

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^{WAF1/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.

Received: May 14, 2013.

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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, Qiamap DNA Mini Kit.

P21^{CIP1/MAF1} 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 MgCl₂, 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% confidence 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				OR	95% CI	p
	CC	CT	TT	Recessive Model			
				T-Carrier			
N (%)	N (%)	N (%)	N (%)				
Controls n = 191	178 (93.2)	10 (5.2)	3 (1.6)	13 (6.8)	1	Refer- ence	
Cases n = 102	90 (88.2)	11 (10.8)	1 (1.0)	12 (11.8)	1.826	0.800–	0.148
Undifferenti- ated n = 100	88 (88.0)	11 (11.0)	1 (1.0)	12 (12.0%)	1.867	0.818–	0.133
						4.261	

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: $p = 0.001$).

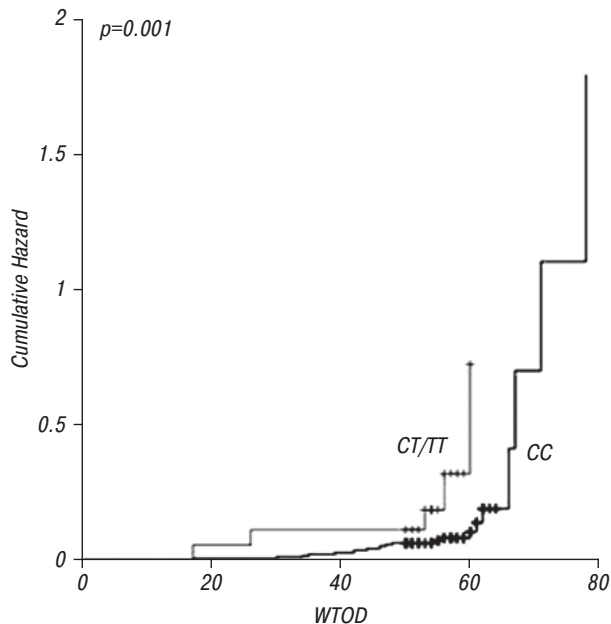


Figure. Association between CC or T carrier (CT/TT) genotypes and the waiting time to onset of disease (WTOD) for stages I and II undifferentiated histologic type of nasopharyngeal carcinoma group

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 asso-

ciated 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.

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