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Oncogenic virus-associated neoplasia: A role for cyclin D1 genotypes influencing the age of onset of disease?

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ABSTRACT

Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. The purpose of our study was to assess the role of *CCND1* genotypes influencing the age of onset of oncogenic virus-associated neoplasia. We conducted a hospital-based case-control study of 581 individuals, including 247 controls and 334 cases (108 nasopharyngeal and 226 cervical cancer cases). The polymorphism analysis was performed in blood samples by PCR–RFLP methodology. Age-adjusted logistic regression analysis indicates that individuals carrying two G-alleles have an increased genetic susceptibility for the development of oncogenic virus-associated cancers (aOR = 2.02, 95% CI 1.30–3.14, $P = 0.002$). Moreover, our results indicate that the waiting time for onset of oncogenic virus-associated neoplasia in patients homozygous (GG) for *CCND1* genotypes (52 years) was 12 years earlier in comparison with patients carrying AG or AA genotypes (60 years) (log-rank test: $P = 0.0003$). Our results may be important in contributing to a more extensive knowledge of the mechanisms involved in oncogenic virus-associated carcinogenesis, as CCND1 may be an important target for the development of new strategies for cancer treatment and prevention.

Oncogenic viruses are currently considered the second most important cause of cancer in humans and contribute to 15–20% of all cancers in the world, some of them being very common, like cervical carcinomas. Human recognized cancer viruses include Human Papillomavirus (HPV) and Epstein–Barr virus (EBV) and although each virus has unique features, it is becoming clearer that all these oncogenic agents target multiple cellular pathways to support malignant transformation and tumor development [1].

Oncogenes encoded by tumor viruses play integral roles in the viral conquest of the host cell by subverting crucial and relatively no-redundant regulatory circuits that regulate cellular proliferation, differentiation, apoptosis, and life span. Some viral oncoproteins subvert cellular safeguard mechanisms intended to eliminate cells that have acquired abnormalities that interfere with normal cell division. Viruses that encode such activities can contribute to initiation as well as progression of human cancers [2].

HPV and EBV are common infectious agents that persist after primary infection in a latent state with occasional shedding of virus. Therefore, one of the fundamental questions in the etiology of cervical cancer (associated with high risk HPV infection) and nasopharyngeal cancer (associated with EBV infection) that are linked to infection with such ubiquitous viruses is why cancer develops in a few people when many are infected. These tumors share a DNA viral etiology and present similar histopathological findings. Moreover, there are several similar aspects of infection with HPV and EBV [3].

Although it is well known that HPV and EBV are directly involved in cervical and nasopharyngeal cancer development, respectively, some researchers acknowledge that viral infection is a necessary but not sufficient factor for cancer development. The progression from infection to cancer involves other environmental and host factors. Single-nucleotide polymorphism (SNP) markers should be considered in the determination of the combinations of genetic factors involved in precancerous changes to cancer development [4–8].

A frequent target in carcinogenesis is the deregulation of G1–S phase progression of the cell cycle. The transition through G1 to S phase is regulated by cyclins, cyclin-dependent kinases and their inhibitors [9]. Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. It forms complexes with CDK4 or CDK6, and is responsible for the phosphorylation of the

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retinoblastoma tumor suppressor protein, resulting in the release of E2F transcription factors that allow cells to enter into S-phase [10–12]. The G1/S checkpoint is frequently altered in many epithelial tumors and may confer growth advantage and enhance tumorigenesis [13]. Amplification of *CCND1* and altered expression of the protein have been reported in a variety of tumors [12,14].

It has been reported that CCND1 mRNA is alternatively spliced to produce two transcripts (*a* and *b*), which are present simultaneously in a variety of normal tissues and cancer cells [15]. Bettticher and colleagues [15] identified a single base pair polymorphism (A870G) in *CCND1*, and *CCND1* genotypes have been significantly associated with carcinogenesis and clinical outcome in a variety of cancers [16].

The purpose of our study was to assess the role of *CCND1* genotypes influencing the age of onset of oncogenic virus-associated neoplasia.

Patients and methods

We conducted a hospital-based case-control study of 581 individuals, including 247 controls and 334 cases (108 nasopharyngeal and 226 cervical cases) diagnosed at the Portuguese Oncology Institute-Porto, Portugal, during the period from 2000 to 2004. The median age at diagnosis of nasopharyngeal cancer patients was 49.0 years (mean age 47.5; standard deviation 14.5). Considering the cervical cases, the median age at diagnosis was 47.0 years (mean age 47.2; standard deviation 12.3). The control group consisted of 247 healthy individuals, with a median age of 55.5 years (mean age 51.1; standard deviation 16.8), without clinical history of cancer, from the same geographic area as the case group. All samples were taken after informed consent according to the declaration of Helsinki. For clinical and statistical purposes, we defined two groups of malignant cases, with a strong association with oncogenic virus: oncogenic virus-associated nasopharyngeal cases (OVNC), and oncogenic virus cervical cases (OVCC). In OVNC group, 84 undifferentiated nasopharyngeal cancers were included (24 were differentiated nasopharyngeal cancers, and thereby not associated with EBV). The OVCC group included 202 cervical malignant cases (50 High-Grade Squamous Intraepithelial Cervical Lesions of the cervix—HSIL—and 154 invasive epidermoid cervical cancers).

Polymerase chain reaction/restriction fragment length polymorphism (PCR–RFLP) analysis. DNA was extracted from peripheral blood leukocytes from each study subject using a salting out protocol [17]. The detection of the A870G polymorphism of *CCND1* was carried out essentially as previously described [15]. The PCR consisted of nearly 0.2 μg of genomic DNA, 30 ρmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1× Taq Buffer, and 1 U of Taq DNA polymerase to a final volume of 50 μl. Primers used in the analysis were CY26 (5′GTG AAG TTC ATT TCC AAT CCG C 3′) and CY27 (5′ GGG ACA TCA CCC TCA CCC TCA CTT AC 3′). Thirty five cycles were performed, consisting of an initial heating at 95 °C for 10 min to activate the enzyme, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 2 min.

PCR products (15 μl) were digested with 1 U *ScrF1* at 37 °C for 4 h, and visualized by electrophoresis on 3% agarose gels containing 0.5 μg/ml ethidium bromide. The 167 bp PCR product generated is not cut by *ScrF1* if the A allele is present, whereas the product from the G allele is cut to produce fragments of 145 and 22 bp.

Statistical analysis. Analysis of data was performed using the computer software SPSS for Windows (Version 12.0) and Epi Info (version 6.04). Chi-square analysis was used to compare categorical variables and 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 *CCND1* genotypes and ICC and NPC risk. Logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI for the influence of the *CCND1* genotypes in the risk for cancer with adjustment for age. The Hardy–Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed versus the expected genotype frequencies.

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 cervical and nasopharyngeal cancers at birth [18]. To address this question, we hypothesized that CCND1 genotypes may alter the onset of these cancers. To analyse the data, we defined age of onset for cancer as the outcome and *CCND1* genotype as an independent variable. We tested the association between age of onset and the A870G *CCND1* polymorphism by comparing Kaplan–Meier survival curves according to *CCND1* genotype. We therefore considered the waiting time for onset of disease (WTOD) as the interval between the time of initial exposure to the risk factor (CCND1) and the time of onset of disease. We estimated the cumulative probabilities for having cervical and nasopharyngeal cancers by the Kaplan–Meier methodology [19]. 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 significance.

Results

CCND1 genotyping

The frequencies of *CCND1* AA, AG and GG genotypes were 27.9, 55.9 and 16.2%, respectively, in normal controls, and 31.1, 54.9 and 14.0%, respectively, in the female only control group. The frequencies of the polymorphism in case groups were: 27.7% for the AA genotype, 45.5% for AG genotype and 26.7% for GG genotype in oncogenic virus-associated cervical cases (OVCC) group and 25.0, 47.6 and 27.4% for AA, AG and GG genotypes, respectively, in the oncogenic virus-associated nasopharyngeal cases (OVNC) group. The genotype distribution of both groups was in the Hardy–Weinberg equilibrium ($P = 0.679$ in the case group and $P = 0.341$ in the control group).

Age-adjusted logistic regression analysis indicates that individuals carrying two G-alleles have a 2.44-fold increase in the risk for the development OVCC (aOR = 2.44, 95% CI 1.38–4.30, $P = 0.002$) and a 2.09-fold increased risk for the development of OVNC (aOR = 2.09, 95% CI 1.15–3.79, $P = 0.016$). For all cases, the presence of the GG *CCND1* genotype was also associated with an increased genetic susceptibility for the development of oncogenic virus-associated cancers (aOR = 2.02, 95% CI 1.30–3.14, $P = 0.002$) (Table 1).

WTOD and CCND1 genotype

The waiting time for onset of disease, the cumulative probabilities of having disease, according to *CCND1* genotypes for the OVCC group are shown in Fig. 1. The mean WTOD was 60 years for carri-

Table 1
Age-adjusted logistic regression analysis of the GG *CCND1* genotype regarding the genetic susceptibility to oncogenic virus-associated cancers

	P	aOR	95% CI
Oncogenic virus-associated cases			
Cervical	0.002	2.44	1.38–4.30
Nasopharyngeal	0.016	2.09	1.15–3.79
All cases	0.002	2.02	1.30–3.14

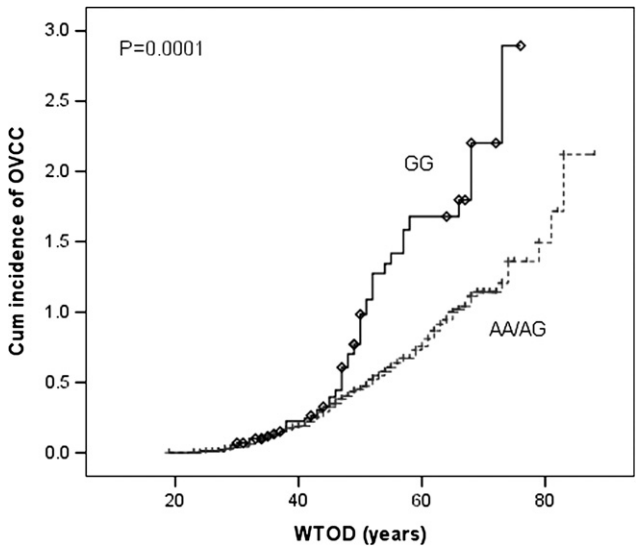

Fig. 1. Association between GG *CCND1* genotype and the waiting time to onset of disease (WTOD) for the OVCC (oncogenic virus-associated cervical cases) group. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($P = 0.0001$).

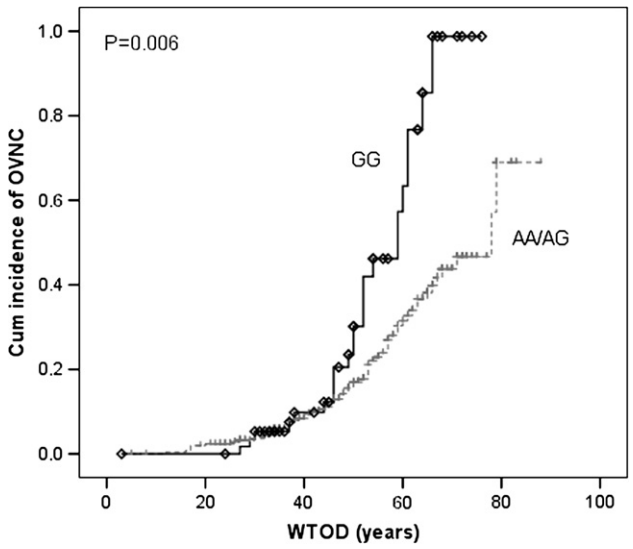

Fig. 2. Association between GG *CCND1* genotype and the waiting time to onset of disease (WTOD) for the OVNC (oncogenic virus-associated nasopharyngeal cases) group. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($P = 0.006$).

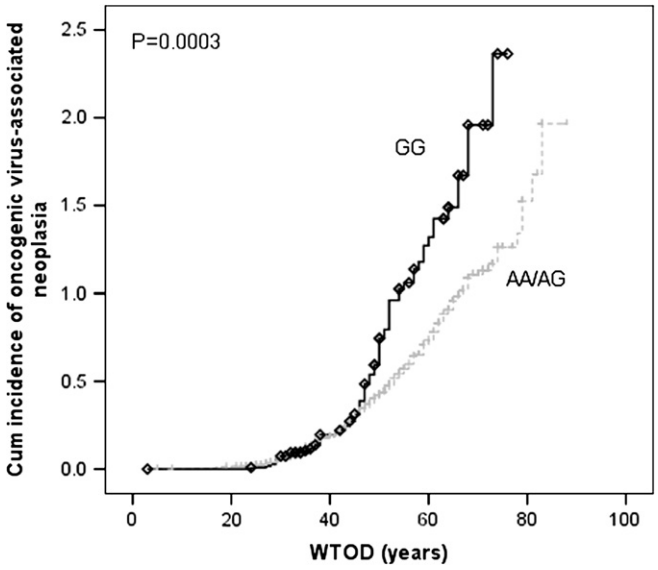

Fig. 3. Association between GG *CCND1* genotype and the waiting time to onset of oncogenic virus-associated cancers. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($P = 0.0003$). WTOD, waiting time to onset of disease.

ers of the AA or AG genotypes and 50 years for carriers of the GG *CCND1* genotype (log-rank test: $P = 0.0001$). Regarding the OVNC group, the mean WTOD for patients carrying AA or AG genotypes was 73 years and 61 years for patients carrying the GG genotype (log-rank test: $P = 0.006$) (Fig. 2). Considering all oncogenic virus cases, Kaplan–Meier analysis (Fig. 3) showed that the WTOD in patients homozygous (GG) for *CCND1* genotypes (52 years) was 12 years earlier in comparison with patients carrying AG or GG genotypes (60 years) (log-rank test: $P = 0.0003$).

Discussion

Oncogenic viruses trigger persistent infections, which can stimulate uncontrolled cell growth by inducing cell transformation. Most oncogenic DNA viruses integrate transforming sets of genes into the host chromosome and encode proteins that bind and inactivate cell growth regulatory proteins. Tumor viruses play an important role for the development of a substantial fraction of human malignancies, including common cancers, such as carcinomas of the cervix uteri, nasopharyngeal cancers, hepatocellular carcinomas, or lymphomas [20]. Malignant transformation typically requires additional genetic alterations of the host cell, to which tumor viruses can contribute by destabilizating the cellular genome [21].

Several genetic polymorphisms contributing to individual's susceptibility to cancer have been studied regarding their association with cancer risk [22–24]. In this study, a single-nucleotide polymorphism in *CCND1* was analysed in order to evaluate its importance in the development and disease onset of cervical and nasopharyngeal cancers. The A870G polymorphism at codon 242 within the conserved splice donor site of exon 4 of the gene appears to modulate the splicing of CCND1 mRNA, originating two transcripts (*a* and *b*), which are present in a variety of tissues [15,25]. The transcript *a* is identical to the reported CCND1 cDNA. However, transcript *b* fails to splice at the exon 4/intron 4 boundary, does not contain exon 5, and terminates downstream of exon 4. The main difference in the cyclin D1 proteins encoded by the two transcripts (*a* and *b*) is in the C-terminal PEST-rich region (destruction box) encoded by exon 5 which is responsible for rapid intracellular degradation and turnover of the G1 cyclins [25,26].

Our results indicate that individuals carrying the *CCND1* GG genotype have increased genetic susceptibility for the development oncogenic virus-associated neoplasia (aOR = 2.02). In this study, we also demonstrate that the GG genotype is associated with an earlier age of onset of oncogenic virus-associated cancers, compared to *CCND1* AG and AA genotypes. These results are consistent with previous findings suggesting that *CCND1* GG genotype is associated with cancer development [16,27–36].

It has been suggested that the variant A allele is a major source of variant transcript *b* in several types of cancer cells. The AA genotype increases the products of transcript *b* in tumor tissue cells, resulting in an increase of an altered protein that lacks the PEST-region with increased half-life [15,25]. The presence of the A allele has been reported to be positively associated with increased risk for several cancers [12,37,38]. However, a recent study reported, surprisingly, that cyclin D1b protein does not inappropriately accumulate in cells and exhibits stability comparable to cyclin D1a. This study also suggests that cyclin D1a is a better catalyst of RB (retinoblastoma protein) phosphorylation/inactivation [39]. These data support the emerging view that CCND1 alternate transcripts encode proteins with differing independent biological functions.

The direction of the biological impact of cyclin D1 expression depends on the state of the cell in accordance with its checkpoint function. While cyclin D1 is best known for its proliferating effect [40,41], experimental evidence suggests that under conditions such as oxidative stress [42–45] or senescence [46,47], cyclin D1 can exhibit S-phase entry and DNA replication and promote growth arrest, as well as apoptosis. The context-dependent dual role of cyclin D1 in cell proliferation and growth arrest may explain the inconsistent associations observed between *CCND1* genotype and cancer risk.

Oxidative stress, primarily due to increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), is a feature of many viral infections, including HPV and EBV infections [48–51]. ROS and RNS modulate the permissiveness of cells to viral replication, regulate host inflammatory and immune responses, and cause oxidative damage to both host tissue and progeny virus [48]. *In vitro* experiments of breast tumor and other tumor cells exposed to oxidative stressors demonstrate that cyclin D1 activation and overexpression is also able to activate molecular pathways

resulting in cell-cycle arrest and apoptosis [45,52]. Turner and coworkers [44] provided *in vivo* evidence of cyclin D1 as a caretaker gene offering downstream protection against oxidative damage. Our findings extend this evidence to situations of more moderate oxidative burden and suggest that modulation of the biological function of cyclin D1 by tumor viruses may lead to differential impact of the *CCND1* polymorphism on oncogenic virus-associated malignant lesions.

It is possible that these conflicting results in part reflect the many different mechanisms through which deregulated expression of CCND1 can occur in cancer, and the direction and magnitude of the CCND1 effect in cancer development. Functional studies in the future may help to elucidate the conflicting experimental findings and influence of *CCND1* genotypes on tumor behaviour in different cell types.

In conclusion, our results may be important in contributing to a more extensive knowledge of the mechanisms involved in oncogenic virus-associated carcinogenesis, as CCND1 may be an important target for the development of chemoprevention or therapeutic strategies.

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