GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Short Communication

Influence of Arg72Pro polymorphisms of *TP53* on the response of buccal cells to radiotherapy

L. Pereira[1], M.R.S. Carvalho[1], C.G. Fonseca[1], S.S.S. Lima[2], E.M.M. Cerqueira[3], W. Jorge[1] and M.C.L. Castro[1†]

[1]Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[2]Hospital Luxemburgo, Belo Horizonte, MG, Brasil
[3]Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Feira de Santana, BA, Brasil

[†]In memoriam
Corresponding author: E.M.M. Cerqueira
E-mail: eneida.cerqueira@gmail.com

Genet. Mol. Res. 10 (4): 3552-3558 (2011)
Received February 7, 2011
Accepted August 3, 2011
Published December 14, 2011
DOI http://dx.doi.org/10.4238/2011.December.14.6

ABSTRACT. Polymorphisms in the *TP53* gene codon 72 (Arg72Pro) influence apoptosis induction and DNA damage repair. We evaluated how variants of protein p53 (p53Arg and p53Pro) affect cell death and DNA damage repair by analyzing the frequencies of karyorrhexis and micronuclei. There were significant differences in the frequency of karyorrhexis between the three p53 genotypes (Arg/Arg, Arg/Pro, and Pro/Pro), between samples taken before and after radiotherapy, and between patients and controls. The frequency of micronucleated cells increased significantly after radiotherapy. There were no significant differences in the micronucleus frequency in healthy tissues of these patients compared to controls, or in the comparisons between the three genotypes. We conclude that Arg72Pro polymorphism influences cell apoptotic capacity. This is the first study investigating karyorrhexis and micronuclei, as indicators of apoptosis after radiotherapy, and how

these indicators are influenced by the *TP53* polymorphism Arg72Pro.

Key words: *TP53*; Karyorrhexis; Micronuclei; Apoptosis; rs56275308; Arg72Pro

In half of all human cancers, the function of tumor suppressor p53 protein is altered as a result of somatic mutations in tumor cells (Green and Kroemer, 2009; Whibley et al., 2009). A common polymorphism (rs56275308) in the p53 encoding gene (*TP53*) results in substitution of a proline (CCC) for an arginine (CGC) at codon 72 in exon 4. The variant p53Arg induces apoptosis more efficiently than does p53Pro, and it has been suggested that this difference may influence the risk of developing cancer (Dumont et al., 2003; Ezzikouri et al., 2007). When the cell does not promote DNA repair or apoptosis, morphological nuclear abnormalities called micronuclei (MN) can persist (Figure 1). MNs are smaller than the normal nuclei and originate from chromosome fragments or whole chromosomes that lag behind in their migration to the cell poles or migrate irregularly during anaphase (Fenech and Bonassi, 2011).

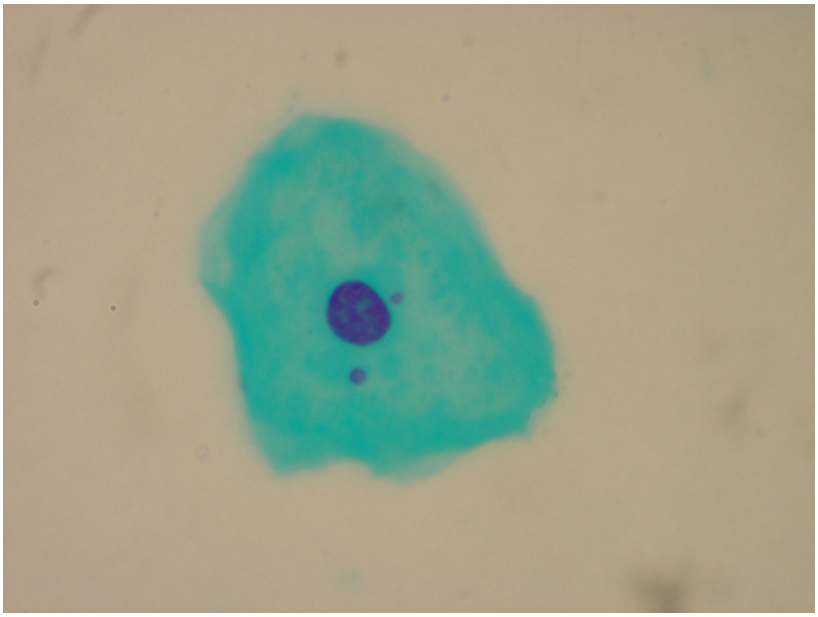

Figure 1. Cell exfoliated mucosal cells containing two micronuclei (stained with Schiff dye and counter-stained with fast green - optical magnitude 1000X).

The main cellular lesions caused by ionizing radiation are breaks in double-stranded DNA. MN and other abnormalities may appear when such lesions are not repaired. A correlation has been found between a high number of MN in tumor cells and a favorable response to radiation treatment (Shidnia et al., 1990). Abnormalities such as MN, multinucleation, kary-

olysis, karyorrhexis, nuclear budding and pyknosis can be used as prognostic parameters for the radiosensitivity of oral cancers (Bindu et al., 2003). Patients with more than 19.5 MN/1000 cells have an increased risk of developing cancer (Bloching et al., 2000). Higher frequencies of cancer have been found in individuals with medium to high frequencies of MN, and the survival of those who develop cancer is lower in these individuals (Bonassi et al., 2007).

The MN test is therefore a valuable tool for evaluating biological damage and for helping to determine the radiosensitivity of tumors (Bonassi et al., 2011). The sensitivity of the MN test is increased if, in addition to evaluating the MN, the nuclear changes indicative of apoptosis, such as karyorrhexis, are also assessed (Tolbert et al., 1992). This test can also aid in decision-making concerning appropriate treatment, can allow doctors to monitor the response to treatment and may be useful in defining the prognosis at the end of therapy. The purpose of this study was to evaluate the influence of Arg72Pro polymorphism on the response of buccal cells to radiotherapy, monitoring the number of cells with micronuclei and karyorrhexis in patients with head and neck cancer. The sample used consisted of mucosa buccal cells from 38 individuals. Nineteen of these were patients diagnosed as having head and neck cancer who had undergone radiotherapy. The control group was composed of 19 healthy individuals. The cases comprised individuals of both genders, from 20 to 80 years old.

All sample collections were done at the Radiotherapy Center of Hospital Luxemburgo, Belo Horizonte City, Minas Gerais State, Brazil. The project was approved by Ethics Research Committees of both Hospital Luxemburgo and Universidade Federal de Minas Gerais. Samples were collected after obtaining signed informed consent. For controls, we collected a single sample of oral mucosa. For cancer patients, we collected one sample from the perilesional area both before and 10 days after the first session of radiotherapy (Cerqueira et al., 2004), and from a clinically healthy area (before radiotherapy).

Cells were mixed with a drop of physiological saline and spread directly onto a microscope slide. Samples were fixed with 3:1 methanol/acetic acid for 10 min. After 24 h, the samples were hydrolyzed with 5 N hydrochloric acid for 30 min, stained with Schiff dye for 90 min (Feulgen and Rossenbeck, 1924) and counterstained with fast green for one minute. We blindly counted MN in at least 2000 cells per individual (Tolbert et al., 1992) and occurrence of karyorrhexis in 1000 cells per individual in patient (Table 1) and control (Table 2) samples.

For the molecular analyses, DNA was extracted from clinically healthy buccal mucosa cells (Miller et al., 1988). A fragment containing *TP53* exon 4 was amplified by PCR using the primers forward: 5'-TCCCCCTTGCCGTCCCAA-3' and reverse: 5'-TCAGGCGGCTCATAGGGC-3'. PCR was carried out in a total volume of 25 µL, with 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 8% DMSO, 50-200 ng genomic DNA, 10 pmol of each primer and 1 U Taq DNA polymerase. Cycling parameters were as follows: an initial denaturation step at 94°C for 5 min and 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a last extension step at 72°C for 5 min. PCR amplifications were checked by electrophoresis on 1% agarose gels, stained with ethidium bromide. Next, 10 µL of the amplification product were digested with 10 U of the restriction enzyme *Acc*II and analyzed on 1% agarose gels.

The PCR product, a 1411-bp amplicon, contains one monomorphic *Acc*II restriction site, which works as an internal control for the digestion and produces a 280-bp band. An additional *Acc*II recognition site is present in those chromosomes carrying the *TP53* allele 72Arg. Therefore, the *TP53* 72Arg allele produces three bands (the internal PCR control band

of 280 bp and two polymorphic bands of 109 and 1022 bp). The allele that encodes for Pro72 produces two bands: the internal control (280 bp) and a 1131- bp band.

Table 1. Age, genotype and number of cells with micronuclei (MN) and karyorrhexis in the patient samples.

Patient	Age (years)*	Genotype	Sample	MN	Cells/Slide	Karyorrhexis/slide	Cells/slide
1	75	Arg/Arg	healthy mucosa	0	2020	43	1003
			perilesional area before	0	1873	18	1000
			perilesional area after	3	1621	9	1000
2	80	Arg/Pro	healthy mucosa	0	2020	10	1009
			perilesional area before	3	2006	0	1004
			perilesional area after	7	2013	119	1011
3	53	Arg/Arg	healthy mucosa	1	1843	28	1000
			perilesional area before	2	2029	37	1000
			perilesional area after	29	2000	8	1001
4	56	Arg/Pro	healthy mucosa	0	2024	141	1011
			perilesional area before	0	2025	66	1001
			perilesional area after	31	2008	20	1011
5	29	Arg/Pro	healthy mucosa	0	1329	75	1000
			perilesional area before	0	1243	49	1001
			perilesional area after	5	692	51	1005
6	49	Arg/Pro	healthy mucosa	0	2085	16	1001
			perilesional area before	2	2038	380	1001
			perilesional area after	1	2180	218	1017
7	49	Arg/Arg	healthy mucosa	0	2021	55	1002
			perilesional area before	0	2084	29	1001
			perilesional area after	15	1995	27	1005
8	61	Arg/Arg	healthy mucosa	0	2028	147	1015
			perilesional area before	0	2042	103	1001
			perilesional area after	2	2029	28	1001
9	46	Arg/Pro	healthy mucosa	0	1047	39	1000
			perilesional area before	0	2037	6	1001
			perilesional area after	3	2100	98	1037
10	75	Arg/Pro	healthy mucosa	0	2039	7	1001
			perilesional area before	0	2068	7	1002
			perilesional area after	6	2014	9	1006
11	56	Arg/Pro	healthy mucosa	2	2095	5	1031
			perilesional area before	0	2036	15	1000
			perilesional area after	23	2034	14	1005
12	65	Arg/Pro	healthy mucosa	1	2028	0	1043
			perilesional area before	1	2008	0	1007
			perilesional area after	40	2006	19	1011
13	59	Pro/Pro	healthy mucosa	0	2016	46	1001
			perilesional area before	0	2025	3	1004
			perilesional area after	3	2012	0	1001
14	74	Arg/Pro	healthy mucosa	0	2009	6	1002
			perilesional area before	1	2059	0	1029
			perilesional area after	0	2037	6	1011
15	47	Arg/Pro	healthy mucosa	0	2013	12	1009
			perilesional area before	5	2035	29	1013
			perilesional area after	60	2005	22	1008
16	51	Arg/Pro	healthy mucosa	0	2011	29	1004
			perilesional area before	7	2039	56	1001
			perilesional area after	18	2010	11	1001
17	42	Pro/Pro	healthy mucosa	1	2035	164	1005
			perilesional area before	0	2052	326	1018
			perilesional area after	3	2006	36	1002
18	49	Arg/Pro	healthy mucosa	1	2014	0	1031
			perilesional area before	4	2013	6	1005
			perilesional area after	0	2007	85	1020
19	56	Pro/Pro	healthy mucosa	0	2071	4	1000
			perilesional area before	0	2007	0	1002
			perilesional area after	0	2007	1	1010

*Mean (and standard deviation): 56.5 (12.9).

Table 2. Age, genotype and number of cells with micronuclei (MN) and karyorrhexis in the control samples.

Control	Age (years)*	Genotype	MN	Cells/slide	Karyorrhexis/Slide	Cells/slide
1	42	Arg/Pro	0	2010	5	1002
2	24	Arg/Pro	0	2025	2	1001
3	52	Arg/Pro	0	2023	6	1004
4	61	Arg/Pro	1	2020	3	1008
5	30	Arg/Arg	0	2007	11	1001
6	42	Arg/Pro	0	2027	8	1011
7	39	Arg/Arg	0	2036	6	1001
8	21	Arg/Arg	0	2015	18	1014
9	20	Arg/Arg	1	2030	12	1002
10	28	Arg/Arg	0	2027	12	1002
11	46	Arg/Arg	0	2053	369	1031
12	25	Arg/Pro	0	2005	54	1000
13	35	Arg/Pro	0	2032	33	1000
14	41	Arg/Arg	0	2029	34	1000
15	50	Arg/Arg	2	2008	21	1003
16	54	Arg/Arg	0	2013	0	1001
17	54	Arg/Arg	0	2020	3	1012
18	52	Pro/Pro	0	2009	0	1019
19	53	Arg/Arg	0	2015	18	1004

*Mean (and standard deviation): 42.3 (10.2).

We made the following observations. 1) Allelic and genotypic frequencies met Hardy-Weinberg equilibrium in both patient and control samples (Table 3). 2) Head and neck cancer patients showed a higher proportion of the Pro72 allele (Table 3, P = 0.047 and 0.041 for allele and genotype frequency tests, respectively, using the exact test implemented in the software Genepop version 3.3 (Raymond and Rousset, 1995)).

Table 3. Allelic and genotypic absolute frequencies of the germline polymorphism Arg72Pro of *TP53*.

	Arg	Pro	Arg/Arg	Arg/Pro	Pro/Pro	P*
Patients	20	18	4	12	3	0.38
Controls	29	9	11	7	1	1

*Significance of Hardy-Weinberg equilibrium model assessed using the Guo-Thomsom test implemented in the Genepop software version 3.3 (Raymond and Rousset, 1995).

There was a significant difference in the number of cells with karyorrhexis between the three genotypes (P = 0.03), suggesting that the Arg72Pro polymorphism affects the cell's apoptotic activity. The number of cells with karyorrhexis was also significant and positively correlated with age (P = 0.0005).

Because the number of micronucleated cells did not fit the normal distribution required by analysis of variance, we normalized the observed values using the formula $(\sqrt{Y+0.5})$ (1), where Y represented the observed values (Sokal and Rohlf, 1995). The baseline number of MN in healthy buccal mucosa were not different between patients and controls, but after radiotherapy (perilesional area both before and after radiotherapy) the number of cells with MN rose significantly (P = 0.0007). This increase was also found by other researchers (Jianlin et al., 2004), and unlike our results, Burgaz et al. (2011) found a significantly higher number of MN in patients than in controls.

We did not find any relationship between the frequency of micronucleated cells and patient age, as also observed in another study (Ramírez and Saldanha, 2002). However, other researchers have reported higher frequencies of micronuclei in older patients, thus suggesting that the genome is increasingly vulnerable with advancing age (Norppa and Falck, 2003; Fenech and Bonassi, 2011).

In general, our results suggest that *TP53* Arg72Pro polymorphism can affect cellular apoptotic activity. Further studies on the effect of this *TP53* polymorphism should be conducted using a larger number of individuals to obtain an adequate number of each of the three genotypes.

Conflict of interest statement

None declared.

ACKNOWLEDGMENTS

Research supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). M.R.S. Carvalho was the recipient of CNPq fellowship (#307975/2010-0). We thank our patients, who contributed to our study.

REFERENCES

Bindu L, Balaram P, Mathew A, Remani P, et al. (2003). Radiation-induced changes in oral carcinoma cells - a multiparametric evaluation. *Cytopathology* 14: 287-293.

Bloching M, Hofmann A, Lautenschlager C, Berghaus A, et al. (2000). Exfoliative cytology of normal buccal mucosa to predict the relative risk of cancer in the upper aerodigestive tract using the MN-assay. *Oral Oncol.* 36: 550-555.

Bonassi S, Znaor A, Ceppi M, Lando C, et al. (2007). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis* 28: 625-631.

Bonassi S, El-Zein R, Bolognesi C and Fenech M (2011). Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* 26: 93-100.

Burgaz S, Coskun E, Demircigil GC, Kocabas NA, et al. (2011). Micronucleus frequencies in lymphocytes and buccal epithelial cells from patients having head and neck cancer and their first-degree relatives. *Mutagenesis* 26: 351-356.

Cerqueira EMM, Gomes-Filho IS, Trindade S, Lopes MA, et al. (2004). Genetic damage in exfoliated cells from oral mucosa of individuals exposed to X-rays during panoramic dental radiographies. *Mutat. Res.* 562: 111-117.

Dumont P, Leu JI, Pietra ACD, George DL, et al. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat. Genet.* 33: 357-365.

Ezzikouri S, El Feydi AE, Chafik A, Benazzouz M, et al. (2007). The Pro variant of the p53 codon 72 polymorphism is associated with hepatocellular carcinoma in Moroccan population. *Hepatol. Res.* 37: 748-754.

Fenech M and Bonassi S (2011). The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes. *Mutagenesis* 26: 43-49.

Feulgen R and Rossenbeck H (1924). Mikorskopisch-chemischer nachweis einer nucleinsaure von typus der thymonucleisaure und die darauf beruhende elektive farbung vom zellkernen in mikroskopischen praparaten. *Z. Phys. Chem.* 135: 203-248.

Green DR and Kroemer G (2009). Cytoplasmic functions of the tumour suppressor p53. *Nature* 458: 1127-1130.

Jianlin L, Jiliang H, Lifen J, Wei Z, et al. (2004). Measuring the genetic damage in cancer patients during radiotherapy with three genetic end-points. *Mutagenesis* 19: 457-464.

Miller SA, Dykes DD and Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16: 1215.

Norppa H and Falck GC (2003). What do human micronuclei contain? *Mutagenesis* 18: 221-233.

Ramirez A and Saldanha PH (2002). Micronucleus investigation of alcoholic patients with oral carcinomas. *Genet. Mol.*

Res. 1: 246-260.
Raymond M and Rousset F (1995). GENEPOP (version 1.2) population genetic software for exact tests and ecumenicism. *J. Heredity* 86: 248-249.
Shidnia H, Crabtree W, Hornback N, Young P, et al. (1990). Micronuclei assay - a predictive variable for tumor response to treatment. *Adv. Exp. Med. Biol.* 267: 51-55.
Sokal RR and Rohlf FJ (1995). The Normal Probability Distribution. In: Biometry. 3rd edn. W.H. Freeman and Company, New York, 116-123.
Tolbert PE, Shy CM and Allen JW (1992). Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat. Res.* 271: 69-77.
Whibley C, Pharoah PD and Hollstein M (2009). p53 polymorphisms: cancer implications. *Nat. Rev. Cancer* 9: 95-107.