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THE ROLE OF nt590 *P21* GENE POLYMORPHISM IN THE SUSCEPTIBILITY TO NASOPHARYNGEAL CANCER

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***Aim*: The purpose of this study was to assess if the P21 nt590 polymorphism is associated with the susceptibility to nasopharyngeal cancer and with the age at diagnosis. *Materials and Methods*: We analyzed the frequency of 3'UTR P21 polymorphisms in blood samples from 102 nasopharyngeal cancer patients and 191 controls, with no known oncologic disease, using PCR–RFLP. *Results*: The polymorphism genotype frequencies were 93.2% (CC), 5.2% (CT) and 1.6% (TT) in the control group and 88.2% (CC), 10.8% (CT) and 1.0% (TT) in the cases group. We found no statistically significant association between the different P21 polymorphism genotypes and risk of nasopharyngeal cancer ($p = 0.201$). However, approximately a four-fold increased risk of undifferentiated nasopharyngeal carcinoma in early stages was observed for P21 T carriers (OR = 3.734; 95% IC 1.289–10.281; $p = 0.01$). Furthermore, our results indicate that the waiting time for onset of neoplasia in T carriers patients was 12.4 years earlier (56.5 years old), comparing with those carrying CC genotype (68.9 years old). *Conclusions*: Our findings suggest that the 3'UTR P21 polymorphism may play an important role in the pathogenesis and initiation, but not in the progression, of undifferentiated nasopharyngeal carcinoma. Moreover, the polymorphism seems to contribute to a significantly earlier age at diagnosis.**
***Key Words*: genetic polymorphisms, nasopharyngeal cancer, P21.**

Nasopharyngeal cancer is usually a multifactorial disease and genetic susceptibility has a crucial role in the etiology of this pathology, as well as environmental, viral and dietetic factors.

The $p21^{1WAF1/Cip1}$ protein belongs to the Cip/Kip family of cyclin-kinase inhibitors (CKI). It is also known as CIP1 (CDK-interacting protein 1) or WAF1 (wild-type p53-activated fragment 1), as p21 protein expression is usually regulated by p53 protein transcriptional levels [1, 2]. In fact, a possible change in TP53 tumor suppressor gene (found in 50% of all tumors) will prevent gene P21 transcription and G1 cell cycle arrest, thus allowing continuous activity of cyclin-CDK complexes and autonomous proliferating capacity.

The p21 protein is codified by gene P21 and it is localized on chromosome 6 (6p21.2). This gene consists of 3 exons (68, 450 e 1600 pb), but the first one is not transcribed. p21 protein has a preferential nuclear location, containing 164 amino acids, with a molecular weight of 21 kDa [3]. It has cell cycle regulation functions, inhibiting CDKs in G1/S and G2/M. The p21 protein binds to cyclin-CDK complexes, inhibiting phosphorylation of the retinoblastoma protein [4]. Thus, the activity of E2F transcription factor is blocked and cell cycle arrests in G1/S transition [5, 6]. In different studies it was concluded that it promotes or inhibits apoptosis and differentiation [7–9].

The CIP/KIP family (p21, p27 and p57) has similarities in the amino terminal of its structure. The amino terminal of these proteins is necessary and sufficient to inhibit the activity of cyclin-CDK complex. The carboxyl terminal of p21 is associated with proliferating cell nuclear antigen (PCNA), interlinked with tumor growth suppression through inhibition of PCNA dependant replication [10]. It also has a nuclear localization signal (NLS) [3] proximal to that terminal.

P21 mutations are rare in neoplasia [11], but a decrease in expression is usually associated with worse prognosis in humans [12, 13]. It is therefore possible that the carcinogenesis process is potentiated by genetic variations in *P21* gene that affect the expression of p21 protein.

The aim of this study was to investigate if the *P21* nt590 polymorphism is associated with the susceptibility for nasopharyngeal cancer and with the age at diagnosis.

MATERIALS AND METHODS

Population. A case-control study was carried out to assess the possible association between the presence of the polymorphism and susceptibility for nasopharyngeal cancer.

Samples were collected from 102 patients with nasopharyngeal cancer, admitted and treated at the Portuguese Institute of Oncology, Porto, Portugal (median age 47.5 years, SD 3.90 years). The control group consisted of 191 healthy volunteers without clinical history of cancer (median age 55.7 years, standard deviation 15 years). Histological type and grade of tumors are presented in Table 1.

All blood samples were collected after informed consent, according to the Declaration of Helsinki, and after local ethics committee approval at the Portuguese Institute of Oncology. During laboratory proceedings, the clinical status and outcome of patients was not known by the investigators.

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Abbreviations used: PCR-RFLP – polymerase chain reaction-restriction fragment length polymorphism; WTOD – waiting time for onset of disease.

Samples. Blood samples were collected in EDTA containing tubes. Genomic DNA was extracted from the white blood cell fraction of each case, using Qiagen® extraction kit, Qiamp DNA Mini Kit.

$P21^{CIP1/WAF1}$ exon 3 genotype analysis. Amplification of a 300 bp DNA fragment was obtained after a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method adapted from a previously described protocol [14]. The primers used were E3A: 5' CCC AGG GAA GGG TGT CCT G-3' and E3B: 5'GGG CGG CCA GGG TAT GTA C-3'. DNA was amplified in a 50 µl mixture including 1xTaq Buffer, 1.5 mM $MgCl_2$, 0,2 nM deoxynucleotide triphosphates, 0.3 µM from each primer, and 1 Unit Taq DNA polymerase. Thermocycler parameters were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 60 s, 60 °C for 45 s and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. The 300 bp fragment, verified by electrophoresis on a 1.5% agarose gel, was digested overnight at 37 °C with 1 Unit of PstI enzyme. This enzyme recognizes the CT polymorphism leading to the loss of a PstI site. In the presence of the C allele, two fragments of 174 and 126 bp were expected. The restriction-digested products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under UV light.

Finally, we considered a basic question: "For a newborn individual, what is the probability that he will experience onset of advanced disease before the age of X, supposing he survives that long?", assuming that all cases were at an identical risk of nasopharyngeal cancer at birth [15]. To address this question, we hypothesized that nt590 P21 genotypes may alter the onset of these cancers. We define the age of the onset for cancer as the outcome and nt590 *P21* genotype as an independent variable.

Statistical analysis. Data analysis was performed using the computer software Statistical Package for Social Sciences — Version 15.0, SPSS Inc, 2004.

Chi-square analysis was used to compare categorical variables. A 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% confidense interval (CI) were calculated as a measurement of the association between P21 genotypes and NPC risk. The Hardy — Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed versus the expected genotype frequencies.

We tested the association between age of onset and the polymorphism genotype by comparing Kaplan — Meier survival curves. We therefore considered the waiting time for onset of disease (WTOD) as the interval between the time of initial exposure to the risk factor (polymorphism) and the time of onset of disease. We estimated the cumulative probabilities for having cervical and nasopharyngeal cancers by the Kaplan — Meier methodology. The primary analysis of time-to-event for WTOD was performed with the use of two-sided log-rank test at the 5% level of statistical significance.

RESULTS

We analyzed P21 3' UTR genotypes in 102 cases of nasopharyngeal cancer and 191 healthy volunteers using the PCR-RFLP method. Clinicopathological information is described in Table 1.

Table 1. Clinicopathological characteristics of nasopharyngeal carcinoma patients and control group

	Cases	Controls
	n = 102	n = 191
Gender	n (%)	n (%)
Male	75 (73.5)	145 (75.9)
Female	27 (26.5)	46 (24.1)
Total	102 (100.0)	191 (100.0)
Age		
Mean	47.49	55.68
Standard deviation	14.966	3.904
Median	50	56
Histological type		
Undifferentiated	100 (98)	-
Other	2 (2)	-
Stage		
I	1 (1.0)	-
II	27 (26.4)	-
III	29 (28.4)	-
IV	40 (39.2)	-
Unknown	5 (4.9)	-
Total	102 (100.0)	-

Regarding histology, 98% of cases were undifferentiated carcinomas and polymorphism analysis was performed in these cases. According to clinical stages, stage I represented 1%, stage II — 26.4%, stage III —28.4% and 39.2% were stage IV. Genotype frequencies in the controls group were 93.2% (CC), 5.2% (CT) and 1.6% (TT). In nasopharyngeal cancer 88.2% patients presented CC genotype, 10.8% were heterozygous (CT) and 1.0% presented TT genotype (Table 2).

Table 2. Genotype frequencies of nt590 P21 polymorphism and risk for nasopharyngeal cancer

	Genotype						
				Recessive Model			
	CC	CT	TT	T-Carrier	OR	95% CI	p
	N (%)	N (%)	N (%)	N (%)			
Controls n = 191	178 (93.2)	10 (5.2)	3 (1.6)	13 (6.8)	1	Reference	
Cases n = 102	90 (88.2)	11 (10.8)	1 (1.0)	12 (11.8)	1.826	0.800–4.164	0.148
Undifferentiated n = 100	88 (88.0)	11 (11.0)	1 (1.0)	12 (12.0%)	1.867	0.818–4.261	0.133

No association was found between the presence of the genotypes studied and risk for the development of nasopharyngeal cancer. A statistically significant association was found between the presence of T allele and cancer susceptibility in the early stages, with T allele carriers presenting a four-fold increase in the risk for the development of nasopharyngeal cancer (OR = 3.734; 95%IC 1.289–10.281; $p = 0.01$) (Table 3). This association was not found for more advanced stages.

Table 3. Analysis regarding the susceptibility of T allele carriers to the development of undifferentiated histologic type of nasopharyngeal carcinoma, according to the tumor stage

	CC	CT/TT	OR T-carrier genotype	95% CI	p (χ^2 test)
Controls	178 (93.2%)	13 (6.8%)	–	–	–
Stages I and II	22 (78.6%)	6 (21.4%)	3.734	1.289–10.821	0.01
Stages III and IV	63 (91.3%)	6 (8.7%)	1.304	0.475–3.577	0.605

For early stages, the estimated average for T allele carriers was 56.5 years, and 68.9 years for CC genotype.

Figure shows the Kaplan — Meier curves relating to the WTOD for undifferentiated nasopharyngeal cancer, stages I and II. The log-rank test was used to compare the curves, with a confidence interval of 95%. This curve shows that T allele carriers present a trend for developing undifferentiated nasopharyngeal cancer (stages I and II) in earlier ages than patients without the same allele (log-rank test: $p = 0.001$).

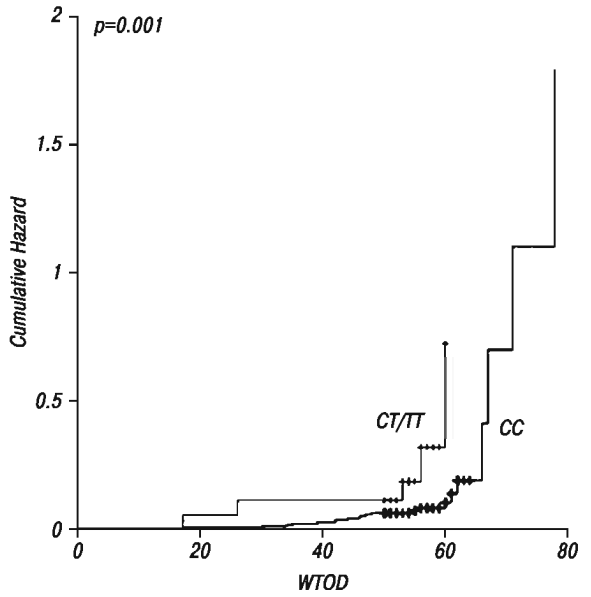

Figure. Association between CC or T carrier (CT/TT) genotypes and the waiting time to onset of disease (WTOD) for stages I and II undifferentiated histologic type of nasopharyngeal carcinoma group

For more advanced stages (III–IV), the average was 57.7 years for allele T carriers, and 61.9 years for CC genotype. These values do not show statistically significant differences (log-rank test: $p = 0.529$), Therefore, there is no association between the genotype of the polymorphism and risk for undifferentiated nasopharyngeal cancer in more advanced stages.

DISCUSSION

It is now accepted that p21 protein is a universal inhibitor of CDKs, preventing phosphorylation of the retinoblastoma protein, promoting cell cycle arrest in G1/S. It also induces normal and tumor cell differentiation, promotion/inhibition of apoptosis, inhibition of DNA replication by binding with PCNA and repair functions, especially those mediated by p53. *P21* gene expression is regulated by protein p53 with increased levels in genotoxic stress situations leading to cell cycle arrest or cell death [16–18]. Several studies associated changes in this gene with carcinogenesis. Most of these studies involve changes in protein expression levels, but several authors focused on the impact of different gene polymorphisms in the carcinogenesis process of different cancers, such head and neck, oral cavity or nasopharynx cancer [19–25] (Table 4).

It has been established that some genetic variations in *P21* gene such as polymorphisms can be associated with different levels of susceptibility for cancer. Few studies are available for nasopharyngeal cancer.

Table 4. Studies on the association between *P21* gene polymorphisms and risk of head and neck, oral cavity and nasopharyngeal cancer

Reference	Year	Country	Cancer Type	Polymorphism	Association
Sun et al.	1995	China	Nasopharynx	Ser31Arg	No
Ralhan et al.	2000	India	Oral Cavity	Codon 149	Yes
Tsai et al.	2002	China	Nasopharynx	Ser31Arg	No
Li et al.	2005	USA	Head and Neck	Ser31Arg	Yes
Bau et al.	2007	China	Oral Cavity	Ser31Arg	Yes
Gomes et al.	2008	Brasil	Oral Cavity	Ser31Arg	Yes
Lei et al.	2010	USA	Head and Neck	Ser31Arg	Yes

The nt590 polymorphism of gene *P21*, commonly associated with codon 31 polymorphism [26], is located in the 3' UTR region (non transcribed region), that is associated with cell differentiation and proliferation as well as tumor and metastatic suppression, because it is frequently related to mRNA stability and degradation [27, 28]. Therefore, although this polymorphism is not included in a transcription region, it can increase susceptibility for cancer, when causing functional changes in the protein and the cell cycle [29].

In our study, no direct association was found between nt590 *P21* polymorphism genotypes and the risk to nasopharyngeal cancer. Nevertheless, a significant association was found between onset of the disease and the polymorphism with T allele carriers (CT and TT genotypes) presenting a four-fold higher risk for the development of undifferentiated carcinoma in early stages when compared with wild-type (CC) allele carriers. This association was not observed for advanced stages of the disease, thus suggesting the relevant role of the polymorphism in the initial stage of carcinogenesis rather than in disease progression, or in less aggressive subtypes of the neoplasia. Additionally, individuals carrying the risk genotype (CT and TT genotypes) are younger at the time of diagnosis, thus supporting the possible role of the polymorphism as a genetic determinant in the pathogenesis of undifferentiated nasopharyngeal carcinoma.

The genetic association between gene *P21* polymorphism and undifferentiated nasopharyngeal carcinoma is biologically plausible. This particular histological type of cancer is associated with EBV [30–32]. With p21 unchanged, cell proliferation is inhibited but it is possible that the presence of T allele in nt590 of gene *P21* changes protein function. This would prevent the B7-H4 cellular protein-induction of G0/G1 cell block, which occurs after EBV infection [33], and therefore perpetuating cell division and contributing to a cancer phenotype. In the same way, the change from allele C to T in nt590 may lead to a change in the p21 function, decreasing the efficacy of apoptosis-induced by BRLF1 viral protein and mediated by E2F1 transcription factor [33]. These points towards the possible impact of polymorphism in nt590 in the initiation stage of the disease, a time intimately related to uncontrolled proliferation triggered by EBV induced cell cycle abnormalities.

P21 expression is high in genotoxic stress situations induced by an increase in p53 levels, leading to cell cycle arrest or cell death. In fact, genotoxic stress is one of the characteristics of viral infections, such as EBV

infections, primarily due to the increase of oxygen and nitrogen reactive species [16–18]. This corroborates the possibility of nt590 polymorphism potentiate the onset of the disease in undifferentiated nasopharyngeal carcinoma, associated with EBV, in relation to all the changes in cell cycle key proteins resulting from this infection. The association between nt590 polymorphism and the risk for undifferentiated nasopharyngeal carcinoma in early stages allows us to hypothesize that T allele can function as a promoter for the initiation stage of the neoplasia. Other factors related to tumor microenvironment may exist that define the evolution of the neoplasia. Alternatively, the presence of T allele in this polymorphism may be related with less tumor aggressiveness, originating an increased number of T allele carriers in the less aggressive early stages of the disease.

REFERENCES

1. **el-Deiry WS, Tokino T, Waldman T, *et al.*** Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. Cancer Res 1995; **55**: 2910–9.

2. **Ball KL.** p21: structure and functions associated with cyclin-CDK binding. Prog Cell Cycle Res 1997; **3**: 125–34.

3. **Javelaud D, Besançon F.** CDKN1A (cyclin-dependent kinase inhibitor 1A). Atlas Genet Cytogenet Oncol Haematol, 2001.

4. **Costanzi-Strauss E, Strauss BE, Naviaux RK, *et al.*** Restoration of growth arrest by p16INK4, p21WAF1, pRB, and p53 is dependent on the integrity of the endogenous cell-cycle control pathways in human glioblastoma cell lines. Exp Cell Res 1998; **238**: 51–62.

5. **Erber R, Klein W, Andl T, *et al.*** Aberrant p21(CIP1/WAF1) protein accumulation in head-and-neck cancer. Int J Cancer 1997; **74**: 383–9.

6. **Griffin SV, Shankland SJ.** Not just an inhibitor: a role for p21 beyond the cell cycle — "The truth is rarely pure and never simple". J Am Soc Nephrol 2004; **15**: 825–6.

7. **Gervais JL, Seth P, Zhang H.** Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis. J Biol Chem 1998; **273**: 19207–12.

8. **Harvat BL, Wang A, Seth P, *et al.*** Up-regulation of p27Kip1, p21WAF1/Cip1 and p16Ink4a is associated with, but not sufficient for, induction of squamous differentiation. J Cell Sci 1998; **111**: 1185–96.

9. **McKay BC, Ljungman M, Rainbow AJ.** Persistent DNA damage induced by ultraviolet light inhibits p21waf1 and bax expression: implications for DNA repair, UV sensitivity and the induction of apoptosis. Oncogene 1998; **17**: 545–55.

10. **Viallard JF, Lacombe F, Belloc F, *et al.*** Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology. Cancer Radiother 2001; **5**: 109–29.

11. **Roninson IB.** Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. Cancer Lett 2002; **179**: 1–14.

12. **Pruneri G, Pignataro L, Carboni N, *et al.*** Clinical relevance of expression of the CIP/KIP cell-cycle inhibitors p21 and p27 in laryngeal cancer. J Clin Oncol 1999; **17**: 3150–9.

13. **Liu J, Hu Y, Hu W, *et al.*** Expression and prognostic relevance of p21(WAF1) in stage III esophageal squamous cell carcinoma. Dis Esophagus 2012; **25**: 67–71.

14. **Law JC, Deka A.** Identification of a PstI polymorphism in the p21Cip1/Waf1 cyclindependent kinase inhibitor gene. Hum Genet 1995; **95**: 459–60.

15. **Medeiros R, Vasconcelos A, Costa S, *et al.*** Steroid hormone genotypes ARStuI and ER325 are linked to the progression of human prostate cancer. Cancer Genet Cytogenet 2003; **141**: 91–6.

16. **Butz K, Geisen C, Ullmann A, *et al.*** Uncoupling of p21WAF1/CIP1/SDI1 mRNA and protein expression upon genotoxic stress. Oncogene 1998; **17**: 781–7.

17. **Dalpke AH, Thomssen R, Ritter K.** Oxidative injury to endothelial cells due to Epstein — Barr virus-induced autoantibodies against manganese superoxide dismutase. J Med Virol 2003; **71**: 408–16.

18. **Catarino R, Pereira D, Breda E, *et al.*** Oncogenic virus-associated neoplasia: a role for cyclin D1 genotypes influencing the age of onset of disease? Biochem Biophys Res Commun 2008; **370**: 118–22.

19. **Sun Y, Hildesheim A, Li H, *et al.*** No point mutation but a codon 31ser-->arg polymorphism of the WAF-1/CIP-1/p21 tumor suppressor gene in nasopharyngeal carcinoma (NPC): the polymorphism distinguishes Caucasians from Chinese. Cancer Epidemiol Biomarkers 1995; **4**: 261–7.

20. **Ralhan R, Agarwal S, Mathur M, *et al.*** Association between polymorphism in p21(Waf1/Cip1) cyclin-dependent kinase inhibitor gene and human oral cancer. Clin Cancer Res 2000; **6**: 2440–7.

21. **Tsai MH, Chen WC, Tsai FJ, *et al.*** Correlation of p21 gene codon 31 polymorphism and TNF-alpha gene polymorphism with nasopharyngeal carcinoma. J Clin Lab Anal 2002; **16**: 146–50.

22. **Li G, Liu Z, Sturgis EM, *et al.*** Genetic polymorphisms of p21 are associated with risk of squamous cell carcinoma of the head and neck. Carcinogenesis 2005; **26**: 1596–602.

23. **Bau DT, Tsai MH, Lo YL, *et al.*** Association of p53 and p21(CDKN1A/WAF1/CIP1) polymorphisms with oral cancer in Taiwan patients. Anticancer Res 2007; **27**: 1559–64.

24. **Gomes CC, Drummond SN, Guimarães AL, *et al.*** P21/WAF1 and cyclin D1 variants and oral squamous cell carcinoma. J Oral Pathol Med 2008; **37**: 151–6.

25. **Lei D, Sturgis EM, Liu Z, *et al.*** Genetic polymorphisms of p21 and risk of second primary malignancy in patients with index squamous cell carcinoma of the head and neck. Carcinogenesis 2010; **31**: 222–7.

26. **Liu B, Zhang Y, Jin M, *et al.*** Association of selected polymorphisms of CCND1, p21, and caspase8 with colorectal cancer risk. Mol Carcinog 2010; **49**: 75–84.

27. **Fan H, Villegas C, Huang A, *et al.*** Suppression of malignancy by the 3' untranslated regions of ribonucleotide reductase R1 and R2 messenger RNAs. Cancer Res 1996; **56**: 4366–9.

28. **Facher EA, Becich MJ, Deka A, *et al.*** Association between human cancer and two polymorphisms occurring together in the p21Waf1/Cip1 cyclin-dependent kinase inhibitor gene. Cancer 1997; **79**: 2424–9.

29. **Mousses S, Ozcelik H, Lee PD, *et al.*** Two variants of the CIP1/WAF1 gene occur together and are associated with human cancer. Hum Mol Genet 1995; **4**: 1089–92.

30. **Raab-Traub N.** Epstein — Barr virus in the pathogenesis of NPC. Semin Cancer Biol 2002; **12**: 431–41.

31. **Wei WI, Sham JS.** Nasopharyngeal carcinoma. Lancet 2005; **365**: 2041–54.

32. **Tse LA, Yu IT, Mang OW, *et al.*** Incidence rate trends of histological subtypes of nasopharyngeal carcinoma in Hong Kong. Br J Cancer 2006; **95**: 1269–73.

33. **Park GB, Song H, Kim YS, *et al.*** Cell cycle arrest induced by engagement of B7-H4 on Epstein — Barr virus-positive B-cell lymphoma cell lines. Immunology 2009; **128**: 360–8.