Polymorphisms of cell cycle control genes influence the development of sporadic medullary thyroid carcinoma

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Abstract

Background: The role of key cell cycle regulation genes such as, *CDKN1B*, *CDKN2A*, *CDKN2B*, and *CDKN2C* in sporadic medullary thyroid carcinoma (s-MTC) is still largely unknown.

Methods: In order to evaluate the influence of inherited polymorphisms of these genes on the pathogenesis of s-MTC, we used TaqMan SNP genotyping to examine 45 s-MTC patients carefully matched with 98 controls.

Results: A multivariate logistic regression analysis demonstrated that *CDKN1B* and *CDKN2A* genes were related to s-MTC susceptibility. The rs2066827*GT + GG *CDKN1B* genotype was more frequent in s-MTC patients (62.22%) than in controls (40.21%), increasing the susceptibility to s-MTC (OR = 2.47; 95% CI = 1.048–5.833; *P* = 0.038). By contrast, the rs11515*CG + GG of *CDKN2A* gene was more frequent in the controls (32.65%) than in patients (15.56%), reducing the risk for s-MTC (OR = 0.174; 95% CI = 0.048–0.627; *P* = 0.0075). A stepwise regression analysis indicated that two genotypes together could explain 11% of the total s-MTC risk. In addition, a relationship was found between disease progression and the presence of alterations in the *CDKN1A* (rs1801270), *CDKN2C* (rs12885), and *CDKN2B* (rs1063192) genes. WT rs1801270 *CDKN1A* patients presented extrathyroidal tumor extension more frequently (92%) than polymorphic *CDKN1A* rs1801270 patients (50%; *P* = 0.0376). Patients with the WT *CDKN2C* gene (rs12885) presented larger tumors (2.9 ± 1.8 cm) than polymorphic patients (1.5 ± 0.7 cm; *P* = 0.0324). On the other hand, patients with the polymorphic *CDKN2B* gene (rs1063192) presented distant metastases (36.3%; *P* = 0.0261).

Conclusion: In summary, we demonstrated that *CDKN1B* and *CDKN2A* genes are associated with susceptibility, whereas the inherited genetic profile of *CDKN1A*, *CDKN2B*, and *CDKN2C* is associated with aggressive features of tumors. This study suggests that profiling cell cycle genes may help define the risk and characterize s-MTC aggressiveness.

European Journal of
Endocrinology
(2014) 171, 761–767

Introduction

Although responsible for no more than 3–5% of all thyroid cancers, medullary thyroid carcinoma (MTC) is responsible for more than 14% of thyroid cancer-related deaths (1, 2). The majority of MTC cases (>75%) are sporadic MTC (s-MTC), even though up to 40% of these so-called sporadic cases may be caused by somatic RET mutations (3). Despite the importance of the RET receptor, it is clear that other signal transduction pathways, tyrosine kinase receptors, and tumors suppressor genes are involved in MTC tumorigenesis

and progression (4). In fact, the molecular basis of s-MTC is still poorly understood (5). It is plausible that, similar to other malignancies, s-MTC is caused by multiple common genetic variants in different susceptibility genes commonly called ‘low-penetrance genes’ (6).

Dysregulation of the cell cycle is a hallmark of many cancers (7, 8, 9). Control and timing of the cell cycle involve checkpoints and regulatory pathways that ensure the fidelity of DNA replication and chromosome

segregation (10). Alterations of genes involved in the G1 phase of the cell cycle, including the cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs), are common events in neoplastic development of a series of different types of tumors (11). Their role in s-MTC is still largely unknown.

Thyroid tumors show low expression of the CDKI P27 (Kip1), and recent evidence has demonstrated that P27 is downregulated by the active *RET* mutant (12). These data suggest that a decreased P27 (CDKN1B) activity is an important event during thyroid tumorigenesis. However, *p27*^{-/-} mice develop MEN-like tumors only in combination with the loss of another CDKI, *p18* (Ink4c). Hence, it is possible that *p18* (*Cdkn2c*) and *p27* are functional collaborators in the suppression of tumorigenesis, and that the loss of both may also be important to MTC development (12).

There have been some reports associating polymorphisms of cell cycle genes with s-MTC (13, 14, 15, 16). The *CDKN1B* V109G polymorphism was reported to influence the clinical course of patients presenting sporadic MTC (15). In addition, cell cycle and apoptosis regulators have long been recognized as critical in the initiation of malignant cell proliferation and this study has recently demonstrated the influence of the presence of a C homozygous allele for the *TP53* gene on the susceptibility to s-MTC (17).

This study aimed to evaluate the role of genetic variations in key cell cycle regulation genes *CDKN1A*, *CDKN1B*, *CDKN2A*, *CDKN2B*, and *CDKN2C* in sporadic medullary thyroid cancer pathogenesis.

Patients and methods

This study was approved by the Ethics and Research Committees of the Federal University of São Paulo (Unifesp) and the State University of Campinas (Unicamp).

Patients

A total of 45 patients diagnosed with s-MTC based on cytology and elevated serum calcitonin levels, and who have signed an informed consent form, were enrolled in this study. As described previously, none of the s-MTC patients had any other types of malignant tumor or any history of thyroid tumor in first-degree relatives (14). All patients were sequenced for the complete *RET* gene and no mutation was identified. Ninety-eight healthy individuals from the same region and matched for sex and age with the enrolled patients served as controls. Individuals with a history of past thyroid disease and antecedents of

malignancy were excluded. An in-person questionnaire, described previously, was used to collect demographic and health condition information (17). All data, including nodule size, tumor histological features, and laboratory test results, were confirmed using the hospital records of the patient. All patients were managed using the same standard protocol according to the recommendations of current guidelines (18, 19).

Genotyping

DNA was extracted from peripheral blood leukocytes obtained from both patients and controls using a standard phenol-chloroform protocol. Nine polymorphisms were genotyped: *CDKN1A* (rs1801270 and rs1059234), *CDKN1B* (rs2066827 and rs34330), *CDKN2A* (rs11515), *CDKN2B* (rs2069426, rs3731239, and rs1063192), and *CDKN2C* (rs12885) using the TaqMan system (Applied Biosystems), as described previously (17). These SNPs were chosen because they have previously been associated with thyroid carcinoma or other tumor risks as described in Table 1 (13, 15, 16, 20, 21, 22).

Statistical analysis

The *t*-test and the Fisher exact test were employed in order to evaluate differences in age and sex using the SAS statistical software (Statistical Analysis System, version 9.1.3, 2002–2003). For comparison of continuous or orderable variables between two groups, we applied the Mann–Whitney *U* test. The Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between SNPs were performed using the HAPLOVIEW software (23). Logistic and stepwise regressions were used to analyze the association between polymorphisms and genes using the

Table 1 Sequence-specific primers used for genotyping *CDKN1B*, *CDKN1A*, *CDKN2C*, *CDKN2A*, and *CDKN2B* genes and the corresponding reference of their description in the risk of other tumors.

Genes	SNP	Registration applied
<i>p27</i> – <i>CDKN1B</i>	rs2066827 rs34330	C_11916245_10 C_2402292_10
<i>p21</i> – <i>CDKN1A</i>	rs1801270	C_14977_20
<i>p18</i> – <i>CDKN2C</i>	rs12885	C_1452499_10
<i>p16</i> – <i>CDKN2A</i>	rs11515 rs3088440	C_12096259_10 C_16008027_10
<i>p15</i> – <i>CDKN2B</i>	rs2069426 rs3731239 rs1063192	C_15858974_10 C_27974751_10 C_2618046_10

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R software (24). All *P* values were adjusted for multiple comparisons by the Bonferroni correction. *Post-hoc* statistical power of the sample was evaluated using the GPower software (25), with a statistically significant level of ≤ 0.05 for all tests.

Results

Sporadic MTC patients (15 males and 30 females; 42.20 ± 12.30 years old) and controls (30 males and 68 females; 40.30 ± 11.20 years old) were similar concerning age ($P=0.356$) and sex ($P=0.745$). The rs1059234 polymorphism of *CDKN1A* was not in the HWE, possibly because of the relatively small size of the studied groups and the high genetic heterogeneity of the Brazilian population. Thereby, this polymorphism was excluded from further statistical analyses.

Influence of cell cycle control gene polymorphisms on the susceptibility to s-MTC

A univariate logistic regression, given in Table 2, showed associations between s-MTC and the following genotypes:

rs2066827*GT ($P=0.0014$; OR=3.48; 95% CI=1.67–7.29) and rs2066827*TT ($P=0.0299$; OR=0.41; 95% CI=0.20–0.86), in the *CDKN1B* gene; and rs11515*C/C ($P=0.0324$; OR=2.51; 95% CI=1.02–6.16) and rs11515*CG ($P=0.0432$; OR=0.42; 95% CI=0.17–1.03), in the *CDKN2A* gene.

A multivariate logistic regression analysis confirmed the importance of the inheritance of *CDKN1B* and *CDKN2A* gene variants (OR=2.472 and OR=0.174 respectively), as detailed in Table 3.

The rs2066827*GT+GG *CDKN1B* genotype was over-represented in s-MTC patients (62.22%) when compared with controls (40.21%; $P=0.038$), increasing substantially the susceptibility to s-MTC (OR=2.47; 95% CI=1.048–5.833; $P=0.038$). By contrast, the polymorphic rs11515*CG+GG of the *CDKN2A* gene was more expressed in the control population (32.65%) than in s-MTC patients (15.56%; $P=0.0075$), reducing the risk of developing s-MTC (OR=0.174; 95% CI=0.048–0.627; $P=0.0075$).

A stepwise regression analysis indicated that, in our sample, *CDKN1B* rs2066827*GT genotype contributed to 8% for the total risk of developing an s-MTC, whereas *CDKN2A* rs11515*CC contributed to 3%. These two

Table 2 Genotyping characteristics of patients with sporadic medullary thyroid cancer (s-MTC) compared with the group of controls.

Genes	SNP	Genotype	s-MTC (%)	Controls (%)	<i>P</i> value ^a	OR	95% CI	
							High	Low
<i>CDKN1B</i>	rs2066827	GG	37.78	59.79	0.3155	0.39	0.09	1.68
		GT	57.78	27.84	0.0014	3.48	1.67	7.29
		TT	4.44	12.37	0.0299	0.41	0.20	0.86
	rs34330	CC	60.00	51.04	0.5940	1.46	0.71	2.98
		CT	37.78	39.58	1.0000	0.95	0.46	1.96
<i>CDKN1A</i>	rs1801270	TT	2.22	9.38	0.2070	0.28	0.05	1.74
		AA	82.93	67.01	1.0000	1.17	0.28	4.84
		AC	17.07	27.84	0.4381	0.59	0.25	1.41
		CC	0.00	5.15	0.5577	1.53	0.69	3.39
		GG	70.73	77.32	0.3949	0.71	0.32	1.57
<i>CDKN2C</i>	rs12885	GT	26.83	22.68	0.5631	1.27	0.56	2.86
		TT	2.44	0.00	0.2058	6.64	0.07	622.36
		CC	84.44	67.35	0.0324	2.51	1.02	6.16
<i>CDKN2A</i>	rs11515	CG	15.56	31.63	0.0432	0.42	0.17	1.03
		GG	0.00	1.02	0.8335	0.71	0.01	66.93
		AA	86.67	75.53	1.0000	0.75	0.23	2.42
<i>CDKN2B</i>	rs2069426	CA	4.44	14.89	0.1915	0.31	0.07	1.28
		CC	8.89	9.57	0.1858	2.34	0.90	6.05
		AA	55.00	57.89	1.0000	0.72	0.36	1.46
	rs3731239	AG	45.00	30.53	0.8044	1.51	0.73	3.15
		GG	0.00	11.58	1.0000	0.94	0.31	2.82
		CC	55.56	53.13	0.4257	2.14	0.77	5.96
	rs1063192	TC	26.67	37.50	0.6055	0.61	0.28	1.33
		TT	17.78	9.38	1.0000	1.10	0.54	2.24
		GG	55.56	53.13	0.4257	2.14	0.77	5.96

^aCorrected by the Bonferroni adjustment.

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Table 3 Comparison between genotyping profiles *CDKN1B* and *CDKN2A* of patients with sporadic medullary thyroid carcinoma (s-MTC) and control population, using multivariate logistic regression analysis.

Genes	SNP	Genotype	s-MTC (%)	Controls (%)	P value ^a	OR	95% CI	
							High	Low
<i>CDKN1B</i>	rs2066827	GG	37.78	59.79	0.0388	2.472	1.048	5.833
		GT+TT	62.22	40.21				
<i>CDKN2A</i>	rs11515	CC	84.44	67.35	0.0075	0.174	0.048	0.627
		CG+GG	15.56	32.65				

^acorrected by Bonferroni adjustment.

genotypes together could explain 11% of the total s-MTC risk (Fig. 1).

Genotype relationship with features of aggressiveness in patients with s-MTC

For these analyses, patients were divided into two groups: WT (homozygous normal alleles) and polymorphic (a combination of homozygous polymorphic + heterozygous alleles), for each gene. Clinical and pathological characteristics, including age, sex, tumor stage (T, N, and M), and size, extra thyroidal extension, serum calcitonin levels, and recurrence in patients were analyzed and compared with their genetic profile.

The Mann–Whitney *U* test demonstrated that patients with WT gene presented extrathyroidal tumor extension more frequently (92%) than patients with polymorphic gene in rs1801270 *CDKN1A* gene (50%; *P*=0.0376).

Patients with WT *CDKN2C* gene (rs12885) presented larger tumors (2.9 ± 1.8 cm) than patients with polymorphic gene (1.5 ± 0.7 cm; *P*=0.0324). By contrast, only patients with polymorphic *CDKN2B* gene (rs1063192) presented distant metastases (36.3%; *P*=0.0261).

We were unable to find any other associations between the profile of the studied genes and the clinical or pathological characteristics of patients.

Discussion

This case–control study is, to the best of our knowledge, the first to investigate the association of *CDKN1B*, *CDKN1A*, *CDKN2C*, *CDKN2A*, and *CDKN2B* polymorphisms and the susceptibility to s-MTC. Besides the demonstration that *CDKN1B* and *CDKN2A* genes were related to s-MTC susceptibility, this study also found evidences that *CDKN1A*, *CDKN2B*, and *CDKN2C* gene variants are associated with tumor features of aggressiveness.

Recent studies have indicated that SNPs of genes in cell cycle control play an important role in carcinogenesis and may lead to altered susceptibility to different cancers (26, 27, 28). An association of *CDKN1B* (rs2066827) and *CDKN2A* (rs11515) gene variants was found with the presence of s-MTC. It was demonstrated that the rs2066827*GT+GG *CDKN1B* genotype was over-represented in s-MTC patients when compared with controls, increasing the susceptibility to this tumor.

The codon 109 in the *CDKN1B* gene SNP results in a valine to glycine (T>G) substitution (V109G – rs2066827) (29). The G allele of the *CDKN1B* gene (rs2066827) has been associated with an increased risk for thyroid (13), squamous cell (28), prostate, and breast cancers (27, 30). However, whether this polymorphism is associated with a better or worse prognosis remains uncertain (13, 21, 28, 31). Pasqualini *et al.* also found differences in the frequency of the WT (TT – 53.6%) and polymorphic alleles

Figure 1 Graphical representation of the relative contribution of the *CDKN1B* and *CDKN2A* genes to sporadic medullary thyroid carcinoma risk according to a stepwise regression analysis.

(TG+GG – 46.4%) between patients and controls ($P=0.048$), suggesting a risk of the disease in individuals bearing the *CDKN1B* rs2066827 polymorphic allele (13). The V109G polymorphism falls within a region (amino acids 97–151) that physically interacts with the activation of the domain-binding protein, which triggers the proteolytic degradation of P27 (32). In fact, a reduced P27 expression has been associated with a poor clinical prognosis in head and neck tumors (33). In addition, it has been demonstrated that amino acid changes around position 108, such as the ones induced by V109G polymorphism, can reduce P27kip1 cytosolic translocation, increasing its nuclear stability (31, 34). However, no evidence has been provided to support the association of *CDKN1B* polymorphisms with tumor progression or outcome in MTC (15).

In the study population, polymorphic rs11515*CG+GG of *CDKN2A* gene was expressed more in the control population than in s-MTC patients, hence presenting a protective effect to s-MTC development. *CDKN2A* gene polymorphisms have largely been investigated in different types of cancers (20, 22, 35). The SNP (rs11515 – C98A) *CDKN2A* gene determines a non-synonymous serine-to-arginine substitution located in the 3'-UTR of exon 3 (36). In fact, similarly, we observed for s-MTC, *CDKN2A* gene rs11515 polymorphism was also considered a protective factor against cervical cancer (35). *CDKN2A* gene (rs11515) polymorphism may affect the DNA-binding zinc finger motif, thus modifying p21 expression and function (37). Additionally, the overexpression of p21 has been demonstrated to prevent mammalian cell proliferation and inhibit all cyclin-CDK complexes, suggesting that p21 is a universal inhibitor of cyclin-CDK complexes (38).

We also found a relationship between tumor aggressiveness and the presence of variants of the *CDKN1A* (rs1801270), *CDKN2C* (rs12885), and *CDKN2B* (rs1063192) genes.

Patients presenting polymorphic *CDKN1A* (rs1801270) less frequently have extrathyroidal tumor extension, suggesting that the inheritance of this *CDKN1A* polymorphism may be associated with a better outcome. A study of cervical cancer in a Chinese population also showed a significant association between the polymorphic allele of the *CDKN1A* (rs1801270) gene and a decreased risk of disease (39). *CDKN1A* (rs1801270) polymorphisms were demonstrated to increase the risk of second primary malignancy in patients with index squamous cell carcinoma of head and neck (31). The SNP (rs1801270) within the *CDKN1A* codon 31 results in a substitution of

arginine for serine in a conserved region of the protein and may affect the DNA-binding affinity and thus the physiological function of p21 (40). The p21 protein plays a role in cell cycle regulation. It binds to cyclin-CDK complexes to arrest cell cycle progression at the G1 phase. The transcription of p21 is partially regulated by p53, which binds to the p21 promoter and induces expression of p21. Therefore, alterations in the functional domains as well as in the promoter region of p21 could be affected by SNPs and affect p21 functionality (41). *CDKN1A* is often misregulated in human cancers, but depending on the cellular context and other circumstances, it can act both as a tumor suppressor and as an oncogene (42).

The molecular mechanism underlying a possible protective effect of the *CDKN1A* (rs1801270) and *CDKN2C* (rs12885) polymorphic alleles in cancer is unclear. The *CDKN2C* gene, involved in cell cycle regulation, is located at 1p32. A frequent finding in MTC tumors is the loss of heterozygosity at chromosome 1p, indicating a potential tumor suppressor gene in this locus (4). Homozygous deletion of the *CDKN2C* gene was also observed in the human MTC cell line (TT) bearing a RET mutation (16). These data indicate that the *CDKN2C* gene may act as a tumor suppressor and may facilitate MTC development (4).

Regarding *CDKN2B* gene polymorphism (rs1063192), we found distant metastases only in polymorphic patients, suggesting an association of this polymorphism with tumor aggressiveness. The polymorphism rs1063192 is located within a gene encoding *CDKN2B*, which is also known as *p15* (*CDKN2B*), a well-known tumor suppressor gene involved in the retinoblastoma (Rb) pathway (43). This region on chromosome 9p21 was implicated as a hotspot locus showing the association with various diseases, including myocardial infarction (44, 45), coronary artery disease (46, 47), type 2 diabetes (48, 49), glioma (50), and endometriosis (51); however, there is no report of a possible effect in thyroid cancer.

In conclusion, we found evidence that variations in some cell cycle genes are associated with both susceptibility and progression of s-MTC. As usually observed in studies concerning the effects of polymorphisms, no one demonstrated a highly significant association (52). Although functional evaluation and larger studies are needed to confirm the observed associations, our findings contribute to the identification of the various factors involved in the pathogenesis of s-MTC. It is suggested that profiling cell cycle genes may help define the risk for s-MTC and characterize tumor aggressiveness.

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.

Funding

This study was supported by the State of São Paulo Research Foundation (FAPESP) under the grant number 07067-5. L S Ward, R M B Maciel, and J M Cerutti are researchers of the National Council for Scientific and Technological Development (CNPq).

Acknowledgements

The authors thank the team of statisticians of the Faculty of Medical Sciences and Espaço da Escrita – Coordenadoria Geral da Universidade – UNICAMP – for the language services provided and for all their valuable suggestions and insights.

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Received 6 June 2014

Revised version received 5 September 2014

Accepted 2 October 2014