

# Allelic variant at –79 (C > T) in *CDKN1B* (p27<sup>Kip1</sup>) confers an increased risk of thyroid cancer and alters mRNA levels

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## Abstract

The aim of this study is to assess if common genetic variants located in the *CDKN1B* locus, coding for the cell cycle inhibitor p27<sup>Kip1</sup>, are involved in thyroid cancer susceptibility. Based on the literature and functional predictions, we selected three polymorphisms within the *CDKN1B* gene (rs2066827 (T326G, V109G), rs34330 (–79C > T) and rs36228499 (–838C > A)) to perform the first case–control study in thyroid cancer involving this locus. We had 649 Spanish patients with sporadic thyroid cancer and 385 healthy representative controls available. Luciferase reporter gene assays, real-time quantitative reverse transcription-PCR and immunoblot experiments were carried out to demonstrate the putative effect of the associated variant. The polymorphism rs34330 (–79C > T) was identified as a risk factor for developing the follicular variant of papillary thyroid carcinoma (FVPTC), fitting a recessive model (odds ratio = 2.12; 95% confidence interval = 1.09–4.15; *P* value = 0.023). The risk allele (T) of this single nucleotide polymorphism led to a lower transcription rate in cells transfected with a luciferase reporter driven by the polymorphic p27<sup>Kip1</sup> promoter (*P* value < 0.001). This effect was observed in –79TT genotype control carriers, who showed a tendency towards lower *CDKN1B* mRNA levels in lymphocytes, as well as at the protein level. This is the first study that identifies *CDKN1B* as a low-penetrance gene in thyroid cancer, and specifically in FVPTC subtype. We propose a reduced *CDKN1B* gene transcription depending on the genotype of the –79C > T (rs34330) variant as a novel mechanism underlying p27<sup>Kip1</sup> downregulation.

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## Introduction

Cancers that arise in the thyroid gland account for 1% of all neoplasias. More than 95% of thyroid carcinomas are derived from follicular cells or thyrocytes, the thyroid hormone-producing cell lineage in the gland (DeLellis 2004). Among the neoplasias arising from thyroid follicular cells, ‘papillary thyroid carcinoma’

(PTC) and ‘follicular thyroid carcinoma’ (FTC) represent the two most common subtypes (85 and 10% respectively; Kondo *et al.* 2006). A minority of the thyroid tumours (3%), referred to as ‘medullary thyroid carcinoma’ (MTC), arise from a different cell lineage, the parafollicular or C-cells, which are involved in calcitonin secretion (Randolph & Maniar 2000).

Different genetic alterations, all of them involving the RET/MAP kinase pathway, have been described as subtype-specific somatic causal changes in PTC and FTC (Santoro *et al.* 1990, Greco *et al.* 1992, Davies *et al.* 2002, Nikiforov 2002, Kondo *et al.* 2006). Although some genes related to thyroid cancer development as part of familial syndromes are known (Mulligan *et al.* 1993, Hofstra *et al.* 1994), there are few data on genetic risk factors related to the susceptibility for developing sporadic thyroid carcinomas (Cebrian *et al.* 2005, Ruiz-Llorente *et al.* 2007). The identification of low-penetrance genes (LPGs) related to follicular cell-derived carcinoma is an arduous task due to the heterogeneity and relative rarity of the disease. A well-known approach, which compensates this problem and increases the probability of finding genetic risk factors related to the disease, is the selection of single nucleotide polymorphisms (SNPs) within candidate genes or *loci* for the development of thyroid cancer.

The *CDKN1B* gene encodes p27<sup>Kip1</sup>, an inhibitor of the cyclin/cyclin-dependent kinase (Cdk) complexes (Sa *et al.* 2005), which are essential for cell cycle progression. Loss of expression of p27<sup>Kip1</sup> protein has been described as a frequent event in several human cancers (Slingerland & Pagano 2000, Chu *et al.* 2008), apparently conferring a proliferative advantage that can lead to tumour formation. This behaviour first suggested a tumour suppressor role of p27<sup>Kip1</sup>, but it does not fit all classic features, since it is rarely mutated in cancer (Kawamata *et al.* 1995, Kossatz & Malek 2007, Lindberg *et al.* 2007). Alternative mechanisms acting at the post-transcriptional level have been suggested, including a lower translation rate of *CDKN1B* mRNA (Hengst & Reed 1996); an increase in protein degradation, specifically by the Scf-Skp2 E3 ubiquitin-protein ligase (Pagano *et al.* 1995, Chiappetta *et al.* 2007); and the cytosolic mislocalization of p27<sup>Kip1</sup> protein (Viglietto *et al.* 2002, Min *et al.* 2004).

*CDKN1B* plays a key role in the aetiology of thyroid cancer. Several lines of evidence from clinical and *in vitro* studies demonstrate that somatic loss, under-expression (Khoo *et al.* 2002) or mislocalization (Baldassarre *et al.* 1999) of p27<sup>Kip1</sup> may contribute to the process of thyroid carcinogenesis. In addition, it has been demonstrated that the phosphatidylinositol-3-kinase (PI3K)/AKT pathway controls thyroid cell proliferation by regulating the expression and subcellular localization of p27 (Baldassarre *et al.* 1999, Khoo *et al.* 2002, Motti *et al.* 2005), and therefore, *CDKN1B* (p27<sup>Kip1</sup>) represents a good candidate gene for a classical case-control study. In view of these antecedents, the aim of the present study was to assess whether common, potentially

functional, genetic variants within the *CDKN1B* gene could influence the risk for thyroid cancer. Thus, based on a scan of the literature and the use of bioinformatics tools, three potentially functional SNPs in the *CDKN1B* gene were selected: -838C>A (rs36228499), -79C>T (rs34330) and 326T>G (V109G, rs2066827).

Here, we propose that the -79C>T (rs34330) polymorphism acts as a genetic risk factor for thyroid cancer by affecting the transcription rate, as indicated by the functional assays performed in this study.

## Subjects and methods

### Patients

A total of 649 Spanish patients with sporadic thyroid cancer were recruited for a multicentre hospital-based study. The main thyroid cancer subtypes were represented in this series: 274 MTC, 328 PTC, composed of 193 'classic PTC' (cPTC) and 129 'follicular variant PTC' (FVPTC), and 47 FTC. Diagnoses were assessed by pathologists from the different institutions that participated in the study.

### Controls

We selected a series of 385 healthy, representative Spanish controls, all of them cancer free and without any thyroid pathology, according to their responses to a general clinical questionnaire.

Mean age and gender distribution were similar in controls and cases (Mann-Whitney's *U* and Kruskal-Wallis associated *P* values >0.05).

Informed consent was obtained from all subjects included in the study.

### DNA and RNA isolation

Genomic DNA was extracted from the blood of 591 patients following a standard method (Sambrook *et al.* 1989). The remaining 58 DNA samples were obtained from patients' saliva using the Oragene DNA Self-Collection Kit (DNA Genotek, Ottawa, Ontario, Canada). Control DNAs were isolated from peripheral blood lymphocytes using automatic DNA extraction according to the manufacturer's instructions (Magnapure, Roche).

Total RNA was isolated from lymphocytes of 97 controls following the standard TRIzol Reagent method (Invitrogen).

### SNP selection

Fifteen *CDKN1B* polymorphisms are described in the International HapMap Project database (<http://www.hapmap.org/>, release 21a/phaseII), considering the

region that spans from 5 kb upstream to 1 kb downstream of the gene. We applied two filters in the SNP selection: the allele frequency (at least a minor allele frequency (MAF) of 0.05 in the HapMap-CEU population) and a predicted functionality. Seven of the fifteen variants passed the first filter, and only three of these ( $-838C>A$  (rs36228499),  $-79C>T$  (rs34330) and  $326T>G$  (V109G, rs2066827)) showed consistent functional predictions (at least two different algorithms predicted the same result) according to the PupaSuite (Conde *et al.* 2006) and F-SNP (Lee & Shatkey 2008) bioinformatics tools. Nucleotide positions are numbered relative to the start of translation according to the Genbank accession number AB003688. Predicted functionality was reinforced by previously published data as described below. The  $-838C>A$  lies within the promoter region of p27<sup>Kip1</sup>, and it has been associated with an increased risk of myocardial infarction (Gonzalez *et al.* 2004). The  $-79C>T$  is a polymorphism within the 5' untranslated region (5' UTR) of p27<sup>Kip1</sup>, which has been shown to contain promoter activity (Minami *et al.* 1997, Ito *et al.* 1999, Liu *et al.* 2005), and has been previously associated with cancer (Chang *et al.* 2004, Wang *et al.* 2007a, Driver *et al.* 2008). Finally,  $326T>G$  (V109G) is a non-synonymous variant in exon 1, which has been also associated with susceptibility to cancer (Kibel *et al.* 2003, Li *et al.* 2004, Gayther *et al.* 2007).

According to the HapMap, there is some linkage disequilibrium along the *CDKN1B* region between rs36228499 and rs34330, but they do not show a high correlation. Thus, the three mentioned SNPs were assessed as independent putative genetic risk factors.

### Genotyping platforms

Two different genotyping strategies were used to evaluate the three polymorphisms: a TaqMan genotyping assay (Applied Biosystems, CA, USA) for SNPs rs34330 and rs2066827, and a restriction fragment length polymorphism (RFLP) approach for rs36228499.

#### Taqman platform

Probe accuracy to discriminate the two alleles at a specific locus was initially checked in a set of 24 DNA samples. Genotyping specificity was assessed by including two DNA duplicates and a negative control in each 96-well plate genotyped, yielding 100% consistent replication results. In addition, we genotyped cases and controls in the same run. The reaction was performed at the default conditions recommended by the manufacturer: an initial denaturing step of

10 min at 95 °C, followed by 40 cycles of 15 s of denaturation at 92 °C, and 1 min of annealing and extension at 60 °C. Samples without a genotype call under these conditions were excluded for further analyses.

#### RFLP technique

rs36228499 was genotyped as described by Gonzalez *et al.* (2004). To improve the amplification intensity and, thus, the assay resolution, we designed a nested PCR, using the following primer sequences and PCR conditions: first PCR:  $-838\_F$ : 5'-GACCTTCGCG-GTCCTCTG-3';  $-838\_R$ : 5'-GTGACTGCTGGAG-GGGTACT-3'; temperature of annealing,  $T_a=58$  °C, 40 cycles; second PCR:  $-838N\_F$ : 5'-TCCAGGTC-CCGGCTTCCCGGt-3';  $-838N\_R$ : 5'-CCTGCTCT-GGCTGGCCTCGGAG-3';  $T_a=65$  °C, 35 cycles. The  $-838N\_F$  primer included a mismatch (shown in lower case) to create a TaqI restriction endonuclease site when  $-838C$  was present. Amplicons were subsequently digested by TaqI (Fermentas, Burlington, Ontario, Canada), and the products were separated in a 3.5% agarose D1 medium (Pronadisa, Torrejón de Ardoz Spain) gel electrophoresis run (data not shown). Enzymatic digestion yielded fragments of 173 bp (undigested, corresponding to the polymorphic AA genotype), 152+21 bp (digested, wild-type CC genotype) or both (CA heterozygotes).

#### DNA sequencing

Accuracy of both TaqMan and RFLP genotyping was confirmed by direct sequencing of randomly selected samples that represented 5% of the total samples. Regions containing the studied SNPs were amplified by PCR prior to sequencing using the following additional primers and conditions:  $-79\_F$ : 5'-CCA-TTTGATCAGCGGAGACT-3';  $-79\_R$ : 5'-CACTC-GCACGTTTGACATCT-3';  $T_a=58$  °C, for  $-79C>T$ ; and  $109\_F$ : 5'-AAGAGTTAACCCGGGAC-TT-3';  $109\_R$ : 5'-CTCTCCCAAAGCTAAATCAG-3',  $T_a=56$  °C, to interrogate the V109G variant. DNA sequencing was carried out using an ABI 3730xl sequencer (Applied Biosystems).

### Construction of p27<sup>Kip1</sup> reporter vector

The  $-79C>T$  SNP lies within the promoter/5' UTR of the *CDKN1B* gene. Therefore, we cloned the minimal regulatory region of the *CDKN1B* gene that has been shown to be able to efficiently drive transcription of a downstream reporter gene (Minami *et al.* 1997, Ito *et al.* 1999, Liu *et al.* 2005). DNA fragments from the 5' region of the *CDKN1B* gene

between nucleotides  $-575$  and  $-1$  (AB003688), containing either C or T at position  $-79$ , were generated through PCR using genomic DNA of homozygous CC and TT individuals with forward (GGTACCGGTACCCACCTTAAGGCCGCGCTCG) and reverse (AAGCTTAAGCTTCTTTCTCCCGGGTCTGCACG) primers containing KpnI and HindIII sites respectively. PCR products were cloned into the pGL3-Basic promoterless plasmid (Promega) to generate pGL3-p27<sup>Kip1</sup><sub>C</sub> ( $-79C$ ) and pGL3-p27<sup>Kip1</sup><sub>T</sub> ( $-79T$ ) in order to drive the expression of the downstream luciferase gene. Plasmids generated for each genotype were confirmed by direct sequencing.

### Cell culture and transfections

HeLa cells (American Type Culture Collection, Manassas, VA, USA) were incubated at  $37^{\circ}C$  in a humidified 5% CO<sub>2</sub>–95% O<sub>2</sub> atmosphere in DMEM – high glucose medium (Sigma–Aldrich) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% foetal bovine serum. The rat thyroid cell line PCC13 was cultured in F-12 Coon's medium (Euroclone, Pero-Milano, Italy) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5% newborn calf serum, 20 ng/ml glycyl-histidyl-lysine (GHL; Sigma), 3.6 ng/ml hydrocortisone (Sigma), 10  $\mu$ g/ml insulin from bovine pancreas (Sigma), 5  $\mu$ g/ml human apo-transferrin (Sigma), 10 ng/ml somatostatin (Sigma) and 0.5 mU/ml TSH (Sigma). Transfections were performed with Trans IT-LT1 Transfection Reagent (Mirus, Madison, WI, USA) following the manufacturer's protocol.

### Luciferase reporter gene assays

For luciferase activity assays, cells were transfected with 1  $\mu$ g of the promoter/luciferase reporter gene (pGL3-p27<sup>Kip1</sup><sub>C</sub> or pGL3-p27<sup>Kip1</sup><sub>T</sub>), with 100 ng of pRL Renilla as the control vector (Promega) to correct for differences in transfection efficiency. Cells were collected 24 and 48 h after transfection by centrifugation, washed twice with PBS and lysed with 100  $\mu$ l of cell lysis reagent (Dual-Luciferase Reporter Assay System, Promega). Cell lysates were dispensed into luminometer plates, followed by sequential auto-injection of the Luciferase Assay Reagent for firefly luciferase activity measurement, and Stop & Glo Reagent for Renilla luciferase activity measurement (Dual-Luciferase Reporter Assay System, Promega). Basal luciferase activity was checked in cells transfected with an empty vector (pGL3 Basic Vector). Three independent transfections were performed for each construct (pGL3 Basic Vector, pGL3-p27<sup>Kip1</sup><sub>C</sub> or pGL3-p27<sup>Kip1</sup><sub>T</sub>) in triplicate.

### cDNA synthesis and real-time quantitative reverse transcription-PCR

We obtained cDNA from RNA that was isolated from lymphocytes of 97 healthy volunteers. We used it to evaluate the genotype–phenotype correlation by means of real-time quantitative PCR (qPCR) to compare the CDKN1B mRNA levels as a function of rs34330 polymorphism genotype. One microgram of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and an oligo dT18 primer following the manufacturer's instructions. The amount of CDKN1B mRNA was quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems) using the specific primers (p27QF: 5'-CCCTAGAGGGCAAGTACGAGT-3' and p27QR: 5'-AGTAGAACTCGGGCAAGCTG-3'), a labelled probe (Universal ProbeLibrary #39, Roche), and the Universal Master Mix (PE Applied Biosystems). The amplification conditions consisted of an initial step at  $95^{\circ}C$  for 10 min, followed by 40 cycles of 15 s at  $95^{\circ}C$  and 1 min at  $60^{\circ}C$ . Standard curves were generated with serial 1:4 dilutions of a sample showing high CDKN1B expression of mRNA. Normalization was carried out with the internal standard  $\beta$ -glucuronidase (GUS) using as a forward primer 5'-GAAAATATGTGGTTGGA-GAGCTCATT-3'; as a reverse primer 5'-CCGAGT-GAAGATCCCCTTTTAA-3'; and as a probe 5'-FAM-CCAGCACTCTCGTCGGTGACTGTTCA-3BQ1-3'. All reactions were in the initial part of the exponential amplification phase, and the cycle at which each sample crossed the threshold ( $C_t$ ) was recorded for CDKN1B and GUS. The  $\Delta\Delta C_t$  method was used for the calculation of the different amounts of mRNA (Livak & Schmittgen 2001). Negative controls were included in all PCRs, and assays were carried out in triplicate.

### Protein isolation and immunoblot

Nine healthy volunteers carrying rs34330 genotypes equally (CC, CT and TT, three each) were randomly selected to check whether this promoter variant could have an effect at the p27<sup>Kip1</sup> protein level. Whole protein extraction from blood lymphocytes was performed using RIPA (Invitrogen) buffer according to the manufacturer's recommendations.

Proteins were separated by 12% SDS-PAGE using the XCell SureLock Mini-Cell electrophoresis system (Invitrogen), and were transferred to polyvinylidene fluoride membranes (Immobilon-P Membrane, Millipore, Billerica, MA, USA). Equal loading of proteins was verified by Ponceau S staining.

The membranes were blocked, and were then incubated with a 1:500 dilution of the sc-776 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) following the manufacturer's instructions. This is a commercial polyclonal antibody raised against amino acids 1–197 representing full-length p27<sup>Kip1</sup>. After washing, the membranes were incubated with a 1:10 000 dilution of Alexa Fluor 680 anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) secondary antibody, and the corresponding fluorescence signal was visualized using a LI-COR Odyssey Blot scanner. Equal protein loading was assessed by using a 1:1000 dilution of anti- $\alpha$ -tubulin (DM1A; Abcam, Cambridge, UK).

Protein quantification was done using Odyssey 2.1 software. Both p27<sup>Kip1</sup> and  $\alpha$ -tubulin ( $\alpha$ TUB) band intensities were corrected by subtracting the intensities of their respective backgrounds. Final p27<sup>Kip1</sup> expression was represented as relative units, resulting from p27<sup>Kip1</sup>/ $\alpha$ TUB protein intensity ratios.

### Statistical analysis

Departure from Hardy–Weinberg equilibrium (HWE) for all SNPs was tested in the controls using Fisher's exact test (command available online at <http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>). Associations between each SNP and thyroid cancer risk were assessed using Pearson's  $\chi^2$  test. Genotype frequencies in cases and controls were compared, and odds ratios (ORs) were estimated directly from contingency tables using homozygotes of the more frequent allele in controls as the reference group. For each SNP, the best fitting model was determined by parsimony based on the corresponding associated *P* values.

When the unadjusted *P* value was found to be significant, further analyses were performed to adjust for putative confounding factors such as age and gender. Age was recodified as a new variable using the mean as the cut-off value. Mantel–Haenszel test was

performed stratifying by gender and age. OR homogeneity was also tested by executing Tarone's test. Finally, stepwise conditional logistic regression method was used to discard age and gender as confounders.

For analyses of luciferase assays, comparisons between means were performed using Student's *t*-test. We used Mann–Whitney's *U* test to compare medians from qPCR assays by genotype.

Statistical tests were performed using SPSS for Windows 11.0 and GraphStat programs.

## Results

### Polymorphism rs34330 (–79C>T) is significantly associated with the FVPTC

The –838C>A (rs36228499), –79C>T (rs34330) and 326T>G (V109G, rs2066827) SNPs among the available Spanish cases and controls were genotyped to determine whether a significant association existed between them and thyroid cancer. Controls fulfilled HWE for all three SNPs (Table 1).

While V109G and –838C>A variants did not show significant associations with any of the thyroid cancer subtypes analyzed, rs34330 (–79C>T) was identified as a risk factor for developing the FVPTC. According to these results, the polymorphic TT genotype of rs34330 was significantly overrepresented in FVPTC patients. The variant fit a recessive model (OR = 2.12; 95% confidence interval (CI) = 1.09–4.15; *P* value = 0.023; Tables 2 and 3).

The previous association remained statistically significant after correcting for gender and age. We first proved that OR remained homogeneous (*P* value > 0.05) by Tarone's test. Then, we demonstrated that neither gender nor age was acting as a confounding factor in our results by both Mantel–Haenszel test and stepwise conditional logistic regression, which yielded an adjusted OR = 2.01 and *P* value = 0.041.

**Table 1** *CDKN1B* polymorphisms and samples genotyped

Polymorphisms selected			Number of samples ( <i>n</i> ) genotyped in each group <sup>a</sup>					
SNP (nucleotide change)	Location	Major/minor allele (MAF)	Controls (HWE <sup>b</sup> )	MTC	PTC	cPTC	FVPTC	FTC
rs2066827 (T326G)	Exon 1 V109G	T/G (0.20)	334 (0.27)	244	313	181	100	25
rs34330 (–79C>T)	5' UTR/promoter	C/T (0.21)	385 (1.00)	274	328	178	129	47
rs36228499 (–838C>A)	Promoter	C/A (0.46) <sup>c</sup>	296 (0.64)	178	326	193	108	23

MAF, minor allele frequency; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma; cPTC, classic PTC; FVPTC, follicular variant of PTC; FTC, follicular thyroid carcinoma.

<sup>a</sup>Samples without a genotype call were excluded.

<sup>b</sup>Hardy–Weinberg equilibrium *P* value in controls.

<sup>c</sup>In our control series (no data available in public databases).

**Table 2** Thyroid cancer risk and rs34330 (–79C>T) genotype

Group	n	Codominant model				Recessive model		P values <sup>b</sup>
		CC versus CT		CC versus TT		(CC + CT) versus TT		
		OR <sup>a</sup>	95% CI	OR <sup>a</sup>	95% CI	OR <sup>a</sup>	95% CI	
Controls	385							
MTC	274	1.00	0.72–1.38	0.93	0.48–1.82	0.93	0.49–1.79	0.834 NS
PTC	328	0.96	0.70–1.32	1.44	0.81–2.56	1.46	0.83–2.56	0.186 NS
cPTC	178	0.91	0.63–1.33	1.05	0.51–2.18	1.09	0.53–2.23	0.819 NS
FVPTC	129	1.08	0.70–1.65	2.20	1.10–4.38	2.12	1.09–4.15	0.023 <sup>a</sup>
FTC	47	1.39	0.75–2.59	0.78	0.17–3.49	0.67	0.15–2.92	0.590 NS

OR, odds ratio; CI, confidence interval; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma; cPTC, classic PTC; FVPTC, follicular variant of PTC; FTC, follicular thyroid carcinoma.

<sup>a</sup>OR obtained by comparing cases against controls and considering T as the risk allele.

<sup>b</sup>The P values are derived from the recessive model.

### Polymorphism –79C>T decreases CDKN1B transcription rate in luciferase assays

Since the CDKN1B –79TT genotype was associated with a statistically significant increased risk in FVPTC patients, we sought to determine whether the –79C>T genetic variation had functional consequences for p27<sup>Kip1</sup> expression.

In a first set of experiments, we determined the transcriptional capacity of the two CDKN1B variant promoters by transfecting the pGL3 Basic Vector, pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub> plasmids into HeLa cells. After 48 h in the presence or absence of serum, cells were collected and the transcriptional activity of the two promoter variants was determined by luciferase assay (Fig. 1A). We observed that the transcriptional activity shown by the variant promoter (p27<sup>Kip1</sup><sub>T</sub>) was significantly lower than that shown by the wild-type promoter (p27<sup>Kip1</sup><sub>C</sub>), both in the presence (P value=0.001) and absence (P value=0.002) of serum.

Then, we determined the activity of the different variants of the p27 promoter. To this end, HeLa

cells were transfected with pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub> constructs and starved. Starved cells were induced to re-enter the cell cycle by serum administration, and were analyzed for luciferase activity after 0, 12 and 24 h. After normalizing luciferase activity for the internal Renilla control, the average transcriptional activity of pGL3-p27<sup>Kip1</sup><sub>T</sub> was consistently lower (by about 50%) than that of pGL3-p27<sup>Kip1</sup><sub>C</sub> (Fig. 1B). The activity of the wild-type CDKN1B promoter (pGL3-p27<sup>Kip1</sup><sub>C</sub>) increased in mid-G1 (12 h after serum administration) and decreased at the G1/S transition (24 h after serum administration), whereas the mutant p27 promoter (pGL3-p27<sup>Kip1</sup><sub>T</sub>) showed similar kinetics but significantly reduced transcriptional activity (P value<sub>0 h</sub> = 0.002, P value<sub>12 h</sub> < 0.001 and P value<sub>24 h</sub> < 0.001 respectively).

Subsequently, we analyzed the activity of the p27 promoter in thyrocytes. To this end, we used PCC13 cells, an established, differentiated rat cell line, as a model of thyroid cells. PCC13 cells were transfected with pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub>, placed in a medium with or without TSH for 24 and 48 h, and

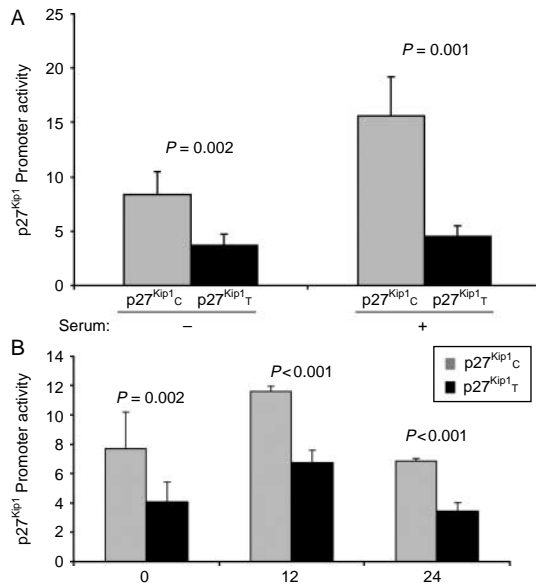
**Table 3** Risks estimated for the CDKN1B polymorphisms genotyped in the follicular variant of papillary thyroid carcinoma (FVPTC)

SNP (location)	Minor (MAF)	FVPTC (n)	Codominant model				Recessive model		P values <sup>b</sup>
			WW versus WM		WW versus MM		(WW + WM) versus MM		
			OR <sup>a</sup>	95% CI	OR <sup>a</sup>	95% CI	OR <sup>a</sup>	95% CI	
rs36228499 (–838C>A)	A (0.46)	108	1.00	0.55–1.79	1.16	0.61–2.19	1.16	0.72–1.87	0.544 NS
rs34330 (–79C>T)	T (0.21)	129	1.08	0.70–1.65	2.20	1.10–4.38	2.12	1.09–4.15	0.023 <sup>a</sup>
rs2066827 (T326G)	G (0.20)	100	0.80	0.50–1.28	1.24	0.52–2.98	1.37	0.58–3.20	0.474 NS

W, wild-type allele; M, minor allele; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

<sup>a</sup>OR obtained by comparing cases against controls and considering the minor allele (M) as the risk allele.

<sup>b</sup>The P values are derived from the recessive model.



**Figure 1** Transcriptional activity of constructs driven by the *CDKN1B* gene promoter containing the  $-79C$  or  $T$  allelic variant in HeLa cells. (A) The transcriptional activity of the pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub> constructs in HeLa cells was evaluated in the presence or absence of serum. Luciferase activity was normalized to the internal Renilla control, after subtraction of pGI3D Basic Vector activity. Data represent the means  $\pm$  s.d. of three independent transfections. (B) Cell cycle-dependent transcriptional activity of the pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub> constructs in HeLa cells. HeLa cells were transfected with pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub> constructs, and were subsequently starved in serum-free medium for 24 h. Cells were induced to re-enter the cell cycle by the addition of fresh medium supplemented with 10% FCS for 0, 12 and 24 h. At these time points, the cells were harvested, lysed and analyzed for luciferase activity.

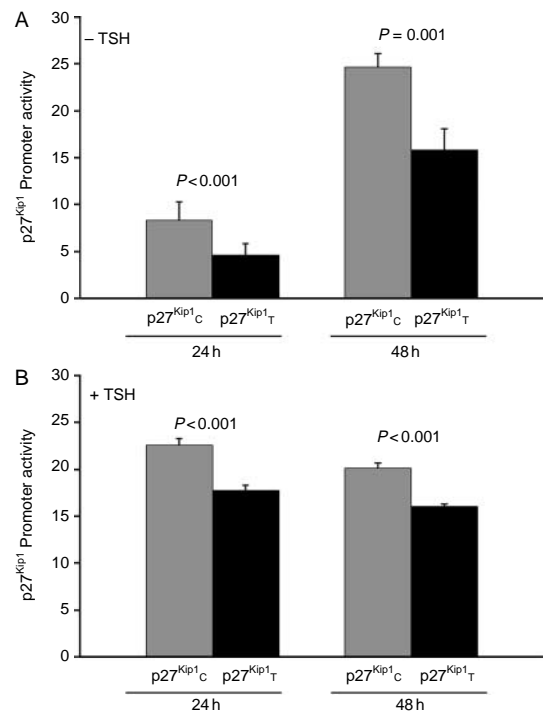
analyzed for luciferase activity. After normalizing luciferase activity for the internal Renilla control, the average transcriptional activity of pGL3-p27<sup>Kip1</sup><sub>T</sub> in thyrocytes was  $\sim 50\%$  compared with that of pGL3-p27<sup>Kip1</sup><sub>C</sub> ( $P$  values  $< 0.001$ ; Fig. 2). Interestingly, the difference in the transcriptional activity of the two promoter variants was more evident when the cells were deprived of TSH.

### The $-79TT$ risk genotype exhibits diminished *CDKN1B* mRNA levels and a tendency towards lower protein levels in lymphocytes from control individuals

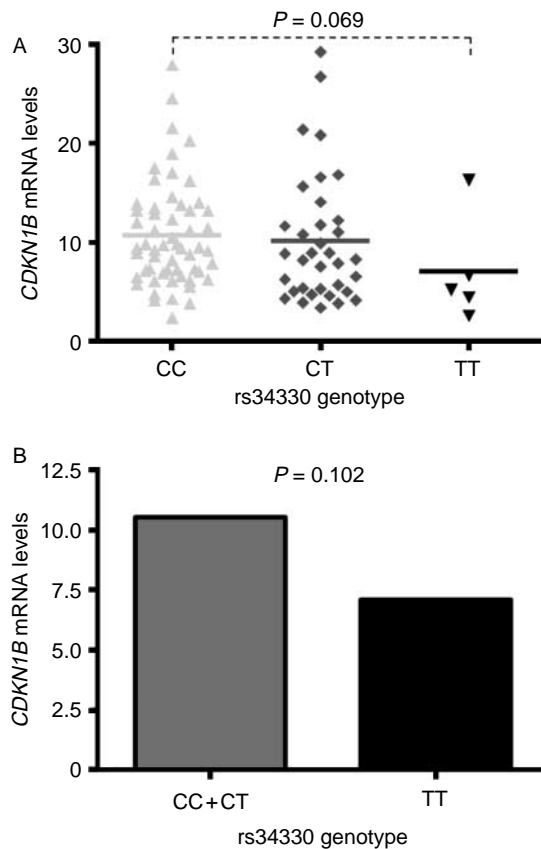
To confirm the *in vitro* data from the transfection experiments, we evaluated *CDKN1B* mRNA expression by real-time quantitative reverse transcription-PCR in lymphocytes from a series of 97 healthy controls with known  $-79C > T$  variant genotype.

Genotypic frequencies in this subset were  $CC = 0.59$ ,  $CT = 0.36$  and  $TT = 0.05$ , which were similar to the frequencies exhibited by the HapMap-CEU population. The mean  $C_t$  values obtained for *CDKN1B* and *GUS* were 29.4 and 27.7 respectively. We observed a marginal statistically significant difference between the average *CDKN1B* mRNA expression of the  $CC$  and  $TT$  genotypes ( $P$  value = 0.069, Fig. 3A). This tendency was also observed when we clustered the non-risk  $CC$  and  $CT$  genotypes, and compared them against the  $TT$  risk group ( $P$  value = 0.102, Fig. 3B). These findings further suggest that the  $-79TT$  genotype is associated with decreased *CDKN1B* mRNA expression.

In addition, immunoblot experiments were performed to further check this downregulation, which suggest a modest effect of  $-79C > T$ -dependent p27<sup>Kip1</sup> regulation at the protein level in lymphocytes (Fig. 4).



**Figure 2** Transcriptional activity of constructs driven by the *CDKN1B* gene promoter containing the  $-79C$  or  $T$  allelic variant in rat thyroid cells. Rat thyroid PCC13 cells were transfected with pGL3-p27<sup>Kip1</sup><sub>C</sub> and pGL3-p27<sup>Kip1</sup><sub>T</sub> constructs, and were subsequently placed in TSH-devoid (panel A) or complete medium (panel B). Cells were collected after 24 and 48 h, and luciferase activity was determined as described. Luciferase activity was normalized to the internal Renilla control after subtraction of pGI3D Basic Vector activity. Data represent the means  $\pm$  s.d. of three independent transfections.



**Figure 3** Differential expression of *CDKN1B* in human lymphocytes, depending on  $-79C>T$  (rs34330) genotype. (A) *CDKN1B* mRNA levels in lymphocytes from 97 control individuals, depending on the genotype of the promoter  $-79C>T$  polymorphism. Data points represent the average normalized mRNA amounts from three measurements for each sample, assessed by real-time quantitative RT-PCR. Horizontal bars represent the mean value for each genotype group. (B) *CDKN1B* mRNA levels of CC+CT against TT carrier individuals show a significant difference between these groups, which backs up the recessive model proposed.

## Discussion

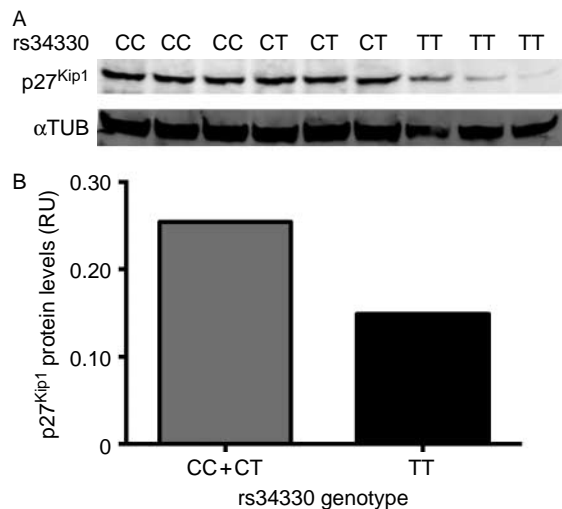
This is the first association study to investigate the effects of common genetic polymorphisms of the cell cycle regulator gene *CDKN1B* ( $p27^{Kip1}$ ) in thyroid cancer. Regression analysis allowed us to determine that the SNP rs34330 ( $-79C>T$ ), located in *CDKN1B* promoter, was significantly associated with the FVPTC under a recessive model (OR=2.12;  $P$  value=0.023). Furthermore, the functional assays designed to analyze its effect indicated that this SNP plays a role in *CDKN1B* transcription. This finding is of special relevance because although  $p27^{Kip1}$  has been widely studied in tumours (Slingerland & Pagano 2000, Chu et al. 2008) and specifically in thyroid cancer (Erickson et al. 1998, Tallini et al. 1999,

Vitagliano et al. 2004), attention has been focused so far on  $p27^{Kip1}$  translation, degradation and mislocalization. Here, we report for the first time a differential transcription rate of *CDKN1B* depending on a genetic variant located in its promoter.

The polymorphism rs34330 has been previously associated with some other neoplasias. Chang et al. (2004) described an association of this SNP with hereditary prostate cancer. They reported that the C allele of the variant was over-transmitted from parents to their affected offspring. They also suggested that the polymorphism was able to disrupt a CpG site, a supposition that has not been demonstrated so far.

Moreover, a two-step large-scale association study, involving more than 2200 patients and more than 2200 controls, found the same variant to be associated with an increased risk of breast cancer in the British population (Driver et al. 2008). They described the same risk genotype (TT) reported here, also fitting a recessive model (OR=1.22;  $P$  value=0.013).

In another recent study, this variant was related to the risk of lung cancer (OR=1.27; 95% CI=1.01–1.60; Wang et al. 2007a), and it was especially strong among heavy smokers (OR=6.24; 95% CI=1.67–23.34).



**Figure 4** Immunoblot experiment assessing  $p27^{Kip1}$  protein levels in human lymphocytes, depending on  $-79C>T$  (rs34330) genotype. (A) Immunoblot evaluating the rs34330-dependent expression of  $p27^{Kip1}$  in whole-cell lysates from healthy volunteers' lymphocytes. Fifty micrograms of proteins were loaded in each lane. Equal loading was verified using  $\alpha$ -tubulin as a reference. (B)  $p27^{Kip1}$  protein expression depending on  $-79C>T$  genotype. A trend towards lower  $p27^{Kip1}$  protein levels in  $-79TT$  carriers against non-risk genotypes (CC+CT) was observed.  $p27^{Kip1}$  protein levels are given in relative units (RU), and they resulted from the correction of  $p27^{Kip1}$  expression with  $\alpha$ -tubulin levels in each case ( $p27^{Kip1}/\alpha$ TUB ratio).

The authors proposed that due to the location of this SNP, its functional effect might be associated with a reduced p27<sup>Kip1</sup> protein production. Indeed, they remarked that the SNP was located in the 5' UTR, where Millard *et al.* (2000) had described a 'U-rich element' that may bind several factors (HuR, hnRNP C1 and C2) involved in mRNA stability, processing and translation. However, the polymorphism rs34330 lies 27 nucleotides upstream of the consensus U-rich element defined by Millard *et al.* This fact, along with some contradictory *in silico* predictions that we obtained for the above-mentioned binding factors (data not shown), leads us to believe that this is not the mechanism underlying the observed SNP effect.

We hypothesized that rs34330 could alter *CDKN1B* transcription. In fact, two independent tools (CON-SITE and TFSEARCH), based on different algorithms and integrated into the F-SNP database (Lee & Shatkey 2008), predicted that rs34330 is located within an important transcription factor binding site. Moreover, both tools predicted a differential affinity, and thus binding, of the human transcription factors AP-2 $\alpha$  and SP1 to the variant sequences. Especially, for the first factor, binding is predicted to be strongly influenced by the presence of the polymorphism.

The foregoing predictions were backed by a regulation mechanism described for some genes, which contain overlapping AP-2 and SP1 DNA-binding sites (Hilger-Eversheim *et al.* 2000). In these cases, gene transcription is regulated by the SP1/AP-2 ratio. This is of special interest, keeping in mind that AP-2 $\alpha$  is known to bind *CDKN1A*<sup>p21/cip1</sup> (Zeng *et al.* 1997), a gene encoding a cell cycle inhibitor that belongs to the same family as *CDKN1B*<sup>p27/Kip1</sup>. Overall, it is tempting to speculate that the above factors contribute to the functional effect of the polymorphism at least in the FVPTC, but specific functional studies focused on these factors will be necessary to prove this hypothesis.

The functional assays performed in the present study provide sufficient evidence to conclude that rs34330 (–79C>T) significantly influences *CDKN1B* transcription rates.

Thus, it should be considered as an alternative mechanism of p27<sup>Kip1</sup> downregulation in thyroid cancer, independently of the precise transcription factors involved.

Regarding the two polymorphisms that were not found to be associated with thyroid cancer in the present study, there are no studies directly assessing the influence of rs36228499 (–838C>A) in cancer. Only a study by Spurdle *et al.* (2009) provides indirect evidence that this SNP, tagged by the neighbouring

polymorphism rs3759216, does not modify breast cancer risk in *BRCA1* and *BRCA2* mutation carriers. In contrast with the –838 variant, there are numerous studies that found a significant association between the *CDKN1B*-coding SNP rs2066827 (V109G) and cancer. Among them, those related to advanced prostate cancer (Kibel *et al.* 2003), oral squamous cell carcinoma (Li *et al.* 2004), invasive epithelial ovarian cancer (Gayther *et al.* 2007), high-grade breast tumours (Tigli *et al.* 2005) and lymph node metastasis in breast cancer (Naidu *et al.* 2007) are worth mentioning. However, our data, based on a large Spanish population, suggest that this amino acid change in p27<sup>Kip1</sup> is not associated with any of the subtypes of thyroid cancer.

Taken together, we suggest that *CDKN1B* acts as a LPG in thyroid cancer, specifically associated with FVPTC. We propose that the –79C>T transition (rs34330) is the causal variant of a differential transcriptional expression of *CDKN1B*. These conclusions are supported by the following observations: a significant association in this first case–control study performed in thyroid cancer (risk genotype TT over-represented in FVPTC; OR=2.12, *P* value=0.023); the absence of a high correlation with neighbouring SNPs around this polymorphism, and the non-significant results for rs2066827 and rs36228499, which minimize the possibility of alternative variants being the causal ones; and the functional assays carried out show a tendency towards lower expression of *CDKN1B* in the presence of the risk genotype (TT) of rs34330, at both mRNA and protein levels. These findings point to an alternative downregulation mechanism of p27<sup>Kip1</sup>, which should be further explored in the cancer field.

Although these results have to be replicated in independent, representative and well-characterized thyroid cancer series, we propose the SNP rs34330, located in the *CDKN1B* promoter, as a genetic risk factor related to susceptibility for developing FVPTC. A biological explanation could be that the genetic alterations characterizing FVPTC are similar to those described in FTC, and are therefore related to the PI3K/Akt pathway. Regarding this, there are several genetic evidences suggesting that dysregulated PI3K/Akt pathway plays a significant role in the pathogenesis of thyroid tumours, particularly FTC (Hou *et al.* 2007, Riesco-Eizaguirre & Santisteban 2007, Wang *et al.* 2007b, Liu *et al.* 2008). Since p27<sup>Kip1</sup> belongs to this pathway, an alteration in *CDKN1B* transcription rate caused by rs34330 could be of fundamental importance in these tumours.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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