p27 variant and corticotropinoma susceptibility: a genetic and *in vitro* study

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Abstract

Germline mutations in *p27*^{kip1} are associated with increased susceptibility to multiple endocrine neoplasias (MEN) both in rats and humans; however, the potential role of common polymorphisms of this gene in endocrine tumor susceptibility and tumorigenesis remains mostly unrecognized. To assess the risk associated with polymorphism rs2066827 (p27-V109G), we genotyped a large cohort of Brazilian patients with sporadic endocrine tumors (pituitary adenomas, $n=252$; pheochromocytomas, $n=125$; medullary thyroid carcinoma, $n=51$; and parathyroid adenomas, $n=19$) and 885 population-matched healthy controls and determined the odds ratios and 95% CIs. Significant associations were found for the group of patients with pituitary adenomas ($P=0.01$), particularly for those with ACTH-secreting pituitary adenomas ($P=0.005$). In contrast, no association was found with GH-secreting pituitary tumors alone or with the sporadic counterpart of MEN2-component neoplasias. Our *in vitro* analyses revealed increased colony formation and cell growth rate for an AtT20 corticotropin mouse cell line overexpressing the p27-V109G variant compared with cells transfected with the WT p27. However, the genotypic effects in genetic and *in vitro* approaches were divergent. In accordance with our genetic data showing specificity for ACTH-secreting pituitary tissues, the overexpression of p27-V109G in a GH3 somatotropin rat cell line resulted in no difference compared with the WT. Pituitary tumors are one of the major clinical components of syndromes associated with the *p27* pathogenic mutations

Key Words
- endocrine tumor
- p27
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MENX and MEN4. Our genetic and *in vitro* data indicate that the common polymorphism rs2066827 may play a role in corticotropinoma susceptibility and tumorigenesis through a molecular mechanism not fully understood thus far.

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Introduction

In the last two decades, genetic studies of endocrine tumor syndromes have identified a number of susceptibility genes including several tumor suppressors (multiple endocrine neoplasia type 1 (*MEN1*), *VHL*, *NF1*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *PRKAR1A*, *AIP*, *TMEM127*, *MAX*, $p15^{INK4B}$ (*CDKN2A*), $p18^{INK4C}$ (*CDKN2C*), $p21^{CIP1}$ (*CDKN1A*), and $p27^{Kip1}$ (*CDKN1B*)) and two protooncogenes (*RET* and *HIF2A* (*EPAS1*)) (Pellegata *et al.* 2006, Agarwal *et al.* 2009, Zhuang *et al.* 2012, Toledo *et al.* 2013 and Supplementary refs 1–13 see section on supplementary data given at the end of this article). The genes predisposing to endocrine tumors encode transcription factors or molecules that are involved in a variety of processes such as hypoxia, cell-cycle inhibition, the citric acid cycle and the electron transfer chain, cAMP and Ras pathways, and in both mTOR and myc and glial cell line-derived neurotrophic tyrosine kinase signaling. Among the recently described tumor susceptibility genes for endocrine tumors is the *CDKN1B* gene, which codes for the cyclin kinase inhibitor $p27^{Kip1}$ (p27). p27 inhibits cyclin and cyclin-dependent kinase (Cdk) complexes which are essential for cell-cycle progression from G1 to S phases. p27 is mutated in a MEN syndrome both in rats and human patients (Pellegata *et al.* 2006, Marinoni & Pellegata 2010). The germline mutations in *CDKN1B* so far identified in patients (12 in total) affect the stability of p27 protein, its localization, its ability to interact with partner proteins, or the efficiency of transcription of the mutated *CDKN1B* allele (Pellegata 2012). Usually, p27 is not somatically mutated, but its expression is reduced or lost in more than 50% of all human tumors, including endocrine tumors (Chu *et al.* 2008). In addition to the pathogenic mutations, *CDKN1B* harbors single-nucleotide polymorphisms (SNPs) within or in the vicinity of the coding sequence, several with a low allelic frequency (<5%) and three of which are potentially functional: −838C>A (rs36228499), −79C>T (rs34330), and 326T>G (V109G, rs2066827). Indeed, patients with the variant AA genotype at position −823 of *CDKN1B* present low risk of restenosis following coronary stenting, and the variant −838A allele is associated with augmented gene transcription (González *et al.* 2004), whereas the −79C>T allelic variant is related to reduced transcription in *in vitro* studies (Landa *et al.* 2010). Furthermore, the coding 326T>G (V109G) allelic variant has been associated with cancer risk and progression of different tumors including 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 tumors (Tigli *et al.* 2005), and pancreatic carcinoma (Chen *et al.* 2010). A recent study has shown that the variant G allele at position 326 protects against thyroid cancer development (Pasquali *et al.* 2011), so that the role of the V109G polymorphism in increasing/decreasing tumor risk remains to be determined.

In the present study, we investigated the potential influence of the p27 rs2066827 (V109G) polymorphism on the risk of developing endocrine tumors in a large cohort of 447 Brazilian patients presenting the following sporadic endocrine tumors: pituitary adenomas ($n=252$), pheochromocytomas ($n=125$), medullary thyroid carcinoma (MTC; $n=51$), and parathyroid adenomas/hyperparathyroidism (HPT; $n=19$). The results were compared with those for 885 healthy patients, representative of Brazilian controls. *In vitro* analyses were performed to further assess the role of p27-V109G in pituitary adenoma cells.

Subjects and methods

Study population

All samples were collected in research centers in the Sao Paulo region of Brazil. Patients and controls were derived from matched population ancestry, composed of majority White and White-Latins, and also Asians, African-Americans, and individuals with mixed (undetermined) ancestry.

Demographic information

The patients were treated in three different hospitals (Hospital das Clinicas, Hospital Brigadeiro, and Hospital Santa Casa) all located within Sao Paulo city. The controls were collected from two different centers: one in

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the same hospital where the patients were treated (Hospital das Clinicas) and the other one located in the Basic Science Building of the University of Sao Paulo (IB-USP).

Characterization of the control group

The samples were obtained from two DNA banks of healthy individuals at the Medical School (FM-USP, Department of Oncology) and at the Biosciences Institute (IB-USP, Human Genome Research Center) at the University of Sao Paulo.

The control group comprised 885 tumor-free adult/elderly subjects distributed as 54% females, 46% males, with a mean age of 65.2 years (677 of them were 30 years old or older). In order to prevent the analysis of 'controls' that had no tumors at a young age but who could eventually develop them later, we collected samples of people still healthy at the average age of 65 years. Thus, the cohort of tumor-free controls is 25 years older than the group of patients.

To exclude from our analysis a possible effect of sex hormones, the sex distribution of the patients (59% females and 41% males) and controls (54% females and 46% of nine males) was similar.

We further characterized our study population with regard to the ethnicity and genotype/allele frequencies comparing with public databases and previous genetic studies of rs2066827. The frequencies of White/White Latinos (79%) and African/Mulatos (10%) in our control group were very similar to those assessed by The Brazilian Institute of Geography and Statistics (IBGE, Instituto Brasileiro de Geografia e Estatística), which reported frequencies of 78 and 13% of White/White Latinos and African/Mulatos, respectively, in the Sao Paulo area in the 2000 Census (www.ibge.gov.br). Allele frequencies found in our population study were compared with those reported in eight papers, which genotyped rs2066827 from controls of Caucasian and Asian ethnicity and from the USA, Canada, Malaysia, and China, as well as from four populations analyzed by the 1000 Genomes Project (CAUC1, AFR1, HISP1, and PAC1). As expected, the frequencies of the Brazilian population (G, 36% and T, 64%) were more similar to those for Caucasians (G, 24% and T, 76%) and Hispanics (G, 30% and T, 70%), than to those for Asians (G, 12% and T, 88%) and Africans (G, 36% and T, 34%).

Patients

The study analyzed 447 patients from matched population ethnicity that were clinically diagnosed with several types of sporadic endocrine tumors, including two different types of pituitary adenomas ($n=252$): GH-secreting/acromegaly ($n=161$) and adrenocorticotropic hormone (ACTH)-secreting/Cushing's disease ($n=91$); parathyroid adenomas/HPT ($n=19$); MTC ($n=51$); and adrenomedullary tumors/pheochromocytomas ($n=125$). Gender distribution was of 59% females and 41% males and the mean age at diagnosis was 40.7 years. They were diagnosed and treated at the Hospital das Clinicas of University of Sao Paulo Medical School (Endocrine Genetics Unit, Neuroendocrinology Unit, and Adrenal Unit), as well as at the Endocrine Divisions of the Hospital Brigadeiro (Sao Paulo) and Hospital Santa Casa (Sao Paulo).

The diagnosis of endocrine tumors was performed by standardized clinical, biochemical, and imaging examinations (Toledo *et al.* 2009, 2010*a*, Lourenco *et al.* 2010). Other possible endocrine tumors were ruled out in each patient. After surgery, tumors were confirmed through pathological criteria and immunostaining for endocrine-specific antibodies. We should mention that all cases with MTC included in the study were examined for the presence of mutations in the hot-spot exons of the *RET* proto-oncogene (exons 10, 11, and 13–16), and none were found (Santos *et al.* 2006, Toledo *et al.* 2010*b*). *TMEM127* and *RET* mutations were ruled out in the 51 cases with pheochromocytomas as previously described (Yao *et al.* 2010). The previously described cases from our MEN1 cohort (Toledo *et al.* 2010*a*) were not included in this study.

Written informed consents were obtained from all subjects in accordance with Institutional Review Board-approved protocols from each center. The protocols from the University of Sao Paulo are numbered as 0425/08, 0549/09, 1231/09, 0050/10, and 0031/10.

SNP genotyping

The genomic DNA of each subject was isolated from peripheral blood using standard salting-out protocol or commercially available DNA extraction kits (DNeasy Blood & Tissue Kit, Qiagen). For the initial 140 DNA samples, the SNP 326T>G (V109G) was genotyped by direct sequencing. PCRs were performed using primers previously described (Pellegata *et al.* 2006) and both DNA strands were sequenced from purified PCR products using Big Dye Terminator v3.1 (Life Technologies) and an automated sequencer (ABI Prism 3130xl DNA Analyzer, Life Technologies).

The presence of the polymorphic G allele creates a restriction site for the enzyme BglI (5′…GCCNNNN^NGGC…3′). Thus, we genotyped the rest of the DNA samples using a PCR–restriction fragment length polymorphism approach

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as previously described (Kibel *et al*. 2003) after having verified that this method had 100% accuracy compared with sequencing (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Amplicons of 762 bp containing codon 109 were generated by using a PTC-200 DNA thermocycler (Peltier thermal cycler; MJ Research, Inc., Waltham, MA, USA) with 10 ng of gDNA as template and the following primers: p27-V109G_F: GTCGGGGTCTGTGTCTTTTG and p27-V109G_R: GCCAGGTAGCACTGAACACC. The digestion step was carried out at 37 °C for 3 h with the enzyme BglI (New England BioLabs, Beverly, MA, USA). After digestion, the enzyme was inactivated at 85 °C for 20 min and the genotypes were identified according to the bands visualized on 3% agarose gels (Supplementary Fig. 1).

Statistical analyses

Hardy–Weinberg equilibrium of the genetic variants at codon 109 in *CDKN1B* (p.V109G) was assessed using χ^2 statistics, and the best fitting model was determined according to the *P* values using parsimony. Assessment of tumor risk was performed through comparison of genotype frequencies between cases and controls using χ^2 statistics and odds ratios (ORs) with 95% CIs using logistic regression models. A modified Bonferroni's-corrected nominal threshold of $P=0.05/n^*$ was used to correct for multiple hypothesis testing, of which n^* is the number of independent comparisons (ACTHoma, GHoma, pheochromocytoma, and MTC) considering G and T as the risk allele. The variants, 'all sporadic endocrine tumors' and 'sporadic counterpart of MEN1/2-component neoplasias'; were not considered independent and were not included in the threshold correction. Age and sex adjustments were performed in the statistical analyses.

Expression vectors, cell lines, and transfections

The p27V109G mutations were introduced by site-directed mutagenesis (Quikchange II Site-Directed Mutagenesis Kit; Stratagene, Waldbronn, Germany) in the WT human *CDKN1B* cDNA cloned in a pYFP backbone as described previously (Pellegata *et al*. 2006). AtT-20/D-16v (kindly provided by Dr M C Zatelli) and HeLa cells (LGC Standards, Wesel, Germany) were maintained in DMEM, respectively, supplemented with 10% FCS, 20 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. GH3 cells (ATCC, Manassas, VA, USA) were grown in F12 medium supplemented with 15% horse serum, 2.5% FCS, 20 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin. Transient transfection was carried out as described previously (Pellegata *et al*. 2006).

Protein extraction and western blotting

To extract total proteins, the cells were collected, washed twice in PBS, and lysed in RIPA buffer essentially as previously reported (Pellegata *et al*. 2006). Protein concentration was assessed by BCA assay (Pierce Chemical Co., Rockford, IL, USA). Total extracts were subjected to PAGE using Bis–Tris 4–12% NuPAGE gels, blotted, and probed with the following antibodies: monoclonal, against p27 (BD Biosciences, Franklin Lakes, NJ, USA); and monoclonal anti-α-tubulin (Sigma). Immunoreactive proteins were visualized using West Pico chemiluminescent substrates (Pierce Chemical Co.). The bands that we obtained by western blotting were quantified using the Molecular Imager ChemiDoct XRS.

Clonogenic assay and growth curve

To examine their clonogenic activity, AtT-20/D-16v were plated in six-well plates (500 000/well) and transfected with p27-wt, p27-V109G, or GFP empty vector using Fugene-6 (Roche). Subsequently, the cells were trypsinized, diluted 1:6, and replated in 10 cm plates. Twenty-four hours later, the cells were selected for 6 weeks by adding 300 μg/ml to the culture medium and stained with 0.3% crystal violet in 30% ethanol. Colonies containing a minimum of 50 cells were scored.

Clones derived from AtT-20/D-16v cells stably expressing exogenous p27-wt, p27-V109G, or GFP were plated in duplicate (100 000/well) in 12-well plates in full medium plus 300 μg/ml G418. The number of cells was counted every 2 days over a 7-day period using a cell counter.

Immunofluorescence

Immunofluorescence was carried out on AtT20 cells grown on coverslips and transfected with p27-wt, p27-V109G, or GFP vector. Twenty-four hours later, the transfected cells were fixed in 2% paraformaldehyde in PBS for 30 min at room temperature. The cell nuclei were stained with 1 μg/ml Hoechst for 5 min at room temperature and mounted on glass slides. Images were generated using a Zeiss Axiovert 200 epifluorescence microscope including an Apotome unit (Zeiss, Jena, Germany) using the YFP and the DAPI channel and processing was carried out using Zeiss Computer Software (AIM 3.2).

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Results

p27-V109G in controls

Genotype frequencies of the SNP rs2066827 were assessed in the control group of 885 representative healthy Brazilian individuals and were distributed as follows: GG=116, 13.1%; GT=406, 45.9%; and TT=363, 41.0%. The allele frequencies (G=0.36 and T=0.64) of our cohort of controls were more similar to those reported in the SNP databases and in previous genetic studies for Hispanic (G=0.30 and T=0.70) and Caucasian (G=0.24 and T=0.76) populations than those for Asians (G=0.12 and T=0.88) or Africans (G=0.72 and T=0.28) (Table 1). No significant deviation from the Hardy–Weinberg equilibrium was observed for the two alleles.

Association of p27-V109G and sporadic counterpart of MEN1-component neoplasia

Genotyping analysis of the overall group including 447 patients with endocrine tumors provided the following genotypic frequencies: GG=49, 11%; GT=200, 44.7%; and TT=198, 44.3%. We observed a significant over-representation of the T allele (valine) in the patients with sporadic counterpart of MEN1-component neoplasias (n=271, parathyroid neoplasias and pituitary adenomas), compared with the 885 controls (OR=1.25; 95% CI=1.02–1.54; P=0.03). Accordingly, the presence of the polymorphic allele G (GT or GG genotypes) was significantly associated with decreased susceptibility to sporadic counterpart of MEN1-component neoplasias (protection), fitting both recessive (OR=0.72; 95% CI=0.55–0.94; P=0.019) as well as dominant (OR=0.72; 95% CI=0.54–0.97; P=0.03) models (Table 2).

Absence of association of p27-V109G and sporadic counterpart of MEN2-component neoplasia

No significant association was observed between the p27-V109G polymorphism and the group of sporadic tumors belonging to the MEN2 spectrum (MTC and pheochromocytoma; P=0.35), as well as the groups containing exclusively MTC (P=0.27) or pheochromocytoma (P=0.57). Also, no significant differences were found between the genotypic and allelic frequencies of the controls and the group of patients with sporadic counterpart of MEN1- and MEN2-component neoplasias (P=0.25; Table 2). Primary HPT was initially included in both MEN1 and MEN2 groups, but its exclusion did not change the results.

Absence of association of p27 rs36228499 and sporadic tumors

In addition to SNP V109G, we analyzed in our controls and patient cohorts the allelic frequencies of another SNP located in the noncoding region of p27 (rs36228499), which has been studied previously and shown not to be associated with endocrine/thyroid tumors (Landa *et al.* 2010), and, according to the HapMap, is not in linkage disequilibrium with the SNP V109G (rs2066827). The genotyping was carried out by PCR and Sanger sequencing (primers −838 F: TGGCCTCGGAGAAATTAAAA and

Table 1 Allele and genotype distribution of the rs2066827 V109G variant of p27 in controls and patients diagnosed with sporadic endocrine tumors investigated

	Total	G	T	GG	GT	TT
Sporadic counterpart of MEN1-component neoplasias	271	168	349	30	108	133
sporadic counterpart of MEN2-component neoplasias	195	143	275	21	101	73
Sporadic pituitary adenomas	252	155	323	28	99	125
Sporadic ACTHomas	91	50	111	10	30	51
Sporadic GHomas	161	105	212	18	69	74
Sporadic HPT	19	13	26	2	9	8
Sporadic MTCs	51	42	69	8	26	17
Sporadic pheochromocytomas	125	88	180	11	66	48
All tumors	447	298	598	49	200	198
Healthy controls	885	638	1132	116	406	363

Sporadic counterpart of multiple endocrine neoplasia type 1 (MEN1)-component neoplasias – types of sporadic endocrine tumors that are included in the clinical phenotype of the MEN1 syndrome as hyperparathyroidism and pituitary adenomas; sporadic counterpart of MEN2-component neoplasias – types of sporadic endocrine tumors that are included in the clinical phenotype of the MEN2 syndrome medullary thyroid carcinoma (MTC) and pheochromocytomas; ACTHomas, ACTH-secreting pituitary adenomas; GHomas, GH-secreting pituitary adenomas, also know as somatotropinomas; HPT, parathyroid adenomas/hyperparathyroidism; G and T represent the alleles that observed in the V109G $p27^{Kip1}$ SNP; and GG, GT, and TT are the observed genotypes.

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Table 2 Endocrine tumor susceptibility and rs2066827 V109G variant of p27

		Allele frequency (G×T)			**Heterozygous** (GG×GT)			**Homozygous** (GG×TT)			**Allele positivity** ((GG+GT)×TT)		
Group	***n***	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Controls	885	1.000			1.000			1.000			1.000		
All sporadic endocrine tumors[a]	455	1.127	0.951–1.335	0.16622	1.166	0.802–1.696	0.42069	1.291	0.886–1.881	0.18222	1.225	0.859–1.748	0.26174
		0.887	0.749–1.051	0.16622	0.903	0.709–1.151	0.40950	0.774	0.532–1.128	0.18222	0.875	0.695–1.100	0.25252
Sporadic counterpart of MEN1-component neoplasias[b]	195	**1.255**	**1.021–1.542**	**0.03091**	1.029	0.653–1.620	0.90322	1.417	0.905–2.217	0.12622	1.212	0.791–1.857	0.37704
		0.797	**0.648–0.980**	**0.03091**	**0.726**	**0.543–0.971**	**0.03054**	0.706	0.451–1.105	0.12622	**0.722**	**0.549–0.948**	**0.01899**
Sporadic counterpart of MEN2-component neoplasias[c]	269	0.973	0.775–1.222	0.81713	1.374	0.822–2.296	0.22354	1.111	0.655–1.884	0.69651	1.250	0.763–2.046	0.37450
		1.027	0.818–1.290	0.81713	1.237	0.887–1.725	0.20972	0.900	0.531–1.527	0.69651	1.162	0.844–1.600	0.35620
Sporadic pituitary adenomas[d]	250	**1.269**	**1.026–1.570**	**0.02787**	1.010	0.633–1.612	0.96605	1.427	0.901–2.260	0.12878	1.207	0.778–1.871	0.40055
		0.788	**0.637–0.975**	**0.02787**	**0.708**	**0.525–0.955**	**0.02348**	0.701	0.442–1.110	0.12878	**0.707**	**0.533–0.936**	**0.01512**
Sporadic ACTHomas	91	**1.488**	**1.059–2.090**	**0.02115**	0.857	0.407–1.805	0.68476	1.630	0.802–3.313	0.17362	1.222	0.616–2.425	0.56605
		0.672	**0.479–0.944**	**0.02115**	**0.526**	**0.338–0.844**	**0.00696**	0.614	0.302–1.247	0.17362	**0.545**	**0.353–0.843**	**0.00575***
Sporadic GHomas	159	1.165	0.905–1.499	0.23592	1.095	0.627–1.914	0.74937	1.314	0.754–2.291	0.33489	1.198	0.707–2.031	0.50093
		0.859	0.667–1.105	0.23592	0.834	0.583–1.192	0.31788	0.761	0.437–1.327	0.33489	0.818	0.583–1.146	0.24184
Sporadic MTCs	51	0.805	0.536–1.208	0.29474	0.929	0.409–2.106	0.85918	0.679	0.286–1.614	0.37841	0.811	0.372–1.768	0.59732
		1.242	0.827–1.100	0.29474	1.367	0.730–2.561	0.32667	1.473	0.619–3.501	0.37841	1.391	0.765–2.528	0.27726
Sporadic pheochromocytomas	125	1.038	0.786–1.369	0.79432	1.714	0.876–3.353	0.11171	1.394	0.701–2.774	0.34152	1.563	0.817–2.991	0.17395
		0.964	0.731–1.272	0.79432	1.229	0.826–1.830	0.30842	0.717	0.361–1.426	0.34152	1.116	0.759–1.639	0.57718

Significant associations were initially observed in the sporadic MEN1-related, sporadic pituitary adenoma and sporadic ACTH-secreting pituitary adenomas (corticotropinomas). After Bonferroni's correction of multiple testing, association with corticotropinomas remained significant, indicating a robust finding. For each tumor type, the upper row represents the results for the T allele and the lower row the results for the G allele. *P* values <0.05 are shown in bold. ***Values that remained significant after use of a modified Bonferroni's-corrected nominal threshold of *P*=0.05/*n** to correct for multiple hypothesis testing where *n** is the number of the independent comparisons (ACTHoma, GHoma, pheochromocytoma, and MTC), and taking account G and T as the risk allele (corrected threshold of *P*=0.05/8=0.00625)**. Age and sex adjustments were performed in the statistical analysis. OR, odds ratio; MTC, medullary thyroid carcinoma.

[a]All sporadic counterpart of MEN1- and MEN2-component neoplasias.

[b]Sporadic counterpart of MEN1-component neoplasias, including hyperparathyroidism and pituitary adenomas.

[c]Sporadic counterpart of MEN2-component neoplasias, including hyperparathyroidism, MTC, and pheochromocytomas.

[d]ACTH- and GH-secreting pituitary adenomas.

−838 R: TTAAGGCTGAGCGAACCATT) and the frequencies observed in our controls (G=0.51 and T=0.49) and patients (G=0.54 and T=0.46) were similar between each other, and both were more similar to the frequencies reported for the European/HapMap-CEU population (G=0.56 and T=0.44) than to those found in African/HapMap-YRI populations (G=0.75 and T=0.25).

The role of p27-V109G specifically in ACTH–pituitary tumors

Protection by the G allele (GT and GG genotypes) was seen overall in the sporadic pituitary adenoma group (OR=0.70; 95% CI=0.53–0.93; P=0.01) and more especially in the ACTH-secreting pituitary adenoma group (OR=0.54; 95% CI=0.35–0.84; P=0.005). The observed association between rs2066827 and corticotropinomas was still significant after correction for multiple testing using the conservative Bonferroni's method, indicating that this finding is robust. In contrast, the presence of the G allele was not associated with protection from acromegaly (P=0.24).

To determine whether the T or G allelic variants at codon 109 of p27 may be associated with specific functions in ACTH-secreting cells, we performed *in vitro* analyses comparing the effects of p27-wt (TT) or p27-V109G (GG) overexpression in a mouse corticotropinoma cell line (AtT20). Following transfection of p27-wt and p27-V109G in these cells, we observed that the p27-V109G variant protein localizes to the nucleus, as the WT protein does (Supplementary Fig. 1). We then assessed the effects of both variants on cell growth by performing colony formation assays. The results were compared with the rat somatotroph adenoma-derived GH3 cell line. We found that AtT20 cells transfected with p27-V109G show a 50% increase in the number of colonies when compared with cells either transfected with p27-wt or with empty vector (Fig. 1). In contrast, both variant proteins show similar growth suppression of GH3 cells (Fig. 1 and Supplementary Figs 2 and 3, see section on supplementary data given at the end of this article). This phenotype is in agreement with the increase in cell proliferation observed in AtT20-derived clones stably expressing exogenous p27-V109G when compared with clones expressing p27-wt or transfected with empty vector (data not shown). Thus, *in vitro*, the two p27 variant proteins show a different behavior in AtT20 corticotrope adenoma cells when compared with GH3 somatotrope adenoma cells.

Discussion

In the last few years, mutations in the *CDKN1B* p27 gene have been identified as the cause of a novel syndrome (named MEN4) that is characterized by multiple endocrine tumors with a spectrum similar to the *MEN1* syndrome

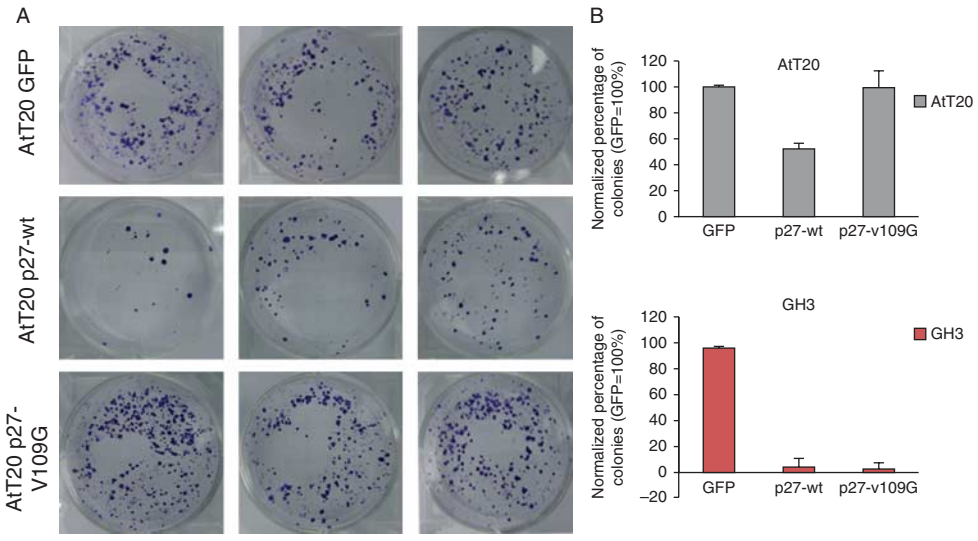

Figure 1
The effect of overexpression of both variants, p27-wt (TT) and p27-V109G (GG), on cell growth in a mouse corticotropinoma cell line (AtT20) and the rat somatotroph adenoma-derived GH3 cell line (A). AtT20 cells transfected with p27-V109G show a 50% increase in the number of colonies when compared with either cells transfected with p27-wt or with empty vector, while both variant proteins show similar growth suppression of GH3 cells (B).

(Pellegata *et al*. 2006, Georgitsi *et al*. 2007, Agarwal *et al*. 2009, Marinoni & Pellegata 2010, Pellegata 2012). While this article was under revision, somatic mutations and deletions of *CDKN1B* were reported in a subset of neuroendocrine tumors of the small intestine (Francis *et al*. 2013). In addition to these rare pathogenic mutations, *CDKN1B* contains numerous SNPs, including a frequent polymorphism (rs2066827, V109G), which has been found to be associated with the risk of or protection from cancer, depending on the tumor type. Specifically, the V109G polymorphism has been shown to have a protective effect on overall survival of patients with sporadic pancreatic carcinoma (Chen *et al*. 2009), while it is associated with increased risk of progression and poor prognosis for prostate and breast cancer patients (Kibel *et al*. 2003, Tigli *et al*. 2005).

In this study, we analyzed the role of the V109G genetic variant in *CDKN1B* in a case–control study, including 447 Brazilian patients with different types of sporadic endocrine tumors and 885 adult healthy representative Brazilian controls. We found an association between rs2066827 and susceptibility to tumors belonging to the MEN1 tumor spectrum (i.e. pituitary adenomas), while no role in the risk of developing tumors belonging to the MEN2 tumor spectrum (i.e. MTC and pheochromocytoma) could be demonstrated (Table 2). We are aware that the ethnic intermix of the Brazilian population can be an important issue for case–control studies like ours, so we addressed this point by further characterizing our cohorts. According to the Brazilian government database (IBGE) for the Sao Paulo area, where all the samples were collected and the study took place, our cohort is composed for the most part of whites and presented no sampling bias. In accordance, the allele frequencies we found for this genetic variant and for a second SNP in the noncoding sequence of *CDKN1B* (rs36228499) were similar to those reported in public databases and in previous genetic studies for Caucasians and Hispanics.

Positive association with rs2066827 was found in the pituitary adenoma group, including both somatotropinomas and corticotropinomas ($P=0.01$); however, more specific association was observed with the corticotropinomas ($P=0.005$) (Table 2). The association between rs2066827 and corticotropinomas remained significant after the calculation of the adjusted P value cut-off using the conservative Bonferroni's method as a correction for multiple testing ($P=0.00625$). In contrast, rs2066827 was not associated either with sporadic counterpart of MEN2-component neoplasias or with MTC or pheochromocytoma groups ($P>0.05$ in all models tested). To our knowledge, this is the first study that investigated p27 polymorphisms in pheochromocytoma, while two independent reports that were recently published analyzed the role of p27 variants in European MTC cohorts (Landa *et al*. 2010, Pasquali *et al*. 2011). In accordance to our results for Brazilian MTC patients, no association of p27-V109G with increased risk of sporadic MTC was observed in a large cohort of Spanish MTC cases, while a borderline significance was observed in the analysis of tumor risk of Italian MTC patients (Landa *et al*. 2010, Pasquali *et al*. 2011).

We could demonstrate that rs2066827 associates with a specific molecular phenotype in corticotrope cells but not in somatotrope cells. Indeed, the ectopic overexpression of p27-V109G in AtT20 cells, as opposed to p27-wt, does not inhibit cell growth and actually seems to promote cell proliferation. In contrast, no differences in the cell growth characteristics of p27-wt and p27-V109G were observed in somatotroph cells. As the expression of p27-V109G is associated with increased cell growth and proliferation in AtT20 cells, it is not easy to reconcile these findings with the results of the association studies. The fact that AtT20 cells express high endogenous levels of p27 and still show sustained proliferation may indicate that this experimental model, the only one available for corticotrope adenomas, might not be ideal for reproducing the situation of the expression of p27 variants *in vivo* in individuals.

Patients with germline pathogenic mutations in p27 (MEN4 syndrome) may develop ACTH-secreting adenoma, although rarely (Lee & Pellegata 2013). Previous studies have shown that p27 is consistently and frequently downregulated in corticotropinomas, but not in other types of pituitary adenomas (Dahia *et al*. 1998). Moreover, a crucial role for p27 in the regulation of the proliferation of pituitary proopiomelanocortin (POMC) cells producing ACTH has also been demonstrated in animal studies, and p27-deficient mice develop POMC tumors at high frequencies (Fero *et al*. 1996).

The exact molecular mechanism involved in p27-V109G change is still not clear. While the structure of the N- and C-terminal domains of p27 has been resolved and the interactions with key partners such as cyclins and CDKs have been mapped, the structure of the central domain of the protein (amino acids 97–151) is still unknown. The amino acid valine 109 is located in the central portion of the protein, within a domain that mediates the binding of p27–p38^{Jab1} (Tomoda *et al*. 1999). The interaction of the two proteins mediates the nuclear export of p27 and its subsequent degradation.

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Interestingly, the substitution of the aspartic acid at position 108 by glycine impairs the formation of this complex (Tomoda *et al*. 2002) and it has been speculated that this variant might interfere in the interaction with $p38^{Jab1}$, and thereby increase the stability of p27 in the nucleus (Schöndorf *et al*. 2004). Experimental evidence is nevertheless required to confirm this hypothesis.

In addition to our finding of a potential role of rs2066827 in corticotropinomas, another p27 polymorphic variant, −79C>T (rs34330), was specifically correlated with a high risk of developing the follicular variant of papillary thyroid carcinoma (Landa *et al*. 2010), indicating that reduced *CDKN1B* gene transcription could be involved in the molecular mechanism that mediates the pathogenic effects of this variant.

There are conflicting findings in the literature regarding the risk/protection associated with rs2066827 T/G alleles. A recent meta-analysis has evaluated the association data of eight studies encompassing 3799 controls and 3591 patients with non-endocrine tumors (oral squamous cell, prostate, breast cancer, and pancreatic cancer) and found no correlation between the rs2066827 variant and the overall cancer risk in the general population (Wei *et al*. 2012). Since p27 is a tumor susceptibility gene for multiple endocrine tumors in both humans and rats, we decided to investigate specifically endocrine tumors. Based on our results, it seems worthwhile to assess whether the V109G genetic variant may modify individual susceptibility to additional endocrine tumors, i.e. entero-pancreatic tumors, prolactinomas, nonfunctioning pituitary adenomas, parathyroid carcinomas, or adrenocortical carcinomas, and to investigate cohorts of patients from different populations.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0486.

Declaration of interest

M D Bronstein declares an association with the following companies: Ipsen, Novartis, and Pfizer (consultant, speaker, and grant/research support). The remaining authors have nothing to disclose.

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Author contribution statement

N S Pellegata, S P A Toledo, and R A Toledo designed the project; M D Bronstein, R S Jallad, M C Machado, T D Goncalves, L H Osaki, J Viana-Jr, C Kater, B Liberman, M C B V Fragoso, and S P A Toledo provided clinical data and DNA samples from patients; G Francisco, R Chammas, M S Naslavsky, D Schlesinger, Y A O Duarte, M L Lebrão, and M Zatz provided clinical data and DNA samples from healthy controls; T Sekiya, V C Longuini, L H Osaki, and R A Toledo performed and analyzed the genetic experiments; B Katiuscia and N S Pellegata performed and analyzed the *in vitro* assays; O Meirelles and R A Toledo performed the statistical analysis; T Sekiya, M D Bronstein, M C B V Fragoso, P Gama, S P A Toledo, N S Pellegata, and R A Toledo contributed to discussions; and R A Toledo wrote the paper with inputs from N S Pellegata, T Sekiya, and S P A Toledo.

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