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RESEARCH

Parkin Gene Alterations in Ovarian Carcinoma from Northern Indian Population

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Abstract *Parkin*, a tumor suppressor gene located on chromosome 6q25-27, has been identified as a target for mutation in many human malignancies like breast, ovaries, cervical and lungs etc. After a preliminary report on the loss of heterozygosity and altered *Parkin* expression in breast and ovarian tumors, we aimed to study loss of heterozygosity in the *Parkin* gene associated microsatellite markers and its expression in human ovarian cancer patients from Indian population. We examined 102 paired normal and ovarian cancer samples for allelic loss in *Parkin* gene locus using *Parkin* gene associated microsatellite markers through loss of heterozygosity and changes in its expression through semiquantitative RT-PCR. Loss of heterozygosity identified common region of loss in *Parkin* locus with highest frequency for the intragenic marker D6S1599 (53%) whereas, 49 of 102 (48%) specimens showed decreased or no expression of *Parkin* in ovarian tumors. The study revealed that presence of loss of heterozygosity was significantly higher in both the intragenic markers (D6S1599 and D6S305) as compared with the locus of flanking region (D6S1008) with their p value 0.000001 and 0.00008, respectively. It also revealed that *Parkin* inactivation is probably a combination of loss of heterozygosity coupled with downregulation of *Parkin* gene through an alternative means like epigenetic mechanism. These data strongly supports the previous study and argue that *Parkin* is a tumor suppressor gene whose inactivation may play an important role in ovarian carcinoma.

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Introduction

Ovarian cancer is one of the leading causes of cancer deaths unique to women [1]. Ovarian cancer afflicts ~204000 women worldwide each year, including ~21650 Americans [2–4]. Despite its relatively low incidence rate, ovarian cancer is an extremely lethal disease. Globally, it claims ~125000 lives per year, making it the seventh leading cause of cancer-related deaths among women [3]. In the United States, ovarian cancer mortality is even higher; it ranks as the fifth deadliest malignancy among women, with an estimated 15520 deaths per year [2]. In general terms, it is much more common in developed countries. In India, ovarian cancer is the third most common among woman-related cancers, followed by breast and cervical cancer, with an estimated 28080 new cases and 19558 deaths [5].

As in the case of other types of tumors, inactivation of tumor suppressor genes and activation of oncogenes are most likely involved in the multi-step process of ovarian carcinogenesis. Chromosome 6q have been reported in the pathogenesis of a number of human malignancies, including breast carcinoma [6], malignant melanoma [7], renal cell carcinoma [8], salivary gland adenocarcinoma [9], acute lymphoblastic leukemia & nodal non-Hodgkin's lymphomas [10], gastric carcinoma [11], hepatocellular carcinoma [12], small-cell lung carcinoma [13], prostate carcinoma [14], parathyroid adenoma [15], capillary hemangioblastomas [16], thymoma [17], cervical cancer [18] and ovarian carcinoma [19]. Loss of heterozygosity (LOH) analysis of chromosome 6q has identified several regions of loss: 6q21-23 [20], 6q25.1-q25.2 [21] and 6q 25-27 [22,

23]. Moreover, deletions at 6q27 are present in benign ovarian tumors [24], suggesting that alterations in one or more genes mapped on this region represent an early event in ovarian tumorigenesis.

Parkin, a gene implicated in autosomal recessive juvenile Parkinsonism [25], was found to be a target of LOH at chromosome 6q25-q27 in breast and ovarian carcinomas [26, 27]. Although various deletions and point mutations have been reported in patients with early onset of Parkinsonism [28], no somatic point mutations were identified in any of the breast or ovarian tumors with LOH at the *Parkin/ FRA6E* locus examined [26]. 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 [26]. Allelic loss and reduced *Parkin* expression was also observed in non-small cell lung carcinoma [29]. We also performed a LOH with 105 cervical cancer specimens and identified a common minimal deleted region, which includes the markers *D6S305* and *D6S1599* present within the large *Parkin* gene [30]. *Parkin* expression was also found to be 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. Furthermore, it was found that loss of *Parkin* expression is frequent in hepatocellular, ovarian and other cancers [31, 32]. In addition, somatic mutations and frequent intragenic deletions of *Parkin* were also studied in human malignancies such as glioblastomas [33].

The focus of this study was to investigate *Parkin* gene locus as a target for LOH in ovarian cancer patients from northern Indian population. We also confirm the data reported by Cesari *et al*. that *Parkin* is downregulated in ovarian tumors [26].

Materials and Methods

Tissue Samples One hundred and two samples of surface epithelial ovarian tumors (SEOTs) and their matched control samples (blood/normal tissue) were collected from Batra Hospital, New Delhi and was immediately stored in −80°C. All histological diagnoses of ovarian epithelial tumors were confirmed by gynecological pathologist. Tumors were staged according to the International Federation of Gynecologists and Obstetricians (FIGO) criteria [34] and classified as follows: 58 serous carcinoma (SC), 10 serous borderline (SB), 10 endometroid carcinoma (EC), 8 mucinous carcinoma (MC), 9 clear cell carcinoma (CC) and 7 undifferentiated carcinoma (UC). The work presented here was approved by the Institutional Ethical and Biosafety Committee. Table 1 summarizes the clinicopathological variables.

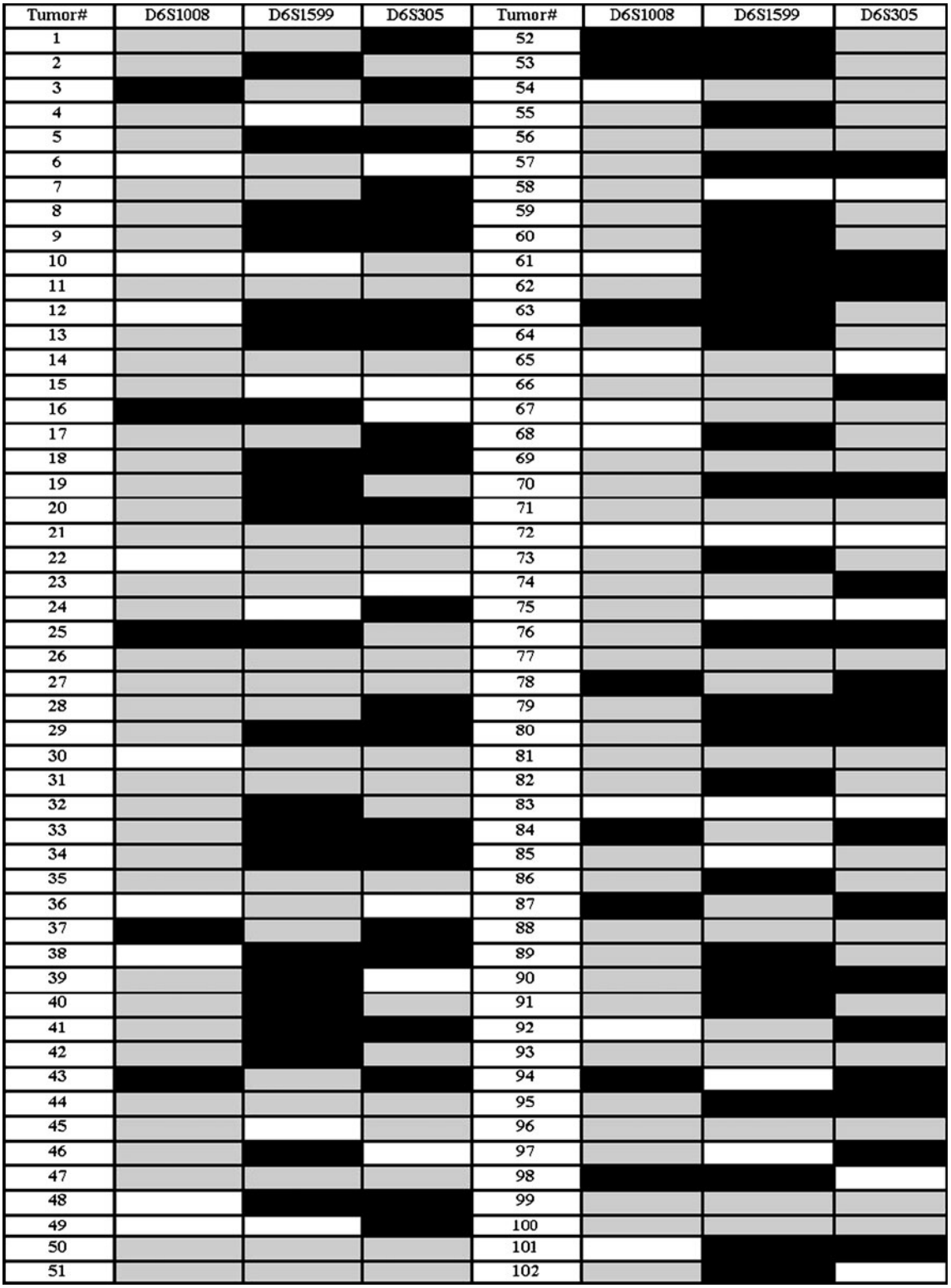
Table 1 Demographic and pathological features of the patient population

Characteristics	No. of patients
Number of patients	102
Mean age (years)	52 (35–70)
Pathologic Stage	
I	24 (24%)
II	47 (46%)
III	23 (23%)
IV	8 (8%)
Tumor Differentiation	
Well-differentiated	15 (15%)
Moderately differentiated	55 (54%)
Poorly differentiated	32 (32%)
Menopausal Status	
Pre Menopausal Stage	34 (33%)
Post Menopausal Stage	68 (67%)

DNA/RNA Extraction DNA was extracted from the ovarian tumor samples and their matched control samples by SDS/ proteinase K treatment, phenol–chloroform extraction, and ethanol precipitation as described previously [35] and then dissolved and stored in TE buffer. Total RNA was isolated using NP-P Total RNA Extraction Kit (Taurus & Scientific, USA). Finally, purity and concentration of extracted DNA/ RNA were analyzed by gel electrophoresis and ultraviolet spectrophotometry.

PCR and LOH Studies Three microsatellite marker sites: D6S1599, D6S305 and D6S1008 in chromosome 6q25-27 were selected to detect LOH of *Parkin* gene. D6S1599 and D6S305 are intragenic markers which are present in *Parkin* introns 2 and 7, respectively where as D6S1008 is present at the telomeric end. Primer sequences are available at the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov/). PCR and LOH analysis was performed as described previously [30, 35]. The heterozygous genomic allele was targeted for LOH information analysis. LOH was defined as a complete loss or up to 40% decreased relative density of silver staining bands of PCR products in ovarian cancer samples compared to their matched control samples [36].

Reverse Transcriptase-PCR for Parkin mRNA Expression One μg of total RNA was used for cDNA synthesis using RevertAid^{TM} first strand cDNA synthesis kit (Fermentas Life Sciences, USA) with random hexamers. After cDNA synthesis, RT-PCR was performed to detect mRNA expression of *Parkin* gene. PCR was carried out in a total volume of 25 μl, using 2 μl of cDNA, 1 U Taq DNA Polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 20 pmol of primers.



Tumor#	D6S1008	D6S1599	D6S305	Tumor#	D6S1008	D6S1599	D6S305
1				52			
2				53			
3				54			
4				55			
5				56			
6				57			
7				58			
8				59			
9				60			
10				61			
11				62			
12				63			
13				64			
14				65			
15				66			
16				67			
17				68			
18				69			
19				70			
20				71			
21				72			
22				73			
23				74			
24				75			
25				76			
26				77			
27				78			
28				79			
29				80			
30				81			
31				82			
32				83			
33				84			
34				85			
35				86			
36				87			
37				88			
38				89			
39				90			
40				91			
41				92			
42				93			
43				94			
44				95			
45				96			
46				97			
47				98			
48				99			
49				100			
50				101			
51				102			

Fig. 1 Detail LOH analysis of 102 ovarian specimens. Black boxes represent Loss of Heterozygosity, Grey shading represents Heterozygous condition and white boxes represents uninformative condition respectively

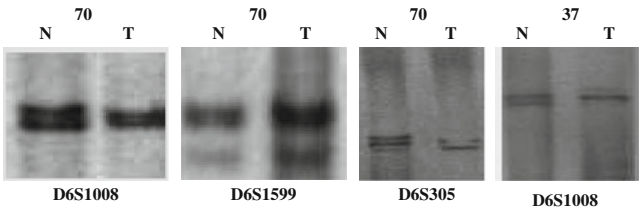


Fig. 2 Representative examples of microsatellite analysis of *Parkin* specific markers at 6q25-27. DNAs of tumor (T) and corresponding normal (N) tissues are shown with the microsatellite markers indicated at the bottom and sample numbers on the top

The sense primer for *Parkin* was (5′-AGAGCTCCAT CACTTCAGGATT-3′), and the antisense primer was (5′-CCCCTTCATGGTACGCTTCT- 3′). The expected amplified fragment for *Parkin* was 230 bp. As an internal control, the sense primer for β-actin gene was (5′-TGGACTTCGA GCAAGAGATGG-3′), and the antisense primer was (5′-ATCTCCTTCTGCATCCTGTCG-3′). The expected amplified fragment for β-actin was 289 bp. PCR conditions were 94°C for 10 min, followed by 28 cycles at 94°C for 1 min, 58°C for *Parkin* and 60°C for β-actin for 1 min and 72°C for 1 min. The final extension was at 72°C for 10 min. The amplified DNA products were separated on 2% agarose gel, stained with ethidium bromide, visualized and photographed with Gel Documentation System (BioRad, USA).

Statistical Analysis LOH incidence and mRNA expression levels of *Parkin* gene was compared with the clinicopathological parameters using the *Chi-Square test* [37]. LOH found in two intragenic markers (D6S1599, D6S305) was also compared with the marker at telomeric end (D6S1008). $P<0.05$ was considered statistically significant.

Results

A total of 102 ovarian cancer specimens were analyzed for allelic loss of *Parkin* gene locus using three *Parkin* specific primers pairs located on the long arm of chromosome 6 and mRNA expression of *Parkin*. Patients had a mean age of 52 and were predominantly with postmenopausal stage. All stages of disease were represented in the group: 24 (24%) patients had stage I, 47 (46%) stage II, 23 (23%) stage III, and 8 (8%) stage IV.

LOH Analysis and Identification of a Common Minimal Region of Loss at 6q25-27

Three polymorphic microsatellite markers were used to test for LOH in 102 ovarian cancer samples. A case is considered to be informative if the normal control tissue is heterozygous at that site. If the control is homozygous, it is not possible to detect LOH and is, therefore, uninformative. Out of 102 tumors examined, 100 were heterozygous for at least one microsatellite markers studied. The percentage informative cases were 82, 87, 86 for the three different microsatellite markers; D6S1008, D6S1599, D6S305 respectively. The incidence and frequency of LOH for each primer pair is summarized in Fig. 1. Overall,

Table 2 Correlation between clinicopathological parameters ovarian cancer specimens and LOH at 6q25-27 (*PARKIN* gene locus)

Clinicopathological parameters	Total number of cases tested	LOH at 6q25-27		*p* value
	n-102	Positive (%) n-64	Negative (%) n-38	
Age (years)				
≤ 49	30	18 (60%)	12 (40%)	0.7
≥ 50	72	46 (64%)	26 (36%)	
Pathological Grade				
1	15	9 (60%)	6(40%)	0.9
2	55	35 (64%)	20 (36%)	
3	32	20 (63%)	12 (37%)	
Clinical Stage				
I	24	13(54%)	11(46%)	0.4
II	47	30(64%)	17(36%)	
III	23	14(61%)	9(39%)	
IV	8	7(88%)	1(12%)	
Menopausal Status				
Pre-Menopausal Status	34	22 (65%)	12 (35%)	0.7
Post Menopausal Status	68	42 (62%)	26 (38%)	

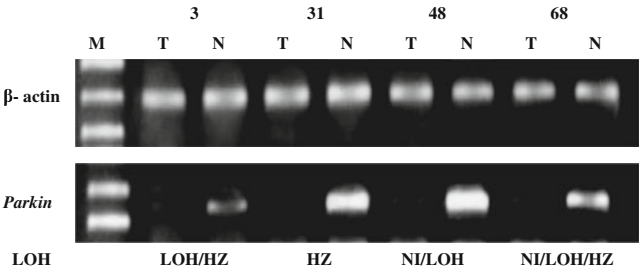


Fig. 3 Reverse transcription-PCR analysis of *Parkin* gene expression in ovarian cancer. The loss of heterozygosity (LOH) data are shown for each case. *NI*, not informative; *HZ*, heterozygous; *LOH*, loss of heterozygosity

64 of 102 (62%) ovarian samples showed LOH in at least one locus in the region examined. The number of markers at which a single tumor displayed LOH ranged from one to two, whereas none of the tumors demonstrated LOH at all loci. 23 samples have shown LOH in both the intragenic markers. The percentage of LOH across each of the three markers ranged from 18% (D6S1008) to 53% (D6S1599). The highest rate of LOH was observed at intragenic markers D6S1599 and D6S305 which is located towards centromeric end between exons 2 and 3 where as the other is located in the 5′ end of the *Parkin* gene, between exons 7 and 8 respectively. Figure 2 show an example of a silver stained gel depicting LOH and the normal control in the adjacent lane.

The correlation between the clinicopathological parameters of 102 cases of surface epithelial ovarian tumors and LOH at 6q25-27 was shown in Table 2. LOH on 6q25-27 of the *Parkin* gene was observed in 18 of 30 (60%) patients below the age of 50 years whereas 46 out of 72 (64%) showed LOH at the age group of more than 50 years. LOH was also identified in 9 of 15 (60%) cases in pathologic grade I, 35 of 55 (64%) cases in grade II, and 20 of 32 (63%) cases in grade III. 13 out of 24 (54%) cases were found to be homozygously deleted in clinical stage I, 30 of 47 (64%) cases in stage II, 14 of 23 (61%) cases in stage III and 7 of 8 (88%) in stage IV. LOH was also identified in 22 of 34 (65%) pre-menopausal and 42 of 68 (62%) post-menopausal status specimens.

However, no statistically significant relationship was found between the presence of LOH at 6q25-27 and patient age (P=0.7), degree of tumor grade (P=0.9), tumor stage (P=0.4), and menopausal status (P=0.7) respectively.

Parkin Expression Analysis

Semiquantitative RT-PCR was performed to analyze *Parkin* expression in all the ovarian tumor specimens. Forty nine of one hundred and two samples (48%) showed decreased or no expression of *Parkin* transcript relative to normal ovarian tissue, whereas fifty three of one hundred and two (52%) showed nearly identical levels of expression (Fig. 3). The correlation between the clinicopathological parameters of 102 cases of surface epithelial ovarian tumors and *Parkin* mRNA expression are also summarized in Table 3. Decreased or no expression of *Parkin* transcript relative to normal ovarian tissue was observed in 12 of 30 (40%) patients below the age of 50 years

Table 3 Correlation between the clinicopathological parameters of ovarian cancer specimens and the mRNA expression of *Parkin* gene

Clinicopathological parameters	Total number of cases tested	*PARKIN* gene expression		*p* value
	n-102	Positive (%) n-49	Negative (%) n-53	
Age (years)				
≤ 49	30	12 (40%)	18 (60%)	0.2
≥ 50	72	37 (51%)	35 (49%)	
Pathological Grade				
1	15	9 (60%)	6(40%)	0.4
2	55	27 (49%)	28 (51%)	
3	32	13 (41%)	19 (59%)	
Clinical Stage				
I	24	13(54%)	11(46%)	0.1
II	47	18(38%)	29(62%)	
III	23	15(65%)	8(35%)	
IV	8	3(37%)	5(63%)	
Menopausal Status				
Pre-Menopausal Status	34	15 (44%)	19 (56%)	0.5
Post Menopausal Status	68	34 (50%)	34 (50%)	

whereas 37 out of 72 (35%) showed decreased or no expression of *Parkin* transcript at the age group of more than 50 years. Decreased or no expression of *Parkin* mRNA was identified in 9 of 15 (60%) cases in pathologic grade I, 27 of 55 (49%) cases in grade II, and 13 of 32 (41%) cases in grade III. 13 out of 24 (54%) cases were found to have decreased or no expression of *Parkin* transcript in clinical stage I, 18 of 47 (38%) cases in stage II, 15 of 23 (65%) cases in stage III and 3 of 8 (37%) in stage IV. Decreased or no expression of *Parkin* mRNA was also identified in 15 of 34 (4%) pre-menopausal and 34 of 68 (50%) post-menopausal status specimens. No statistically significant relationship was also found between *Parkin* mRNA expression and patient age (P=0.2), degree of tumor grade (P=0.4), tumor stage (P=0.1), and menopausal status (P=0.5) respectively.

LOH at 6q25-27, the *Parkin* Gene Locus is Associated with Its Reduced mRNA Expression

To determine the effect of LOH at 6q25-27 on gene expression, we analyzed mRNA expression levels of *Parkin* by RT-PCR in all ovarian tumor samples. Out of 102 ovarian tumor samples, 30 (29%) samples exhibited both LOH and reduced *Parkin* gene expression. Interestingly, 10 specimens demonstrated either reduced *Parkin* gene expression or no expression in those samples which showed common region of loss in both the intragenic markers D6S305 and D6S1599 which involves *Parkin* exons 2-10 and suggests that the expression of *Parkin* transcript are the result of genomic deletions. In addition, 16 out of 102 (16%) ovarian tumor samples, which either retained or lost heterozygosity, respectively in our LOH analysis defined by the intragenic markers D6S305 and D6S1599, both exhibited a reduction in *Parkin* gene expression. It indicates that a mechanism other than deletion may account for the reduction in the levels of *Parkin* in these tumors.

Discussion

LOH at the long arm of chromosome 6 constitutes an important role in the development of various cancers, including ovarian tumors. 6q27 is already reported to be one of the regions commonly deleted in ovarian carcinomas [38, 39]. Involvement of the locus of *Parkin* gene, 6q25-27 have been studied in the carcinomas of breast [26], ovary [26, 31], small cell lung [29] liver [32] and cervical cancer [30]. In this study, we confirm the work reported by Cesari *et al*. (2003) and examined the region within *Parkin* gene locus using three microsatellite markers *viz*. D6S1008, D6S1599, and D6S305 located at 6q25-27 [26]. The present study shows that LOH was significantly higher in both the intragenic markers (D6S1599 and D6S305) as compared with the locus at telomeric end (D6S1008) with their p value 0.000001 and 0.00008, respectively [37]. Subsequently, analysis of *Parkin* gene expression found transcript levels to be reduced or absent in > 45% of the samples examined. This analysis revealed a very high rate of LOH in those cases which belongs to the stage IV, grade 2 and 3 (Table 2). On the contrary, no or reduced expression of *Parkin* transcript was found in the specimens of grade I and stage III, suggesting that deletion of the *Parkin* gene may have a unique association with a histological subtype (Table 3).

Although, *Parkin* function is not entirely understood, *Parkin* protein was found to be a ubiquitin-protein ligase (E3). It is therefore possible that mechanisms related to the ubiquitin function are involved in the tumorigenic process and to elucidate the role of *Parkin* in tumorigenesis, it is necessary to identify substrates of *Parkin* E3 ubiquitinating activity and their potential relationship to apoptosis and/or cellular proliferation. In Autosomal juvenile recessive parkinsonism (AR-JP) affected individuals, *Parkin* is inactivated by point mutations or more frequently, by exon deletions or amplification [28] where as in cancer *Parkin* has undergone intragenic deletions, which may contribute to tumor initiation and development [26]. Studies have also shown that *Parkin* was down-regulated in 60% of the primary ovarian tumors analyzed [27]. 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 [40].

Our findings supports the previous study and revealed that *Parkin* is a putative tumor suppressor gene at human chromosome 6q25-27 and microsatellite analysis of this gene specific markers revealed that its reduced expression and inactivation may play an important role in the progression of ovarian carcinoma and other human cancers.

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