Analysis of somatic mutations in *BRAF*, *CDKN2A/p16* and *PI3KCA* in patients with medullary thyroid carcinoma

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Received December 31, 2014; Accepted October 14, 2015

DOI: 10.3892/mmr.2015.4731

Abstract. Medullary thyroid carcinoma (MTC), a neuroendocrine tumor originating from thyroid parafollicular cells, has been demonstrated to be associated with mutations in *RET*, *HRAS*, *KRAS* and *NRAS*. However, the role of other genes involved in the oncogenesis of neural crest tumors remains to be fully investigated in MTC. The current study aimed to investigate the presence of somatic mutations in *BRAF*, *CDKN2A* and *PI3KCA* in MTC, and to investigate the correlation with disease progression. DNA was isolated from paraffin-embedded tumors and blood samples from patients with MTC, and the hotspot somatic mutations were sequenced. A total of 2 novel *HRAS* mutations, p.Asp33Asn and p.His94Tyr, and polymorphisms within the 3' untranslated region (UTR) of CDKN2A (rs11515 and rs3088440) were identified, however, no mutations were observed in other genes. It was suggested that somatic point mutations in *BRAF*, *CDKN2A* and *PI3KCA* do not participate in the oncogenesis of MTC. Further studies are required in order to clarify the contribution of the polymorphisms identified in the 3'UTR of *CDKN2A* in MTC.

Introduction

Medullary thyroid carcinoma (MTC), a neuroendocrine tumor originating from thyroid parafollicular cells, accounts for ~4% of thyroid cancer cases (1). The majority are sporadic cases, however, 20-25% occur as a hereditary syndrome termed multiple endocrine neoplasia type 2 (MEN 2A and MEN 2B) and as familial MTC, both of which are associated with germline mutations in the *RET* oncogene (2).

Mutations in the *RET* oncogene have previously been identified in the tumor tissue of up to 64% of sporadic MTC cases (3). In addition, *RAS* gene mutations are observed in 10% of *RET*-negative cases and are associated with a subset of tumors with less aggressive behavior (4). While certain studies identified that ~90% of sporadic MTCs exhibited mutually exclusive mutations in *RET*, *HRAS* and *KRAS* (4-8), Moura *et al* (3) reported the presence of the *RAS* mutation in one case with *RET*-positive sporadic MTC and Rapa *et al* (9) identified no *RAS* mutations in 49 examined cases. Nevertheless, the clinical phenotype of sporadic and inherited MTCs is heterogeneous even in the presence of the same mutation; however the molecular mechanisms underlying the pathology remain to be fully elucidated.

In addition, it remains unclear whether there is a modulatory role in MTC tumor progression for additional genes such as *BRAF*, *CDKN2A* and *PI3KCA*. These genes participate in the tumorigenesis of several types of human malignancies such as tumors derived from neural crest cells, including melanoma, pheochromocytoma and paraganglioma (10-12).

BRAF, like *RET* and *RAS*, is involved in the mitogen-activated protein kinase pathway and has a well-established role in the pathogenesis of malignancies such as melanoma and papillary thyroid cancer (13). Nevertheless, the contribution in the tumorigenesis of MTC remains controversial. A previous study reported a high prevalence of the p.Val600Glu *BRAF* mutation in sporadic MTC cases (14); however, subsequent studies did not confirm this observation (3,9,15,16).

An additional tumor suppressor gene, *CDKN2A/p16*^{INK4A}, is involved in the G_1/S transition in the cell cycle. Mutations and deletions have been identified in melanoma, and polymorphisms in its 3' untranslated region (UTR) have been associated with earlier progression from primary to metastatic disease (17). By contrast, polymorphisms in another tumor suppressor gene, *CDKN1B*, which is in the same *CDKN* family, are associated with improved outcomes (18).

Additionally, *PI3KCA* is a gene that serves an important role in signaling pathways and cell growth, and contributes to tumorigenesis in several types of human malignancy (19,20).

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Key words: medullary thyroid cancer, somatic mutation, *RET*, *BRAF*, *RAS*, *CDKN2A*, *PI3KCA*

Table I. Primers used in the present study.

Gene	Forward primer	Reverse primer
BRAF exon 15	5'-AACTCAGCAGCATCTCAGGG-3'	5'-CTTCATAATGCTTGCTCTGATAG-3'
CDKN2A exon 1	5'-ACCCTGGCTCTGACCATTC-3'	5'-CAGGTCACGGGCAGAC-3'
CDKN2A exon 2	5'-GACCTCAGGTTTCTAACGCC-3'	5'-CATATATCTACGTTAAAAGGCAGGAC-3'
PI3KCA exon 9	5'-TGGCAGTCAAACCTTCTCTC-3'	5'-GAGAAAGTATCTACCTAAATCCACAGA-3'
PI3KCA exon 20	5'-AAATGTTTTGGTGTTCTTAATTTATTC-3'	5'-GCAGCCAGAACTCTTTATTTTG-3'
C-kit exon 9	5'-GCCAGGGCTTTTGTTTTCTT-3'	5'-AGCCTAAACATCCCCTTAAATTG-3'
C-kit exon 11	5'-AACCATTTATTTGTTCTCTCTCCA-3'	5'-CCACTGGAGTTCCTTAAAGTCA-3'
C-kit exon 17	5'-TGGTTTTCTTTTCTCCTCCAAC-3'	5'-GGACTGTCAAGCAGAGAATGG-3'
HRAS exon 2	5'-GGCAGGAGACCCTGTAGGAG-3'	5'-AGCTGCTGGCACCTGGAC-3'
HRAS exon 3	5'-GTCCCTGAGCCCTGTCCTC-3'	5'-CAGCCTCACGGGGTTCAC-3'
HRAS exon 4	5'-CTCTCGCTTTCCACCTCTCA-3'	5'-GGGTGGAGAGCTGCCTCA-3'
KRAS exon 2	5'-TTAACCTTATGTGTGACATGTTCTAA-3'	5'-GGTCCTGCACCAGTAATATGC-3'
KRAS exon 3	5'-AGACTGTGTTTCTCCCTTCTCA-3'	5'-TGGCATTAGCAAAGACTCAAA-3'
KRAS exon 4	5'-GATATTTGTGTTACTAATGACTGTGCT-3'	5'-TTATGATTTTGCAGAAAACAGATC-3'
NRAS exon 2	5'-TCGCCAATTAACCCTGATTAC-3'	5'-TCCGACAAGTGAGAGACAGG-3'
NRAS exon 3	5'-TGGGCTTGAATAGTTAGATGC-3'	5'-AGTGTGGTAACCTCATTTCCC-3'

However, the role of this gene in the tumorigenesis of MTC remains to be fully understood.

Therefore, the current study aimed to verify the prevalence of somatic mutations in *BRAF*, *CDKN2A* and *PI3KCA*, which have already been described in other neural crest-derived tumors, and to determine the possible supporting role of these genes in the tumorigenesis of MTC.

Patients and methods

Patients and tissue samples. From 128 patients with MTC assessed at the Multiple Endocrine Neoplasia outpatient clinic at the Universidade Federal de Sao Paulo (Sao Paulo, Brazil) between February 2007 and June 2013, formalin-fixed paraffin-embedded (FFPE) tumor tissues were selected from 31 patients on the basis of the availability of tumor tissues, with no other selection criteria. DNA extraction was subsequently performed, using an in-house method as previously described (21). Subsequent to DNA extraction, 20 samples (from 13 males and 7 females; mean age, 40.55±16.74 years) provided the appropriate quantity and quality of DNA. The study was approved by the Ethics and Research Committee of the Universidade Federal de Sao Paulo (protocol number 1945/10), and all patients provided informed consent. Additionally, 1,092 genotypes of variant frequencies (single nucleotide polymorphisms; SNPs) were obtained from the 1000 Genomes database (http://www.1000genomes.org/) as a population genetics control.

DNA extraction and genotyping. DNA from peripheral blood and somatic DNA from 10-μm sections of FFPE MTC tissues was extracted using an in-house method as previously described (21). Polymerase chain reaction (PCR) was performed to amplify DNA corresponding to hotspot exons 2, 3 and 4 of *HRAS*; 2, 3 and 4 of *KRAS*; 2 and 3 of *NRAS*; 15 of *BRAF*; 9 and 20 of *PI3KCA;* and exons 2, 3 and the 3'UTR of the *CDKN2A* gene. The sequences of the primers are listed in Table I. The reactions were performed using 10 pM of each specific primer, 2.5 μl PCR buffer, 200 μM dNTP, 1.5 μM $MgCl_2$ and 0.2 units *Taq* DNA polymerase (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) in a 25-μl total reaction volume. The cycling conditions were as follows: 5 min at 95˚C, 38 cycles of 45 sec at 95˚C, 45 sec for annealing and 1 min at 72˚C, and a final elongation for 10 min at 72˚C. The PCR products were purified using the Illustra GFX PCR DNA and Gel Purification kit (GE Healthcare Life Sciences, Chalfont, UK) and were subject to sequencing using the Sanger method, with the Big Dye™ Terminator Cycle Sequencing Ready Reaction kit and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific). Gel electrophoresis of the PCR products was performed to analyze product quality and yield using a 1.8% agarose gel and a DNA ladder.

In silico analysis of HRAS mutations and CDKN2A polymorphisms. Mutational analysis of *HRAS* was performed by the use of Project HOPE to obtain structural information from the analysis of PDB-file 1CTQ (22). The *in silico* analysis for the *CDKN2A* polymorphisms was performed using the Functional Single Nucleotide Polymorphism database (http://compbio.cs.queensu.ca/F-SNP/) as previously described (23). This database provides information regarding potential deleterious effects of SNPs with respect to splicing, transcription, translation and post-translation based on SNP functional significance (FS). The FS score for neutral SNPs is 0.1764, whereas the FS score for disease-associated SNPs is in the range of 0.5-1.

Statistical analysis. The allele and genotype frequencies were compared between patients with MTC and the 1000 Genomes database controls using a χ^2 test. The clinicopathological features of patients carrying each of the polymorphisms rs11515 and rs3088440 were compared with those of patients without such

Table II. Summary of patient clinicopathological features and molecular analysis.

Patient	Gender	Age at diagnosis (y)	pTNM[a]	Germline *RET* allele	Somatic *RET* allele	Somatic *H-*, *K-*, *NRAS* allele	Somatic *CDKN2A*
1	M	28	T2N1bMx	WT	WT	HRAS_p.Asp33Asn	rs11515
2	F	25	T3N1bMx	WT	p.Met918Thr	-	WT
3	M	38	T1N1aMx	WT	WT	NA	rs11515/rs3088440
4	M	56	T3N1bMx	WT	WT	WT	WT
5	F	49	T2NxMx	WT	p.Gln681Stop	-	WT
6	M	69	T2N0Mx	WT	WT	HRAS_p.Gln61Arg	rs3088440
7	M	27	T4N1Mx	WT	WT	WT	WT
8	M	51	T3N1bMx	WT	p.Met918Thr	-	rs11515
9	F	56	T1N1bMx	WT	WT	HRAS_p.Asp33Asn	WT
10	M	41	T4N1bMx	WT	WT	HRAS_p.His94Tyr	WT
11	M	27	T1N1aMx	p.Cys634Arg	-	-	rs11515/rs3088440
12	F	21	T1N1aMx	p.Gly533Cys	-	-	rs11515
13	M	61	T1N1aMx	p.Gly533Cys	-	-	WT
14	F	22	T2N0Mx	p.Cys634Arg	-	-	rs11515/rs3088440
15	M	43	T2N0Mx	p.Cys634Arg	-	-	rs11515
16	M	72	T1N0Mx	p.Cys634Arg	-	-	WT
17	M	45	T1N1aMx	p.Cys634Arg	-	-	rs3088440
18	F	31	T1NxMx	p.Cys634Arg	-	-	rs3088440
19	F	15	T1N1aMx	p.Cys634Arg	-	-	rs3088440
20	M	40	T1N0Mx	p.Gly533Cys	-	-	WT

[a]TNM (Tumor, Node, Metastasis)/American Joint Committee on Cancer staging system. M, male; F, female; y, years; NA, not available; WT, wild-type.

polymorphisms using the χ^2 test or the Student's unpaired t-test as appropriate. $P<0.05$ was considered to indicate a statistically significant difference, and the Hardy-Weinberg equilibrium was evaluated. Statistical analyses were performed using SPSS, version 22.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism, version 3.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Screening of the RET, HRAS, KRAS and NRAS genes. Mutational screening of the *RET* gene was performed on all 20 patients. A total of 10 cases were identified to be familial tumors as confirmed by the presence of a germline mutation. In total, 30% of the sporadic cases (3/10) presented with a *RET* somatic mutation. The clinicopathological features and molecular analysis, including tumor staging based on the American Joint Committee in Cancer staging system (24), are summarized in Table II.

To investigate exclusive causative mutations in cases of sporadic MTC other than *RET* mutations, *HRAS*, *KRAS* and *NRAS* were screened for somatic mutations in the hotspots. The majority of these patients had been previously analyzed for RET germline mutations as part of our routine evaluation, and for RET somatic mutations in a previous study (25) Two novel *HRAS* mutations, p.Asp33Asn and p.His94Tyr, were detected in *RET*-negative MTC tumors. Mutational analysis using Project HOPE suggests that the p.His94Tyr mutation is deleterious, and that the p.Asp33Asn mutation is likely to be damaging (Fig. 1). No differences in the clinical presentation or histological observations were noted between patients with MTC that had a mutation in the *RAS* gene (Table II).

No somatic mutations were identified in exon 15 of *BRAF* or in exons 9 and 20 of *PI3KCA*. Patient 9 was not analyzed for somatic mutations in *PI3KCA* due to an insufficient number of tumor samples.

Despite not having identified somatic mutations in *CDKN2A* hotspots, two polymorphisms in the 3'UTR regulatory region, 500 C→G (rs11515) and 540 C→T (rs3088440), were identified in the patients observed. The heterozygotic pattern of the two SNPs was observed in the same proportion, 7/20 MTC (35%). The genotype distribution was identified to be in the Hardy-Weinberg equilibrium and was not identified to exhibit linkage disequilibrium. To investigate whether the observed polymorphisms were limited to a somatic event, they were further analyzed in the peripheral blood, which confirmed germline inheritance. The *in silico* analysis demonstrated that the *CDKN2A* polymorphisms rs11515 and rs3088440 are located in the transcriptional regulatory region and that the nucleotide alterations may affect the binding of transcription factors.

In seven cases, it was possible to detect the presence of these polymorphisms in the secondary tumors in the lymph nodes (tumor metastases), however no differences between the genotypes of the primary and secondary tumors were observed, indicating that there was no additional somatic event in *CDKN2A* involved in the metastatic process. This analysis

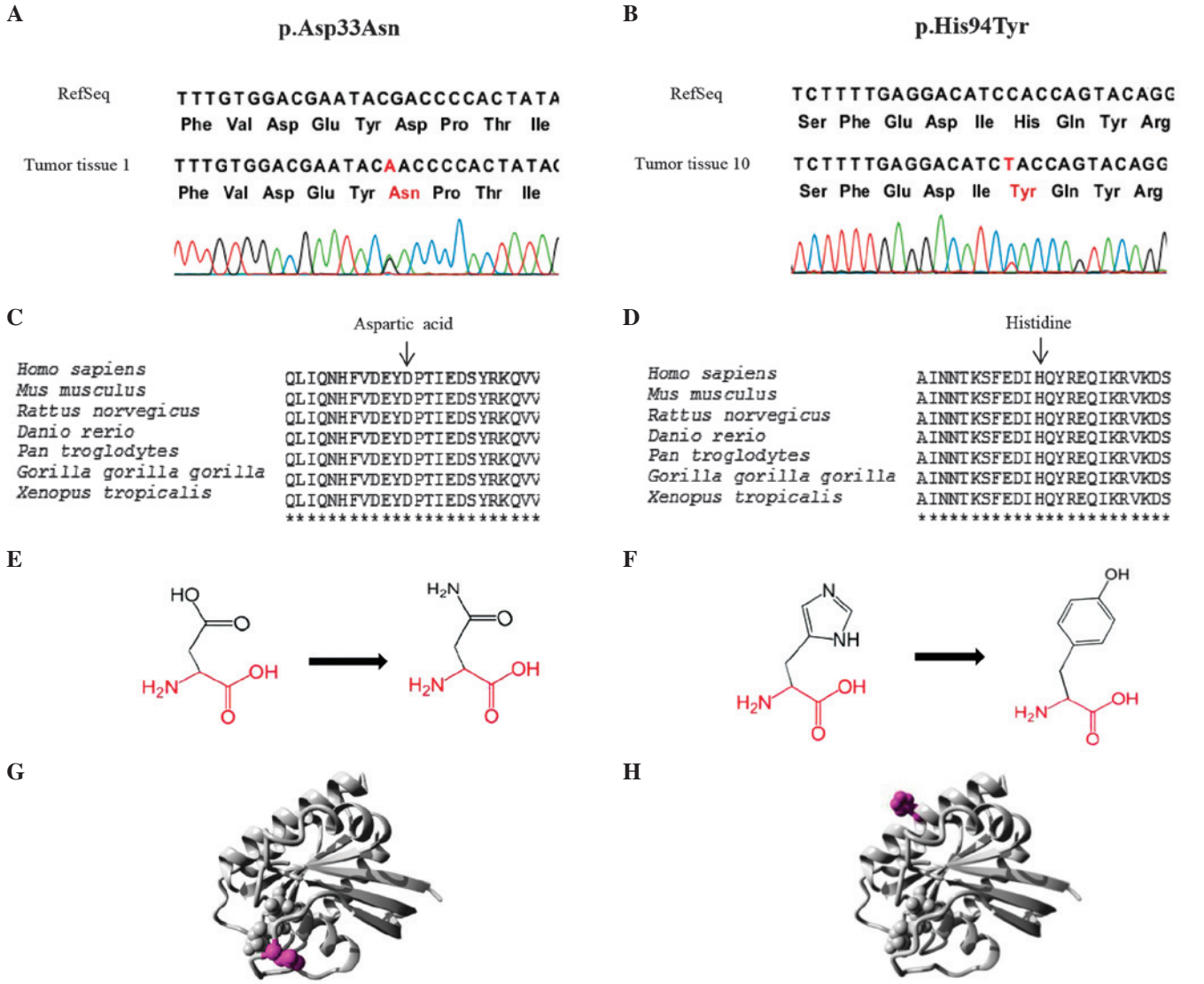

Figure 1. Mutational analysis of the *HRAS* somatic mutations p.Asp33Asn and p.His94Tyr. Electropherogram of tumor tissues (A) 1 and (B) 10; (C and D) sequence alignment of human *HRAS* protein residues in which the position of the conserved amino acids are indicated (arrows); multiple sequence alignment was generated with Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/), *indicates that the residues in the column were identical in all sequences in the alignment; schematic structures of the (E) original and (F) mutant amino acids in the two *HRAS* mutations; (G and H) structure of the *HRAS* proteins in ribbon-presentation; gray, protein; magenta, side chain of the mutation (p.Asp33Asn and p.His94Tyr).

was additionally performed for *BRAF* and *PI3KCA* in metastatic tissues.

No associations between the polymorphisms and the clinicopathological features observed were identified (Table III). In addition, the frequency of the SNPs was compared with a population genetics control, and there was no significant difference between the two populations (Table IV).

Discussion

The adjuvant role of additional genes in the tumorigenesis of MTC was investigated in the current study through analysis of tumor tissues from 20 patients. Screening in hotspot regions of *BRAF*, *CDKN2A* and *PI3KCA* did not identify any somatic mutations in the coding region. In addition, the results of the current study were not in agreement with the *BRAF* mutation frequency of 68.2% observed by Goutas *et al* (14). This suggests that *BRAF* does not serve an important role in the tumorigenesis of MTC. The observations of the current study concerning MTC are consistent with a previous study that demonstrated that somatic mutations in genes other than *RET* and *RAS* are very rare or even absent (5). Notably, the present study identified two novel *HRAS* mutations.

Additionally, two common polymorphisms in the 3'-UTR non-coding region of the gene *CDKN2A* were identified, rs11515 and rs3088440 (26). It is known that protein synthesis can be modulated by regulatory elements located in the 5'-UTR and 3'-UTR regions. The 3'-UTR, the site of the polymorphisms identified in the current study, serves an important role in translation and mRNA stability. Alterations in this region may be associated with the onset or progression of disease (27).

These polymorphisms have been investigated in various tumor types including urinary bladder neoplasm (28), esophageal adenocarcinoma (29) and cervical cancer (30) as presented in Table V. The two identified polymorphisms have

Table III. Correlation between *CDKN2A* SNPs and clinicopathological features in the patient cohort.

Clinicopathological feature	rs11515 (n=20)			rs3088440 (n=20)		
	CC (n=13)	CG (n=7)	P-value	CC (n=13)	CT (n=7)	P-value
Gender			0.526			0.474
Male (n=13)	8/13 (61.5%)	5/7 (71.4%)		9/13 (69.2%)	4/7 (57.1%)	
Female (n=7)	5/13 (38.5%)	2/7 (28.6%)		4/13 (30.7%)	3/7 (42.9%)	
Age at diagnosis			0.088[a]			0.272[a]
Mean ± SD (y)	45.41±17.49	31.53±11.36		44.062±17.49	31.53±11.36	
Tumor type			0.500			0.175
Sporadic (n=10)	7/13 (53.8%)	3/7 (42.9%)		8/13 (61.5%)	2/7 (28.5%)	
Familial (n=10)	6/13 (46.1%)	4/7 (57.1%)		5/13 (38.5%)	5/7 (62.5%)	
T category			0.464			0.291
T1	7/13 (53.8%)	2/7(28.5%)		5/13 (38.5%)	4/7 (57.1%)	
T2	2/13 (15.3%)	3/7 (42.9%)		3/13 (23.1%)	2/7 (28.5%)	
T3	2/13 (15.3%)	2/7 (28.5%)		3/13 (23.1%)	1/7 (14.4%)	
T4	2/13 (15.3%)	0/7 (0%)		2/13 (15.3%)	0/7 (0%)	
Tumor size			0.421[a]			0.689[a]
Mean ± SD (cm)	1.954±1.11	2.34±1.03		2.315±1.22	1.671±0.59	
<2	8/13 (61.5%)	2/7(28.6%)	0.378	7/13 (53.8%)	4/7 (57.1%)	0.339
≥2	5/13 (38.5%)	5/7 (71.4%)		6/13 (46.1%)	3/7 (42.9%)	
Lymph node metastases			0.742			0.742
N0	4/13 (30.8%)	5/7 (71.4%)		3/13 (23.07%)	3/7 (42.9%)	
N1	9/13 (69.2%)	2/7 (28.5%)		10/13 (76.9%)	4/7 (57.1%)	
AJCC stage			0.742			0.742
I and II	4/13 (30.7%)	2/7 (28.5%)		4/13 (30.7%)	2/7 (28.5%)	
III and IV	9/13 (69.2%)	5/7 (71.4%)		9/13 (69.2%)	5/7 (71.4%)	

P-values were obtained using the χ^2 test; [a]continuous variables analyzed with Student's t-test. SNPs, single nucleotide polymorphisms; SD, standard deviation; y, years; AJCC, American Joint Committee on Cancer.

Table IV. Comparative analysis of the frequency of the non-coding *CDKN2A* germ line single nucleotide polymorphisms in patients with MTC and the control.

A, rs11515

Population	Genotype frequency			Allele frequency		P-value
	CC	CG	GG	C (32)	G (8)	
MTC	0.60	0.40	-	0.80	0.20	0.25
1,000 genomes[a]	0.79	0.19	0.02	0.88	0.12	

B, rs3088440

Population	Genotype frequency			Allele frequency		P-value
	CC	CT	TT	C (31)	T (9)	
MTC	0.55	0.45	-	0.78	0.22	0.65
1,000 genomes[a]	0.73	0.24	0.03	0.85	0.15	

[a]Sequences obtained from the 1000 Genomes database used as a population control. MTC, medullary thyroid carcinoma. The numbers in parentheses represent the frequency of each allele type in this locus in the studied cohort.

Table V. Summary of the studies on *CDKN2A* polymorphisms in different tumor types.

Source, year (ref)	rs11515 (%)	rs3088440 (%)	Tumor type	n	Sample	Method used
Sauroja *et al*, 2000 (17)	16.67	16.67	Melanoma	48	Frozen/FFPE tissue	PCR-SSCP/ sequencing
Kumar *et al*, 2001 (26)	25	27.27	Melanoma	229	FFPE tissue	PCR-SSCP
Sakano *et al*, 2003 (28)	18.1	12.9	Bladder	309	Blood	PCR-SSCP
Geddert *et al*, 2005 (29)	13.3	-	ADC	315	FFPE tissue	PCR-RFLP
Chansaenroj, *et al* 2013 (30)	7.1	17.9	Cervical	56	Cervical swab	Sequencing
Straume *et al*, 2002 (31)	25	23	Melanoma	185	FFPE tissue	PCR-SSCP/ sequencing
Boonstra *et al*, 2011 (32)	22.07	-	EAC	214	FFPE tissue	Sequencing
	21.05	-	ESCC	97	FFPE tissue	Sequencing
Pinheiro *et al*, 2014 (33)	15.63	-	HNSCC	96	FFPE tissue	PCR-RFLP
Jin *et al*, 2012 (34)	-	16.7	SGC	156	Blood	PCR-RFLP
Polakova *et al*, 2008 (35)	25.98	13.07	Colorectal	612	Blood	PCR-RFLP
Royds *et al*, 2011 (36)	31.78	-	GBM	107	Blood	Sequencing
Thakur *et al*, 2012 (37)	13.64	-	Cervical	150	Fresh tissue	PCR-RFLP
Zhang *et al*, 2011 (38)	-	17.0	SCCHN	1,287	Blood	PCR-RFLP
Zhang *et al*, 2013 (39)	-	20.5	DTC	303	Blood	PCR-RFLP
	-	20.9	PTC	273	Blood	PCR-RFLP
De Giorgi *et al*, 2014 (40)	16.67	-	Melanoma	12	Blood	Sequencing
Song *et al*, 2014 (41)	-	33.88	SCCOP	552	Blood	PCR-RFLP
Nascimento *et al*, 2015[a]	35	35	MTC	20	FFPE tissue + blood	Sequencing

[a]Indicates the current study. ADC, gastric and esophageal adenocarcinomas; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; GBM, glioblastoma multiforme; SCCHN, squamous cell carcinoma of the head and neck; SGC, salivary gland carcinoma; DTC, differentiated thyroid carcinoma; PTC, papillary thyroid cancer; HNSCC, head and neck squamous cell carcinoma; SCCOP, squamous cell carcinoma of the oropharynx; FFPE, formalin-fixed paraffin-embedded; PCR; polymerase chain reaction; SSCP, single-strand conformation polymorphism; RFLP, restriction fragment length polymorphism.

been previously associated with an earlier progression from primary to metastatic disease in the case of melanoma (17), and rs3088440 was associated with the mechanism of tumor invasion in bladder cancer (28). Controversially, this polymorphism has been previously associated with a sub-group with reduced vertical growth of melanoma and a favorable outcome (31). However, additional studies have not identified a clinical correlation with tumor behavior (30,32,33).

Using *in silico* analysis, the current study identified that the polymorphisms rs11515 and rs3088440 are located within a transcriptional regulatory region, and the alteration of nucleotides can affect the binding of potential transcriptional factors. For example, the presence of the C allele in rs3088440 favors the binding of the transcription factor c-Myb, which potentially results in the transcriptional repression of the *CDKN2A* gene, compromising its normal function in cell cycle control (42). However, no association was identified between this polymorphism and the clinicopathological parameters investigated in the cohort studied (Table III).

In conclusion, it is suggested that *BRAF*, *CDKN2A* and *PI3KCA*, listed as potential adjuvants in the tumorigenesis of MTC, do not participate through somatic mutations as modulators of oncogenesis. To the best of our knowledge, the current study is the first to investigate these two *CDKN2A* polymorphisms in the pathophysiology of MTC. Therefore, *CDKN2A* and its regulatory regions and the additional genes involved in tumorigenesis warrant further investigation in MTC.

Acknowledgements

The authors would like to thank the team of the Laboratory of Molecular and Translational Endocrinology, particularly Ms. Teresa Kasamatsu, Mr. Gilberto Furuzawa, Dr João Roberto Martins, Dr Ji Hoon Yang, Dr Fausto Germano Neto and Mr. Fernando Soares. The authors would additionally like to acknowledge Mr. Gilmar Miranda from Siratec Ltd. for graphic art design. The current study was supported grants from the Sao Paulo State Research Foundation (grant nos. 2012/11036-3, 2012/02465-8, 2012/01628-0, 2009/50575-4, 2012/00079-3 and 2011/20747-8).

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