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TP53 Arg72Pro polymorphism is associated with increased overall survival but not response to therapy in Portuguese/Caucasian patients with advanced cervical cancer

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Abstract. Identification of mechanisms that influence the therapeutic response and survival in patients with cancer is important. It is known that the genetic variability of the host, including presence of genetic polymorphisms in genes involved in DNA damage response, serves a crucial role in the prognosis of these patients. The present hospital-based retrospective cohort study aimed to evaluate the influence of *TP53* Arg72Pro (rs1042522) polymorphism in the clinical outcome of 260 Caucasian patients diagnosed with cervical cancer and treated with concomitant radiotherapy and chemotherapy. The polymorphism genotyping was assessed using allelic discrimination by quantiative polymerase chain reaction. The results indicate that the *TP53* Arg72Pro polymorphism did not significantly impact the response to therapy (P=0.571) nor disease-free survival (P=0.081). However, the polymorphism did influence overall survival, as increased median survival time was observed for patients carrying Arg/Pro genotype when compared with patients with Arg/Arg and Pro/Pro genotypes (126 months vs. 111 months, respectively; P=0.047). To conclude, the present findings suggest that a pharmacogenomic profile based on the genetic background of patients, including the analysis of the *TP53* genotypes, may individualize treatment nad assist in the selection of therapies that may improve clinical outcome and lower toxicity for the patients.

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Introduction

Cervical cancer is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases and 266,000 deaths worldwide in 2012 (1). Currently, for locally advanced cervical cancer standard therapy is cisplatin-based concurrent chemoradiotherapy, with an overall survival (OS) of approximately 66% at 5 years (2-5).

Chemotherapy and radiotherapy are both considered as DNA damage agents, more precisely capable to introduce DNA double-strand breaks (DSBs) in order to induce cell death (6-8). When cellular DNA damage is not repaired one alternative response is apoptosis, which is the objective of the current therapeutic approach with cytotoxic agents and radiotherapy, but genetic alterations at key proteins in the pathway may result in the development of resistance to therapy (9,10). Therefore, studies involving variations in genes involved in cellular response to damage are important to understand how the development of resistant phenotypes occurs. One example may be the *TP53* gene, the 'guardian of genome' due to its role on cell cycle arrest, DNA repair activation and regulation of apoptosis (11-13). This suppressor gene is located on chromosome 17 (17p13.1) and encodes a phosphoprotein of 393 long amino acids (14,15).

Polymorphic variants are the substitution of a single base which results in alteration of the codon may have different conformation and function, i.e., no changes cannot occur or can be gain or loss of protein function (16,17). Several *TP53* mutant proteins associated with tumors, have gained oncogenic function besides losing the suppressive function (18,19). The most studied polymorphism of the *TP53* gene is the *TP53* Arg72Pro (rs1042522), which influences the protein expression of *TP53* protein expression (20). This variant results from a change of guanine (G) to cytosine (C) in codon 72 in exon 4, that leads

to the replacement of arginine (Arg) by proline (Pro) (21-23). It should be noted that due to the location in the proline-rich region of the *TP53* gene, this single nucleotide polymorphism (SNP) may interfere with protein stability (24,25). The two allelic variants confer different susceptibilities to cancer progression, because they are structurally and functionally different (26). In studies *in vivo* and *in vitro*, the Arg allele *has* a higher capacity to induce apoptosis than the Pro allele. The functions associated with Pro allele include higher induction of cell cycle arrest in G1 and better activation of *TP53* dependent DNA repair (27). It has also been mentioned that this polymorphism can influence the individual response (28).

Concerning cervical cancer, few studies have evaluated the predictive role of *TP53* Arg72Pro polymorphism in clinical outcome and the results are contradictory (29,30). Therefore, we have conducted this study to assess the possible influence of the *TP53* Arg72Pro polymorphism (rs1042522) in OS and disease-free survival (DFS) in patients with advanced cervical cancer.

Materials and methods

Patients. We conducted a retrospective hospital-based study analyzing a total of 260 Caucasians patients with histologically confirmed locally advanced cervical carcinoma (FIGO stage IB2-IVA). These patients were recruited between February 2002 and October 2009, from the north region of Portugal and treated with cisplatin-based chemotherapy (40 mg/m^2 per week) and concomitant external radiotherapy and/or brachytherapy in Portuguese Institute of Oncology Francisco Gentil (Porto, Portugal). All women were selected consecutively according to the following inclusion criteria: Women with histological and cytology diagnosis of cervical cancer, age greater than or equal to 18 years, stage IB2-IVA and QTRT concomitant. Regarding exclusion criteria, these were surgery before treatment; absence of informed consent; failure to comply with any of the inclusion criteria.

Patients' clinical characteristics obtained from medical records are described in Table I. The median age at diagnosis was 48.00 years, the more frequent histological type was squamous cancer cell, the stage more common was IIB and the median follow up time was 63.5 months. The tumor stage was evaluated according to the International Federation of Gynecology and Obstetrics (FIGO) classification system, and the assessment of histology type was based the on system of Bethesda classification. Genomic DNA was extracted from peripheral blood samples by using FavorPrep™ Genomic DNA Mini kit (FABGK® 300; Favorgen Biotech Corp., Ping-Tung, Taiwan), according to the manufacturers protocol. All samples were obtained with the informed consent of the participants prior to their inclusion in the study, according to Helsinki Declaration principles and after approval of the Portuguese Institute of Oncology ethics committee (CES.287/014).

Evaluation of chemoradiotherapy response. The therapy response was evaluated according to RECIST criteria (31). Complete response (CR) indicates disappearance of the disease, partial response (PR) indicates at least 50% reduction in tumor load, stable disease (SD) indicates that the lesion showed ≤25% progression or <50% shrinkage, and progression of disease (PD) indicates >25% enlargement of the lesion, or appearance of a new lesion. CR and PR were considered to be a good response; SD and PD, a poor response.

Genotyping of TP53 Arg72Pro (rs1042522) polymorphism. The selected SNP was chosen from the best evidence from published studies (29,30,32,33), through public databases who provide information on the phenotypic risks, had a minor allele frequency of an at least 10 to 20% and the SNP biological effect. The genotyping was performed using Taqman™ Allelic Discrimination methodology by quantitative polymerase chain reaction (qPCR). This method uses probes labeled with fluorochromes specific for each allele, thus VIC probe is allele C and the FAM probe is allele G (AGGAGCTGCTGGvTGC AGGGGCCACG [C/G] GGGGAGCAGCCTCTGGCATTC TGGG). The allelic discrimination PCR reactions were carried out in 6 μl volumes using 2.5 μl of TaqMan® Universal PCR Master Mix (2X), 0.125 μl of 40x assay mix 2.375 μl of sterile H_2O and 1 μl of genomic DNA. Amplification of DNA was carried out using the following amplification conditions: 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. Data capture and analysis was carried thought the ABI 7300 Real Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the Sequence Detection Systems software (version 1.2.3; Applied Biosystems; Thermo Fisher Scientific, Inc.).

Quality control included the use of negative controls in all runs, double sampling in at least 10% of the samples, genotyping performed blindly regarding to clinical and pathologic characteristics of patients and the results independently evaluated by two researchers. We observed complete concordance among duplicates.

Statistical analysis. Difference in frequencies of the *TP53* Arg72Pro genotypes between the different chemoradiotherapy responses groups were evaluated by χ^2 test. The OS and the overall survival at 5 years was defined from the date of diagnosis to the date of death and the percentage of patients alive after 5 years of diagnosis, respectively. The DFS times were defined from the data from the date of diagnosis to the date of disease recurrence. Patients without progression, lost to follow-up or died from other causes were censored at their last date of record. In the evaluation of OS and DFS was used Kaplan-Meier survival estimate and log-rank test. We applied a multivariate analysis using COX regression method to calculate hazard ratio (HR) and 95% confidence intervals (CI) for the association between the genotypes and the risk of death in advanced cervical cancer patients. This analysis was used to adjust for potential confounders, such as age (<48 years vs. ≥48 years), stage (<IIB vs. ≥IIB), smoking habits (non-smokers vs. smokers and former smokers) and histological type (adenosquamous cell carcinomas and small cell carcinoma vs. adenocarcinoma and squamous cell carcinoma), with *TP53* Arg72Pro genotypes fitted as indicator variables. A level of $P<0.05$ was considered statistically significant. All analysis of data was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 22.0; IBM Corp., Armonk, NY, USA).

As our study was performed based on DNA availability, we did not carry out any power analysis before the study.

Table I. Distribution of patients' clinicopathologic characteristics.

Characteristics	No. of patients (%)
Age (years)	260 (100)
Median, 48.00	
Mean ± SD, 49.00±11.50	
Follow-up time (months)	
Median, 63.5 (range 3-115)	
Number of chemotherapy cycles	
Median, 6 (range 1-6)	
Total dose of radiotherapy (Gy)	
Median, 80 (range 45-88)	
Tumor stage	
IB2	22 (8.5)
IIA2	10 (3.8)
IIB	163 (62.7)
IIIA	5 (1.9)
IIIB	53 (20.4)
IVA	7 (2.7)
Histologic type	
Squamous cell carcinoma	216 (83.1)
Adenocarcinoma	32 (12.3)
Adenosquamous carcinoma	7 (2.7)
Small cell carcinoma	5 (1.9)
Nodal involvement	
Present	14 (5.4)
Not present	246 (94.6)
Smoking habits	
Smoker/former smoker	35 (13.5)
Non-smoker	152 (58.5)
Unknown	73 (28.0)
Response to therapy	
Complete	197 (75.8)
Parcial	45 (17.3)
Persistent/stable	12 (4.6)
Progression	6 (2.3)
Recurrence	
Yes	50 (19.2)
No	210 (80.8)

SD, standard deviation.

Therefore, we cannot report on any original study power. However, two-way analysis of variance followed by post hoc analysis was performed as follows: The power to detect a hazard ratio of 2.001 obtained by multivariate analysis, depended on the distribution of the polymorphism genotypes, patients' median survival time, recruitment period (93 months) and additional follow-up time (63.5 months). Consequently, assuming a type I error probability of 0.05, we estimate a post hoc power higher than 80%. This analysis was performed using the Power and Sample Size program (version 3.1.2).

Results

As mentioned before, the *TP53* Arg72Pro polymorphism studied in this work results from a change of guanine (G) to cytosine (C) in codon 72 in exon 4 that leads to the replacement of arginine (Arg) by proline (Pro). Besides that, all results are presented for an analysis comparing heterozygote genotypes (Arg/Pro) with homozygous genotypes (Arg/Arg and Pro/Pro).

Of the 260 patients included in this study (Table I), only 249 patients have results for genotyping. The frequencies of Arg/Arg, Arg/Pro and Pro/Pro genotypes were 0.10, 0.33 and 0.56, respectively. The allele frequency for Arg allele and Pro allele was 27.11 and 72.89%, respectively. The good treatment response rate was 10.7, 33.9 and 55.4% for Arg/Arg, Arg/Pro and Pro/Pro genotypes, respectively. Poor treatment response rate for Arg/Arg, Arg/Pro and Pro/Pro genotypes was 6.3, 25.0 and 68.8%, respectively. This polymorphism were found to be not associated with response to therapy (P=0.571) (Table II).

Regarding OS rates found using Kaplan-Meier method and log-rank test, we observed that the mean survival rates were not statistically different according to the patients *TP53* Arg72Pro genotypes (P=0.058), age (P=0.630) and histology (P=0.758). Stage (P=0.008) and recurrence (P<0.001) were independent prognostic factors that influenced significantly OS of women with advanced cervical cancer treated with chemoradiotherapy (QTRT). Moreover, there are significant differences in mean survival between heterozygote genotypes (Arg/Pro) and homozygous genotypes (Arg/Arg and Pro/Pro). The group of patients carrying heterozygous genotype present a higher mean survival rate than the other patients (126 vs. 111 months, P=0.047) (Fig. 1).

Concerning smoking history, our results demonstrate that OS time differed according to the Arg/Arg and Pro/Pro homozygous genotype vs Arg/Pro heterozygous genotypes carriers in non-smoker individuals, but these results are in the threshold for statistical significance (P=0.052; Fig. 2A). No statistically significant differences were found in the genotype frequencies and OS rate among smokers and former smokers (P=0.194; Fig. 2B). Using the Cox regression analysis, we found that carriers of *TP53* Arg72Pro homozygous genotypes (Arg/Arg and Pro/Pro) present a 2-fold increase of risk of death which is not statistically significant, when compared with *TP53* Arg72Pro heterozygous genotypes, with tumor stage, median age, histology and smoking history as covariates [hazard ratio (HR), 2.001; 95% CI, 0.917-4.368; P=0.082] (Table III).

No difference was found for DFS according to the distribution of genotypes of *TP53* Arg72Pro polymorphism (P=0.205), same when comparing patients with heterozygous and homozygous genotypes (P=0.081; Fig. 3).

Discussion

The activation of the response to DNA damage aims to cell cycle arrest and DNA repair, and the lesions correction failure can result in the senescence or apoptosis (34). Assuming that cells respond differently to DNA damage taking into account whether or not they are tumor cells, understanding this mechanisms will allow selection of therapeutic strategies to individualize response to DNA damage in altered in

Table II. Response to treatment of the advanced cervical cancer patients treated with chemoradiotherapy according to genotypes of the *TP53* Arg72Pro polymorphism.

TP53 Arg72Pro polymorphism	Good response (CR + PR), no (%)	Poor response (SD + PD), no. (%)	P-value
Genotype			0.571
Arg/Arg	25 (10.7)	1 (6.3)	
Arg/Pro	79 (33.9)	4 (25.0)	
Pro/Pro	129 (55.4)	11 (68.8)	
Allele			0.271
Arg	129 (27.7)	6 (18.8)	
Pro	337 (72.3)	26 (81.2)	

CR, complete response; PR, partial response; SD, stable disease; PD, progression disease.

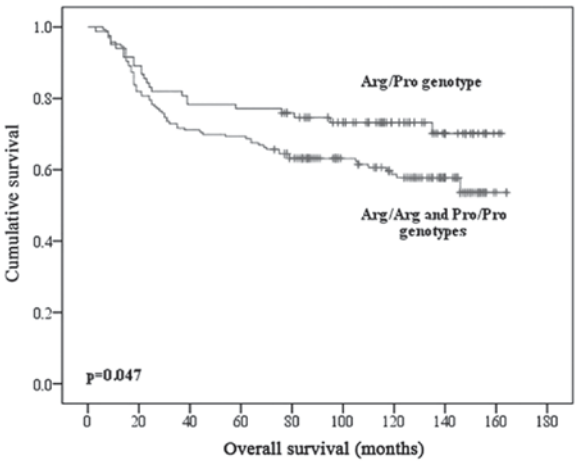

Figure 1. Overall survival by Kaplan-Meier method and log-rank test of cervical cancer patients according to *TP53* Arg72Pro polymorphism.

cancer cells (35). Apoptosis is the main mechanism by which anti-cancer agents originate toxicity (36).

One major mechanism of resistance to therapy and cell survival is the inactivation of the function of *TP53* gene, so observed resistance in tumor cells harboring wild type *TP53* for a large variety of agents, such as ionizing radiation and several classes of cytotoxic drugs, can occur directly by factors that regulate or negate the functional activity of this protein, or indirectly, by deregulation of pathways downstream of this gene (37).

The role of the *TP53* Arg72Pro polymorphism remains controversial (17). The segregation of this polymorphism shows pronounced ethnic differences, so results will be dependent of the study population (32,38). It is also important to refer that in this study we did not consider HPV infections, although it might be a relevant issue for additional studies. However, Medeiros and colleagues (39) studied HPV genotyping profile in squamous cervical lesions in Portugal, and then they find high prevalence of HPV-16 and -18, approximately 80 and 15%, respectively, in cases with invasive cervical cancer. Thus, future studies may include HPV genotyping to evaluate its role in disease progression and clinical outcome under the influence of the genetic background.

In our study, carriers of the heterozygous genotype (Arg/Pro) had a higher mean survival overall than patients with both homozygous genotypes (Arg/Arg and Pro/Pro). Several studies have evaluated the influence of TP53 codon 72 polymorphism in the clinical outcome of cancer patients with controversial results (29,30,33,40). Investigation of Piña-Sánchez *et al* (30) in Mexican women with cervical cancer found a higher survival in heterozygous women (Arg/Pro) than in homozygous women (Arg/Arg and Pro/Pro), however without significant statistical differences. In study of Liu *et al* (29), they did not find association of *TP53* Arg72Pro polymorphism and clinical outcome in Chinese women with cervical carcinoma.

In patients with pancreatic, testicular and prostate cancer no significant effect of this polymorphism was found (40,41). Pro allele homozygosis has been linked with lower sensitivity to chemotherapy in breast and head and neck cancer and lower survival in breast, lung and colorectal cancer (42-46). Moreover other studies show that carriers of Arg genotype have a higher treatment response rate and survival after chemoradiotherapy in advanced head and neck cancer and lung and breast cancer (43,44,47). The presence of one mutated Arg allele may be associated with a reduced sensitivity to cancer therapy in head and neck cancer as well as retention of the Arg allele in heterozygous women with breast cancer are associated with a reduced OS and progression-free disease (48). In this sense, Sullivan and colleagues (44) found that drugs exert their cytotoxic effect in different ways, according to the two codon 72 mutant variant of *TP53* gene, thus verifying a differentiated cell resistance.

There are four possible reasons that may explain the fact that homozygous patients had lower survival than heterozygous patients: i) As seen in other types of cancer, patients with homozygous Pro allele have lower survival than heterozygous carriers, since this allele has a major role in cell cycle arrest and DNA repair than Arg allele (27). Wild type *TP53* Pro variant activates several genes involved in DNA repair more effectively than *TP53* Arg variant. At the same time, cells expressing the *TP53* Pro allele were able to repair the DNA damage much more effectively than cells expressing *TP53* Arg allele (25); ii) Patients with homozygous Arg allele showed lower survival rates compared to heterozygous. It is important refer that the Arg variant has been correlated with a higher affinity of binding and degradation of TP53 protein by E6 oncoprotein of HPV-16/HPV-18 (38,49-51). One of the better well-known functions of the HPV E6 is the ability to

Table III. Multivariate analysis of death risk at 5 years by Cox regression for the *TP53* genotypes, adjusted to different clinical and pathological variables.

Clinicopathological characteristics	HR	95% CI	P-value
Median age (<48 years/≥48 years)	1.319	0.693-2.511	0.400
Tumor stage (<IIB/≥IIB)	3.212	0.767-13.445	0.110
Tobacco (non-smokers/smokers and former smokers)	0.938	0.817-1.077	0.364
Histology (adenosquamous cell carcinomas and small cell carcinoma/adenocarcinoma and squamous cell carcinoma)	0.680	0.092-5.001	0.705
TP53 Arg72Pro genotypes (Homozygous/heterozygous)	2.001	0.917-4.368	0.082

HR, hazard ratio; CI, confidence interval.

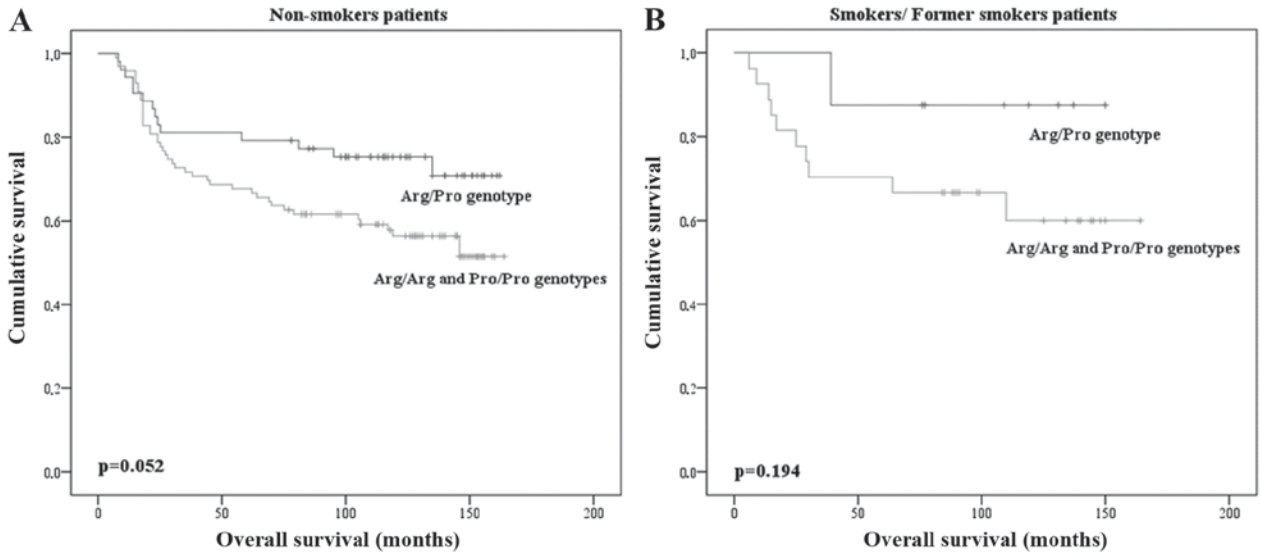

Figure 2. Overall survival by Kaplan-Meier method and log-rank test of cervical cancer patients according to *TP53* Arg72Pro genotypes, adjusted to smoking habits: (A) Non-smokers group; (B) smokers/former smokers group.

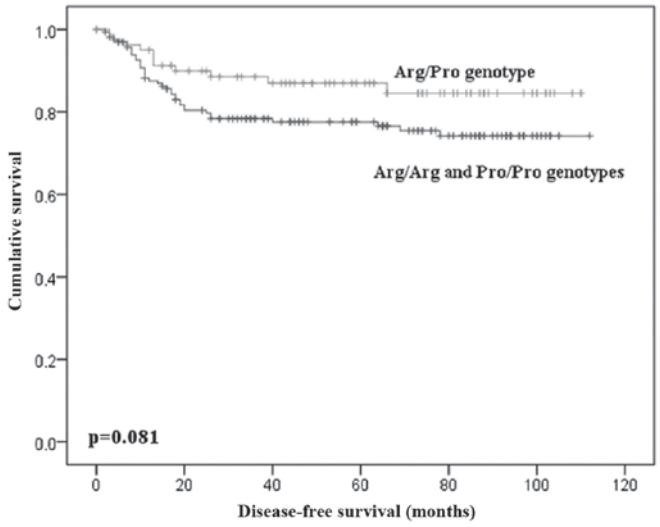

Figure 3. Disease-free survival by Kaplan-Meier method and log-rank test in cervical cancer patients according to genotypes of *TP53* Arg72Pro polymorphism.

increase the tolerance of normal response to DNA damage or then the independent regulation of cellular growth (52,53). These actions are promoted by *TP53* degradation as well as the inhibition of the gene and the multiple repair pathways. Furthermore, the expression of E6 decreases the ability to repair DSBs (53). In this sense, it is believed that carriers of the Arg/Arg homozygous genotype have lower apoptotic capacity, which results in poor survival; iii) the low survival for Arg/Arg homozygous patients have a greater affinity for the mutant *TP53* creating mutants with gain-of-function. Furthermore this variant seems to inhibit the pro-apoptotic activity of the *TP73* gene, which determines the cellular response to different anticancer drugs. In head and neck tumors where *TP53* gene is frequently mutated it is noted that Arg allele carriers had higher resistance to chemotherapy leads to shorter survival. It should be noted that the Pro allele is more frequent with *TP53* wild type and tumors are less sensitive to apoptosis (25); d) it is known that tumors with Arg allele were associated with insufficient or absence of apoptosis, because it was observed the absence of coexpression of Fas and FasL, as well as

high expression of Bcl-2 protein. In heterozygous carriers of Arg72Pro polymorphism the absence of expression of Bcl-2 and co-expression of Fas/FasL is not found. The Bcl-2, Fas and FasL are three apoptosis-related proteins and the down regulation of Fas expression is common in vary type of cancers, including gynecological cancers (54).

This study also indicates that the influence of Arg72Pro polymorphism in treatment response of cervical cancer patients seems to be modulated by smoking history. Our results demonstrate that non-smoker carriers of homozygous genotype present a lower mean OS time comparing with patients with heterozygous genotype (P=0.052), but these results are in the threshold for statistical significance. However, this potential association was not observed in smoker or former-smokers (P=0.194). In a similar study in lung cancer no association was found between genotype of the polymorphism and OS of patients, taking into account their smoking history (P=0.850) (55). Moreover, the biological mechanism that may explain these differences in results is not yet known.

One of the possible limitations of the present study was the exclusion of participants with cervical intraepithelial neoplasia (CIN) or uninfected controls. Therefore, future studies including these types of participants will be relevant to make this study more complete, so to compare the impact of the *TP53* Arg72Pro polymorphism in clinical outcome between pre-invasive cancer patients and advanced cervical cancer patients.

In conclusion, our results demonstrate survival advantage in heterozygous carriers of Arg72Pro polymorphism and a trend to greater risk of death in the homozygous carriers of this polymorphism. Furthermore, *TP53* genotypes could be a useful molecular tools for predicting the clinical outcome of cervical cancer patients and may allow to evaluate optional therapeutic regimens in patients with lower survival. Therefore, in the attempt of optimizing responses and minimizing toxicities associated with chemoradiotherapy, the analysis of a wide range of genetic polymorphisms in DNA damages response genes may indicate the more suitable therapeutic procedure for each cancer patient.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AC and AN conceived and designed the experiments. AC and AN performed the experiments. AC, AN and RM analyzed the data. SS, JA, IB and RC contributed to the interpretation of results obtained and manuscript construction. DP analyzed and interpreted the patients' data regarding the clinical characteristics. AC and AN wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All samples were obtained with the informed consent of the participants prior to their inclusion in the study, according to Helsinki Declaration principles and after approval of the ethics committee of Portuguese Oncology Institute of Porto (CES.287/014).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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