RESEARCH ARTICLE

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Modulatory Role of Single Nucleotide Polymorphisms of Distinct Genetic Pathways on Clinical Behavior of Medullary Thyroid Carcinoma

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Abstract

Background: Role of *RET* proto-oncogene as predisposing gene for Medullary Thyroid Carcinoma is well established which provides the basis for clinical management of patients. However clinical behavior of MTC varies considerably among patients. Several studies have investigated whether SNPs in low penetrance genes could modulate the clinical behavior of MTC but with conflicting or inconclusive results. The present study aimed to investigate the modifier effect of 13 SNPs of three distinct genetic pathways -Detoxification, Cell cycle regulation and RET on the clinico-pathological features of hereditary and sporadic MTC. **Methods:** SNPs were genotyped using RFLP or TaqMan method. The genotypes were correlated with various clinico-pathological parameters (age and calcitonin levels at MTC diagnosis, tumor volume, nodal and distant metastasis). **Results:** Nodal metastasis was the only clinico-pathological parameter showing significant association with any SNP. In the hereditary MTC group (n=77), incidence of nodal metastases was significantly higher in wild type allele for *Cyp1A1m1*, *CDKN2A* and *CDKN2C* (p=0.01 for all three). In sporadic MTC group (n=361) *CDKN2C* wild type allele had higher nodal metastasis (p=0.03). **Conclusion:** In this largest MTC cohort with comprehensive analysis of modulatory role of 13 most frequently studied SNPs with MTC clinical outcome, we observed a statistically significant association of few SNPs with nodal metastasis. However as these SNPs did not show association with any other clinico-pathological parameters like tumor volume or Calcitonin, they may not be true modifier of MTC. Additional large cohort studies with clinico-pathological details and long-term follow-up are needed to identify genetic modifiers of MTC behavior.

Keywords: Medullary Thyroid Carcinoma- MTC- *RET*- SNP- association-study

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Introduction

Gain-of-function germline point mutations in the RET proto-oncogene have long been established as the predisposing factor for hereditary Medullary Thyroid Carcinoma (MTC) which presents as a part of an autosomal dominant inherited cancer syndrome MEN2 (De Groot et al., 2006; Donis-Keller et al., 1993; Eng et al., 1996; Figlioli et al., 2013). The site specific mutations in the 'hotspot' regions of *RET* gene govern phenotypic variability observed in the mutation carriers provide the basis for clinical management of proband along with prophylactic interventions in healthy carriers (Kloos et al., 2009; Wells et al., 2015). Despite the strong genotype-phenotype association, the clinical behavior of MTC varies considerably among patients and clinical heterogeneity is observed in individuals harboring the same *RET* mutations (Barbieri et al., 2013). Similarly for sporadic MTC, somatic mutation in *RET* gene is not found in all cases (Agrawal et al., 2013; Marsh et al., 1996) and appear not to occur uniformly among the different subpopulation of cells in the tumor unlike germline mutations (Eng et al., 1996).

Several studies have investigated whether the presence of variants such as Single Nucleotide Polymorphisms (SNPs) in low penetrance genes could modulate the clinical behavior of the disease. Low penetrance gene variants are the polymorphic sequence variants which are known to have a relatively moderate effect in disease progression compared to pathogenic variants. If the presence of an SNP correlates with a change in clinical presentation of the disease then they may be considered as disease modifiers (Robledo et al., 2003). However, it is still a matter of debate to what extent these neutral

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polymorphisms could have modifying roles in the pathogenesis of MEN2 or sporadic MEN2-related tumors.

A series of studies in different population have attempted to correlate presence of SNPs in RET gene with MTC progression but the findings have been contradictory or inconclusive possibly because of small cohort sizes of this relatively rare cancer or due to differences in geo-ethnic background (Fugazzola et al., 2008; Lesueur et al., 2006; Machens et al., 2012; Robledo et al., 2003; Siqueira et al., 2010; Sromek et al., 2010; Tamanaha et al., 2009). Recently SNPs in detoxification and Cell cycle regulatory genes have also been studied as modulators of both hereditary and sporadic MTC behavior (Barbieri et al., 2013, 2014, 2012; Pasquali et al., 2011). However none of the studies have examined the association of SNPs of all these distinct genetic pathways together in a single cohort of MTC cases. Using the largest cohort of 438 MTC cases (361 sporadic and 77 hereditary) we therefore undertook a comprehensive gene dose-response relationship analysis between 13 SNPs of genes of three distinct genetic pathways (Table 1). These include SNPs from genes of detoxification (*Cyp1A1m1, Cyp1A2*F, NAT2, GSTP1*), cell cycle regulation (*CDKN1A, CDKN1B, CDKN2A, CDKN2B, CDKN2C*) and the RET gene (*G691S, L769L,S836S, S904S*). This gene dose-response relationship aimed to investigate the clinic-pathological differences between the wild-type versus heterozygous versus homozygous variant for each SNP separately in hereditary and sporadic MTC cases.

Materials and Methods

Patient cohort

The 438 Indian MTC patients enrolled in this study were registered at the Cancer Genetics Clinic, Tata Memorial Hospital over a period of 12 years (2006 to 2018), under an Institutional Ethics Committee approved study. For each patient, personal and family history with clinico-pathological details was recorded using a standard case-record form. Blood sample was collected after a written informed consent and detailed genetic counseling. Based on clinical details, family history and RET genetic test result, the patients were classified as hereditary MTC cases or sporadic. In our cohort of 438 MTC patients, we have 77 hereditary and 361 sporadic MTC patients. All the 77 hereditary MTC cases harbored mutations in the hotspot region of the *RET* gene.

Clinical and Pathological examination

The clinical diagnosis of MTC was made on biochemical evaluation of serum Calcitonin levels at diagnosis (reference range: 2-10mg/ml) and histopathological examination of surgically resected tumor tissues. The tumor volume was calculated using the ellipsoid volume formula $\frac{1}{2} \times l \times w \times h$ cm^3 (Jensen et al, 2008). The diagnosis of nodal metastasis was made on the histopathological examination of lymph nodes resected at the time of total thyroidectomy. Distant metastasis was confirmed by either ultrasonography, computed tomography, magnetic resonance imaging, positron emission tomography or their combination. The age at MTC diagnosis was recorded for each patient.

Genotyping

DNA was extracted from peripheral blood sample using Qiagen QIAmp DNA Mini kit (Cat#51304). Germline *RET* mutation analysis was performed for 6 hotspot exons of RET (10, 11, 13, 14, 15 16) by PCR and Sanger Sequencing. Sanger Sequencing was performed using BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) on ABI 3500 and 3730 DNA Sequencer (Applied Biosystems) and electropherograms were analyzed by Chromas Lite version 2.6.4 using reference sequence of RET gene from National Centre for Biotechnology Information (NCBI) NG_007489.1.

SNP genotyping was performed by Restriction Fragment Length Polymorphism (RFLP) for *Cyp1A1m1, Cyp1A2*F, GSTP1, NAT2, CDKN1A, CDKN1B, CDKN2A, RET L769L, S836S* and *S904S* polymorphisms and by TaqMan SNP genotyping method for *CDKN2B, CDKN2C* and *RET G691S* polymorphisms. For RFLP, the digested products were visualized on 2 % agarose gel and the genotypes were inferred from band sizes in the gel. For TaqMan SNP genotyping, realtime PCR was performed on QuantStudio 5.0 and genotypes were inferred from amplification plot and allelic discrimination plots. Around 5% of all the genotyping results were confirmed on Sanger Sequencing.

Statistical Analysis

All Statistical analysis was performed on SPSS v21.0. The clinico-pathological variables used for correlation studies included age at MTC diagnosis, serum Calcitonin levels at diagnosis (Pre-Op), tumor volume (cm^3), nodal and distant metastasis. To examine gene dose-response relationship, the clinic-pathological variables were stratified between wild-type, heterozygous and homozygous variants and compared. Categorical data was tested with Pearson Chi-Square test and two-tailed Fisher exact test whereas for continuous data, the means were compared using one-way analysis of variance (ANOVA) and medians was compared using Kruskal-Wallis test. The level of significance was set at <0.05.

Results

The 438 Indian MTC cases in our cohort included 239 males and 199 females. The mean age at MTC diagnosis was 40.64 + 14.24; median: 40 years (range: 8-80years). All the SNPs except Cyp1A1m1 and CDKN2A maintained the Hardy-Weinberg equilibrium (Table 2).The genotype frequency of the wild-type, heterozygous and homozygous variants for all the 13 SNPs are given in Table 3. For a few SNPs, the genotype frequency of the homozygous variant is either absent or very low limiting their analysis between the wildtype and heterozygous variants.

In gene dose-response association, the only clinic-pathological parameter with which any SNP showed significant association was nodal metastasis at diagnosis. In hereditary MTC group, *Cyp1A1m1, CDKN2A* and *CDKN2C SNP* showed this association (Supplementary

Table 1. List of SNPs of Distinct Genetic Pathways Selected for the Study

Gene/SNP	Reference ID	Variant	Nucleotide change	Ancestral Allele	Amino acid Change
Genes of Detoxification					
Cyp1A1m1	rs4646903	3′UTR	T/C	T	-
Cyp1A2	rs762551	Intronic	A/C	A	-
NAT2	rs1041983	Synonymous	C/T	C	Y94Y
GSTP1	rs1695	Missense	A/G	A	I105V
Cell Cycle regulatory genes					
CDKN1A	rs1801270	Missense	C/A	C	S31R
CDKN1B	rs2066827	Missense	T/G	T	V109G
CDKN2A	rs11515	3′ UTR	G/C	G	-
CDKN2B	rs1063192	3′UTR	T/C	T	-
CDKN2C	rs12885	3′ UTR	G/T	G	-
RET Proto oncogene					
G691S RET	rs1799939	Missense	G/A	G	G691S
L769L RET	rs1800861	Synonymous	T/G	T	L769L
S836S RET	rs1800862	Synonymous	C/T	C	S836S
S904S RET	rs1800863	Synonymous	C/G	C	S904S

Table S1). Patients with wildtype *Cyp1A1m1* showed higher rate of regional nodal metastasis compared to the heterozygous and homozygous variants (95.6% vs 86.2% vs 33.3%; p=0.01). Similarly patients with wildtype *CDKN2A* showed a higher incidence of nodal metastasis compared to their variant counterparts (91.3% vs 83.3% vs 33.3%; p=0.01). For *CDKN2C*, the comparison could only be made between wildtype and heterozygote as the homozygous variant for this SNP was absent. We observed that cases heterozygous for *CDKN2C SNP* showed higher rate of lymph node metastasis compared to wildtype *CDKN2C* (100% vs 86.3%; p=0.01) (Supplementary Table S1).

In sporadic MTC group, this association was observed between the wildtype and the homozygous variant for *CDKN2C SNP*. The cases wildtype for *CDKN2C SNP* showed higher rate of nodal metastasis compared to homozygous variant genotype (83.8% vs 40%; p = 0.03). The rate of nodal metastasis for heterozygous variant was similar to the wildtype *CDKN2C* (84.4% vs 83.8%) (Supplementary Table S2). No other significant association was observed between any of the SNPs and the patient's clinico-pathological behavior.

Table 2. Hardy-Weinberg (HWE) Calculation for SNPs in MTC Cohort

Gene/SNP	MTC cases (n=438)	
	MAF	HWE *P*-value
Cyp1A1m1	0.31	0.006
Cyp1A2	0.43	0.65
NAT2	0.45	1.00
GSTP1	0.26	0.65
CDKN1A	0.09	0.14
CDKN1B	0.29	0.57
CDKN2A	0.07	0.003
CDKN2B	0.25	0.11
CDKN2C	0.10	0.31
G691S RET	0.27	0.17
L769L RET	0.35	0.60
S836S RET	0.08	0.33
S904S RET	0.29	0.05

Discussion

The role of RET variants as modulators of clinical behavior of MTC has been studied in different populations in both hereditary and sporadic MTC patients. A few studies have reported a significant dose-response relationship between the RET SNPs and the age at MTC diagnosis (Cardot-Bauters et al., 2008; Robledo et al., 2003; Siqueira et al., 2010; Sromek et al., 2010) whereas many others have failed to establish these associations(Fugazzola et al., 2008; Lesueur et al., 2006; Machens et al., 2012; Tamanaha et al., 2009). Recently, Barbieri et al., (2012, 2013) have studied the modulatory role of SNPs of genes of detoxification (*Cyp1A1m1, Cyp1A2*F, NAT2* and *GSTP1*) in both hereditary and sporadic MTC patients in Brazilian population (Barbieri et al., 2012, 2013). In their study on sporadic MTC patients, they failed to establish any significant relationship between the genetic profiles of these SNPs and patient's clinical outcome which they concluded to be a result of their small cohort size (47 sporadic MTC cases) (Barbieri et al., 2012). Further in their study on hereditary MTC cohort of 132 patients, they demonstrated that the inherited profile of *Cyp1A2*F, NAT2* and *GSTP1* contributed significantly to the clinical behavior of the patients (Barbieri et al., 2013). They showed that inheritance of *Cyp1A2*FSN*P was associated with lesser tumor burden in patients and inheritance of *GSTP1 SNP* was associated with later age at MTC diagnosis. The same group also studied the influence of cell cycle regulation genes (*CDKN1A, CDKN1B, CDKN2A, CDKN2B* and *CDKN2C*) in a small cohort of

Table 3. Genotype Frequency of SNPs in Hereditary and Sporadic MTC Groups

Gene/ SNP	Genotype Frequency – Hereditary MTC cases (n=77)			Genotype Frequency – Sporadic MTC cases (n=361)		
	Wild-type	Heterozygous	Homozygous	Wild-type	Heterozygous	Homozygous
Cyp1A1m1	37 (48.1%)	36 (46.7%)	4 (5.2%)	161 (44.6%)	175 (48.4%)	25 (6.9%)
Cyp1A2	28 (36.4%)	34 (44.2%)	15 (19.5%)	117 (32.4%)	176 (48.7%)	68 (18.8%)
NAT2	31 (42.2%)	34 (44.2%)	12 (15.6%)	103 (28.5%)	183 (50.7%)	75 (20.7%)
GSTP1	35 (45.5%)	35 (45.5%)	7 (9.0%)	208 (57.6%)	129 (35.7%)	24 (6.6%)
CDKN1A	65 (84.4%)	11 (14.3%)	1 (1.3%)	295 (81.7%)	66 (18.3%)	0 (0%)
CDKN1B	35 (45.5%)	33 (42.8%)	9 (11.6%)	182 (50.4%)	153 (42.4%)	26 (7.2%)
CDKN2A	67 (87%)	7 (9.0%)	3 (3.8%)	310 (85.8%)	47 (13.0%)	4 (1.1%)
CDKN2B	46 (59.8%)	27 (35.0%)	4 (5.2%)	206 (57.1%)	125 (34.6%)	30 (8.3%)
CDKN2C	62 (80.5%)	14 (18.1%)	1 (1.3%)	297 (82.3%)	59 (16.3%)	5 (1.4%)
G691S RET	37 (48.1%)	31 (40.2%)	9 (11.6%)	201 (55.6%)	131 (36.3%)	29 (8.0%)
L769L RET	35 (45.5%)	33 (42.8%)	9 (11.6%)	146 (40.4%)	172 (47.6%)	43 (11.9%)
S836S RET	64 (83.1%)	13 (16.8%)	0 (0.0%)	311 (86.1%)	46 (12.7%)	4 (1.1%)
S904S RET	36 (46.8%)	30 (38.9%)	11 (14.3%)	194 (53.7%)	133 (36.8%)	34 (9.4%)

45 sporadic MTC cases (Barbieri et al., 2014). They have reported that patients with wildtype *CDKN1A* presented extrathyroidal tumor extension more frequently (p=0.037) and patients with wildtype *CDKN2C* presented larger tumors (p=0.032) whereas patients with polymorphic *CDKN2B* presented higher rate of distant metastasis (p=0.026).Further, one study on Italian population by Pasquali et al., (2011) reported *CDKN1B V109G* polymorphism to be a prognostic marker in sporadic MTC (Pasquali et al., 2011). The study was performed in a cohort of 84 sporadic MTC patients and 90 matched controls. They had demonstrated that *CDKN1B V109G* polymorphism was associated with a more favorable disease progression than the wildtype allele.

Although statistically significant associations were observed in some of these studies, their cohort size was comparatively small. A small cohort study is likely to produce false-positive results than the conclusions drawn from a larger cohort study. The two comparatively larger studies investigating the role of these SNPs was by Barbieri et al., (2013) in a cohort of 132 hereditary MTC patients (discussed above) and the other by Machens et al., (2012). Machens et al., (2012) have investigated the clinical relevance of *SNPs* of *RET* gene (*G691S, L769L, S836S* and *S904S*) in a cohort of 150 sporadic MTC cases. They investigated the differences in clinic-pathological characteristics of sporadic MTC patients with or without these *RET SNPs*. They have failed to identify any significant dose-response relationship between the *RET SNPs* and *MTC* clinical behavior. The findings of the two studies had called for another comprehensive and larger cohort study to decipher definitive conclusions.

The present study represents a large cohort of MTC including both the hereditary and sporadic MTC cases. Here we have undertaken a comprehensive investigation of 13 SNPs of all the above discussed genetic pathways in a cohort of 361 sporadic and 77 hereditary MTC cases. Although a few SNPs show a statistically significant association ($p<0.01$) with regional nodal metastasis, they may not represent a true gene dose-response relationship as they were not associated with any other clinico-pathological parameters. Our findings on *RET SNPs* were consistent with Machens et al., (2012) group as we too have failed to establish any gene dose-response relationship between the carriers and non-carriers of *RET* gene SNPs in our large MTC cohort. Further, our study have failed to replicate the findings of Pasquali et al group as we have not identified any significant association between the *CDKN1B SNP* and the patients clinical outcome. This discrepancy might have arisen as we did not have the follow-up data of the patients with which Pasquali et al have done their correlation studies and reported strong associations.

Taken together, this study is the first single cohort study to perform a comprehensive analysis of modulatory role of 13 most frequently studied SNPs with clinical outcome of MTC patients. We have extended our analysis on both the hereditary and sporadic MTC cohort and reported no significant association of these SNPs with clinical behavior of MTC. There is a need for additional genetic association studies on large cohort of MTC cases with clinic-pathological details and long-term follow-up data in order to draw consistent and definitive conclusions.

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Disclosure

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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