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ORIGINAL ARTICLE

p53 gene alterations and human papillomavirus type 16 infection in early stages of cervical carcinoma in Serbia

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Summary

Purpose: *The incidence rate (age-standardized) of cervical carcinoma in Serbia is the highest in Europe. p53 is mainly inactivated at protein level in carcinomas associated with human papillomavirus (HPV) infection, such as cervical carcinomas. These tumors show low rate of p53 mutations. It is not clear if p53 mutations confer additional impact on disease prognosis. The role of polymorphic variant at codon 72 of p53 gene on patient's prognosis is controversial. The aim of this study was to determine the frequency of p53 mutations and to assess polymorphic variants of codon 72 among cervical carcinoma patients.*

Patients and methods: *53 patients, mainly FIGO stage I (n=50), with squamous cell carcinoma (n=49) were included. 30/32 (94%) patients who received adjuvant radiotherapy were followed-up (median 15 months, range 4-39). DNA was isolated by the salting out method from tumor tissue (n=53) and blood (42/53). p53 mutations were detected by PCR-SSCP (polymerase chain reaction – single-stranded conformational polymorphism) electrophoresis. Codon 72 polymorphism was assessed by the restriction fragment-length polymorphism method.*

Results: *Six p53 mutations were detected in 5/53 (9%) patients with FIGO stage I squamous cell carcinoma (one patient had double mutations). 25/42 (60%) patients exhibited Arg/Arg genotype. HPV16 type was detected in 29/51 (57%) cervical carcinoma samples. Relapse of disease occurred in only 2 patients- both with Arg/Arg genotype and HPV16 positive. One of them exhibited p53 mutation.*

Conclusion: *Our results showed low incidence of p53 mutations and prevalence of Arg/Arg genotype polymorphic variant of codon 72 of p53 gene in early stages of cervical carcinoma.*

Key words: cervical carcinoma, human papillomavirus, p53

Introduction

Cervical cancer is the second most common malignant disease among women worldwide, with more than 80% of cases arising in less developed countries. It is a major cause of death in women of reproductive age in parts of the developing world. The incidence rate (age-standardized) of cervical carcinoma in Serbia is the highest in Europe (incidence with 1,400 newly diagnosed cases per year, translated in 27.2/100,000 females per year and mortality with 450 cases per year, translated in 7.2/100,000 per year; data of 2002) [1,2].

It is estimated that 15% of all cancer types are etiologically linked to viral infection [3]. Infection with HPVs plays a central role in the development of cervical cancer. HPVs can be grouped as high-risk and low-risk HPV types, based on their association with cervical cancer and its precursor lesions. High-risk HPVs include types 16, 18, 31 and 45. Approximately 99% of all cervical cancers contain high-risk HPVs types [4].

Like in other malignant tumors, alterations in oncogenes and tumor suppressor genes may contribute to cervical carcinogenesis. p53 mutations occur in the majority of human tumors and are often associated with advanced tumor stage and poor patient's prognosis. Tumor with mutated p53 can be more anaplastic, have a higher rate of proliferation, and have more aggressive phenotype than a similar tumor with wild-type p53.

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p53 gene is key regulator in a wide range of cellular processes including cell cycle control, DNA repair, genome stability, programmed cell death (apoptosis), differentiation, senescence and angiogenesis [5]. These activities are mediated through a variety of biochemical functions, such as transcription activation, transrepression and 3'-5' exonuclease activity, and they involve a large set of target genes and interacted proteins [5]. Anticancer therapies such as radiotherapy and DNA-damaging chemotherapy act throughout p53-dependent apoptosis. It can be presumed that loss of p53 function may correlate with insensitivity to these therapies.

In cervical carcinomas, p53 is mainly inactivated at protein level by association with E6 oncoprotein of high-risk HPV. E6 proteins from high-risk HPV types first bind to cellular ubiquitin-ligase, termed E6-associated protein (E6-AP). The dimeric complex then binds p53 and induces multi-ubiquitination of p53 in the presence of ubiquitin complex of enzymes and its degradation by the proteasome [6]. Amino-acid changing mutation in the DNA-binding domain, corresponding to exons 5-8, is crucial in the multiple mechanisms of p53 inactivation. It prevents binding to specific DNA sequence and activates the adjacent genes. About 95% of all p53 mutations are located in this gene region [7], and that is why we analyzed exons 5-8. Exon 4 was included in this study because of the impact of this region on p53 protein function in apoptosis [8].

It seems that cervical carcinomas are characterized by low rate of p53 mutations. However, it is not clear if p53 mutations in HPV-related carcinomas confer additionally to poor disease prognosis [9].

p53 gene shows a polymorphism at codon 72 with single-base change that codes either arginine (CGC) or proline (CCC) in the transactivation domain of protein. These two allelic variants are structurally and functionally different, and may confer different susceptibilities for cancer development. Single nucleotide polymorphism (SNP) at codon 72 of the p53 gene has been associated with risk of developing various neoplasms, such as lung, esophageal, ovarian, endometrial and cervical cancer in the last decade [10].

The rationale for the potential use of a polymorphic variant of codon 72 of p53 gene as risk or prognostic factor in HPV-infected tumors is connected with the observation that p53 protein with arginine at position 72 is more susceptible to degradation by E6 protein of high-risk HPVs. This data suggests that individuals homozygous for arginine allele are at higher risk of HPV-related cervical cancers [11]. However, subsequent studies failed to confirm this association [12].

The majority of the studies on p53 polymorphism has been based on case-control designs to assess the risk of cervical cancer, but data on the prognostic value of 72 codon polymorphism in cervical cancer patients is missing [13].

This study was aimed to determine the frequency of p53 mutations in Serbian patients with cervical carcinoma. Due to the potential significance of polymorphic variants of 72 codon of p53 gene as risk factor for disease, we also assessed its genotypes in order to determine the frequencies of different genotypes among patients with cervical carcinoma in Serbia. As the majority of patients were followed-up, we tried to correlate p53 gene polymorphisms and mutations with the course of the disease.

Patients and methods

Patients

53 patients with early-stage cervical carcinoma (FIGO stage I n=50 and II n=3) were included in our study. The patient age ranged from 30 to 73 years (median 48). Patient characteristics are shown in Table 1.

Thirty patients were followed-up from 4 to 39 months (median 15) after adjuvant radiotherapy. For all

Table 1. The characteristics of patients with cervical carcinoma and distribution of p53 mutations within the examined characteristics

Characteristic	*Patients n*	*p53 mutations n*	*p-value*
Age (years)			
Median	48		
Range	30-73		
Follow up (months)			
Median	15		
Range	4-39		
Menopausal status			
Premenopausal	29	4	0.049*
Postmenopausal	24	2	
FIGO stage			
I	50	6	NA
II	3		
Histological type			
Squamous cell carcinoma			NA
Adenocarcinoma			
Histological grade			
I	19	1	0.139*
II	22	4	
III	5	1	0.418**
Unclassified	7		
Adjuvant therapy			
Radiotherapy	30	6	NA
Radiotherapy plus carboplatin	2		

*x^2 with Yates correction, **Fisher exact test
NA: not applicable

patients except one, radiotherapy consisted of external beam irradiation up to 40 Gy (20-25 sessions) and intracavity brachytherapy up to 30 Gy in 4 or 5 sessions. One patient received intracavitary brachytherapy only. Two of 30 patients received concurrent radiotherapy and carboplatin.

DNA isolation

DNA was isolated by the salting out method from tumor tissue and blood of patients with cervical carcinomas. DNA was dissolved in distilled water and stored at –20°.

p53 mutation detection

p53 mutation detection was done by PCR-SSCP analysis in all 53 samples. DNA exons 4-8 were amplified by PCR in automated thermocycler. Genomic DNA (400 ng) was amplified in 50 µl reaction volume (25 µl 2X AmliTaq Gold PCR Master Mix, 1.25 µl 10 µM sense primer and 1.25 µl 10 µM antisense primer).

Primers sequences were: exon 4 S: 5'-ATCTA-CAGTCCCCCTTGCCG-3' and AS: 5'-GCAAC-GACCGTGCAAGTCA-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. Control DNA was isolated from peripheral blood lymphocytes (PBL) of healthy donors. 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 for exon 4 was 4 h at 150 V on cold (4° C) and for exons 5-8 2.5 h at 200 V on cold (4° C). The gels were silver-stained. SSCP-PCR analysis of p53 exons 5-8 is shown in Figure 1.

HPV 16 detection

E7 viral gene and β globin gene, as reference gene, were amplified by PCR reaction. Genomic DNA (400 ng) was amplified in 50 µl reaction volume (25 µl 2X AmliTaq Gold PCR Master Mix; 3 µl 2.5 µM sense primer and 3 µl 2.5 µM antisense primer for E7 gene or 1.4 µl 8 µM sense and 1.4 µl 8 µM antisense primer for β globin gene).

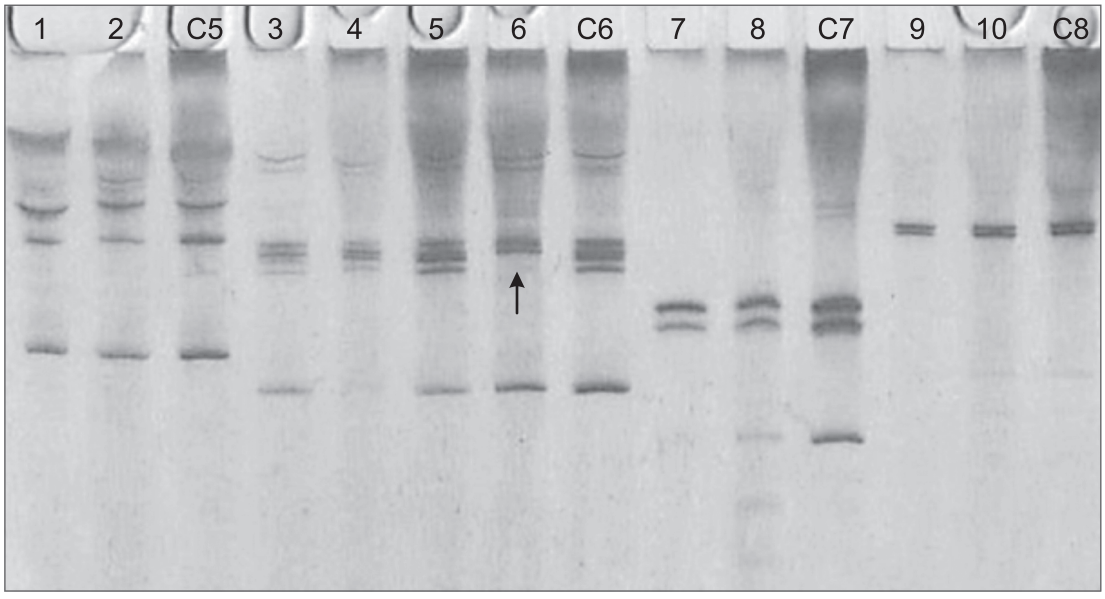

Figure 1. SSCP-PCR analysis of p53 exons 5-8: line 1-2 exon 5; line 3-6 exon 6; line 7-8 exon 7; line 9-10 exon 8. C5, C6, C7, C8 were controls for exon 5, 6, 7 and 8, respectively. Mutation was found in exon 6 (line 6; arrow).

Primers sequences were: HPV 16 E7 S 5'-CATGGAGATACACCTACATTGC-3' and AS: 5'-CTGAGAACAGATGGGGCACAC-3'; β globin S 5'-GAAGAGCCAAGGACAGGTAC-3' and AS: β globin AS: 5'-CAACTTCATCCACGTTCACC -3'. PCR conditions were 95° C, 30 sec; 55° C, 60 sec; 72° C, 120 sec; 40 cycles. Pre-PCR step for E7 was 10 min at 95° C. PCR products were separated on 8% polyacrylamide gel for 3 h at 200V and correlated with positive HPV 16 control and DNA molecular weight marker pUC18 HaeIII Digest DNA marker in a concentration of 249 μg/ml.

Polymorphism at codon 72 of p53 gene

This polymorphism was assessed by the RFLP method in 42/53 blood samples, since blood samples from 11 patients were not available. 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 3 h at 200V 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 2.

Statistical analysis

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

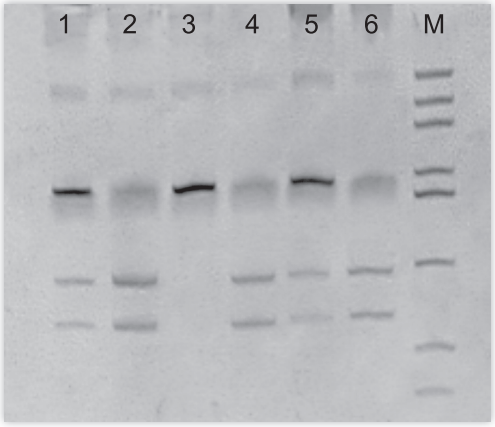

Figure 2. Gel electrophoresis of PCR products of exon 4 after digestion with enzyme Bsh1236I. Lines 1 and 5=Arg/Pro heterozygote; lines 2, 4 and 6=Arg/Arg homozygote; and line=3 Pro/Pro homozygote. M line=molecular weight marker pUC18 HaeIII Digest.

Results

p53 mutations were present in 5/53 (9%) patients. Six mutations were detected (one patient has double mutation - 1 in exon 6 and the other in exon 8).

p53 mutations were located in exon 5 (n=1), exon 6 (n=2) and exon 8 (n=3).

Analysis of distribution of mutations within host /tumor characteristics was not possible for FIGO stage, as well as for histological type, since all of the patients with detected p53 mutations were in FIGO stage I and had squamous cell carcinoma. Concerning histological grade (I to III), p53 mutations were not significantly differently distributed (I vs. II: x^2=0.139, p >0.05; II vs. III: Fisher exact test, p=0.418) (Table 1). No significantly different distribution of p53 mutations was observed between pre and postmenopausal women (x^2=0.049, p >0.05).

During the follow-up period (median 15, range 4-39 months), disease relapse occurred in 2 of 30 patients. In one of them p53 mutation was found.

The pattern of 3 different genotypes at codon 72 of p53 gene, obtained by RFLP analysis, is shown in Figure 2. The ratio between the 3 different genotypes was: Arg/Arg 25/42 (60%), Arg/Pro 16/42 (38%) and Pro/Pro 1/42 (2%) (Figure 3).

Concerning 72 codon polymorphisms, 4/6 p53 mutations were associated with Arg/Arg genotype and 2/6 with Arg/Pro genotype. There was no statistically significant difference between them (x^2=0.02, p >0.05) (Figure 4). None of the mutations were detected in Pro/Pro genotype.

Among patients being followed (n=30), polymorphism of codon 72 of p53 gene was determined in 25/30 (83%) cases. Analysis of disease relapse within different genotypes of codon 72 showed that both patients with relapse exhibited Arg/Arg genotype. However, it was not statistically significant (Arg/Arg genotype vs. Arg/Pro plus Pro/Pro, 2/15 vs. 0/10; Fisher exact test, p=0.350) (Figure 5).

HPV-16 testing was performed on 51 samples. Among them, 29 (56.9%) tumor samples were HPV-16-positive. In 3/5 patients we detected both HPV infec-

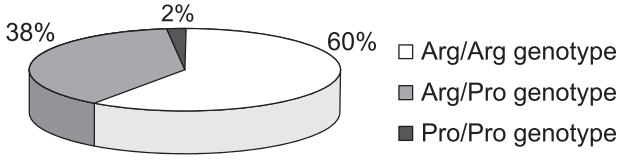

Figure 3. Distribution of different genotypes at codon 72 of p53 gene in the tested group of patients.

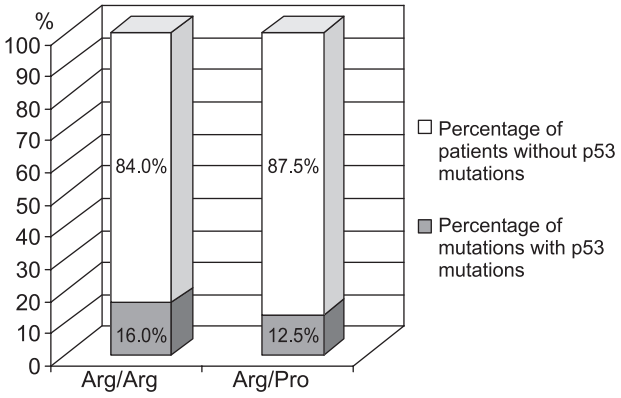

Figure 4. The frequency of p53 mutations in Arg/Arg vs. Arg/Pro groups of patients.

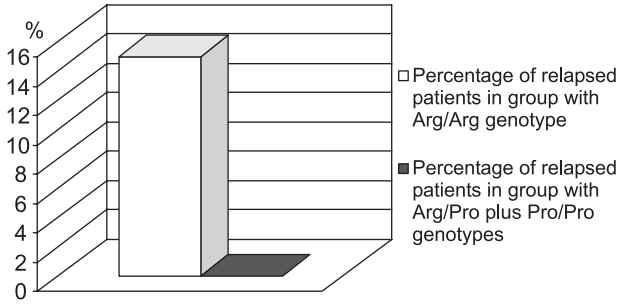

Figure 5. Relapsed patients with Arg/Arg genotype vs. Arg/Pro plus Pro/Pro genotypes.

tion and p53 mutation. The distribution of the different genotypes at codon 72 of p53 gene in HPV-positive and HPV-negative tumors is shown in Table 2. There was no statistically significant difference in the frequency of Arg/Arg genotype between HPV-16 positive and HPV-16 negative patients (Fisher exact test, p=0.525). Concerning relapse of disease, both relapsed patients with Arg/Arg genotype were also HPV-16 positive.

Discussion

The p53 tumor suppressor gene is mutated in over 50% of human cancers. Unlike other cancers in which p53 gene is often mutated, low incidence of p53 mutations is found in cervical cancers. Literature indicates that the frequency of p53 mutation is less than 10% in cervical carcinomas [14]. In our study, mutations in p53 gene were detected in 9% (5/53) cervical carcinoma patients, so our data is in the range of reported results.

Type 16 of HPV accounts for about half of the cases in the United States and Europe and types 18, 31, and 45 together account for an additional 25-30% of the cases [15]. We found HPV-16 in 56.9% of cervical carcinoma samples.

However, when p53 mutations occur, they can be found in both HPV-positive and HPV-negative cervical tumors, indicating that there is no correlation between HPV infection and p53 gene status [6]. Nevertheless, it is now considered that more than 99% of cervical carcinomas are HPV-positive. Therefore, it is interesting to find out if the presence of p53 mutation in an already HPV infected tumor, in which p53 is inactivated on protein level by HPV E6 oncoprotein, adds value to the prognosis of disease. In our study one of two relapsed patients had p53 mutation, while both were HPV-16 infected.

p53 mutations are often associated with more aggressive phenotypes than similar tumors with wild type p53. Usually, in order to estimate the role of p53 in disease prognosis, relations between p53 mutations and tumor/host characteristics have been investigated. Literature data showed that altered p53 could be found in early stages of cervical carcinomas, indicating its role in cervical carcinogenesis [16]. Our results do not confirm this data, since we detected only a small number of p53 mutations (n=6) and all were found in patients with FIGO stage I. This discrepancy can be caused by the methodology used for altered p53 screening - in the majority of studies p53 mutations were measured at protein level by immunochemistry, while we performed DNA mutation analysis. Although mutation in p53 gene may lead to increased stability of p53 protein, wild type p53 protein also can be accumulated in tumors. This indicates that p53 expression and p53 mutation do not have a good correlation [17]. Furthermore, it was not possible to compare the presence of p53 mutations in different histological tumor types in our study, since all 6 mutations were detected in squamous cell carcinoma (Table 1). Nevertheless,

Table 2. Distribution of p53 codon 72 polymorphism in HPV-positive and HPV-negative tumors

HPV status	*Cases, n (%)*	*Arg/Arg Cases, n (%)*	*Arg/Pro Cases, n (%)*	*Pro/Pro Cases, n (%)*
HPV-16 positive	21/40 (52)	13/21 (62)	7/21(33)	1/21 (5)
HPV-16 negative	19/40 (48)	11/19 (58)	8/19 (42)	0/19 (0)

Fisher exact test p=0.525

the obtained small number of p53 mutations in our study may be the consequence of the high proportion of squamous cell carcinoma, since it has been reported that the proportion of mutated p53 is higher in adenocarcinomas than in squamous cell carcinomas [16].

We correlated the presence of mutations within different histological grades. The results obtained showed no difference between frequencies of p53 mutations in tumors with different histological grade. The majority of authors that correlated the presence of p53 mutations with histological grade concluded that p53 mutations are predominantly found in high grades of various tumors, including gynecological ones [17].

Resistance to radiotherapy is one major therapeutic problem for cervical carcinoma patients. Tumors of the same histological type and grade, as well as stage of disease, may be extremely heterogeneous in their sensitivity to radiotherapy [18]. Experimental data showed that p53-dependent apoptosis would be the main acting mechanism of many anticancer treatments, including radiotherapy. Exploiting data from research to clinical oncology was in some ways disappointing, due to lack of significance [19]. The explanation lies in the fact that wild type p53 can induce either cell cycle arrest or apoptosis, thus influencing response or resistance to therapy. It is not perfectly clear when the one or the other mechanism is induced - it is presumed that p53 response depends on cell type, level of damage, environmental conditions etc [20]. The presence of mutant p53 is related to radioresistance in a variety of tumor types. In cervical cancer, the results are controversial. Some studies reported that the presence of mutant p53 was significantly associated with radioresistance in cervical carcinomas [21] while others showed no relationship between p53 and response to radiotherapy [22]. Since 32/53 of our cervical carcinoma patients were treated with radiotherapy and 30/32 of them were followed-up at our Institute, we intended to correlate the clinical course of disease with p53 status. But, only 2 relapses after radiotherapy were observed in the follow-up period and statistical analysis was not performed. Consequently, the influence of p53 alteration on radiotherapy response can not be discussed on the basis of the obtained results.

Due to potential significance of polymorphic variants at codon 72 of p53 gene as risk factor for cervical carcinomas, we assessed its genotypes in order to determine the frequencies of different genotypes among patients with cervical carcinoma in Serbia. Because of lack of data for distribution of these genotypes among healthy women in our population, we couldn't determine if a particular genotype was predominantly associated with malignant disease. Since both of our relapsed patients exhibited Arg/Arg genotype, but only one had p53 mutation, no relevant conclusion can be reached.

Marked ethnic differences in frequencies of polymorphic variants at codon 72 have been observed. In the Northern hemisphere, the Pro72 allele shows a North-South gradient. The Pro72 allele increases near the Equator. The Arg72 allele is more frequent in the north of Europe and in the United States [23]. Thus, we chose to compare our data concerning the frequencies of 3 different genotypes in cervical carcinoma with data from literature for populations ethnically and geographically close to Serbia. We found data for Polish and Czech populations as Slavic ones, and also for Hungarian population which is geographically close (Table 3).

The frequency of Serbian Arg/Arg genotype is the most close to the value obtained for Hungary (60 vs. 63%), whereas the frequency of Arg/Pro genotype is the most similar with Czech population (38 vs. 39%). The frequency of Pro/Pro genotype was the most similar to Polish population (2 vs. 3%) (Table 3).

The majority of studies of p53 polymorphism at codon 72 were directed to determine the risk for cervical carcinogenesis, since in HPV-related tumors the Arg variant is more susceptible to E6-mediated protein inactivation. Some reports indicated that cells expressing the wild type Arg variant on codon 72 of p53 exposed to anticancer drugs exhibited higher apoptosis [27]. On the contrary, another study suggests that Arg allele prevents apoptosis. Namely, tumors having Arg72 constitution showed lack of co-expression of Fas and FasL and high expression of Bcl-2 protein, which are associated with impairment or lack of apoptosis [28]. Also, Arg allele may be more efficient in the suppression of the multiple drug resistance gene MDR1 and induction of the proapoptotic oncogene BAX, than Pro allele. Pro allele is more efficient in promoting cell cycle arrest and DNA repair via induction of p21-Waf and GADD45 [10]. In addition, the Arg allele may enhance mutant p53 binding to p73, thus neutralizing p73-induced apoptosis independently of HPV-related mechanism [29, 30].

Table 3. Percentage of different genotypes at codon 72 of p53 gene among cervical carcinoma patients in different populations

Population (Reference)	*Arg/Arg %*	*Arg/Pro %*	*Pro/Pro %*
Serbia	60	38	2
Poland [24]	70	27	3
Czech Republic [25]	52	39	9
Hungary [26]	63	27	10

Our results concerning the distribution of Arg/Arg genotype among HPV-positive vs. HPV-negative tumors showed no statistically significant difference. Theoretically, we can expect a higher proportion of Arg/Arg genotype among HPV-positive patients. The reason for the lack of difference may be the fact that our HPV screening was only directed towards HPV-16 type. There is no reported data about the frequency of different HPV oncogenic types in cervical carcinoma in Serbia. At least HPV-31 testing has to be performed, since there is data showing that the most frequent HPV type, besides HPV-16 in precancerous lesions in Serbia, is HPV-31 [31].

According to the obtained data, we can presume that the profile of cervical carcinoma patients would be the following: mainly HPV-16-positive, with predominantly Arg/Arg genotype at codon 72 of p53 gene, and low rate of p53 mutation. Both relapsed patients exhibited Arg/Arg polymorphic variant and both were HPV-16-positive. One of them exhibited p53 mutation.

In conclusion, our preliminary results showed low incidence of p53 mutations in early-stage cervical carcinoma. Arg/Arg genotype is the prevalent polymorphic variant of codon 72 of p53 gene in cervical carcinoma patients in Serbia. The results also indicate that the investigated p53 alterations do not correlate with the clinical course of disease. However, due to the small number of the relapsed patients during the follow-up period (2/30), further confirmation of the results obtained is required.

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