Implication of alterations in Parkin gene among North Indian patients with colorectal cancer

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ABSTRACT

Background/Aims: *Alterations in Parkin (PRKN) have been described in many cancers; however, the molecular mechanism that contributes to loss of Parkin expression in colorectal cancer (CRC) remains unclear. The aim of this study was to investigate the involvement of PRKN mutation and loss of heterozygosity (LOH) in loss of Parkin expression. To understand the role of PRKN in cancer progression, we also evaluated the association of Parkin expression with clinicopathological parameters in North Indian population.*

Materials and Methods: *We studied 219 CRC samples and their adjacent normal tissues (control) obtained from North Indian patients with CRC. The expression of Parkin was analyzed by immunohistochemistry (IHC). PRKN mutations were analyzed by single-stranded conformational polymorphism (SSCP) and sequencing. For loss of heterozygosity (LOH), we employed two intragenic, D6S305 and D6S1599, and one telomeric marker, D6S1008.*

Results: *In our study, we found four novel somatic mutations, namely, C166G, K413N, R420P (exon 4), and V425E (exon 11). Both mutation in Parkin ($p = 0.0014$) and LOH ($p = 0.0140$) were significantly associated with loss of Parkin expression. Additionally, Parkin mutations were not associated with the clinicopathological parameters of the patients. Furthermore, both, LOH in Parkin and Parkin expression were significantly correlated with different clinicopathological variables ($p<0.05$).*

Conclusion: *Our results indicate that Parkin expression is not regulated by a single mechanism, but both mutation and LOH contribute to loss of Parkin expression. We also provide evidence of involvement of Parkin in metastasis and cancer progression. We, therefore, suggest Parkin as a potential prognostic marker and warrant further analysis in this direction.*

Keywords: *Parkin gene, mutation, LOH, expression, colorectal cancer*

INTRODUCTION

Colorectal cancer (CRC) is the most frequently diagnosed cancer in both men and women around the world (1). CRC is a multifactorial disease, including various factors from lifestyle to genetic and environmental factors (2). The process of CRC development is characterized by a multistep sequence of phenotypic changes that involves many molecular signaling pathways (3). Molecular changes in various genes are associated with different stages of oncogenic transformation (2). A most important feature of CRC is genetic instability, which mainly involves two different mechanisms. The most common (~84% of sporadic CRC) is chromosomal instability (CIN), which is characterized by gross changes in chromosome number and structure such as deletions, gains, translocations, and other chromosomal rearrangements. The second mechanism (~13-16% of sporadic CRC) involves hypermutation and microsatellite instability (MSI) owing to defective DNA mismatch repair (MMR) (3).

Numerous molecular markers have been identified for the diagnosis, prognosis, and treatment of CRC. Molecular genetic studies of CRC have revealed several genes that show a high frequency of CRC associated mutations (4). Investigating the gene alterations may provide predictive value for assessing prognosis and sensitivity to various drugs. Thus, there is a need for development of novel and more precise prognostic tools that can be employed to improve both prognostic prediction and management of treatment.

Parkin (*PRKN*) is an RBR (ring-between-ring) containing E3 ubiquitin-protein ligase, which functions in the ubiquitin-proteasome system and facilitates ubiquitination of

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target proteins (5). *PRKN* is mainly associated with autosomal recessive Parkinsonism but many reports have suggested it as a potential tumor suppressor gene (5-8). *PRKN* is mapped within one of the most common fragile sites regions, FRA6E (6q26), and it is found to be altered in various types of malignancies including ovarian, cervical, breast, pancreatic, colorectal, and lung cancers (5, 9-12). Interestingly, Parkin has been found to play key roles in several hallmarks of cancer (6). Several studies have reported that the expression of Parkin is significantly lowered in various tumors (5, 10-13)**.** Conversely, overexpression of Parkin has been found to inhibit cell growth in different cell lines (9, 12-15)**.** A loss of heterozygosity (LOH) within the chromosomal region 6q25-q27 surrounding *PRKN* is frequently observed in various human tumors, including ovarian, breast, and lung cancers (5, 9). Moreover, mutations in *PRKN* lead to loss of its well-documented E3 ubiquitin-ligase activity (12). Remarkably, several studies have revealed the potential role of alterations in *PRKN* and its subsequent expression, predicting the survival of patients with CRC (12, 16, 17). Nevertheless, how other mechanisms like mutation and LOH are associated with aberrant expression of Parkin in CRC is still a topic of debate. Based on the above observations, we were curious to assess the status of Parkin in North Indian population. In the present study, we evaluated somatic mutations in and LOH of *PRKN* and determined how they are associated with its protein expression. In addition, we also examined the association of alterations in *PRKN* with clinical variables.

MATERIALS AND METHODS

Sample collection

Cancer tissues of 219 North Indian patients with colorectal adenocarcinoma who underwent surgery between 2013 and 2017 at the Hospital and their matched normal/control tissues were obtained from the Pathology Department of the Hospital. Ethical approval was obtained from the ethical committee of the concerned hospital. Biopsies of the cancer tissues and their matched normal samples (>5 cm away from the margin of tumors) were snap-frozen, following which a portion of the biopsies was collected in phosphate buffered saline (PBS) for molecular analysis and the remaining part was stored in 10% buffered formalin for IHC. The exclusion criteria included age >80 years or <18 years, metastasized cases from other organs, prior history of cancer, and patients previously treated with radiotherapy or chemotherapy. Histopathological and clinical variables were obtained from the hospital (Supplementary Table 1). The clinicopathological variables were considered according to histologic classification of World Health Organization and TNM staging, as given by American Joint Committee on Cancer (AJCC) (16, 17).

MAIN POINTS

- *PRKN gene mutations and LOH are independent events in North Indian CRC patients.*
- *In loss of Parkin expression, its somatic mutations and LOH both are involved in North Indian CRC patients.*
- *Loss of Parkin protein expression and LOH are associated with poor differentiation and tumor progression of colorectal cancer.*

Extraction of DNA

DNA was extracted from 219 colorectal tumor samples and their adjacent normal tissues by phenol-chloroform method as described earlier (19). Concentration and purity of the extracted DNA were analyzed by NanoDrop (ND1000) Spectrophotometer. Furthermore, electrophoresis was performed on 0.8% agarose gel using standard ethidium bromide stain.

PCR based single-strand conformational polymorphism (PCR-SSCP)

All exons of *PRKN* were studied using single strand conformation polymorphism PCR (SSCP-PCR). The primer sequences used for the amplification were as described in a previous study (20). The protocol and conditions for SSCP were assessed as described previously (20). Normal tissue and tumor samples from same patient were loaded in parallel lanes in the gel to compare their mobility. The samples with difference in the migration of bands between the normal tissue and tumor samples were identified as mutants. The band shift was confirmed in three independent PCR reactions and the regions were re-amplified in 40 µL of reaction mixture for DNA sequencing. To minimize sequencing artifacts, amplicons from at least two different PCRs were sequenced using forward and reverse primers. The BLAST tool was employed for pair-wise nucleotide sequence alignment.

Loss of heterozygosity (LOH) and PCR amplification

To identify LOH in *PRKN* gene, we selected three microsatellite marker sites, D6S1599, D6S305, and D6S1008, in chromosome 6q25-27 (10). D6S1599 and D6S305 are intragenic markers present in introns 2 and 7 of *PRKN*, while D6S1008 is situated at the telomeric end. The primer sequences used in this study and their annealing temperatures are summarized in Supplementary Table 2.

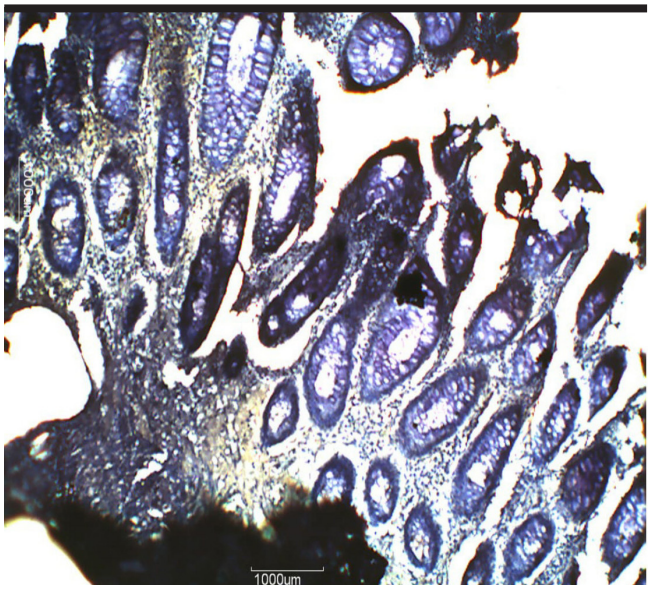

Figure 1. Representative images of immunostaining of Parkin protein in samples obtained from North Indian patients with colorectal cancer. Colorectal cancer tissue showing negative Parkin expression.

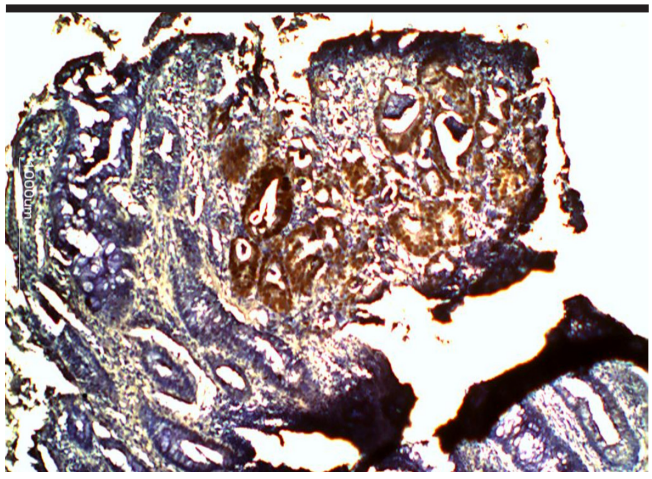

Figure 2. Representative images of Parkin protein expression in samples obtained from North Indian patients with colorectal cancer. Normal colorectal tissue (control) showing strong cytoplasmic expression of Parkin, (magnification: 100×, scale bar 1000 µm). Parkin-positive staining is generally detected in cytoplasm, which can easily be distinguished from Parkin-negative cases. For the present images, 3, 3′-Diaminobenzidine (DAB) was used as a chromogen.

The amplification conditions used in PCR were as mentioned earlier (21). The amplified PCR products were denatured at 95°C for 6 min and run on 10% denaturation polyacrylamide gel, prepared in 1X TBE buffer, at 50 V for 14-16 h. Silver staining was performed as mentioned earlier (21). LOH was defined as complete loss or up to 50% decreased relative density of silver staining bands of PCR products of CRC samples compared to their matched normal control samples (22).

Immunohistochemical analysis (IHC)

For each case, the expression of Parkin protein was confirmed by immunohistochemical performed on formalin-fixed paraffin-embedded tissue sections as per our lab protocol (23). The tissue sections were placed on poly-l-lysine coated slides, deparaffinized using xylene, gradually rehydrated with ethyl alcohol, and rinsed with distilled water. Endogenous peroxide activity was blocked by incubating the slides in hydrogen peroxide (0.3%) for 30 min. Later, heat-mediated antigen retrieval was performed using citrate buffer (10 mM, pH 6.0) in a microwave oven for 15-20 min. The slides were then incubated with anti-PARKIN polyclonal antibody (cat #ab15954, Abcam) overnight at 4°C. The slides were treated with secondary antibody followed by treatment with avidin-horseradish peroxidase for 25 min. The reaction was visualized using 3, 3′-diaminobenzidine (DAB) and hematoxylin as a counterstain. Normal colorectal tissues served as positive controls while negative controls were obtained by omitting the primary antibodies. The stained slides were examined by an expert pathologist under a light microscope and images were captured at 100× magnification. The samples with ≤5% staining were considered as samples with negative/ low expression and the samples with >5% staining were considered as samples with positive expression, where 5%-25% staining was considered as moderate expression and >25% staining was considered as highly positive expression.

Statistical analysis

All statistical data were analyzed using the Statistical Package for Social Sciences (SPSS) version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Fisher's exact test was performed for all the comparisons to assess the statistical significance and odds ratios (ORs) were calculated at 95% confidence intervals (CIs). The $p<0.05$ were considered significant.

RESULTS

Parkin expression

Out of 219 CRC tissue samples, 58% (127/219) samples showed loss of Parkin protein expression while 42% (92/219) samples showed low to moderate expression. In the immunohistochemical images, predominant cytoplasmic expression of Parkin protein was also observed (Figures 1, 2).

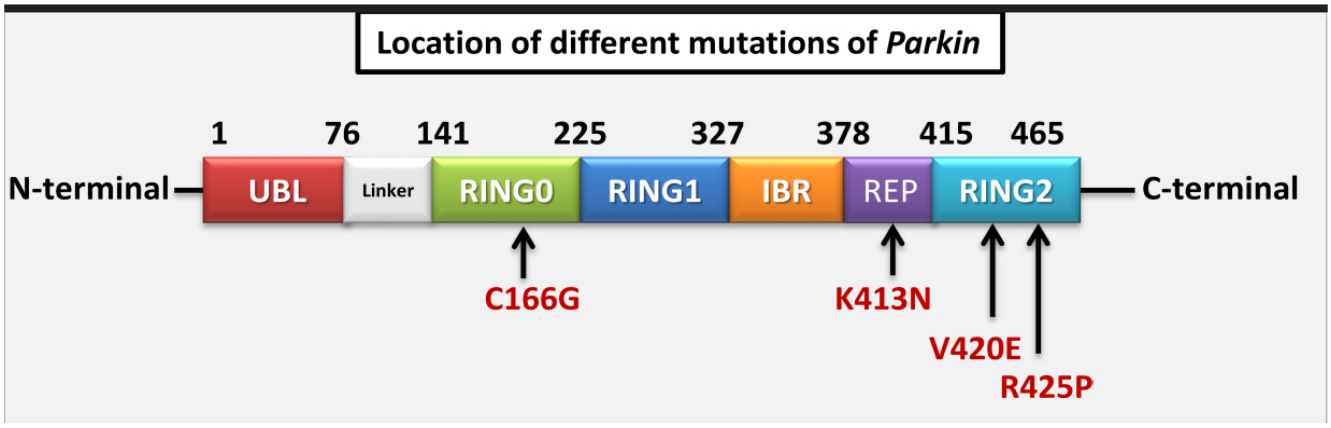

Figure 3. *PRKN* somatic mutations. PRKN 2D structure showing the position of four mutations in various domains.

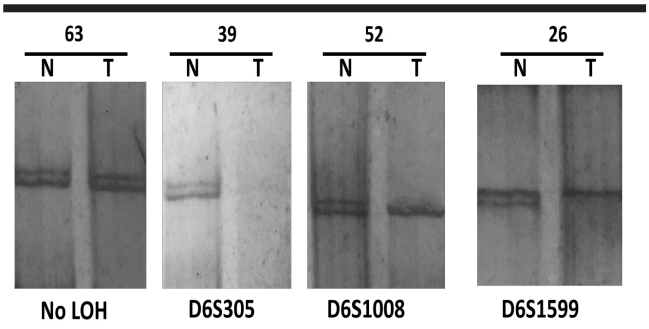

Figure 4. Loss of heterozygosity (LOH) at each of the three loci in 6q26 region in colorectal cancer. Silver-stained polyacrylamide gel containing amplified products of the normal control (N) and tumor (T) tissues of patients (identified by numbers) with colorectal cancer. The tumors from patients 39, 52, and 26 showed loss of heterozygosity at different loci in the *PRKN* gene while patient 63 did not show LOH.

Mutational analysis of Parkin gene

In our study, mutational analysis of all the CRC tissues samples (n=219) showed that, overall, 13.2% (29/219) cases had mutations in exon 4 and exon 11 of *PRKN* (Figure 3). Out of 29 mutations, six samples demonstrated T to G transversion at nucleotide position 627 leading to conversion of cysteine to glycine at codon 166 in exon 4 (Table 1, Supplementary Figure S1). In exon 11, five samples showed A to G transition at nucleotide position 1365 leading to conversion of lysine to asparagine at codon 413, eight samples showed C to T transition at nucleotide position 1383 accounting for the conversion of asparagine to proline acid, and remaining 10 samples demonstrated transversion of T to A resulting in conversion of valine to glutamic acid at codon 425 (Table 1, Supplementary Figure S2).

LOH in PRKN

In this study, a case was considered as informative if the normal control tissue was heterozygous at a particular site. If the control is homozygous, it is not possible to detect LOH and it is, therefore, uninformative. Out of the 219 tumors examined, 119 (54%) were heterozygous for at least one of the microsatellite markers studied (Figure 4). The highest rate of LOH was observed at intragenic marker D6S305 (44%) compared with the other intragenic marker D6S1599 (35%) while the lowest rate of LOH was observed at the locus D6S1008 (23%) in the telomeric region of *PRKN* gene (Supplementary Table 3). Out of 119 samples, 49 (41%) samples showed LOH in both the intragenic markers. In addition, only 19 (16%) samples exhibited an allelic loss at all the three markers. The remaining samples showed LOH in at least one of the loci examined.

Significance of Parkin protein expression

While evaluating the correlation of Parkin protein expression with clinical factors of the patients, we observed a significant association between negative Parkin expression with clinical stage ($p=0.0004$), histological group ($p<0.0001$), lymph node ($p<0.0001$), and angiolymphatic/perineural invasion ($p=0.0115$) (Table 2). There was, however, no significant association of Parkin expression with other clinical parameters ($p>0.05$).

Significance of PRKN mutation

We could not find a significant correlation between *PRKN* mutation and any available clinical variables of the patients. However, a significant association was observed between *PRKN* mutation and loss of Parkin protein ($p=0.0014$) (Table 3).

Table 1. PRKN mutations found in exons 4 and 11.

PARK gene: Exon 4						
Sr. No.	Age (Years)	Tumor grade	Nucleotide alteration	Amino acid alteration	LOH in *PRKN*	Parkin expression
1	56	II	627-T→G	C166G (Cys 166Gly)	Present	Absent
2	62	III	627-T→G	C166G (Cys166Gly)	Present	Absent
3	59	III	627-T→G	C166G (Cys166Gly)	Present	Absent
4	42	IV	627-T→G	C166G (Cys166Gly)	Present	Absent
5	65	IV	627-T→G	C166G (Cys166Gly)	Present	Absent
6	51	II	627-T→G	C166G (Cys166Gly)	Absent	Absent
PRKN gene: Exon 11						
Sr. No.	Age (Years)	Tumor grade	Nucleotide alteration	Amino acid alteration	LOH in *PRKN*	Parkin expression
1	53	II	1365-A→G	K 413N (Lys413Asp)	Present	Absent
2	39	III	1365-A→G	K413N (Lys413Asp)	Present	Absent
3	40	III	1365-A→G	K413N (Lys413Asp)	Present	Absent
4	51	II	1365-A→G	K413N (Lys413Asp)	Absent	Absent
5	42	IV	1365-A→G	K413N (Lys413Asp)	Present	Absent
6	62	IV	1383-C→T	R 420P (Arg420Pro)	Present	Absent
7	55	II	1383-C→T	R420P (Arg420Pro)	Present	Present
8	59	III	1383-C→T	R420P (Arg420Pro)	Absent	Absent
9	46	III	1383-C→T	R420P (Arg420Pro)	Present	Absent
10	61	II	1383-C→T	R420P (Arg420Pro)	Present	Absent
11	56	III	1383-C→T	R420P (Arg420Pro)	Absent	Absent
12	48	II	1383-C→T	R420P (Arg420Pro)	Present	Absent
13	37	IV	1383-C→T	R420P (Arg420Pro)	Absent	Absent
14	47	II	1383-C→T	R420P (Arg420Pro)	Present	Absent
15	57	III	1383-C→T	R420P (Arg420Pro)	Present	Absent
16	43	III	1400-T→A	V 425E (Val425Glu)	Present	Present
17	38	III	1400-T→A	V425E (Val425Glu)	Present	Absent
18	49	IV	1400-T→A	V425E (Val425Glu)	Absent	Absent
19	54	IV	1400-T→A	V425E (Val425Glu)	Present	Absent
20	56	II	1400-T→A	V425E (Val425Glu)	Present	Present
21	62	II	1400-T→A	V425E (Val425Glu)	Present	Absent
22	47	III	1400-T→A	V425E (Val425Glu)	Present	Absent
23	56	III	1400-T→A	V425E (Val425Glu)	Present	Absent

Cysteine, Glycine, Lysine, Asparagine, Arginine, Proline

Table 2. Correlation of Parkin protein expression and LOH with the clinical variables of patients with colorectal cancer (n=219).

Variables	Total no. (n=219)	Parkin expression		p	Loss of heterozygosity		p
		Negative (%)	Positive (%)		Positive (%)	Negative (%)	
Gender							
Male	117	69 (59)	48 (41)	0.7522	69 (59)	48 (41)	0.1408
Female	102	58 (57)	44 (43)		50 (49)	52 (51)	
Age (years)							
<50	56	27 (46)	29 (53)	0.0874	25 (45)	31 (55)	0.1076
≥50	163	100 (62)	63 (38)		92 (56)	69 (42)	
Tumor Size (cm)							
<3 cm	48	23 (48)	25 (52)	0.1115	21 (43)	27 (57)	0.0976
>3 cm	171	104 (61)	67 (39)		98 (57)	73 (43)	
Clinical Stage							
I+II	114	53 (46)	61 (54)	0.0004	51 (45)	63 (55)	0.0032
III+IV	105	74 (70)	31 (30)		68 (65)	37 (35)	
Degree of Differentiation							
Low/moderate	183	121 (66)	62 (34)	<0.0001	107 (58)	76 (42)	0.0070
Well-differentiated	36	6 (17)	30 (83)		12 (33)	24 (67)	
Angiolymphatic/perineural Invasion							
Present	138	89 (65)	49 (35)	0.0115	81 (58)	57 (42)	0.0920
Absent	81	38 (47)	43 (53)		38 (47)	43 (53)	
Lymph node status							
Present	144	98 (68)	46 (32)	<0.0001	98 (68)	46 (32)	<0.0001
Absent	75	29 (39)	46 (61)		21 (28)	54 (72)	
Location of tumor							
Colon	126	68 (54)	58 (46)	0.1611	68 (54)	58 (46)	0.8983
Rectum	93	59 (63)	34 (36)		51 (55)	42 (45)	
Mucin production							
Mucinous	36	17 (47)	19 (53)	0.1548	17 (47)	19 (53)	0.3498
Non-mucinous	183	110 (60)	73 (30)		102 (56)	81 (44)	
Side of tumors							
Right	60	33 (55)	27 (45)	0.5819	35 (58)	25 (42)	0.4663
Left	159	94 (59)	65 (41)		84 (53)	75 (47)	

Valine, Glutamic acid, Through immunohistochemistry, Fisher's exact test

Significance of LOH at PRKN region

We investigated the correlation of LOH in *PRKN* with clinical variables and found significant correlations of LOH in *PRKN* with clinical stage (p=0.0032), histological group (p=0.0070), and lymph node (p<0.0001). Interestingly, LOH in *PRKN* showed a significant correlation with loss of Parkin protein expression (p=0.0140) (Table 2).

Parkin expression status in mutation and LOH cases

There were 11% (23/219) cases in the present study, which harbored double hit (i.e. they had both LOH and *PRKN* mutation). Out of these, 70% (16/23) cases also showed a dramatic loss in Parkin protein expression, thereby indicating complete inactivation of *PRKN* gene as shown in Table 4. Although there were 30% (7/23) cases that showed positive expression of Parkin in spite

Table 3. Correlation of Parkin protein expression with mutation and LOH in PRKN among patients with colorectal cancer (n=219).

		Parkin Expression			
Molecular Event	Total no. (n=219)	Negative (%)	Positive (%)	p[11]	OR (95%) CI
PRKN Mutation					
Present	29	26 (90)	3 (10)	0.0014	7.3795
Absent	190	101 (53)	89 (47)		(2.1587-25.2275)
LOH at PRKN					
Present	119	78 (65)	41 (35)	0.0140	1.9801
Absent	100	49 (49)	51 (51)		(1.1485-3.4138)

PRKN: Alterations in Parkin; LOH: Loss of heterozygosity
[11]Fisher's exact test

Table 4. Correlation of Parkin protein expression with both mutation and LOH in PRKN among patients with colorectal cancer.

		Parkin Expression[12]		
Variables	Total no.	Negative (%)	Positive (%)	p[13]
LOH Present	119	78 (65)	41 (35)	0.0391
Mutation Present	29	26 (90)	3 (10)	
Both Present (LOH+Mutation)	23	16 (70)	7 (30)	

PRKN: Alterations in Parkin; LOH: Loss of heterozygosity
[12]Through immunohistochemistry
[13]Fisher's exact test
[14]Loss of heterozygosity

of the double hit (Table 4). This positive expression of Parkin protein indicated the involvement of other mechanisms in the regulation of Parkin expression such as promoter methylation. We found that molecular events like LOH and mutation of Parkin gene play an important role in progression of CRC and have an immense impact on clinical variables.

DISCUSSION

PRKN has been found to be involved in various hallmarks of cancer ranging from cell cycle to apoptosis, hence, the loss of expression of *PRKN* could shift the balance towards a growth-promoting effect and consequently cancer formation (6, 22, 23). Many published reports have highlighted the potential role of *PRKN* gene in a wide spectrum of malignancies (5, 7, 9, 14, 19, 23, 25). However, mutations in *PRKN* are not the universal cause of decreased Parkin expression in tumors (10, 12, 21). Thus, the mechanisms involved in the regulation of Parkin expression remain unclear. Hence, promotion of tumorigenesis due to loss of Parkin expression needs larger attention. Recently, we reported that *PRKN* polymorphism has a significant association with CRC risk (15). Consistent with our findings, in the present study, we evaluated the LOH and mutation in *PRKN* gene in human CRC. To the best of our knowledge, no study has (19) demonstrated the association of genetic events like mutation and LOH in North Indian patients with CRC until now, which prompted us to assess the status of *PRKN* and its association with the progression of pathological features of CRC in North Indian population.

In the present study, we observed a significant loss (58%) in Parkin protein expression in CRC, which is consistent with results of several previous studies (5, 16, 24) except one study that reported an increase in Parkin expression in CRC (16). The study population consisted of 102 women and 117 men. A previous study on Brazilian population has shown no correlation of Parkin immunoreactivity with clinicopathological variables such as degree of differentiation, angiolymphatic and/or perineural invasion, and lymph node status (16). Conversely, our study demonstrated a significant correlation of Parkin expression with advanced clinical stage and high degree of differentiation suggesting the possible contribution of loss of *Parkin* expression in the pathogenesis and aggressiveness of this cancer in Indian population. In addition, *Parkin* loss has also been strongly associated with lymph node status and angiolymphatic/perineural invasion, which supports a previous study that showed the role of *PRKN* in metastasis in which *PRKN* gene was found to be interacting with HIF-1α in breast cancer (29) and also our unpublished data. Thus, our study suggests that frequent loss of Par-

kin expression may contribute to malignant progression of CRC. The cause of these disparities between the populations can be explained in manifold terms like genetic variation and dissimilarities in social, economic, and geographical environments. Moreover, we found no significant differences between patients' gender and Parkin protein expression as described by an earlier study(16). Furthermore, the same study also indicated that Parkin expression could be used as an independent predictor of patient survival and found that higher Parkin protein levels in deep tumor region correlated with prolonged survival. Contrarily, in our study, we did not find any difference in Parkin expression within the deep region of tumors; however, we could not evaluate survival importance of Parkin expression due to lack of follow up data. Additionally, the study population was divided into two age groups (<50 years and ≥50 years), but we could not find any correlation between patients' age and expression of Parkin protein.

To acknowledge the genetic mechanism behind loss of Parkin expression, we evaluated both the somatic mutations and LOH in *PRKN* region. Somatic mutations and intragenic deletions of *PRKN* have been reported in glioblastoma, colon cancer, as well as lung cancer (12). In the present study on North Indian patients, 29 out of 219 patients (13%) were found to have mutations in *PRKN*, which is contrary to a previous study suggesting that missense mutations are not common in CRC (28). We found four novel somatic mutations, namely, *C166G, K413N, R420P* (exon 4), and *V425E* (exon 11), which were absent in normal colorectal tissues. Interestingly, variant *C166G* was found to be located in RING0 domain of *PRKN*, which is mainly involved in substrate ubiquitination. Another mutation, *K413N*, was found in REP (Repressor Element of Parkin) domain, which usually blocks the E2 Ub-conjugating enzyme binding site on RING1 domain, and thus, plays a crucial role in *PRKN* activation. Remaining two mutations, *V420E* and *R425P*, were both found in RING2 domain at C terminal of Parkin protein. Therefore, mutations found in our study may alter the activation and substrate binding process of *PRKN*.

To get a closer view of genetic alterations of *PRKN* and their impact on loss of Parkin expression, we analyzed the LOH after mutational study. Several studies have identified that LOH in the chromosome region 6q25-q27 is frequently altered in a variety of human cancers such as ovarian, breast, and lung cancers (5, 9, 26). Moreover, expression of Parkin was found to be decreased or absent in the majority of breast and ovarian samples examined suggesting a role of LOH at 6q25-q27 in the loss of Parkin expression and development of these tumors. We, for the first time, examined LOH at 6q25-q27 in CRC. Overall, we found 58% LOH in CRC, which was in line with other cancers like breast (55%), lung (44%), ovarian (62%), and cervical (56%) cancers as previously reported (5, 9, 19, 26). We also speculated a significant correlation of LOH with loss of Parkin expression as reported in breast and ovarian cancers (5, 26). We used three markers, D6S305, D6S1599, and D6S1008, in the study. The highest rate of LOH was observed at the intragenic markers D6S305 and D6S1599, followed by the LOH rate at the locus D6S1008 found at the telomeric end of the gene. Interestingly our LOH data was similar to that obtained for breast and ovarian cancers (5), as these cancers also have lowest LOH at the telomeric marker D6S1008, unlike lung cancer, which shows highest LOH at the telomeric marker (10). The marker D6S305 is localized within intron 7 of *PRKN*, whereas the marker D61599 is mapped within intron 2 of *PRKN*. A high frequency of LOH was observed at the intragenic markers D6S305 and D6S1599, indicating the vulnerability of these regions in CRC. Furthermore, we observed a significant association of LOH with clinical stage, degree of differentiation, and lymph node of patients, which underlies its pathological significance in North Indian patients. Contrary to this, we did not find any significant correlation of *PRKN* mutation with the available clinical variables; however, *PRKN* mutations are strongly associated with loss of Parkin expression. In line with this, we also checked the combined effect (double hit) of Parkin mutations and LOH on loss of Parkin expression. Notably, out of 11% (23/219) double hit cases, 70% (16/23) cases showed loss of Parkin expression while the remaining 30% (7/23) cases showed positive Parkin expression, suggesting the involvement of other mechanisms like promoter methylation. Hence, our study provides evidence that along with double hit, *PRKN* mutations and LOH both are major independent mechanisms that lead to loss of Parkin protein expression, at least in North Indian patients with CRC.

Our study design has both strengths and limitations, which should be discussed. In contrast to the Brazilian study, which did not find any correlation of Parkin immunoreactivity with clinical variables; however, we found a significant association of loss of *PRKN* expression with tumor grade, stage, and lymph node in North Indian population, which may be considered as a strength of the study. A potential limitation of our study is that we could not examine the variations in intensity of Parkin expression, like in a morphometric study. The second limita-

tion of our study is the lack of follow up data of patients, hence, the impact of loss of Parkin expression on the survival of patients could not be examined in this study. The aim of the present study was to investigate the possible relationship of loss of Parkin expression with its genetic alterations and to evaluate the potential of Parkin as a biomarker in CRC. Nonetheless, we will analyze the functional impact of these mutations and investigate the other genes in this network to fully clarify the role of this important tumor suppressor gene in the genetic etiology of CRC. However, such limitations highlight the need for further studies involving different ethnicities, using population-based sources of cases and controls to clarify the role of *PRKN* in CRC. Our study provides evidence of loss of Parkin expression and its relation with LOH and mutation in *PRKN* among North Indian patients with CRC, which can be used as an initial study for future research.

In summary, we explored the underlying mechanisms contributing to loss of Parkin expression, in which mutation and LOH have their independent roles besides the double hit cases including both. We also demonstrated that both LOH and Parkin expression are noteworthy from a pathophysiologic standpoint. We believe our study has wide implications for understanding the role of *PRKN* in colorectal oncogenesis, which may open up unique opportunities for developing therapeutic approaches for treatment of CRC. We also suggest further evaluation of role of *PRKN* in large, randomized studies of patients with CRC to distinguish the more favorable group for a particular treatment.

Ethics Committee Approval: Ethics committee approval was received fort his study from the Ethics Committee of Jamia Millia Islamia, New Delhi, India (Reference No: IEC/268008/13 Dated: 05/12/2013).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - R.R.T.; Design - R.R.T., K.W.; Supervision - M.A.R., S. S.H.; Resource - M.A.R., S.S.H.; Materials - S.S.S.; Data Collection and/or Processing - R.R.T., A.P.; Analysis and/or Interpretation - R.R.T., K.W., A.P., Z.I.; Literature Search - A.P., Z.I.; Writing - Raj R.T., K.W.; Critical Reviews - M.A.R.

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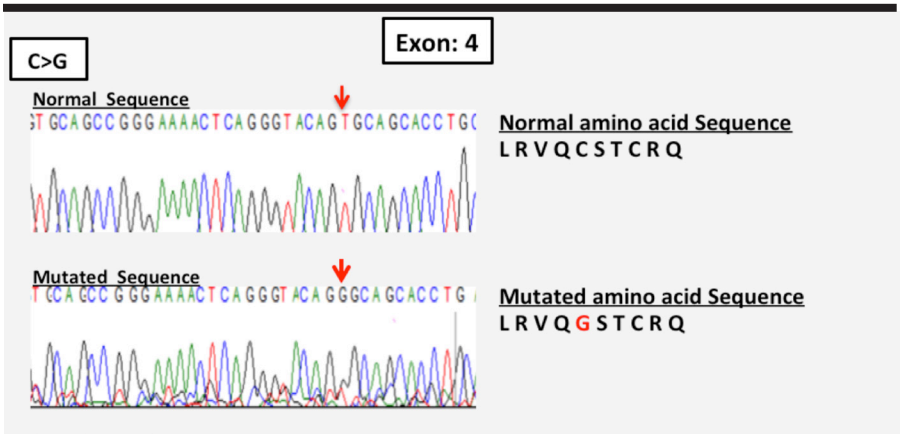
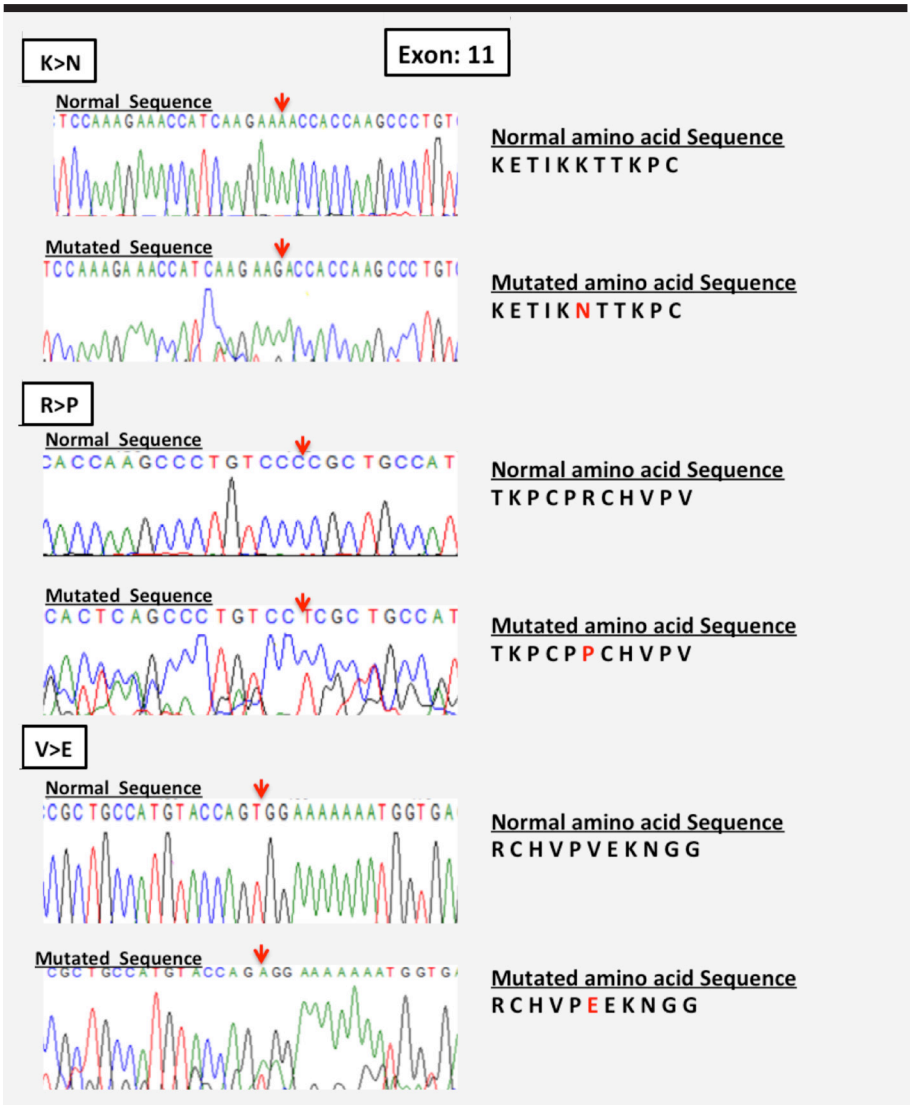


Supplementary Figure S1. *PARKIN* somatic mutations at exon 4: Sequencing histogram of exon 4 showing transition of T→G at nucleotide position 627 leading to the conversion of cysteine (C) to glycine (G) at codon 166.



Supplementary Figure S2. *PARKIN* somatic mutations at exon 11. Sequencing histogram showing transition A→G at nucleotide position 1365 leading to the conversion of lysine (K) to asparagine (N) at codon 413. Second histogram demonstrating with transition C→T at nucleotide position 1383 leading to the conversion of arginine (R) to proline (P) at codon 420. Last histogram showing transversion T→A at nucleotide position 1400 leading to the conversion of valine (V) to glutamic acid (E) at codon 425.

Supplementary Table 1. Clinicopathological characterization of patients with colorectal cancer (n=219).

Variables	Total no. (N)	%
Gender		
Male	117	53
Female	102	47
Age (years)		
<50	56	26
≥50	163	74
Tumor Size		
<3 cm	48	22
>3 cm	171	78
Clinical Stage		
I+II	114	52
III+IV	105	48
Degree of Differentiation		
Low or moderate	183	83
Well-differentiated	36	17
Angiolymphatic/perineural invasion		
Present	138	63
Absent	81	37
Lymph node status		
Present	144	66
Absent	75	34
Location of tumor		
Colon	126	57
Rectum	93	43
Type		
Mucinous	36	16
Non-mucinous	183	84
Side of tumors		
Right	60	27
Left	159	73

Supplementary Table 2. Primer sequences used for LOH markers.

LOH Markers	Primer sequences	Annealing Temp. (° C)	Product Size
D6S305	F5′-CACCAGCGTTAGAGACTGC-3′		
	R5′-GCAAATGGAGCATGTCACT-3′	58	218
D6S1599	F5′-TGTTTTCCACAGGTTCCAG-3′		
	R5′- CTTCAGATGTAGGCTC-CACG-3′	57	133
D6S1008	F5′-AAGAAAGACTAGAGAGACAG-ACAGC-3′		
	R5′-ATCATTTGCCCATTTACCAA-3′	55	246

Supplementary Table 3. Summary of LOH determined at different microsatellite markers.

Microsatellite Markers	D6S1008	D6S1599	D6S305	Cases with both Intragenic Markers	Cases with all Three Markers
LOH	23% (28/119)	35% (42/19)	44% (52/119)	33% (39/119)	16% (19/119)