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CLINICAL STUDY

Association between the *p27* rs2066827 variant and tumor multiplicity in patients harboring *MEN1* germline mutations

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Abstract

Objective To date, no evidence of robust genotype–phenotype correlation or disease modifiers for multiple endocrine neoplasia type 1 (MEN1) syndrome has been described, leaving the highly variable clinical presentation of patients unaccounted for.

Design As the CDKN1B (p27) gene causes MEN4 syndrome and it is transcriptionally regulated by the product of the MEN1 gene (menin), we sought to analyze whether p27 influences the phenotype of MEN1-mutated patients. The cohort consisted of 100 patients carrying germline MEN1 gene mutations and 855 population-matched control individuals.

Methods Genotyping of the coding p27 c.326T>G (V109G) variant was performed by sequencing and restriction site digestion, and the genotypes were associated with clinical parameters by calculating odds ratios (ORs) and their 95% CIs using logistic regression.

Results There were significant differences in p27 V109G allele frequencies between controls and MEN1-mutated patients (OR=2.55, P=0.019, CI=1.013–5.76). Among patients who are ≥30 years old carrying truncating MEN1 mutations, the T allele was strongly associated with susceptibility to tumors in multiple glands (three to four glands affected vs one to two glands affected; OR=18.33; P=0.002, CI=2.88–16.41). This finding remained significant after the Bonferroni's multiple testing correction, indicating a robust association. No correlations were observed with the development of MEN1-related tumors such as hyperparathyroidism, pituitary adenomas, and enteropancreatic and adrenocortical tumors.

Conclusions Our study suggests that the p27 tumor suppressor gene acts as a disease modifier for the MEN1 syndrome associated with MEN1 germline mutations. If confirmed in independent patient cohorts, this finding could facilitate the management of this clinically complex disease.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a challenging syndrome due to its extraordinary clinical complexity. MEN1 was first described by Wermer (1954) (1) as 'the syndrome of adenomatosis of the anterior pituitary, the parathyroids and the pancreatic islets, and the stomach and the duodenum'. As Wermer (2) extended the clinical screening, neoplasias in the adrenal cortex

were also included in the panel of MEN1 clinical features. Additionally, he also delineated the genetics of MEN1, describing this disorder as having a dominant inheritance associated with 'mosaic pleiotropism'.

Since the initial reports by Wermer, the chromosomal locus of this disease has been mapped to 11q13 (3), and the gene, named *MEN1*, has been identified by the positional cloning method (4, 5). To date, more than 1300 *MEN1* mutations have been reported worldwide, and molecular analysis of at-risk asymptomatic relatives is offered to affected families (6, 7, 8, 9). Although the implementation of such testing helps the clinical management of this syndrome, further studies still need to be conducted to reduce the potential of post-genetic analyses uncertainties (8, 9, 10, 11). For example, while in the absence of a detectable mutation, mutation-negative asymptomatic relatives are excluded from the periodic clinical surveillance, the presence of a mutation remains baffling, as patients harboring exactly the same *MEN1* mutation (including first-degree relatives) usually present very different clinical and tumoral outcomes (4, 5, 6, 7, 8, 9, 10, 11, 12).

Owing to the lack of a genotype–phenotype correlation, the current MEN1 guidelines recommend that all patients carrying *MEN1* mutations should be treated equally and subjected to an intense clinical surveillance to diminish the chance of a late diagnosis of MEN1-associated neoplasias and a consequently poor prognosis (10). Such recommendations have proven to be beneficial, but as the tracking of these patients is lifelong and not all mutation-positive individuals develop the full spectrum of MEN1-related tumors, new translational research approaches are required to optimize the follow-up protocols. This strategy could reduce the effort currently spent by both physicians and patients, and thus minimize the financial costs without reducing the quality of care (6, 7, 8, 9). In this context, the identification of genetic–phenotypic modifiers of the disease would facilitate the prediction of outcomes for *MEN1* mutation-positive patients and would provide useful information for the refinement of clinical protocols for these patients (11, 12).

The *CDKN1B* (*p27*) gene that encodes the p27 cell cycle inhibitor has recently been identified as a molecule associated with a MEN1-like phenotype in patients without a *MEN1* germline mutation (MEN4 syndrome) (13, 14). Interestingly, the *CDKN1B* gene is transcriptionally regulated by the product of the *MEN1* gene (the menin protein), suggesting that *MEN1* and *p27* may share a common endocrine tumorigenic pathway (15, 16). Considering the increasingly important role of *p27* in the susceptibility to endocrine neoplasias, we currently sought to investigate whether the *p27* rs2066827 (c.326T>G; V109G) genetic variant of this gene, previously associated with an increased risk for several tumor entities (17), acts to modify the clinical manifestations of patients harboring germline *MEN1* mutations.

Patients and methods

Written informed consent was obtained from the subjects in accordance with the Institutional Review Board-approved protocols from each center. The University of Sao Paulo Ethical Committee protocol numbers were the following: 0425/08, 0549/09, 462/09, 1231/09, 0050/10, and 0031/10. The study was conducted between July 2007 and March 2011.

MEN1 patients

A total of 100 DNA blood samples from Brazilian patients with a clinical diagnosis of MEN1 (50 males and 50 females; average age at diagnosis 36.5, 13–71-year-old) and harboring a germline mutation in the *MEN1* gene were investigated (8, 18, 19). Patients were of heterogeneous ethnic backgrounds, although the majority of them were Caucasians. Patients were followed up and treated by several units of the Division of Endocrinology, Hospital das Clinicas of the University of Sao Paulo Medical School (Endocrine Genetics Unit, Neuroendocrinology Unit, Neurosurgery Unit, and Adrenal Unit). The clinical diagnosis of the MEN1-related endocrine tumors was performed using standardized clinical, biochemical, and imaging procedures, as reported previously (20, 21). After surgery, tumors were confirmed through pathological features and immunostaining with endocrine-specific antibodies.

The frequencies of MEN1-related tumors in the 100 patients were as follows: hyperparathyroidism (HPT; 100%); pituitary adenomas (61.4%);

enteropancreatic neuroendocrine tumors (89.5%); and adrenocortical tumors (59.6%). The overall cohort was used for the comparison of allelic and genotype frequencies in MEN1 patients and healthy control individuals. Additionally, to assess the potential impact of p27 in the phenotypic modulation in MEN1 patients, a cutoff at the age of 30 years was used to avoid age-related issues regarding tumor development.

Seventy two MEN1 patients (≥30 years) MEN1 patients with complete medical information for the four main MEN1-related glands (parathyroids, pituitary, adrenals, and endocrine pancreas/duodenum) were included according to the first criterion (age) in the phenotypic modulation studies, which considered the development of each type of tumor and the total number of affected glands in each patient. Furthermore, the type of *MEN1* mutation was also considered. As described in almost all other MEN1 cohorts reported, *MEN1* truncating mutations were more frequent than missense mutations and were present in 57 out of the 72 ≥30-year-old MEN1 patients (79.2%) (8, 18, 19, 20, 21). The clinical features of this very informative cohort of patients for testing the hypothesis of genetic–phenotypic modulators (all ≥30 years old and carrying truncating *MEN1* mutations) are listed in Supplementary Table 1, see section on supplementary data given at the end of this article and the list of *MEN1* mutations in Supplementary Table 2. Briefly, all patients developed HPT (57/57, 100%), 51 patients had enteropancreatic neuroendocrine tumors (51/57, 89.5%), 35 patients developed pituitary adenomas (35/57, 61.4%), and 34 patients presented adrenocortical tumors (34/57, 59.6%). Regarding the number of affected glands, three, eight, 26, and 20 patients developed tumors in one to four MEN1-related main glands respectively (Supplementary Table 1). Owing to the reduced number of ≥30-year-old MEN1 patients who had developed tumors in only one or two glands, groups with one to two and three to four tumors were combined for statistical purposes.

Control individuals

The control group comprised 885 tumor-free adult/elderly subjects distributed as 54% of females and 46% of males, with a mean age of 65.2 years (677 of them were 30-years-old or older) from the same demographics and ethnicity as patients. In accordance to the Census of the Brazilian Institute of Geography and Statistics (IBGE, Instituto Brasileiro de Geografia e Estatística, www.ibge.gov.br), the frequencies of White/White Latinos and African/Mulatos in the Sao Paulo area are 78 and 13% respectively. We have similar frequencies in our study, White/White Latinos (79%) and African/Mulatos (10%), indicating that our selection has fulfilled the population-matched criteria to case–control studies. In order to prevent the analysis of 'controls' who had no tumors at 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 30 years older than the group of patients. To exclude from our analysis a possible effect of sex hormones, gender distribution between patients (50% of females and 50% of males) and controls (54% of females and 46% of males) was similar.

Blood DNA samples and medical data from healthy individuals were provided by two DNA databanks located at the Department of Oncology, University of Sao Paulo School of Medicine, and at the Human Genome Research Center, Biosciences Institute, University of Sao Paulo.

Single nucleotide polymorphism genotyping

PCRs were performed using previously described primers, and both DNA strands were sequenced from the purified PCR products using the Big Dye Terminator v3.1 Kit and an automated sequencer (ABI Prism 3130xl DNA Analyzer; Life Technologies) (21). The *p27* single nucleotide polymorphism (SNP) V109G was genotyped by direct sequencing in the initial 140 samples. After verifying that the PCR–restriction fragment length polymorphisms (with BglI enzyme, New England Biolabs, Ipswich, MA, USA) showed 100% of accuracy in comparison with the sequencing results, the remaining samples were then genotyped by digestion.

Statistical analyses

Hardy–Weinberg equilibrium was assessed by χ^2 statistics, and the best fitting model was determined according to the *P* values using parsimony. The assessment of tumor risk was performed through a comparison of genotype frequencies between the cases and the controls using χ^2 statistics and odds

ratios (ORs) with 95% CIs in logistic regression models. A number of clinical variants such as the presence of the four main MEN1-related tumors (HPT; pituitary adenomas – ACTH-, GH-, and PRL-secreting and nonsecreting pituitary adenomas; and secreting and nonsecreting enteropancreatic neuroendocrine tumors and adrenocortical lesions) were assessed by logistic regression. *MEN1* gene mutation types (missense or truncating) were also treated as cofactors in the statistics. Multiple testing correction was performed using the conservative Bonferroni's method.

Phenotypic modulation analysis

In the current study, we aimed to evaluate the possible phenotypic modulation of rs2066827 in the tumor multiplicity of MEN1 syndrome. Based on the age-associated penetrance curves for MEN1, young patients are likely to develop additional tumors as they come to adulthood and get older, so in order to avoid age-related bias, we included only patients carrying germline *MEN1* mutations, who were older than 30 years. Therefore, we increased the chances of assessing a clearer sign of the possible modulation effect. In total, 72 patients passed these criteria: 57 patients who are >30-year-old with *MEN1* truncating mutations and 15 patients who are >30-year-old with missense mutations.

Results

rs2066827 in MEN1 patients and controls

As no robust genotype–phenotype correlation has ever been documented for MEN1 syndrome associated with germline mutations in the *MEN1* gene (4, 5, 6, 7, 8, 9, 10, 11), we tested the hypothesis that the tumor suppressor gene *p27* might act as a genetic modifier for clinical manifestations in MEN1 patients. We first compared the allelic frequencies of the V109G polymorphism between MEN1 patients and controls. As these patients carry a germline mutation in the *MEN1* gene (11q13) and *p27* is located in a different locus (12p13.1), no significant differences in these frequencies were initially expected. However, a statistically significant higher frequency of the TT genotype at *p27* V109G (56.0%) was found in the MEN1 patients compared with the controls (41.0%; n=100, P=0.002; Table 1). Such an unanticipated finding can be obtained by chance when analyzing small sample groups (i.e. due to false positives). However, we investigated a large cohort of a total of 985 population-matched individuals, including 885 controls and 100 patients. Thus, the present data supports the occurrence of over-representation of the T allele at SNP rs2066827 in the MEN1 patients.

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Table 1

Genotype frequencies of the p27 V109G variant in the controls and MEN1 patients.

Phenotypic modulation of rs2066827 in MEN1 overall cohort

We then examined whether the higher frequency of the T allele in the MEN1 patient cohort might play a role in modulating their clinical features, which are presented in Supplementary Table 1. Although there was no association between the T allele and the development of specific MEN1-related tumors (HPT, pituitary adenomas, enteropancreatic tumors, and adrenocortical lesions), we found a strong association between the presence of at least one T allele and a higher number of affected neoplastic glands in patients carrying truncating *MEN1* mutations (three to four neoplastic glands vs one to two neoplastic glands; OR=18.33; P=0.002, CI=2.88–16.41). Our data indicated that MEN1 patients older than 30 years carrying a truncating *MEN1* mutation and the *p27* c.326 GT or TT genotypes at the SNP rs2066827 had a striking 18.3 times higher chance of developing tumors in three or all four major MEN1-related glands than patients at the same age, who carried the same type of mutation, but with the third genotype, GG, at the SNP rs2066827 genotype (assuming the co-dominant model). Similar results were obtained in all other models tested, with ORs varying from 4.3 to 20.8 (Tables 2 and 3). Among the 54 patients of our cohort with three to four tumors, only two were homozygous GG (3.7%); five out of 18 (27.8%) patients with one to two tumors showed this genotype, indicating that GG probably acts as a 'protective' genotype (P=0.002). To test the robustness of these findings, we used the conservative Bonferroni's

method to calculate an adjusted and more stringent threshold for significance according to the multiple testing performed (0.05/10=*P*<0.005). Our findings remained significant after this correction, indicating that our data were really robust. Interestingly, we have recently shown that the G allele is associated with the protection against pituitary adenoma development, more specifically, against sporadic corticotropinomas (Sekiya *et al.*, submitted). In addition, another recent study has shown that the same G allele at SNP p27 326 resulting in V109G is a genetic marker of better post-surgical outcomes for medullary thyroid carcinoma in Italian patients (22).

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Table 2

Comparison of p27 V109G allelic and genotype frequencies in the overall cohort of 100 MEN1-mutated patients and 885 population-matched healthy controls. The T (risk) allele was over-represented in MEN1 patients, whereas the G (protective) allele was over-represented in healthy individuals.

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Table 3

A total of 72 MEN1-mutated patients aged 30 years or older were included in the phenotypic modulation analysis. A strong correlation was found between the T allele of the p27 V109G variant (and the corresponding genotypes GT and TT) and the development of three or four MEN1-related tumors within the subgroup of 57 patients carrying truncating mutations. Conversely, the G allele was associated with the development of fewer tumors (one or two). Age was not correlated with the number of tumors developed (P=0.70, data not shown).

It is well accepted that pediatric MEN1 patients usually present fewer MEN1-related tumors than adults. However, as observed in our patient cohort and in others reported previously, the full manifestation of the disease (with the development of all four of the main MEN1-related tumors) does not occur in every adult patient older than 30 years (9, 10, 19, 20, 22, 23, 24, 25). In fact, we found that age was not a risk factor for developing multiple tumors in three or four glands among our patients aged 30 years or more (*P*=0.70). This result further strengthens our finding of the role of p27 V109G in tumor risk modulation in the MEN1 syndrome.

Phenotypic modulation of rs2066827 in MEN1 families

We further evaluated whether our finding was due to some unique SNP genotyping occurring in family(ies) investigated. Our cohort is composed of one large family harboring the c.308delC mutation and several other small families, hence we analyzed the individual families separately and the same pattern observed in the overall cohort (T allele being associated with more tumors) was found in both the large family and the remaining smaller families separably (Supplementary Table 3, see section on supplementary data given at the end of this article). This result indicates that the phenotypic features associated with *p27* SNP rs2066827 are not due to a MEN1 family or *MEN1* mutation specificity, but they are probably the result of a broader, and therefore more interesting, mechanism of regulation.

Discussion

To date, no evidence of robust genotype–phenotype correlation or disease modifiers for MEN1 syndrome has been described. The results obtained in the analysis of our cohort, clinically and genetically selected to be informative, revealed that *p27* rs2066827 polymorphism can influence the clinical outcome of MEN1 patients. This represents the identification of the first potentially strong genetic modifier of the phenotypic features of this complex syndrome.

Notably, previous reports have demonstrated the role of p27 in endocrine tumor risk and tumorigenesis, including studies on naturally occurring (MENX rats)

and engineered animal models with p27 deficiency, in which endocrine tumors develop at a high frequency (13, 26, 27). Moreover, germline *p27* mutations predispose patients to MEN4, also called MEN1-like syndrome (13, 14). Interestingly, *in vitro* studies have shown a functional correlation between the product of the *MEN1* gene (menin protein) and the expression of the *p27* gene that may be associated with the role of *p27* SNP rs2066827 as a phenotypic modifier of *MEN1*-mutated patients reported herein. Additionally, menin forms a transcriptional activation complex together with the MLL2 methyltransferase and RNA polymerase II, and this complex regulates the expression of the *p27* gene in pancreatic β-cells (15, 16, 28, 29). Therefore, it is postulated that the truncation of menin (resulting in a loss of function) in MEN1 patients/tumors consequently leads to decreased *p27* mRNA levels. Functional assessment of the *p27* V109G variant has not been reported so far, and the mechanism by which it might influence tumor susceptibility and tumorigenesis is currently unknown. Such nucleotide change affects an amino acid located in the domain mediating the binding of p27 to the $p38^{Jab1}$ protein, and this interaction mediates the nuclear export of p27 and its subsequent degradation (30). Thus, it has been speculated that this V109G variant might interfere with the interaction of p27 with $p38^{Jab1}$ and could therefore lead to an increased nuclear stability for p27 (31). The combination of *MEN1* truncation and the *p27* SNP V109 may potentially further impair p27 function.

Interestingly and consistent with the data from humans presented herein, the effects of genetic background and modifiers on the phenotype of embryonic lethality in *Men1*-knockout mouse models have been demonstrated previously. By backcrossing $Men1^{+/-}$ mice, the authors generated the C57BL/6 and 129S6/SuEv strains; a significant early lethality in the 129S6/SuEv strain was found after analyzing a large number of embryos (32). These data underline the importance of the genetic background in influencing the MEN1 phenotype and implicate a role for genetic modifiers in this syndrome in mice, in a finding parallel to the data presented herein for humans.

The reason as to why no association was found between SNP rs2066827 and tumor multiplicity in patients carrying missense *MEN1* gene mutations is currently unknown, but may be due to the smaller number of cases with missense mutations in our patient cohort. Alternatively, it is possible that the change of only one amino acid in the menin protein may lead to the activation of downstream molecular mechanisms that are not fully dependent on p27. Recent studies have shown that several *MEN1* missense mutations do not change the protein stability compared with WT menin (33), but may lead to unique gene expression profiles (34).

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 (35). As *p27* is a tumor susceptibility gene for multiple endocrine tumors in both humans and rats, and has recently been reported to be somatically mutated in small intestine neuroendocrine tumors (36), we decided to investigate its role specifically in modulating the risk of endocrine tumors. In conclusion, we identified *p27* rs2066827 as a genetic variant that influences the clinical manifestation of MEN1 adult patients carrying the most frequent type of *MEN1* gene defects, i.e. truncating mutations. To our knowledge, this is the first strong genotype–phenotype correlation found in the MEN1 syndrome and, if confirmed in other cohorts, it may improve genetic counseling and the clinical management of this highly complex syndrome. Furthermore, as *p27* is a downstream gene in the MEN1 tumorigenesis-driven pathway, a disease-modifying mechanism for the 'mosaic pleiotropism' described by Wermer in MEN1 may be involved (Supplementary Fig. 1, see section on supplementary data given at the end of this article), a hypothesis that might be worthy of *in vitro* testing in the future.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-14-0130.

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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Footnotes

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