Parkin expression in H460 cells, we injected 1 × 10⁶ cells infected with either EGFP or Parkin lentiviruses s.c. into the flanks of 6-week-old female nude mice. Tumor cells transduced with virus particles containing Parkin or EGFP alone showed no significant differences in their ability to proliferate over the course of 96 h (Fig. 3). High levels of ectopic gene expression were maintained in nearly 100% of the cells examined by flow cytometry and Western analysis before injection (data not shown). After injection, mice were monitored for up to 5 weeks, and tumor volumes were measured with a metric caliper (Fig. 3). As shown, the tumor volume of H460 cells infected with the Parkin virus was reduced ~3–4-fold relative to the controls. Thus, Parkin alone has significant tumor-suppressor activity in H460 cells deficient in the Parkin protein (11).

Homozygous deletions of exon 2 of Parkin, a gene implicated in autosomal recessive juvenile Parkinsonism (10), were identified in the lung adenocarcinoma cell lines Calu-3 and H-1573 (11). In addition, Western analysis of several lung cell lines positive for Parkin RNA expression failed to detect any Parkin protein, thus prompting our investigation of the possible role of Parkin in NSCLC. Although various deletions and point mutations have been described in patients with early onset of Parkinsonism (13), mutation analysis failed to identify somatic point mutations in any of the lung tumors examined for LOH at the Parkin/FRA6E locus (11). Parkin expression was down-regulated or absent in a significant portion of the lung samples examined, suggesting that Parkin expression is targeted by the LOH observed at 6q25-q27 and may play a role in the development of these tumors. In vivo studies showed that re-expression of Parkin in a lung carcinoma cell line, although having no direct consequences on cell growth or cell cycle, consistently reduced the volumes of tumors in nude mice. Although its function is not entirely understood, Parkin protein was found to be an ubiquitin-protein ligase (E3). It is therefore possible that mechanisms related to the ubiquitin function are involved in the tumorigenic process. Of note, the FHIT gene, a TSG located in FRA3B, was found to be deleted/down-regulated but not mutated in lung cancer (22), and WWOX, another TSG located in a fragile site (FRA16D), is deleted/truncated in several types of cancers, including NSCLC (23).
In summary, we have expanded the histological spectrum of tumors in which the candidate TSG Parkin is genetically altered to include NSCLCs. In addition, using a lentiviral system, we demonstrated that in vivo effects of restored Parkin gene expression are compatible with a tumor suppressor function. These data argue that Parkin is a TSG whose inactivation may play an important role in NSCLC tumorigenesis.

REFERENCES

Alterations of the Tumor Suppressor Gene *Parkin* in Non-Small Cell Lung Cancer

Maria Cristina Picchio, Eric Santos Martin, Rossano Cesari, et al.


Updated version  Access the most recent version of this article at:  
http://clincancerres.aacrjournals.org/content/10/8/2720

Cited articles  This article cites 22 articles, 10 of which you can access for free at:  
http://clincancerres.aacrjournals.org/content/10/8/2720.full#ref-list-1

Citing articles  This article has been cited by 15 HighWire-hosted articles. Access the articles at:  
http://clincancerres.aacrjournals.org/content/10/8/2720.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.