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ORIGINAL ARTICLE

TP53 gene status and human papilloma virus infection in response to platinum plus taxane-based chemotherapy of epithelial ovarian carcinomas

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Summary

Purpose: *Lack of symptoms in early stages of disease and resistance to chemotherapy make epithelial ovarian carcinomas one of the most lethal neoplasms among gynaecological malignancies. The aim of this study was to analyse the impact of TP53 mutations, codon 72 polymorphism and human papillomavirus (HPV) infection on the response to platinum-taxane combination chemotherapy in patients with epithelial ovarian carcinomas.*

Methods: *The study was conducted on 26 ovarian carcinoma patients who received carboplatin plus paclitaxel combination chemotherapy. DNA was isolated by salting-out procedure. Mutations in exons 4-8 of TP53 gene were detected by PCR-SSCP and confirmed by automatic DNA sequencing. Codon 72 polymorphism was assessed by the RFLP method. HPV infection was detected through amplification of one part of L1 viral gene. Genotyping was performed by DNA sequencing. Fisher's exact and log-rank tests were used for statistical analysis.*

Results: *TP53 mutations were present in 5/26 (19.2%) ovarian carcinomas. The distribution of codon 72 TP53 genotypes was: Arg/Arg 38.5%, Arg/Pro 50.0%, Pro/Pro 11.5%. HPV was present in 4/26 (15.4%) ovarian carcinomas. All HPV-positive tumors were HPV16 type.*

Patients with mutations in TP53 gene, Arg/Arg genotype of codon 72 and absence of HPV infection experienced the highest tumor response rate to platinum-taxane chemotherapy. However, no significant correlation between progression free interval (PFI) and the examined biomarkers was observed.

Conclusion: *Our results indicate that, based on the TP53 gene status and the presence/absence of HPV infection, the subgroups of patients having better initial response to platinum-taxane therapy could be distinguished. This might contribute to more adequate treatment and individual therapeutic approach.*

Key words: HPV, ovarian carcinoma, polymorphism, *TP53* gene

Introduction

Epithelial ovarian carcinomas comprise 90% of ovarian malignancies and have the highest mortality rate among gynaecological cancers. The 5-year survival rate at early stages is 80-90% compared to 25% in advanced disease. The early stages are generally asymptomatic and only around 25% of the cases are diagnosed at this point [1]. Additionally, in more than 70% of the patients, relapse of disease appears within 2 years, despite sensitivity to the postoperative platinum-taxane chemotherapy [2].

The factors contributing to the onset of disease are well known and include genetic alterations associated with specific subtypes of ovarian carcinomas [3,4], positive family history of ovarian, breast or colon cancer, old age, number of ovulations, endocrine factors, endometriosis, pelvic inflammation, fat intake etc. [5], but the problem of early detection and chemotherapy resistance remains. Some of the genetic and environmental factors can affect the response to chemotherapy and may represent biomarkers for this disease. Thus, finding such markers is of great importance.

p53 is 53 kDa nuclear phosphoprotein which is essential for preventing inappropriate cell proliferation and maintenance of genome integrity. In response to genotoxic stress, it activates transcription of target genes and causes cell cycle arrest, DNA damage repair

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or induction of apoptosis [6]. The primary mechanism of abrogation of p53 function is the occurrence of mutations in the DNA binding domain (exons 5-8) in which appear around 95% of *TP53* mutations [7]. Mutations in exon 4 have impact on p53 function in apoptosis [8].

TP53 mutation status may have impact on the response to chemotherapy in ovarian carcinomas, since platinum-based drugs act by induction of DNA damage and apoptosis [9]. On the other hand, mutated *TP53* increases the expression of microtubule-associated protein 4 and consequently microtubules polymerization and taxane binding [10] that act via blockage of cell division by preventing the formation of the mitotic spindle.

The most studied polymorphism in *TP53* gene is the one 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 p53 variants are functionally different, based on the ability to bind components of the transcriptional machinery, activate transcription, induce apoptosis and suppress tumor growth [11]. Arg allele is more efficient in inducing apoptosis while the Pro allele induces higher level of G1 arrest [12]. It was indicated that some of polymorphic variants of *TP53* codon 72 are potential prognostic markers in ovarian carcinomas [13], but data about this polymorphism as predictive factor are insufficient.

HPV is the most common sexually transmitted infection [14]. Approximately 40 HPV types infect the genital tract [15]. They are classified as low risk (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) which cause genital warts, high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) which cause precancerous lesions and cancer, and probable high-risk (26, 53 and 66) [16]. One of key events in HPV-mediated carcinogenesis is integration of high-risk HPV into the host genome. That results in uncontrolled expression of E6 and E7 virus oncoproteins which inhibit the function of p53 and Rb tumor-suppressors and other cellular proteins and lead to increased cell proliferation, disruption of DNA repair, differentiation and apoptosis [17].

The role of HPV in the development of precancerous and cancerous lesions of the lower part of the female genital tract (vulva, vagina and cervix) was established. In the last decades, some authors [18,19] were examining the presence and the potential role of HPV infection in ovarian carcinoma development. But data about the impact of HPV infection over the response to chemotherapy is missing.

The purpose of this study was to investigate the impact of *TP53* gene mutations, polymorphism of codon 72 and HPV infection as potential biomarkers in response to platinum plus taxane chemotherapy of patients with ovarian carcinoma.

Methods

Patients

The study was performed on 26 ovarian carcinoma patients. All patients received standard carboplatin plus paclitaxel chemotherapy. The age of patients ranged from 34 to 69 years (median 54).

The tumors were classified and graded according to the World Health Organization (WHO) criteria. Clinical stages were established according to the International Federation of Obstetrics and Gynecology (FIGO) system. The characteristics of patients and tumors are summarized in Table 1.

Before treatment, all patients had a detailed history, physical examination and baseline laboratory parameters. Pretreatment baseline tumor status was evaluated with CT scan. Data collected included patient and disease characteristics, the best tumor response and data of progression. Responses were documented according to Response Evaluation Criteria in Solid Tumors (RECIST). The study was approved by the local Ethics committee.

DNA extraction

Genomic DNA was isolated by the salting-out method from ovarian carcinoma tissue samples collected after surgical treatment and stored in liquid nitrogen at -197° C. The extracted DNA was dissolved in sterile deionized water and stored at -20° C until PCR amplification.

Table 1. Patient and disease characteristics

Characteristics	*Patients (n=26)*	
	n	*%*
Menopausal status		
Premenopausal	14	53.8
Postmenopausal	12	46.2
Histological subtype		
Serous	18	69.2
Mucinous	1	3.9
Endometrioid	3	11.5
Clear cell	3	11.5
Undifferentiated	1	3.9
Histological grade		
G1	5	19.3
G2	15	57.7
G3	3	11.5
Unclassified	3	11.5
FIGO stage		
II	6	23.1
III	16	61.5
IV	4	15.4

Detection of TP53 mutations (analysis of TP53 mutations)

Genomic DNA (400 ng) was amplified in 25 μL reaction volume consisting of 12.5 μL 2X AmliTaq Gold PCR Master Mix (Applied Biosystems, USA), 0.6 μL 10 μM sense and 0.6 μL 10 μM antisense primer for exon 4-8 *TP53* gene (Metabion, Germany). Primers' sequences and PCR conditions are shown in Table 2.

Mutations screening was done by SSCP electrophoresis for 150 min at 100 V at 4° C. DNA isolated from peripheral blood lymphocytes of healthy donors was sequenced and used as a control.

TP53 mutations were confirmed by direct sequencing. PCR products were purified using QIAquick Purification Kit (QIAGEN, Germany). Purified PCR products were labeled with fluorescent dyes using BigDye Terminator v3.1 Cycle Sequencing Kit and the appropriate sequencing primer (Applied Biosystems, USA). Labeled oligonucleotides were precipitated by EDTA/ethanol precipitation. The samples were sequenced by automatic ABI PRISM 310 genetic analyzer (Applied Biosystems, USA). The obtained sequences were aligned by the BLAST program with normal *TP53* sequences (X54156).

Determination of TP53 polymorphism at codon 72

Codon 72 polymorphism was assessed by the RFLP method. 1.0 μL of each PCR product of *TP53* exon 4 was digested with 1.5 μL of the restriction enzyme Bsh1236I (Fermentas, Lithuania) 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. 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).

Detection and genotyping of HPV

HPV DNA were detected by PCR amplification, using general primers GP5+ and GP6+ [20]. These primers have been designed to amplify the 150 bp conserved sequence of L1 viral gene of a broad spectrum of HPV genotypes (6, 11, 13, 16, 18, 30-35, 39, 40, 42, 45, 51-53, 56, 58, 61, 66, 68) [21]. DNA from HeLa cells containing HPV18 was used as PCR positive control. Human β globin amplification was used to test sample DNA quality.

Genomic DNA (400 ng) was amplified in 25 μL reaction volume: 12.5 μL 2X AmliTaq Gold PCR Master Mix; 0.5 μL 10 μM GP5+ and 0.5 μL 10 μM GP6+ primer for L1 gene or 0.5 μL 8 μM sense and 0.5 μL 8 μM antisense primer for β globin gene. Primers' sequences and PCR conditions are displayed in Table 2.

Presence of amplified L1 and β globin gene were checked by electrophoresis on a 2% agarose gel.

HPV-positive PCR products were analyzed by direct DNA sequencing. A 50 bp DNA sequence in the hypervariable region of the L1 gene downstream of the GP5+ binding site was compared with the known HPV genotype sequence stored in the Gen Bank [22].

Table 2. Primers' sequences and PCR conditions

Primer sequences (5'-3')		*PCR conditions*
exon 4	ATCTACAGTCCCCCTTGCCG GCAACTGACCGTGCAAGTCA	95°C, 5 min- initial denaturation, 95°C, 50 sec; 55°C, 50 sec; 72°C, 1 min - 35 cycles
exon 5	TGTTCACTTGTGCCCTGACT CAGCCCTGTCGTCTCTCCAG	95°C, 5 min-initial denaturation 95°C, 1 min; 60°C, 1 min - 35 cycles
exon 6	TGGTTGCCCAGGGTCCCCAG GGAGGGCCACTGACAACCA	
exon 7	ACTGGCCTCATCTTGGGCCT TGTGCAGGGTGGCAAGTGGC	
exon 8	TAAATGGGACAGGTAGGACC TCCACCGCTTCTTGTCCTGC	
GP5+ GP6+	TTTGTTACTGTGGTAGATACTAC GAAAAATAAACTGTAAATCATATTC	95°C, 5 min-initial denaturation, 94°C, 1 min; 55°C to 40°C in 1°C decrements, 2 min; 72°C, 1.5 min - 16 cycles, 94°C, 1 min; 40°C, 2 min; 72°C, 1.5 min - 24 cycles, 72°C, 4 min- final extension*
β globin	GAAGAGCCAAGGACAGGTAC CAACTTCATCCACGTTCACC	95°C, 10 min - initial denaturation, 95°C, 30 sec; 55°C, 1 min; 72°C, 2 min - 40 cycles

*touchdown PCR [20]

Statistical analysis

Response to therapy was assessed based on response rate (PR) and PFI.

RR was defined as the sum of the percentage of patients with complete response (CR) or partial response (PR) to chemotherapy. RR in relation to the presence or absence of *TP53* mutations, the genotype of codon 72 *TP53* gene and the presence of HPV infection was investigated by the Fisher's exact test.

The Kaplan-Meier method with median of survival and 95% confidence interval (CI) was used to estimate PFI. The log-rank test was used to test the difference in PFIs compared with the parameters of interest.

Results

Distributions of TP53 mutations, polymorphism at codon 72 and HPV infection

TP53 mutations were present in 5/26 (19.2%) ovarian carcinomas. Among them 3 were deletions and 2 missense mutations. Deletions were detected in exon 5 (g.13170delA and g.13180delG) (Figure 1) and exon 7 (g.14063_14074del). Missense mutations were found in exon 7 (g.14060G>T, found as homozygous) and exon 8 (g.14493G>T).

The distribution of codon 72 *TP53* genotypes in ovarian carcinoma patients was: Arg/Arg 10/26 (38.5%), Arg/Pro 13/26 (50.0%) and Pro/Pro 3/26 (11.5%).

HPV DNA was present in 4/26 (15.4%) ovarian carcinomas. All HPV-positive tumors were of high-risk HPV16. The quality of electropherographic data obtained by DNA sequencing indicated that none of the HPV-positive patients had been infected with more than one type of HPV.

Response rate

Since p53 can be inactivated on protein level by interaction with the E6 protein of high-risk HPV types, 4 patients whose tumors were infected with HPV were excluded from the analysis of the impact of *TP53* mutations on the response to platinum-taxane chemotherapy. Among HPV-negative patients (n=22), all patients with mutations in the *TP53* gene (n=5) had CR or PR, and 14/17 patients without mutations in the *TP53* gene had CR or PR during chemotherapy. However, this difference in the response to therapy (100 vs. 82.4%) was not statistically significant (Fisher's exact test, p = 0.442). CRs were higher in patients with *TP53* mutations (80.0%) as compared with patients without mutations (47.1%) but without significant difference (Fisher's exact test, p=0.218). In the group of HPV-negative patients with wild type *TP53* gene (n=17), all patients with Arg/Arg genotype of codon 72 *TP53* gene responded to chemotherapy, while in the group of Arg/Pro plus Pro/Pro genotype, 8/11 responded to therapy. The difference in the response to therapy (100 vs. 72.7%) was not statistically significant (Fisher's exact test, p=0.243). CR achieved 3/6 (50.0%) patients with Arg/Arg and 5/11 (45.4%) patients with Arg/Pro plus Pro/Pro genotype.

In the group of patients without *TP53* gene mutations (n=21), 2/4 (50%) patients whose tumors were infected with HPV responded to therapy, and among HPV-negative patients 14/17 (82.4%) responded to therapy. The difference in RR between these groups of patients (HPV-negative *vs*. HPV-positive) was not statistically significant (Fisher's exact test, p=0.229). CR was found in 2/4 (50%) of HPV-positive and 8/17 (47.0%) of HPV-negative patients.

Progression-free interval

In the group of HPV-negative patients (n=22), no statistically significant differences in PFI in patients with mutated *TP53* compared to those with wild type *TP53* gene were found (Figure 2) (log-rank, p=0.997).

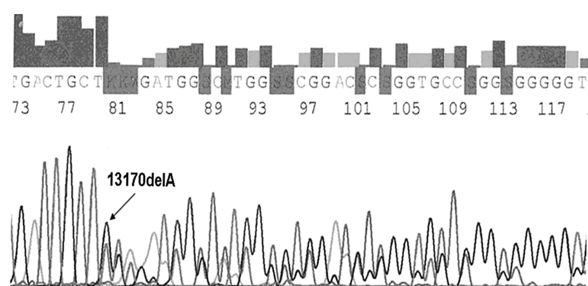

Figure 1. The deletion in exon 5 of *TP53* gene at position 13170 (GenBank sequence no. X54156).

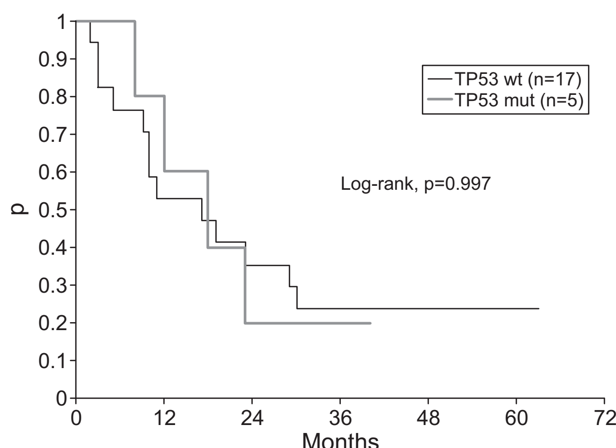

Figure 2. Kaplan-Meier curves of progression free interval in relation to the presence or absence of mutations in the *TP53* gene.

Median time to disease progression in the group of patients with mutations in *TP53* gene was 18 months (95% CI 12; the upper limit could not be estimated) compared to 17 months (95% CI 10; the upper limit could not be estimated) in the group with wild type *TP53*.

In the group of HPV-negative patients with wild type *TP53* gene (n=17), PFI was compared in those with Arg/Arg vs. Arg/Pro plus Pro/Pro genotype of codon 72 *TP53* gene (Figure 3). No statistically significant differences in time to disease progression between these two patient groups was found (log-rank, p=0.620). Median time to disease progression in both groups was 17.5 months, while 95% CI of the median was different among the group of patients with the Arg/Arg (95% CI 9 months; the upper limit of normal could not be estimated) and in the group Arg/Pro plus Pro/Pro (95% CI 10 months; the upper limit could not be estimated).

Among patients without *TP53* mutations (n=21), there was no statistically significant difference in time to disease progression in patients with HPV infection compared to those without (log-rank, p=0.869) (Figure 4). Median time to disease progression in the group of HPV-positive patients was 18.5 months (95% CI 8 months; the upper limit could not be estimated), while in the group of HPV-negative patients it was 17 months (95% CI 10 months; the upper limit could not be estimated).

Discussion

Search for new biomarkers has an important place in molecular oncology. Defining biomarkers that characterize certain types of cancer is essential for early detection of disease and/or adequate monitoring and individual therapeutic approach to each patient.

Platinum derivatives in combination with taxanes are the standard first-line chemotherapy for epithelial ovarian carcinomas. Ovarian carcinomas are initially very sensitive to chemotherapy, but in more than 70% of the cases, relapse occurs within 2 years.

Based on the mechanism of action of platinum derivatives and taxanes, one can speculate that *TP53* gene status may play an important role in the response to these drugs, but this hypothesis has not been clarified. Also, numerous other factors contribute to the response to this treatment.

In addition to *TP53* gene mutations, p53 function can be disrupted by *MDM2* gene amplification, deletion of *P14ARF* gene etc. p53 signaling pathways may be disturbed via changes in the genes that encode proteins downstream of p53. Also, in the absence of functional p53, p73 can induce apoptosis in cells exposed to cisplatin [9].

Besides the loss of p53 function, reduction of cisplatin intake into the cell, enhanced inactivation of cisplatin, loss of recognition of DNA adducts, increased repair of DNA adducts, overexpression of BCL-2, interference with caspase activation and other factors also contribute to cisplatin resistance. It was even shown that tumor cells exposed to cisplatin (depending on the cisplatin dose, energy and metabolic conditions in the cell) can die by necrosis [23], characterized by different morphological and biochemical characteristics compared with apoptosis.

In addition to wild type *TP53* gene, amplification and/or overexpression of *MDR* gene, which encodes P glycoprotein pump that is responsible for efflux of drugs from cytoplasm [24], changes in the structure of beta-tubulin, overexpression of antiapoptotic genes that sustain the survival of damaged cells [10] etc., contribute to the resistance to paclitaxel.

Infection with high-risk HPV types leads to deg-

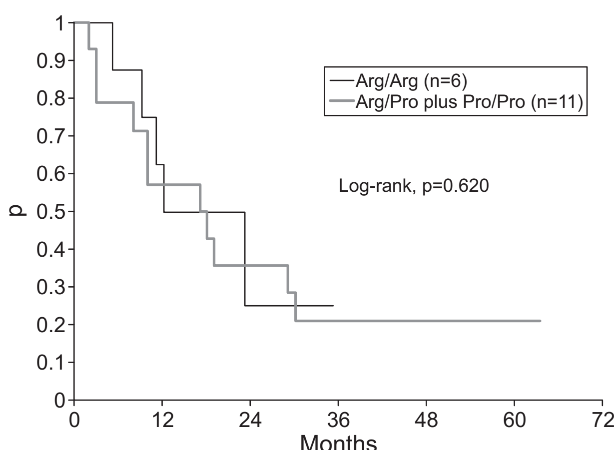

Figure 3. Kaplan-Meier curves of progression free interval in relation to the genotype of codon 72 *TP53* gene.

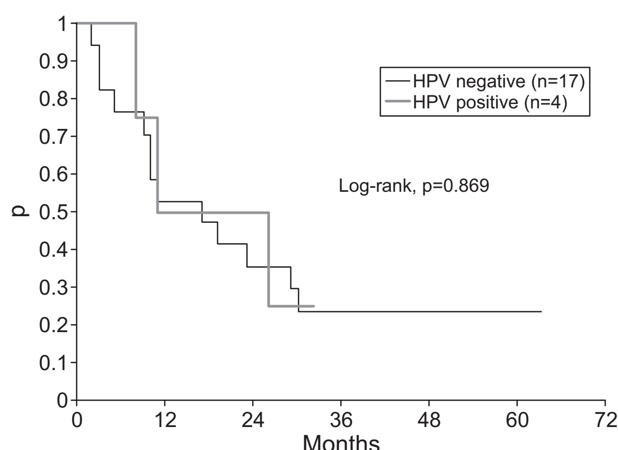

Figure 4. Kaplan-Meier curves of progression free interval in relation to the presence or absence of HPV infection.

radation of p53 protein. Since in the examined sample all HPV-infected ovarian carcinomas were HPV16 type, to investigate the effect of mutations in *TP53* gene on the response to platinum-taxane chemotherapy, we took into consideration only patients whose tumors were HPV-negative. RR, as well as CR were higher in HPV-negative patients with mutant *TP53* as compared to wild type *TP53* (100 vs. 82.4% and 80.0 vs. 47.1%, respectively). This suggests that mutations in the *TP53* gene are associated with better regression of disease during platinum-taxane chemotherapy.

Results of an Italian study in a group of 48 women with ovarian cancer also showed that a higher percentage of patients with mutated *TP53* responded to platinum-taxane chemotherapy compared with patients with wild type *TP53* (86.0 vs. 47.0%). Analysis of overall survival showed no statistically significant difference between patients with mutated and wild type *TP53* [25]. In contrast, the results of another study on 46 patients with serous ovarian cancer showed that patients with wild type *TP53* had a better CR rate than those with mutated *TP53* gene (90.0 vs. 60.8%), as well as better progression-free survival and overall survival [26].

In contrast to wild type p53 with Arg72, which is a better inhibitor of tumor growth (due to increased apoptotic ability), mutant p53 with Arg72 may increase tumor growth by binding to p53 homologue p73 and neutralizing p73-induced apoptosis [27]. Thus, to follow the effect of the influence of polymorphic variants of *TP53* codon 72 gene independent of the potential effect of HPV16 on the degradation of p53 protein and mutations in *TP53* gene, we selected a group of HPV-negative patients with wild type *TP53* gene. In this group, all patients with Arg/Arg genotype of codon 72 *TP53* gene responded to chemotherapy, while in the group of patients with Arg/Pro plus Pro/Pro genotype 72.7% responded to therapy. The percentage of complete responders within Arg/Arg genotype vs. Arg/Pro plus Pro/Pro was similar (50.0 vs. 45.4%).

In regard to prognosis and response to cisplatin-paclitaxel chemotherapy, it was indicated that the Arg/Arg genotype is associated with better prognosis than Arg/Pro or Pro/Pro genotype of codon 72 of *TP53* gene, probably related with higher induction of apoptosis [28]. Consistently, some results showed that patients with ovarian cancer with Pro/Pro genotype had a decreased overall survival compared with patients with one or two Arg alleles [29].

In other tumors such as cancer of breast [30,31], lung [32,33], head and neck [34], patients with Arg variant of p53 had a higher tumor response and/or better survival after chemotherapy and/or radiotherapy. In contrast, Gadducci et al. [26] showed that Pro/Pro homozygotes compared to the heterozygotes had a better CR rate (87.5 vs. 25.0%) and Pro homozygotes compared to Arg homozygotes had a better PFI and overall survival.

In the group of patients with wild type *TP53* gene, 82.4% without HPV infection and 50% of those infected with HPV, responded to therapy. This indicates that HPV infection may have negative impact on response to platinum-taxane chemotherapy. The percentage of patients with and without HPV infection who achieved CR was similar (50.0 vs. 47.0%).

In conclusion, our investigation showed that the RR was higher in patients with *TP53* mutations vs. wild type TP53, Arg/Arg vs. Arg/Pro plus Pro/Pro genotype of codon 72 and absence of HPV infection vs. HPV infection, but the PFI in relation to these parameters did not differ. This could mean that the mentioned biomarkers are associated with better initial response to the platinum-taxane chemotherapy but this effect may disappear with time.

Due to the sample size, our results should be taken as preliminary. If comprehensive analysis finds that some of the examined markers are predictors of good response to platinum-taxane chemotherapy, their routine analysis could distinguish subgroups of patients and individualize therapeutic approaches.

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