Journal of BUON **15:** 101-106, 2010

ORIGINAL ARTICLE

p53 gene mutations and codon 72 polymorphism in ovarian carcinoma patients from Serbia

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Summary

Purpose: *Ovarian cancer is the leading cause of death from gynecological malignancies. The early stages of this disease are asymptomatic and more than 75% of the cases are diagnosed with regional or distant metastases. p53 gene is frequently mutated in some histological subtypes of ovarian carcinomas. The role of p53 mutations and polymorphic variant of codon 72 in the prognosis of disease is still unclear. The aim of this study was to determine the frequency of p53 mutations and polymorphic variants of codon 72 among ovarian carcinoma patients and to correlate them with clinicopathological characteristics of disease.*

Methods: *54 ovarian carcinoma patients were included in the study. DNA was isolated from tumor tissue by the salting-out method. p53 mutations in exons 4-8 were detected by PCR-SSCP (polymerase chain reaction – single-stranded conformational polymorphism) electrophoresis. Codon 72 polymorphism was assessed by RFLP (restriction fragment-length polymorphism) method.*

Results: *p53 mutations were present in 11 out of 54 patients (20.4%). Twenty-four patients (44.4%) exhibited Arg/Arg, 24 patients (44.4%) Arg/Pro and 6 patients (11.2%) Pro/Pro genotype of 72 codon polymorphism. Correlations between p53 mutations and various clinicopathological characteristics were not found. However, we observed that the frequency of Pro/Pro genotype was increasing with higher histological grade as well as in advanced compared to localized disease, but without statistical significance. Distribution of p53 gene mutations between Pro/Pro genotype and Arg/Pro plus Arg/Arg genotypes was not statistically significant.*

Conclusion: *Our study suggests that Pro/Pro genotype of 72 codon polymorphism could be an independent prognostic marker in ovarian carcinomas.*

Key words: codon 72 polymorphism, ovarian carcinoma, p53 mutations

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies. The overall 5-year survival rate is about 50%. The early stages of this disease are asymptomatic and more than two-thirds of cases are diagnosed with regional or distant metastases [1].

Over 90% of these cancers are of epithelial origin. It is very a heterogeneous disease with variability of clinical characteristics such as histological subtypes, differentiation, potential for invasion and metastasis, response to therapy and outcome. Malignant transformation of epithelial ovarian cells is caused by genetic alteration that disrupt the regulation of proliferation, apoptosis and DNA repair [2].

One of the most studied genes in human malignancies is p53. The gene is located on the short arm of chromosome 17 at 17p13.1 position, and encodes a 53 kDa nuclear phosphoprotein that is involved in cell cycle control, DNA repair, genome stability, apoptosis, differentiation, senescence and angiogenesis [3].

p53 gene is mutated in over of 50% of all malignancies and often with high frequency. p53 gene is inactivated in ovarian carcinomas by point mutations, deletions,

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Received 11-06-2009; Accepted 11-08-2009

loss of part of 17p chromosome and hypermethylation of the promotor region [4]. The primary mechanism of p53 dysfunction is the occurrence of point mutations in the evolutionary conserved DNA binding domain (exons 5-8) of the gene. The percentage of p53 mutations vary from 0 to 80%, depending on histological subtypes, stage of disease and pathways of ovarian tumorigenesis [5].

About 95% of all p53 mutations are located in the DNA binding domain [6]. Because of that, we analyzed mutations in exons 5-8. Exon 4 was also included in this study because of the impact of this region on p53 protein function in apoptosis [7].

p53 mutations are associated with worse prognosis in many human cancers. The role of p53 alterations as prognostic factors in ovarian carcinomas remains controversial. The impact of p53 alterations on prognosis in patients with ovarian cancer has been studied, but has been mainly focused in advanced disease. The majority of studies demonstrated that p53 alterations have failed to influence the prognosis in multivariate analysis including known prognostic factors [8].

While p53 mutations are the most frequently studied alterations in human cancer, p53 polymorphisms are poorly investigated. The common polymorphism in p53 gene is at codon 72 with single-base change that codes either arginine (CGC) or proline (CCC) in proline- rich region of the transactivation domain of the protein. These two allelic variants are structurally and functionally different. These differences between the p53 variants are based on the ability to bind components of the transcriptional machinery, activate transcription, induce apoptosis, and suppress the transformation of primary cells [9]. Arg allele is more efficient in inducing apoptosis than the Pro allele which appears to induce higher level of G1 arrest [10]. Data about the role of this polymorphism in ovarian carcinoma development, prognosis and response to chemotherapy are insufficient and controversial.

In the last decade, p53 polymorphism at codon 72 has been associated with risk of developing various neoplasms [11]. A connection between this polymorphism and ovarian cancer has been suggested [12,13], but has not been extensively studied.

Concerning the prognosis and response to cisplatinum/paclitaxel-based chemotherapy some authors reported that Arg/Arg genotype is associated with better prognosis than Arg/Pro or Pro/Pro genotype, probably due to higher induction of apoptosis [14]. In another study women with the codon 72 Pro/Pro had a decreased overall survival compared with women with one or two arginine alleles [15]. The prognostic value of codon 72 polymorphism in ovarian cancer is unclear.

The aim of this study was to determine the frequency of p53 mutations and polymorphic variants of codon 72 and examine the possible correlation with the clinicopathological characteristics of disease.

Methods

Patients

54 patients with ovarian carcinoma were included in our study. The age of patients ranged from 25 to 81 years (median 56). The clinicopathological characteristics of patients are summarized in Table 1.

DNA isolation

Genomic DNA was isolated by the salting-out method from ovarian carcinoma tumor tissue samples collected in liquid nitrogen (–197^{o} C) after surgical treatment.

PCR-SSCP analysis

p53 exons 4-8 were amplified by PCR in automated thermocycler. Genomic DNA (400 ng) was amplified in 25 μl reaction volume (12.5 μl 2X AmliTaq Gold PCR Master Mix, 0.75 μl 10 μM sense primer and 0.75 μl 10 μM antisense primer).

Primers sequences were: exon 4 S: 5′-ATCTA-

Table 1. Clinicopathological characteristics of ovarian carcinoma patients and number of p53 gene mutation detected

Characteristics	*Number of patients (n=54)*	*Number of p53 mutations*	*p-value*
Menopausal status			
Premenopausal	18	4	0.014*
Postmenopausal	36	7	
Histological subtype			
Serous	35	7	0.069*
Mucinous	2	1	
Endometrioid	7	0	
Clear-cell	4	1	
Undifferentiated	2	1	
Adenocarcinoma NOS	3	1	
Mixed (serous/mucinous)	1	0	
Histological grade			
G1	19	2	0.121**
G2	21	6	0.337**
G3	6	1	0.446**
Unclassified	8	2	
FIGO stage			
I	12	0	0.024*
II	9	4	
III	29	6	
IV	4	1	

*chi-square test with Yates correction; ** Fisher's exact test

CAGTCCCCCTTGCCG-3′ and AS: 5′-GCAACGACCGTGCAAGTCA-3′; exon 5 S: 5′-TGT TCA CTT GTG CCC TGA CT-3′ and AS: 5′-CAG CCC TGT CGT CTC TCC AG-3′; exon 6 S: 5′-TGG TTG CCC AGG GTC CCC AG-3′ and AS: 5′-GGA GGG CCA CTG ACA ACC A-3′; exon 7 S: 5′-ACT GGC CTC ATC TTG GGC CT-3′ and AS: 5′-TGT GCA GGG TGG CAA GTG GC-3′; exon 8 S: 5′-TAA ATG GAA CAG GTA GGA CC-3′ and AS: 5′-TCC ACC GCT TCT TGT CCT GC-3′. PCR conditions for exon 4 were 95° C, 50 sec; 55° C, 50 sec; 72° C, 60 sec; 35 cycles and for exons 5-8 of p53 gene were 95° C, 60 sec; 60° C, 60 sec; 35 cycles. Pre-PCR step for exons 4-8 of p53 gene was 5 min at 95° C.

Mutations were detected by SSCP electrophoresis. DNAs isolated from peripheral blood lymphocytes (PBL) of healthy donors were sequenced and used as controls. PCR reaction products (6 µl) were diluted in 3 µl loading dye (0.25% xylene cyanole, 0.25% bromphenol blue, 20% ficol 400 in distilled water) and 10 µl distilled water, and denaturated by heating to 95 ˚C. These aliquots of PCR products were separated on 8% polyacrylamide gel in 0.5 XTBE buffer. SSCP-electrophoresis conditions were: 150 min at 100 V on cold (4˚ C). The gels were silver-stained.

RFLP analysis

This polymorphism was assessed by the RFLP method. 10 µl of each PCR product of p53 exon 4 was digested with 1.5 µl of the restriction enzyme Bsh1236I at 37˚ C for 1 h and 20 min. The digestion reaction was stopped by heating at 65˚ C for 20 min. After digestion, the fragments were separated on 8% polyacrylamide gel for 100 min at 100 V at room temperature. pUC18 HaeIII Digest DNA marker in a concentration of 249 µg/ml was used as DNA molecular weight marker.

Arg allele was cut by Bsh1236I in 2 fragments (126 bp and 170 bp). The Pro allele was not cut by Bsh1236I and had a single 296 bp band. The heterozygote contained 3 bands (126, 170 and 296 bp). RFLP analysis of codon 72 different genotypes is shown in Figure 1.

Statistical analysis

The obtained results were analyzed by the chi-square test with Yates correction and the Fisher's exact test.

Results

p53 mutations were present in 11/54 (20.4%) patients. All patients had single mutation.

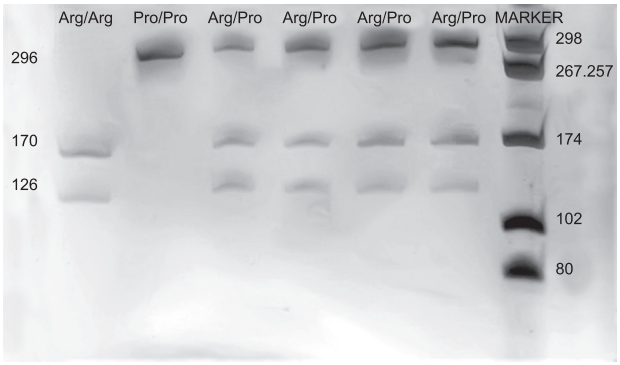

Figure 1. Gel electrophoresis of PCR products of exon 4 after digestion with Bsh1236I enzyme. Line 1: Arg/Arg homozygote; line 2: Pro/Pro homozygote; and lines 3-6 Arg/Pro heterozygote; M line=molecular weight marker pUC18 HaeIII Digest.

p53 mutations were located in exon 4 (n=3), exon 5 (n=2), exon 6 (n=2), exon 7 (n=2) and exon 8 (n=3).

Distribution of p53 mutations between pre and postmenopausal women was not significantly different (x^2=0.014, p >0.05).

Comparing the histological subtypes, distribution of p53 mutations between serous and non-serous (mucinous, endometrioid, clear-cell, undifferentiated, adenocarcinoma NOS and mixed), was not different (x^2=0.069, p >0.05).

p53 mutations did not show statistically significant different distribution between histological grades (G) (I to III) (I vs. II: Fisher's exact test, p= 0.121, p>0.05; II vs. III: Fisher's exact test, p=0.337, p >0.05; and I vs. III: Fisher's exact test, p=0.446, p >0.05), as well as between localized (FIGO stages I and II) and advanced disease (FIGO stages III and IV) (x^2=0.024, p >0.05). However, among 12 patients with FIGO I stage, no p53 gene mutations were detected.

The ratio between the 3 different genotypes was: Arg/Arg 24/54 (44.4%), Arg/Pro 24/54 (44.4%) and Pro/Pro 6/54 (11.2%). The distribution of genotypes at codon 72 of p53 gene among different clinicopathological characteristics of ovarian carcinoma patients is presented in Table 2.

The distribution between p53 codon 72 polymorphic variants in pre and postmenopausal women was similar (x^2=0.211, p >0.05). However, we found Pro/Pro variant being significantly more frequent in non-serous histology (x^2=4.692, p <0.05).

We observed that the frequency of Pro/Pro genotype was increasing with higher histological grade (from 0% in G1, 4.8% in G2 to 33.3% in G3; Figure 2) and with FIGO stage (from 8.3% in FIGO I, 11.1% in FIGO II, 6.9% in FIGO III to 50.0% in FIGO IV; Figure 3).

Statistically significant distribution of Pro/Pro variant was found comparing G1 to G3 (G1 vs. G2:

Table 2. Clinicopathological characteristics of ovarian carcinoma patients and distribution of p53 gene codon 72 genotypes

Characteristics	*Number of patients (n=54)*	*Number of Arg/Arg plus Arg/Pro genotypes*	*Number of Pro/Pro genotype*	*p-value*
Menopausal status				
Premenopausal	18	17	1	0.211*
Postmenopausal	36	31	5	
Histological subtype				
Serous	35	34	1	4.692*
Mucinous	2	1	1	
Endometrioid	7	5	2	
Clear-cell	4	4	0	
Undifferentiated	2	1	1	
Adenocarcinoma NOS	3	2	1	
Mixed (serous/mucinous)	1	1	0	
Histological grade				
G1	19	19	0	0.525**
G2	21	20	1	0.108**
G3	6	4	2	0.050**
Unclassified	8	5	3	
FIGO stage				
I	12	11	1	0.022*
II	9	8	1	
III	29	27	2	
IV	4	2	2	

*chi-square test with Yates correction; **Fisher's exact test

Fisher's exact test p=0.525, p >0.05; G2 vs. G3: Fisher's exact test p=0.108, p >0.05; and G1 vs. G3: Fisher's exact test p=0.050).

There was no difference in the frequency of Pro/Pro genotype comparing the localized (FIGO stages I and II) and the advanced disease (FIGO stages III and IV) (x^2=0.022, p >0.05).

The distribution of p53 gene mutations between Arg/Arg plus Arg/Pro vs. Pro/Pro variant was similar (x^2=1.887, p >0.05).

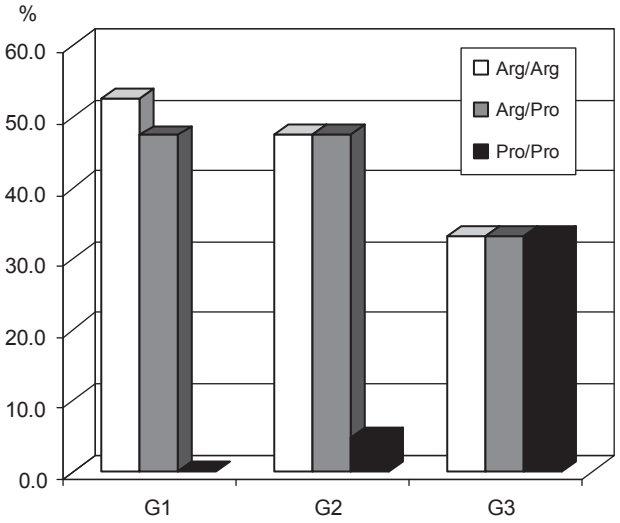

Figure 2. The frequency of genotypes at codon 72 of p53 gene in different histological grades (G) in ovarian carcinomas (n=54).

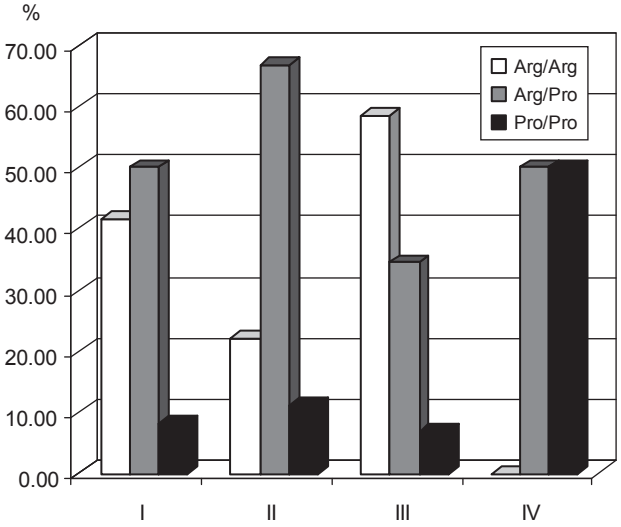

Figure 3. The frequency of genotypes at codon 72 of p53 gene in different FIGO stages (I-IV) in ovarian carcinomas (n=54).

Discussion

Ovarian carcinoma is often described as the silent killer or the disease that expands without symptoms [16]. In more than 75% of the patients, lack of specific/sensitive markers and/or techniques of screening, leads to delayed diagnosis at advanced stages of the disease. Unfortunately, the 5-year survival rate at this point is less than 25% compared to 80-90% for patients diagnosed in early stages [17].

CA125 (Cancer Antigen 125) is the only recognized serum marker for ovarian cancer detection in combination with ultrasound. CA125 is a glycoprotein that is secreted by the ovarian cancer cell. CA125 is elevated (more than 35 U/ml) in over 80% of women with advanced ovarian cancer, but only in 50% of patients with stage I disease [18]. CA125 is also associated with inflammatory cells of the pleura, pericardium, and peritoneum [19], certain benign gynecologic conditions (including endometriosis, uterine leiomyoma, pelvic inflammatory disease, early pregnancy, and benign ovarian cysts) [20] and in some others malignant conditions.

Therefore, finding new markers for early detection and/or prognosis of ovarian carcinomas is very important. p53 gene mutations and codon 72 polymorphic variant are possible candidates.

p53 was found mutated in approximately 40-80% of ovarian carcinomas [21]. In our study p53 gene was mutated in 20.4% of tumors only. This discrepancy could partly be explained with the existing model of ovarian tumorigenesis. Ovarian carcinomas show more morphological heterogeneity than adenocarcinomas of any other body site. The morphologically defined subtypes of ovarian carcinoma are distinct diseases, with different risk factors, different oncogenesis, metastatic potential, responses to chemotherapy, and prognosis [22].

According to the model of ovarian tumorigenesis, ovarian cancer is divided into two groups, designated as Type I and Type II. Type I tumors are slow-growing, generally limited to the ovary at diagnosis and develop from well established precursor lesions, called "borderline" tumors. Type I tumors include low-grade micropapillary serous carcinoma, mucinous, endometrioid, and clear cell carcinomas. They are genetically stable and are characterized by mutations in a number of different genes including KRAS, BRAF, PTEN, and beta-catenin. Type II tumors are fast-growing, highly aggressive neoplasms without well defined precursor lesions; at the time of diagnosis, the majority of tumors are already in advanced stage. Those include high-grade serous carcinoma, malignant mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas. The Type II tumors are characterized by frequent mutation of p53 gene and with high level of genetic instability [23].

In our patient population, we theoretically should expect mutations in p53 gene in less than half of the examined tumors (19 high grade serous subtype and 2 undifferented carcinomas).

We examined the distribution of p53 gene mutation among clinicopathological characteristics such as menopausal status, histological subtype, histological grade and FIGO stage, but significant differences were not revealed.

The frequency of p53 gene mutations among pre and postmenopausal women was nearly equal (22.2% compared to 19.4%), as well as for serous and non- serous tumors (20.0% compared to 21.0%). The highest frequency (28.6%) of mutations was found in G2 tumors comparing to G1 (10.5%) and G3 (16.7%).

Similar frequency of mutations was found in tumors from patients diagnosed with FIGO stages III/IV (21.2%) compared to stages I/II (19.0%). Some authors also indicate the prevalence of p53 mutations in FIGO stages III/IV tumors (49%) compared with stages I/II tumors (31%) [21].

The role of p53 mutations as a prognostic factor is still unclear. There are very few data about a correlation of p53 mutations and menopausal status, as well as tumor grade. For histological subtypes, significant difference in the distribution of mutations among serous vs. non-serous carcinomas has been observed, but only in early disease stages [24]. Another study did not confirm this [25]. p53 mutations were more common in late-stage (III or IV) than in early-stage disease (I or II) [25]. Yet, another study found no relationship between the mutation of p53 gene and FIGO stage in ovarian carcinomas [26].

In our study, we found an equal frequency of Arg/Arg and Arg/Pro genotypes at codon 72 of p53 gene. We found significantly higher distribution of Pro/Pro genotype in non-serous comparing to serous tumors. It was observed that the frequency of Pro/Pro genotype was increasing with higher histological grade (from 0% in G1, 4.8% in G2 to 33.3% in G3) and in advanced stages (FIGO III and IV) compared with localized disease (FIGO I and II).

According to the distribution of the polymorphic variant at codon 72 of p53 gene, there was no correlation between the frequency of the 3 genotypes (Pro/Pro, Arg/Arg and Arg/Pro) and histological subtype or stage of the tumor [27].

Our results indicate that the distribution of p53 mutations among the Pro/Pro variant vs. Arg/Arg plus Arg/Pro did not reach statistical significance. It could mean that the altered p53 protein does not additionally

confer to Pro/Pro variant for aggressive disease. An inverse correlation was evident between the p53 codon 72 polymorphism and mutations at exons 5 to 9, with the latter more frequently found in Arg/Arg cases in serous ovarian carcinomas [28].

In conclusion, our study did not show any correlation between p53 gene mutations and the clinicopathological characteristics in ovarian carcinomas. However, we found an increasing tendency of the Pro/Pro genotype in higher histological grade and in advanced stages of disease. Since, the distribution of p53 mutations between Pro/Pro and Arg/Pro plus Arg/Arg genotypes was not significantly different, we suggest that the Pro/Pro variant of codon 72 could be an independent prognostic marker in ovarian carcinomas.

Acknowledgements

This project is financially supported by Ministry of Science of Serbia (grant No. 145035).

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