Detection of Numerical Abnormalities of Chromosome 9 and p16/CDKN2A Gene Alterations in Ovarian Cancer with Fish Analysis

CHRISTOS ARAVIDIS[1], ANNA D. PANANI[1], ZOI KOSMAIDOU[2], NIKOLAOS THOMAKOS[3], ALEXANDROS RODOLAKIS[3] and ARISTEIDIS ANTSAKLIS[3]

[1]*Critical Care Department, Cytogenetics Unit, Evangelismos Hospital, Medical School of Athens University, Athens, Greece;*
[2]*Department of Genetics, Alexandra Hospital, Athens, Greece;*
[3]*Department of Obstetrics and Gynecology, Alexandra Hospital, Medical School of Athens University, Greece*

Abstract. *Background: The molecular events leading to the development of ovarian cancer are not well-established. Defects of the retinoblastoma protein (pRb)/cyclin-D1/p16 pathway have been shown to play a critical role in the development of human malignancies. In particular, the p16/cyclin-dependent kinase inhibitor 2A (CDKN2A) gene located on chromosomal region 9p21 frequently is altered in several types of cancer. Materials and Methods: To investigate both the presence of numerical abnormalities of chromosome 9 and p16 gene alterations in ovarian cancer, we studied 28 cases by the fluorescence in situ hybridization (FISH) technique using a DNA p16 probe and an a-satellite probe specific for chromosome 9. Results: Numerical abnormalities of chromosome 9 were found in all studied cases. Polysomy 9 was detected in 10 cases while monosomy 9 in seven cases. In 11 cases, there were two cell populations, one with polysomy 9 and the other with monosomy 9. In all cases, the p16 gene deletion was observed. Among them, 25 cases presented deletion of p16 gene in 21.43%-86.3% of the examined cells. Three cases carried deletion of the p16 gene in a lower proportion (12.04%-19.49%). In five cases with p16 gene deletion, homozygous deletion was detected. Conclusion: Numerical aberrations of chromosome 9 and p16 gene deletion are common findings in ovarian cancer. Data suggest that the p16 gene, located in the short arms of chromosome 9, may play a role in ovarian carcinogenesis. In addition, polysomy 9 could lead to activation of a number of oncogenes, thus participating in the neoplastic process in the ovaries.*

Correspondence to: Dr. Christos Aravidis, Department of Cancer Genetics, Centrum for Molecular Medicine, Karolinska University Hospital, L8:02 Stockholm171 76, Sweden. Tel: +46 739572851, e-mail: christos.aravidis@yahoo.gr

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Ovarian cancer is one of the leading causes of death from gynaecological malignancies worldwide. The molecular events leading to the development of ovarian cancer and the molecular factors that may predict response to treatment are not well-established. De-regulation of cell-cycle control, in particular G_1- to S-phase transition, is implicated in the pathogenesis of most types of human cancer, including ovarian cancer (1). The tumor suppressor gene p16/cyclin-dependent kinase inhibitor-2A (CDKN2A) is located within the chromosomal region 9p21 and encodes a cell-cycle protein that is an inhibitor of cyclin-dependent kinases (CDK)-4 and -6. As a result, it negatively regulates cyclin-D-dependent phosphorylation of the retinoblastoma (pRb) gene product, thus blocking cell-cycle progression from G_1-to S-phase (2-4). Loss of function of the *p16* gene has been reported to occur mainly by homozygous deletion, mutation or aberrant DNA methylation of the promoter of the region (5-8).

A number of studies have shown that *p16/CDKN2A* is frequently altered in melanoma (9), pancreatic (10), urinary bladder (11) and lung cancer (12). There are few studies focusing on the molecular analysis of *p16* gene in ovarian cancer (13-16). The aim of the present study was to determine the significance of this tumor suppressor gene for ovarian tumorigenesis, investigating both numerical aberrations of chromosome 9 and *p16* gene alterations in 28 cases of ovarian tumors, by the fluorescence *in situ* hybridization (FISH) technique.

Materials and Methods

Twenty-eight cases of patients who had undergone surgical resection of ovarian cancer between the years 2005 to 2009 were included in the present study. Among the methods used for cytogenetic evaluation was the FISH technique for *p16* gene deletion. Tissue specimens were

Table I. *Numerical aberrations of chromosome 9 and p16 gene alterations in 28 cases of ovarian cancer.*

Case	Age, years	Chromosome 9 numerical aberrations			*p16* Gene alterations				Histological subtype	Stage	Grade
		Monosomy 1g	Disomy 2g	Polysomy >2g	Deletion 1g/1r	Normal 2g/ 2r	Deletion r<g	Gains g=r, >2			
1	63	16.04%	73.8%	10.16%	13.9%	43.85%	36.9%	5.35%	Serous carcinoma	IIIc	3
2	78	58.65%	41.35%	0%	40.38%	26.92%	32.7%	0%	Serous carcinoma	IIIc	3
3	70	0%	9.87%	90.13%	0%	9.87%	28.4%	61.73%	Serous carcinoma	IIIc	3
4	60	7.14%	22.45%	70.41%	7.14%	17.35%	45.92%	29.59%	Clear cell carcinoma	IIIc	3
5	70	5.13%	35.9%	58.97%	4.27%	23.09%	46.15%	26.49%	Serous carcinoma	IIIc	3
6	48	14.56%	64.08%	21.36%	10.68%	49.51%	29.13%	10.68%	Borderline serous carcinoma	Ia	1
7	73	9.52%	24.76%	65.72%	7.62%	16.2%	49.52%	26.66%	Clear cell carcinoma	IIa	3
8	58	33.59%	54.2%	12.21%	25.95%	24.4%	48.85%	0.8%	Serous carcinoma	IIIc	3
9	67	21.69%	49.4%	28.91%	20.48%	46.99%	14.46%	18.07%	Serous carcinoma	IIc	3
10	70	18.05%	40.6%	41.35%	15.04%	33.08%	23.31%	28.57%	Serous carcinoma	IIa	2
11	57	2.38%	43.65%	53.97%	2.38%	34.13%	21.43%	42.06%	Clear cell carcinoma	IV	3
12	68	32.28%	60.63%	7.09%	32.28%	38.58%	29.14%	0%	Serous carcinoma	IIIc	3
13	68	15.27%	70.99%	13.74%	13.74%	13.74%	70.99% * 13.74%	1.53%	Serous carcinoma	IIIc	3
14	59	12.78%	56.39%	30.83%	12.03%	39.1%	41.35%	7.52%	Clear cell carcinoma	IIIc	3
15	65	9.68%	42.31%	48.01%	3.85%	13.46%	82.69% * 60.58%	0%	Serous carcinoma	IIIc	3
16	54	18.18%	45.45%	36.37%	12.88%	22.73%	53.79% * 12.88%	10.61%	Serous carcinoma	IIIc	3
17	63	11.2%	29.6%	59.2%	8%	16.8%	52.8%	22.4%	Serous carcinoma	IIc	3
18	61	82.9%	17.1%	0%	9.4%	4.3%	86.3%	0%	Mucinous carcinoma	Ia	1
19	54	9.3%	40.31%	50.39%	6.2%	25.58%	64.34%	3.88%	Serous carcinoma	IIIc	3
20	85	2.91%	37.86%	59.23%	0.97%	8.74%	82.52% * 55.34%	7.77%	Clear cell carcinoma	IIc	3
21	80	21.32%	77.21%	1.47%	10.29%	22.06%	66.91% * 15.44%	0.74%	Serous carcinoma	IIIc	2
22	44	47.62%	43.81%	8.57%	28.57%	28.57%	37.14%	5.72%	Serous carcinoma	IIIc	3
23	85	16.78%	79.72%	3.5%	9.1%	54.55%	34.95%	1.4%	Granulosa cell tumor	Ia	1
24	85	3.7%	52.78%	43.52%	3.7%	48.15%	12.04%	36.11%	Mixed Mullerian tumor	IIIc	3
25	52	10.17%	77.12%	12.71%	10.17%	61.02%	19.49%	9.32%	Serous carcinoma	IIc	3
26	61	10.23%	75%	14.77%	6.82%	55.68%	30.68%	6.82%	Mixed Mullerian tumor	IIIb	3
27	49	5.36%	16.96%	77.68%	4.46%	14.29%	26.79%	54.46%	Serous carcinoma	IIIc	3
28	50	23.97%	66.94%	9.09%	39.67%	14.88%	44.63%	0.82%	Serous carcinoma	IIIc	3

*Homozygous deletion of *p16* gene; g (green) copies of chromosome 9, r (red) *p16* gene copies.

collected from fresh surgically resected tumors in 27 cases and in one case, ascitic effusion was taken just before a surgery with fine-needle aspiration biopsy (FNA). None of the patients had ever received chemotherapy or radiation prior to surgery. The FISH technique was applied to recently made slides from methanol/acetic acid-fixed cells using a DNA *p16* probe and an α-satellite probe specific for chromosome 9. The p16 probe, (Cytocell Ltd., Cambridge, UK), labeled red, covered a 101-kb region of 9p21 extending from 59 kb 3' of *p16* to the 5' end of *p15*. The probe mix also contained a control probe for chromosome 9 (D9Z3, the heterochromatic block at 9q12 region), labeled green. The technique was carried out according to the manufacturer's instructions. The hybridization was visualized by fluorescence microscope, equipped with selective filters for the fluorochromes used.

As far as the evaluation of the signal is concerned, a normal cell has two copies of chromosome 9 (two green spots) and two copies of *p16* gene (two red spots). Cells with deletion of *p16* gene have fewer red spots than green. Cells with one red and one green spot have deletion of *p16* gene and monosomy 9. A minimum of 150 non-overlapping cells from each slide were evaluated for each case. Signals were scored using well-established criteria (17). To avoid misinterpretation due to technical error, normal lymphocyte nuclei were used as a control. A case was defined as abnormal if more than 10% of cells showed losses or gains of signals for chromosome 9 and the *p16* gene. The present study was approved by the local Ethical Committee.

Results

Results are shown in Table I. Numerical abnormalities of chromosome 9 were found in all studied cases. Polysomy 9 was detected in 10 cases, while monosomy 9 in seven cases. In 11 cases, there were two cell populations, one with polysomy 9 and the other with monosomy 9. In the entire case cohort, *p16* gene deletion was observed (red spots<green spots) (Figure 1). Among them, 25 cases presented deletion of the *p16* gene in 21.43%-86.3% of the examined cells. Three cases carried deletion of *p16* gene in a lower proportion, between 12.04%-19.49% of the examined cells. In five cases with *p16* gene deletion, the gene was deleted homozygously in 12.88%, 13.74%, 15.44%, 55.34% and 60.58% of the examined cells,

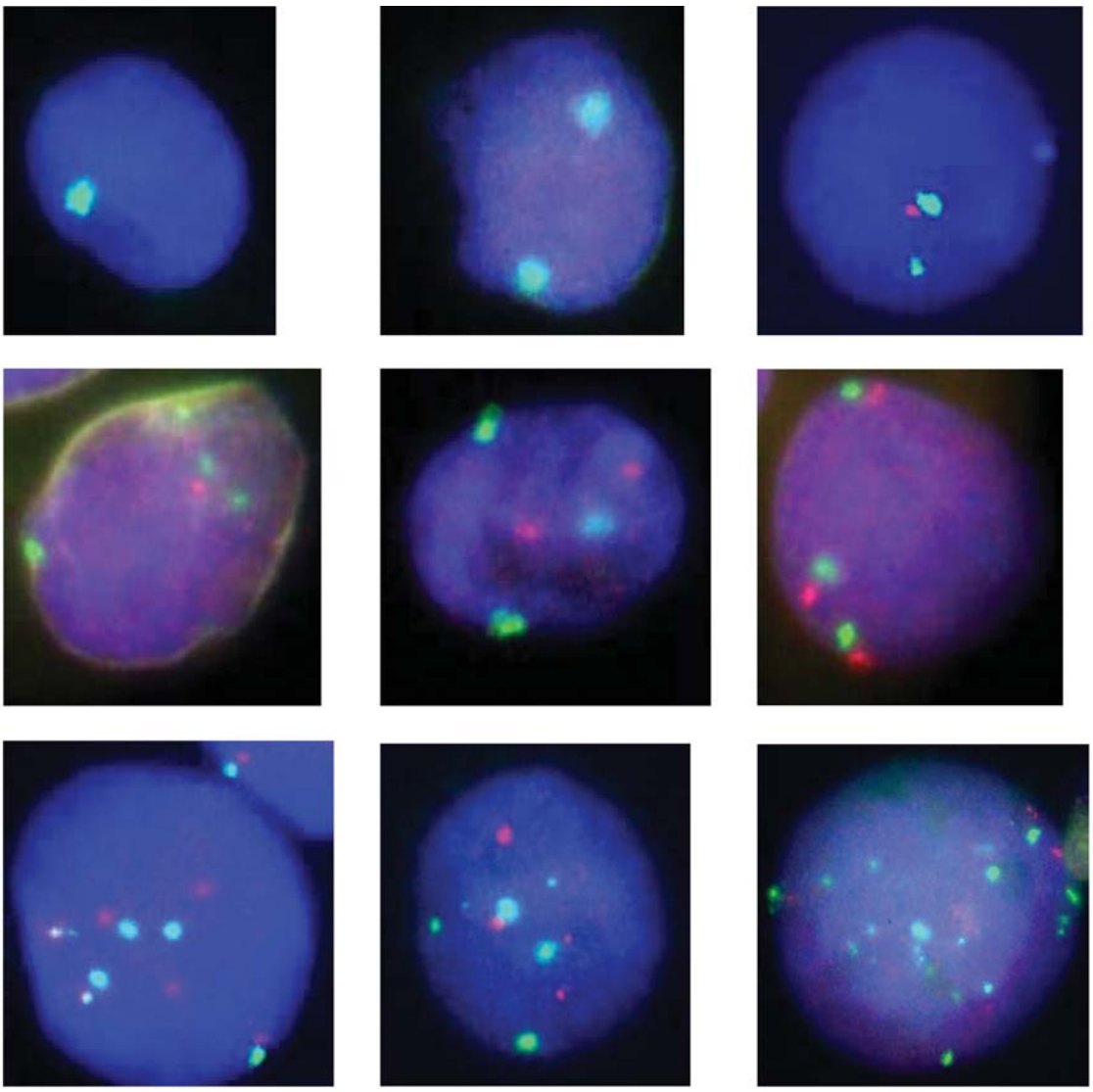

Figure 1. *Copy number of chromosome 9 (green spots) and p16 gene (red spots) detected by fluorescence in situ hybridization (FISH) in ovarian cancer cells from different cases. Cells with one copy (top left image) and two copies (top center) of chromosome 9 and absence of p16 gene. Cell with two chromosomes 9 and only one copy of p16 (top right). Four chromosomes 9 and only one copy of p16 gene (middle left), three copies of chromosome 9 and two p16 gene copies (middle center image) three chromosomes 9 and three copies of p16 (middle right). Multiple copies of chromosome 9 and p16 gene copies (bottom images).*

respectively. Moreover, in 14 cases, there was a cell population with one copy of chromosome 9 and one *p16* gene (one red and one green spot) in 10.17%-40.38% of cells. Finally, in 12 cases, a cell population with *p16* gains equal to chromosome 9 copies (red spots=green spots, >2), was found for 10.61 to 61.73% of the examined cells.

Discussion

Several studies have investigated the genetic changes in ovarian carcinogenesis, in order to identify specific molecular markers, which provide information regarding prognosis and also the development of new therapeutic strategies. Molecular studies focusing on tumor suppressor gene *p16* showed that its inactivation has an important role in carcinogenesis of several types (9-12). The inactivation of *p16* gene is mainly caused by homozygous deletion, mutations or promoter hypermethylation of the gene.

There are a number of studies focused on the *p16* gene alteration in ovarian tumorigenesis, most of which are based on polymerase chain reaction (PCR) analysis techniques and immunohistochemistry for protein expression (18-23).

Nevertheless, results from these studies provided conflicting information concerning the expression of the gene, and the prognostic significance of differential expression levels of cell-cycle regulatory protein-product in ovarian carcinomas. This may be a result either of the size of the samples used for this technique or broad heterogeneity of the patients with respect to stage and postoperative therapy. However, one large-scale prospective study of advanced-stage ovarian cancer indicated that p16-positive tumors have better survival rate compared to p16-negative tumors (24).

In our study, we utilized FISH to determine numerical abnormalities of chromosome 9 and *p16* gene alterations in ovarian cancer. Interestingly, all ovarian cancer cases, regardless of histological subtype, tumour stage or grade, exhibited *p16* gene deletion in a range of proportion from 12.04% to 86.3% of the examined cells. Moreover, numerical aberrations of chromosome 9 were present in every case. Polysomy 9 was detected in 10 cases, while monosomy 9 in seven cases. In 11 cases, two cell populations were found, one with monosomy and one with polysomy 9. Our data show that numerical aberrations of chromosome 9 and *p16* gene deletion are common findings in all subtypes of ovarian cancer. It seems evident that in cases with one red and one green spot, loss of *p16* gene is due to chromosome 9 monosomy. Similarly, in cases with gains of *p16* gene copies equal to those of chromosome 9, the gene copies result from chromosome 9 polysomy.

The FISH technique is considered the most reliable method for detecting homozygous gene deletions. In five of our cases with *p16* gene deletion, the gene was deleted homozygously in 12.88%-60.58% of the examined cells. High frequency of homozygous deletion of *p16* gene has been reported in cell lines from several cancer types (25). Moreover, studies showed that homozygous deletion of the *p16* gene is a common finding in ovarian cancer cell lines (8, 26). However, regarding primary ovarian cancer cells, homozygous deletion of *p16* gene is not a recurrent finding, thus in one study, the gene was homozygously deleted at a very low frequency (1/67 cases) (6). It should be noted that the evaluation of these findings depends on the technique applied for the analysis (PCR, loss of heterozygosity-LOH, representative DNA analysis-RDA).

Besides *p16* gene inactivation, novel data based on techniques of RDA identify alternate pathways for tumorigenesis. It is suggested that genes such as *IFN-α*, *p16*, *p15* and other unknown tumor suppressor genes, which could be implicated in ovarian carcinogenesis, are located in the chromosomal region 9p21 (27). Additionally, data of the present study may indicate that a number of oncogenes, in association with polysomy 9, could contribute to ovarian carcinogenesis.

Moreover, our study suggests that there might not be a correlation between genetic changes and of particular histological subtypes, stage or grade of this neoplastic disease. Hence, the fact that similar recurring genetic aberrations have been observed within different histological subtypes of ovarian cancer might suggest that there are no tissue-specific differences in the genetic changes by which neoplasia is initiated or progresses. However, in order to verify and establish this, more data from cytogenetic studies are required.

In conclusion, the FISH technique using cocktail probes specific for chromosome 9 and the *p16* gene is a valuable method for concurrently detecting numerical abnormalities of chromosome 9 and numerical alterations of *p16* gene copies, events which are common findings in ovarian cancer. The utilization of molecular technologies that allow for high throughput analysis of genes has provided new insights into gene expression profiles in ovarian cancer and may lead to the development of new biomarkers or novel therapies for this neoplasia.

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