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Modifying impact of *RET* gene haplotypes on medullary thyroid carcinoma clinical course

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Abstract

The clinical course of medullary thyroid carcinoma (MTC) associated with the MEN2A syndrome as well as of sporadic MTC shows considerable heterogeneity. The disease picture varies not only between the same *RET* proto-oncogene mutation carriers but also among sporadic MTC patients with no *RET* germinal mutations, which suggests the involvement of additional modulators of the disease. However, genetic factors responsible for this heterogeneity of the MTC clinical course still remain unknown. The aim of this study was to determine if polymorphic variants or specific haplotypes of the *RET* gene may modify the MTC clinical course. We genotyped the following loci: c.73+9277T>C, c.135G>A, c.1296A>G, c.2071G>A, c.2307T>C, c.2508C>T and c.2712C>G in 142 MTC patients and controls. We demonstrated considerable differences in the genotypes distribution within c.73+9277T>C, c.135G>A and c.2307T>C loci. Our results show that the c.73+9277T variant associated with a decreased activity of the MCS+9.7 *RET* enhancer is rare in hereditary MTC patients with primary hyperparathyroidism, and thus, may influence the MTC clinical picture. The decreased activity of the *RET* promoter enhancer reduces *RET* expression level and may counterbalance the activating mutation in this gene. Frequent co-occurrence of the c.73+9277T allele with p.E768D, p.Y791F, p.V804M or p.R844Q *RET* mutations may be associated with their attenuation and milder clinical picture of the disease. Haplotypes analysis showed that C-G-A-G-T(C)-C (c.73+9277T>C – c.135G>A – c.1296A>G – c.2071G>A – c.2307T>G – (c.2508C>T) – c.2712C>G) alleles combination predisposes to pheochromocytomas and primary hyperparathyroidism. We consider that *RET* haplotypes defining may become an auxiliary diagnostic tool in MTC patients.

Key Words

- ▶ medullary thyroid carcinoma
- ▶ MEN2 syndrome
- ▶ *RET* gene polymorphisms
- ▶ haplotypes
- ▶ associations analysis

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Introduction

Medullary thyroid carcinoma (MTC) occurs sporadically (sMTC) or as an inherited cancer (hMTC). The hereditary form may be present as a component of the multiple endocrine neoplasia type 2 syndrome (MEN2) or as an isolated MTC without additional symptoms. However, while germinal mutations of the *RET* proto-oncogene have been shown to be closely associated with the hereditary form of MTC, the molecular bases of sporadic MTC remain unexplained (Elisei *et al.* 2007, Wells *et al.* 2015). Amino acid substitutions in the receptor tyrosine kinase (RET) increasing its activity occur mostly through mutations in the 3' part of the *RET* proto-oncogene. The mutations inducing a strong activation of the RET kinase are associated with more aggressive MTC forms, whereas those resulting in a weaker activation are reported to cause less aggressive MTC with a late onset. Moreover, the course of the disease frequently varies among carriers of an identical *RET* gene mutation as well as among family members, who are diagnosed at different ages, present disease of different aggressiveness, with or without such associated symptoms as pheochromocytoma (PHEO) and primary hyperparathyroidism (PHPT). This suggests the existence of other factors modifying the disease course (Qi *et al.* 2011, Sarika *et al.* 2012, 2015, Wells *et al.* 2015). Research aimed at finding such markers were initiated at the turn of the century. Da Silva *et al.* (2003) have described various MTC clinical courses in a multi-generation family and suggested a possible effect of single-nucleotide polymorphisms (SNPs) in the *RET* gene on the heterogeneous clinical course of the familial MTC. Some other reports have shown that *RET* genetic variants may be associated with a higher risk of aggressive early-onset sporadic MTC (sMTC) (Gimm *et al.* 1999, Ruiz *et al.* 2001, Berard *et al.* 2004, Elisei *et al.* 2004, Baumgartner-Parzer *et al.* 2005, Siqueira *et al.* 2010, Ceolin *et al.* 2016). Recent studies by Ceolin *et al.* (2016) have shown a significant correlation between polymorphisms in the 3'UTR region and the secondary structure of the *RET* mRNA, suggesting their role in posttranscriptional control of the *RET* transcripts and disease presentation in MTC patients. Regarding this fact, it would be reasonable to expand the knowledge considering the proximal part of the gene. One of the most interesting polymorphisms in this part of the gene is c.73+9277T>C localized in intron 1. It was previously reported that this variation is situated within the MCS+9.7 enhancer region (Multispecies Conserved Sequence 9.7kb downstream from the ATG codon) and that the c.73+9277T variant disrupts a binding site for

SOX10, which compromises *RET* transactivation and results in its transcriptional modulation (Emison *et al.* 2010). Formerly Emison *et al.* (2005) showed that the c.73+9277T allele in heterozygotes decreases activity of the *RET* gene enhancer, increasing the risk of the Hirschsprung's disease sixfold, and its homozygous state have even higher impact, rising risk 20-fold. Hirschsprung's disease (aganglionic megacolon, HSCR) has a complex genetic etiology; however, inactivating mutations in the *RET* proto-oncogene are considered as the major HSCR-causing factors (Pan & Li 2012).

In a study on the small group of MTC patients, the c.73+9277T allele has not been detected at all and the authors suggested its protective effect (Boruń *et al.* 2012). More recently, *RET* overexpression has emerged as a new player in the ER-positive (ER+) breast cancer and as a potential target to enhance sensitivity and avoid resistance to tamoxifen treatment. Increased overall survival was observed in carriers of the c.73+9277T variant (Griseri *et al.* 2016).

In view of the fact that the contribution of *RET* SNPs and haplotypes to the development and progression of sporadic MTC remains not well evidenced and the authors of studies that have been published to date emphasize the necessity to continue research, we decided to investigate it.

The aim of this study was to determine the influence of polymorphic variants in the *RET* gene including the polymorphisms in the proximal region of the gene on clinical course of hereditary and sporadic MTC. The frequency of variants: c.73+9277T>C (IVS1+9277T>C, rs2435357) in intron 1, p.A45A (c.135G>A, rs1800858) in exon 2, p.A432A (c.1296A>G, rs1800860) in exon 7, p.G691S (c.2071G>A, rs1799939) in exon 11, p.L769L (c.2307T>C, rs1800861) in exon 13, p.S836S (c.2508C>T, rs1800862) in exon 14 and p.S904S (c.2712C>G, rs1800863) in exon 15 were determined in group of 142 patients with MTC and population control. An association of particular alleles and genotypes with the clinical course of the disease were studied. We have also analyzed the linkage disequilibrium and the correlations of the *RET* gene haplotypes with the MTC clinical picture, and the presence of particular *RET* mutations in association with the haplotypes.

Materials and methods

Patients and controls

We studied DNA samples from a group of 142 patients with MTC from the Great Poland (Wielkopolska) region,

who have been hospitalized in The Department of Endocrinology, Metabolism and Internal Diseases of Poznan University of Medical Sciences in Poland. The group included 51 patients with hereditary MTC and diagnosed *RET* gene mutations (Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article), and 91 sporadic cases with *RET* germinal mutations excluded. Data on the presence of somatic mutations in patients with sMTC were not available.

The demographic and clinical data included gender, age at diagnosis, histopathological examination results with the evaluation of multifocality, the TNM classification and in patients with hereditary MTC also the presence of comorbid diseases: pheochromocytoma (PHEO) and primary hyperparathyroidism (PHPT) were collected (Supplementary Table 1). Controls were unrelated individuals from the Polish population who were received at the Laboratory of Molecular Genetics (Poznan, Poland) for paternity testing. Depending on the tested polymorphism, the control group consisted of 204–678 individuals. All subjects signed an informed consent to genetic testing, and the study was approved by the Ethical Committee of Poznan University of Medical Sciences.

phenol–chloroform extraction using a standard protocol (Słomski 2008). Isolates were dissolved in 1× TE buffer and stored at –20°C until analysis. Then, SNPs in the *RET* gene were analyzed by pyrosequencing or high-resolution melting analysis (HRMA) and Sanger sequencing. Primers were designed using PyroMark Assay Design Software 2.0 (Biotage) and Primer 3 online primer design tool (all primer sequences and melting temperatures are listed in Supplementary Table 2). Templates for pyrosequencing were amplified by PCR as follows: a 5-min initial denaturation at 94°C, then 50 cycles of 94°C for 30s, annealing for 30s and 72°C for 60s, followed by a 5-min final elongation at 72°C. Pyrosequencing reactions were performed on the PSQ96 apparatus using PyroMark Gold Q96 reagents kits (Qiagen) according to the manufacturer's recommendations. HRM analyses were run on the Rotor-Gene Q cyclor using the Type-it HRM PCR Kit (Qiagen). The DNA fragments containing the *RET* gene exons were amplified by PCR (5 min at 95°C, then 40 cycles as follows: 95°C for 10s, 60°C for 30s and 72°C for 10s) and the products were subsequently melted from 75 to 95°C by raising the temperature by 0.1°C at each step. Sanger sequencing was performed on MegaBACE 500 (GE Healthcare) or ABI PRISM 3100 (Applied Biosystems).

Genetic analyses

Genomic DNA was extracted from peripheral blood using guanidine isothiocyanate and following

Statistical analyses

The comparison of the age of diagnosis between the two groups (hereditary vs sporadic) as well as

Figure 1

A diagram showing a fragment of the *RET* gene, where MTC-related mutations are most common. Mutations identified in the study group were marked. The triangles represent individual families, while the numbers in the middle of the triangles indicate the number of family members who inherited the mutation.

between particular alleles was conducted using the Mann–Whitney test because the data did not have a normal distribution as shown by the Shapiro–Wilk test. The comparison of age of diagnosis between genotypes was performed by Kruskal–Wallis test. The nominal scale variables were analyzed using the chi-square independence test or – in case of low observation numbers – Fisher's exact test. Odds ratio (OR) with 95% confidence intervals (CI) and *P* values were also calculated. *P* values below 0.05 were considered as indicative of statistical significance.

All statistical analyses were conducted using Statistica10.0 (StatSoft) and GraphPad 3.06 (GraphPad Software). The concordance with Hardy–Weinberg equilibrium was tested using the calculator on the following website: <https://ihg.gsf.de/cgi-bin/hw/hwa1.pl>. *P* values for HWE were returned by Pearson's chi-square test of independence. In case one of the alleles was not present in the study group, a correction has been applied by adding 0.5 values to each count, according to Deeks & Higgins (2010).

Linkage disequilibrium analysis

The analysis of the linkage disequilibrium for the polymorphisms under study and of the associations between the haplotypes and the clinical data was conducted using the Haploview v.4.2 software (Barrett *et al.* 2005).

Ethical approval

The study was approved by the Bioethical Committee of the University of Medical Sciences in Poznań, Poland, under Resolution No. 627/07 and 829/13. Informed consent was obtained from every participant.

Results

In hereditary MTC patients, we have identified 9 different mutations in *RET* gene in exons: 10, 11, 13, 14 and 16. The most frequent were alterations in codon 634 (Fig. 1 and Supplementary Table 1). Demographic and clinical characteristics of hereditary and sporadic MTC patients compared to controls are summarized in Table 1. Both hereditary and sporadic cases were slightly more frequent in women (56.9% and 63.7%). The average age at diagnosis was statistically lower in patients with hereditary MTC (34.4 vs 52.9; *P*-value <0.001); patients who were diagnosed before symptoms appeared were excluded from these analyses. Sporadic MTC patients presented with a more advanced disease at diagnosis, with a higher proportion of individuals with tumors in T3 and T4 stages (35% of sporadic vs 21.6% hereditary MTC patients). Also lymph nodes metastases seemed to be more frequent in sporadic MTC patients (46.3% vs 37.8%), as were distant metastases (10% vs 5.4%), but these observations were not statistically significant. Multifocality was five times more frequent in hereditary MTC patients than in sporadic cases (OR=5.5; CI=2.18–14.99; *P* value <0.001).

Table 1 Basic characteristics of examined MTC patients – hereditary vs sporadic cases.

	Hereditary MTC		Sporadic MTC <i>n</i> (%)	
	<i>n</i> (%)	<i>n</i> total**	<i>n</i> (%)	<i>n</i> total**
Sex				
Females	29 (56.9)	51	58 (63.7)	91
Males	22 (43.1)		33 (36.3)	
Age at diagnosis (mean ± s.d. *)	34.4 ± 13.8	40	52.9 ± 15.5	88
Tumor stage				
T				
T1 + T2	29 (78.4)	37	52 (65.0)	80
T3 + T4	8 (21.6)		28 (35.0)	
N				
N0	23 (62.2)	37	43 (53.8)	80
N1	14 (37.8)		37 (46.3)	
M				
M0	35 (94.6)	37	72 (90.0)	80
M1	2 (5.4)		8 (10.0)	
Multifocality	15 (40.6)	37	10 (11.2)	89
Pheochromocytoma (PHEO)	22 (47.8)	46	0	–
Primary hyperthyroidism (PHPT)	8 (17.8)	45	0	–
Other malignancies	1 (2.2)	45	7 (7.9)	89

*s.d., standard deviation.

Pheochromocytomas and primary hyperparathyroidism were observed only in hereditary MTC patients.

In an analysis of allele and genotype distribution, we have observed a discordance with Hardy–Weinberg equilibrium among sporadic MTC patients, with statistically significant differences in the genotype distribution between the studied groups for the following four polymorphisms: c.73+9277T>C, c.135G>A, c.1296A>G and c.2307T>G (Table 2).

We have shown a significant inverse correlation between the c.73+9277T allele and primary hyperparathyroidism: the T allele had a protective effect (OR=0.12; CI=0.02–0.98; *P* value=0.032), while allele C was risk allele (OR=8.13; CI=1.02–65.02; *P* value=0.032). Similarly, the c.135A allele was less frequent among patients with PHPT (OR=0.13; CI=0.01–1.05), while c.135G allele predisposed to PHPT (OR=7.65; CI=0.96–61.31), but this association was borderline significant (*P* value=0.055) (Table 3). The analyses performed for sporadic cancer patients showed that the c.73+9277T>C variants have a higher frequency of the c.73+9277C allele (OR=2.43; CI=1.07–5.53; *P* value=0.035) as well as of the wild-type homozygotes (CC) among patients with tumors at advanced stages (T3 and T4) at diagnosis (*P* value=0.051) (Table 4). However, we have made no corrections for the multiple statistical testing.

Imputing genotype data to the Haploview v.4.2 software (Fig. 2 and Supplementary Table 3), we observed a strong linkage disequilibrium ($D'=1$) for the following sets of polymorphisms: c.73+9277T>C and c.135G>A, c.73+9277T>C and c.2071G>A, c.73+9277T>C and c.2712C>G, c.2307T>G and c.2508C>T (only in sporadic MTC patients), and c.2071G>A and c.2712C>G. However, the corresponding values of the r^2 factor (>0.8) were found only for the following pairs of polymorphisms: c.73+9277T>C and c.135G>A among hereditary MTC patients, and c.2071G>A and c.2712C>G among sporadic MTC patients. In both groups, the most frequent allele combination was as follows: C-G-G-G-T-C-C and C-G-G-A-T-C-G (Fig. 2 and Supplementary Table 3). The analysis of correlations between the haplotypes and the clinical course of the disease has shown that the C-G-A-G-T-(C)-C (c.73+9277T>C – c.135G>A – c.1296A>G – c.2071G>A – c.2307T>G – (c.2508C>T) – c.2712C>G) haplotype may predispose to pheochromocytoma (OR=3.41; CI=1.57–7.40; *P* value=0.002) and primary hyperparathyroidism (OR=4.37; CI=1.56–12.28; *P*-value=0.005). This haplotype was also more common in patients with the disease diagnosis set before 40 years of age (OR=1.96; CI=1.01–3.87; *P* value=0.049) and in

individuals with multifocal MTC (OR=3.17; CI=1.03–10.26; *P* value=0.044) (Table 5).

As pheochromocytomas and PHPT are characteristic for the MEN2A syndrome in hereditary MTC patients, we have also analyzed the presence of *RET* mutations on haplotype backgrounds. We have found a strong link of mutations in codon 634 with the C-G-T allele combination (c.73+9277T>C – c.135G>A – c.2307T>G) (OR=4.58; CI=1.75–11.90; *P* value=0.002), while they were rare in association with haplotype block T-A-G (OR=0.18; CI=0.06–0.51; *P* value=0.001). When focusing only on the most frequent mutation p.C634R alone, the association was even stronger, with the OR of 5.65 (CI=2.27–14.04; *P* value <0.001) for the C-G-T haplotype block, and 0.11 (CI=0.03–0.04; *P* value <0.001) for the alternative combination, T-A-G. In contrast, the mutations associated with hereditary isolated MTC (p.E768D, p.Y791F and p.V804M, p.R844Q) co-existed rarely with the C-G-T haplotype block (OR=0.16; CI=0.06–0.47; *P* value <0.001) and frequently with the T-A-G allele combination (OR=4.50; CI=1.54–13.18; *P* value=0.006) (Table 6).

Discussion

Many mutations of the *RET* proto-oncogene leading to MTC and the MEN2 syndrome have been identified so far. However, it is still not known why patients presenting the same genetic alteration vary significantly on clinical course of the disease. Studies beyond routine diagnostics of the most frequent mutations have identified several polymorphisms that may be involved; however, their influence on the clinical course of the disease is not yet clearly understood.

The c.73+9277T>C (IVS1+9277T>C, rs2435357) polymorphism is localized in intron 1 of the *RET* gene, within the MCS enhancer located 9.7 kb from the ATG codon and the presence of one copy of c.73+9277T allele was found to be clinically relevant, causing a sixfold decrease in the *RET* gene enhancer activity (Emison et al. 2005, 2010). In our study, genotype distribution in this locus has shown that the TT genotype was six times more frequent in hereditary MTC patients than in sporadic cases and four times more frequent among controls than in sMTC cohort. These findings may suggest a protective effect of the TT genotype against sporadic MTC.

To the best of our knowledge, an association between the c.73+9277T>C polymorphism and the MTC course has never been reported. In our study, we have observed a higher frequency of the CC (wild-type) genotype in

Table 2 Allele and genotype frequency of *RET* gene polymorphisms in hereditary and sporadic medullary carcinoma (MTC) patients as compared to controls.

Polymorphism rs number NM	Controls (n*)	Hereditary MTC (hMTC) (n=51)	Sporadic MTC (sMTC) (n=91)	Allele frequency (P-value; *OR; 95% CI)		Genotype frequency (P-value; *OR; 95% CI)			
				Controls vs sMTC	Controls vs hMTC	Controls vs sMTC	Controls vs hMTC	Controls vs sMTC	hMTC vs sMTC
065681.1									
rs2435357	n=244	CC=0.54	CC=0.51		(T) vs (C)	(CC+CT) vs (TT) – dominant model, risk allele: C			hMTC vs sMTC
c.73+9277T>C	C=0.72	CT=0.34	CT=0.47	OR=1.11	OR=0.95	OR=1.17	OR=0.66		OR=0.07
Intron 1	T=0.28	TT=0.12	TT=0.02	CI=0.75–1.63	CI=0.59–1.52	CI=0.68–2.02	CI=0.25–1.72		CI=1.18–31.30
	TT=0.08		PHWE=0.030	P=0.597	P=0.818	P=0.565	P=0.388		P=0.016
rs1800858	n=226	GG=0.54	GG=0.52		(A) vs (G)	(GG+GA) vs (AA) – dominant model, risk allele: G			
c.135G>A	G=0.72	GA=0.34	GA=0.46	OR=1.14	OR=0.95	OR=1.21	OR=0.27		OR=5.93
A45A	A=0.28	AA=0.12	AA=0.02	CI=0.77–1.70	CI=0.59–1.53	CI=0.70–2.09	CI=0.09–0.81		CI=1.15–30.61
	AA=0.08		PHWE=0.038	P=0.510	P=0.821	P=0.501	P=0.014		P=0.018
rs1800860	n=204	GG=0.30	GG=0.60		(G) vs (A)	(GG) vs (GA+AA) – recessive model, risk allele: A			
c.1296A>G	G=0.65	GA=0.54	GA=0.26	OR=0.66	OR=1.38	OR=0.48	OR=0.828		OR=0.28
p.A432A	A=0.35	AA=0.16	AA=0.13	CI=0.45–0.97	CI=0.89–2.16	CI=0.28–0.79	CI=0.48–1.43		CI=0.13–0.59
	AA=0.14		PHWE=0.001	P=0.033	P=0.152	P=0.004	P=0.089		P<0.001
rs1799939	n=361	GG=0.70	GG=0.58		(A) vs (G)	(GG+GA) vs (AA) – dominant model, risk allele: G			
c.2071G>A	G=0.81	GA=0.30	GA=0.39	OR=0.80	OR=1.32	OR=0.61	OR=5.13		OR=0.25
p.G691S	A=0.19	AA=n.o.	AA=0.030	CI=0.54–1.18	CI=0.74–2.35	CI=0.32–1.16	CI=0.30–86.65		CI=0.01–4.95
	AA=0.05			P=0.262	P=0.353	P=0.130	P=0.117		P=0.168
rs1800861	n=589	TT=0.60	TT=0.47		(G) vs (T)	(TT+TG) vs (GG) – dominant model, risk allele: T			
c.2307T>G	T=0.71	TG=0.30	TG=0.42	OR=0.84	OR=0.83	OR=0.99	OR=0.94		OR=4.94
p.L769L	G=0.29	GG=0.10	GG=0.02	CI=0.59–1.20	CI=0.52–1.33	CI=0.56–1.73	CI=0.37–2.53		CI=0.92–26.5
	GG=0.10		PHWE=0.049	P=0.340	P=0.432	P=0.960	P=0.294		P=0.041
rs1800862	n=678	CC=1.0	CC=0.92		(T) vs (C)	(CC+CT) vs (TT) – dominant model, risk allele: C			
c.2508C>T	C=0.96	CT=n.o.	CT=0.09	OR=0.83	OR=7.77	OR=0.10	OR=0.23		OR=1.81
p.S836S	T=0.04	TT=n.o.	TT=n.o.	CI=0.39–1.79	CI=0.48–126.86	CI=0.01–1.79	CI=0.01–5.56		CI=0.04–92.70
	TT=0.01	**PHWE<0.001		P=0.638	P=0.054	P=0.054	P=0.786		P=1.000
rs1800863	n=367	CC=0.72	CC=0.58		(G) vs (C)	(CC+CG) vs (GG) – dominant model, risk allele: C			
c.2712C>G	C=0.81	CG=0.28	CG=0.29	OR=0.81	OR=1.45	OR=0.56	OR=5.04		OR=0.25
p.S904S	G=0.19	GG=n.o.	GG=0.03	CI=0.55–1.20	CI=0.80–2.62	CI=0.29–1.09	CI=0.23–85.16		CI=0.01–4.95
	GG=0.05			P=0.295	P=0.220	P=0.084	P=0.120		P=0.194

We have made no corrections for the multiple statistical testing. Statistically significant results were considered for *P* value <0.05 and when confidence intervals did not contain the number 1. Introducing Bonferroni correction the statistically significant result should be considered for *P* value <0.0024.

*Depending on the tested polymorphism, the control group has different size; **allele T was not present in the study group, and therefore we introduced a correction in the calculation by adding 0.5 to each value (Deeks & Higgins 2010).

n.o., not observed; *P*_{PHWE}, *P*-value for Hardy–Weinberg equilibrium.

patients with advanced sporadic MTC (T3 and T4), including patients with metastases. This suggests that lower *RET* gene expression levels associated with the TT genotype counterbalances an increase in the RET kinase activity and thus alleviates the disease. That would explain our observation that wild-type CC homozygote genotype is more frequent in sporadic MTC patients with a more severe disease: earlier onset, larger tumor size and metastases at the moment of diagnosis.

The c.135G>A (p.A45A, rs1800858) polymorphism in exon 2 of the *RET* gene had been described in four publications (Gimm *et al.* 1999, Cebrian *et al.* 2005, Fernandez *et al.* 2006a, Fugazzola *et al.* 2008). In a meta-analysis compiling the results of these studies, Figlioli *et al.* (2013) determined the average frequency of the c.135A allele to be 18.5% in patients and 23.6% in the population controls. In our study, we have found a lower frequency of the c.135A allele in sporadic MTC patients (25.3%) than in hMTC patients (29.0%) and controls (27.9%). The AA genotype was significantly more frequent in hMTC patients (12.0%) than in sporadic MTC patients (2.2%). Moreover, c.135A allele was present in only 18.1% sMTC patients with T3 and T4 tumors in comparison with 30.4% in patients presenting T1–T2 tumors. This finding might suggest a protective effect of the c.135A allele. In patients with the hereditary form of the disease, we have observed an association of the c.135G allele with the primary hyperparathyroidism and the c.135A allele was present only in one out of eight patients with diagnosed PHPT.

Similar to c.73+9277T, the c.135A variant has been shown to be associated with an increased risk of developing the Hirschsprung's disease (Borrego *et al.* 1999, Carter *et al.* 2012, Vaclavikova *et al.* 2014). Studying genetic factors associated with susceptibility to sMTC, Cebrian *et al.* (2005) observed a protective effect of the c.135A allele. They found the presence of this allele in a haplotype that was not associated with sMTC. The authors suggested the involvement of another variant also being in linkage disequilibrium with c.135G>A, which may be localized in the region of the promoter or regulatory elements of the *RET* gene. Haplotype analysis conducted in this study for sporadic MTC patients has shown a nearly complete linkage disequilibrium between the c.135G>A and c.73+9277T>C variant. Taking all this into consideration, we conclude that c.73+9277T>C is a functional variant, which may influence the clinical course of MTC.

The analysis of the c.1296A>G (p.A432A, rs1800860) polymorphism has shown that the c.1296A allele

was significantly less common among sporadic MTC patients as compared to the control group and to hMTC individuals. However, in this sole case, the frequency of the wild-type c.1296G allele obtained for the Polish population in our study was higher than the minor allele frequency (MAF) in the NCBI database (35.3% vs 22.5%). This discrepancy may result from a relatively small size of the group analyzed for this polymorphism in our study (204 individuals). Recent study on the Czech population has shown a similar frequency of the c.1296G allele: 33.9% and its significantly lower occurrence in HSCR patients (25.8%) (Vaclavikova *et al.* 2014). It is worth to emphasize that in this study, we found a significantly higher frequency of the c.1296A allele in hMTC patients as compared to sporadic MTC cohort. Moreover, the AA and GA genotypes in sporadic patients were considerably less common than those in population-based controls. However, there have been no reports describing this polymorphism in MTC patients.

The c.2071G>A (p.G691S, rs1799939) polymorphism in exon 11 of the *RET* gene is particularly interesting, bearing in mind its functional implication. This variant is frequently detected during routine MTC diagnostics and therefore abundantly reported in the literature. The c.2071A allele was associated with an aggressive course of pancreatic cancer (Sawai *et al.* 2005, Donahue & Hines 2009). It has also been shown to be frequent in cell lines derived from neuroendocrine lung tumors (Sosonkina 2014). Cebrian *et al.* (2005) and Elisei *et al.* (2004) evidenced an association between the c.2071A allele and sMTC. In an *in silico* analysis, Lantieri *et al.* (2013) found that the presence of the c.2071G>A variant changes the phosphorylation pattern of the RET protein, which may result in an induction of signal transduction and hyperactivation of the RAS/ERK pathway. These authors also determined the frequency of this polymorphism among Italian patients and showed differences between MTC patients and the general population. However, these were not statistically significant. In a meta-analysis of eleven studies, they showed that the c.2071A variant represents a low-penetrance allele that predisposes to MTC and may also affect the metastatic capacities and the aggressiveness of the tumor. In our study, we did not found statistically significant differences in frequencies of the c.2071G>A polymorphic variants between examined groups; nevertheless, the c.2071A allele seemed to appear a bit more frequently in sporadic than in hereditary MTC patients (22.5% vs 15%) and also a bit more often than in the control population group (22.5% vs 18.8%). We have not confirmed an association of the c.2071A allele with

Table 3 Correlation of the disease clinical picture with receptor tyrosine kinase gene (*RET*) alleles and genotypes in hereditary medullary thyroid carcinoma (MTC) patients.

Genotype/allele	Age at diagnosis: mean $\pm$ s.d. (n (%))			Comorbidities (n (%))			Primary hyperthyroidism (PHPT)			Multifocality (n (%))			Lymph node and distant metastases (n (%))		
	n*	40		No	Yes		No	Yes		No	Yes		No	Yes	
c.73+927TT>C															
CC		31 $\pm$ 11.0 (50%)		11 (47.8%)	10 (45.4%)		17 (46%)	7 (87.5%)		11 (50%)	9 (60%)		13 (52%)	8 (57.15%)	
CT		32 $\pm$ 17.1 (37.5%)		7 (30.4%)	8 (36.4%)		14 (37.8%)	1 (12.5%)		9 (40.9%)	4 (26.7%)		9 (36%)	5 (35.7%)	
TT		37 $\pm$ 14.9 (12.5%)		5 (21.8%)	4 (18.2%)		6 (16.2%)	n.o.		2 (9.1%)	2 (13.3%)		3 (12%)	1 (7.15%)	
P-Value		$P=0.764$		$P=0.237$			$P=0.095$			$P=0.685$			$P=0.714$		
C		33 $\pm$ 13.4 (68.6%)		29 (63%)	28 (63.6%)		48 (64.9%)	15 (93.8%)		28 (63.6%)	22 (73.3%)		35 (70%)	21 (70%)	
T		36 $\pm$ 18.8 (31.4%)		17 (37%)	16 (36.4%)		26 (35.19%)	1 (6.2%)		16 (36.4%)	8 (26.7%)		15 (30%)	7 (30%)	
OR				OR=1.03			OR=8.13			OR=1.57			OR=1.29		
95% CI				CI=0.43–2.42			CI=1.02–65.02			CI=0.57–4.34			CI=0.45–3.67		
P-Value		$P=0.251$		$P=0.954$			$P=0.032$			$P=0.383$			$P=0.638$		
c.135G>A															
GG		34 $\pm$ 11.3 (52.5%)		13 (54.2%)	13 (59.1%)		18 (48.6%)	7 (87.5%)		11 (47.8%)	9 (60%)		13 (52%)	8 (57.15%)	
GA		32 $\pm$ 17.5 (35%)		6 (25%)	8 (36.4%)		13 (35.1%)	1 (12.5%)		11 (47.8%)	3 (20%)		9 (36%)	5 (35.7%)	
AA		42 $\pm$ 11.6 (12.5%)		5 (20.8%)	1 (4.5%)		6 (16.3%)	n.o.		1 (4.4%)	3 (20%)		3 (12%)	1 (7.15%)	
P-Value		$P=0.764$		$P=0.238$			$P=0.124$			$P=0.118$			$P=0.882$		
G		33 $\pm$ 12.8 (68.6%)		32 (66.7%)	34 (77.3%)		49 (66.2%)	15 (93.8%)		33 (71.7%)	21 (70%)		35 (70%)	21 (75%)	
A		34 $\pm$ 16.5 (31.4%)		16 (33.3%)	10 (22.7%)		25 (33.8%)	1 (6.2%)		13 (28.3%)	9 (30%)		15 (30%)	7 (25%)	
OR				OR=1.70			OR=7.65			OR=0.92			OR=1.29		
95% CI				CI=0.67–4.30			CI=0.96–61.31			CI=0.34–2.53			CI=0.45–3.67		
P-Value		$P=0.201$		$P=0.261$			$P=0.055$			$P=0.870$			$P=0.638$		
c.1296A>G															
GG		31 $\pm$ 12.8 (22.5%)		4 (17.4%)	7 (31.8%)		9 (24.3%)	2 (25%)		5 (22.7%)	3 (20%)		6 (24%)	3 (21.4%)	
GA		32 $\pm$ 14.8 (60%)		15 (65.2%)	11 (50%)		21 (56.8%)	5 (62.5%)		14 (63.65%)	8 (53.3%)		15 (60%)	9 (64.3%)	
AA		36 $\pm$ 10.1 (17.5%)		4 (17.4%)	4 (18.2%)		7 (18.9%)	1 (12.5%)		3 (13.65%)	4 (26.7%)		4 (16%)	2 (14.3%)	
P-Value		$P=0.690$		$P=0.568$			$P=0.909$			$P=0.609$			$P=0.731$		
G		33 $\pm$ 14.0 (52.5%)		23 (50%)	25 (56.8%)		39 (52.7%)	9 (56.3%)		24 (54.5%)	14 (46.7%)		27 (54%)	15 (53.6%)	
A		35 $\pm$ 14.3 (47.5%)		23 (50%)	19 (43.2%)		35 (47.3%)	7 (43.7%)		20 (45.5%)	16 (53.3%)		23 (46%)	13 (46.4%)	
OR				OR=1.32			OR=1.15			OR=0.73			OR=0.98		
95% CI				CI=0.57–3.02			CI=0.39–3.43			CI=0.29–1.85			CI=0.39–2.50		
P-Value		$P=0.908$		$P=0.517$			$P=0.797$			$P=0.506$			$P=0.971$		
c.2071G>A															
GG		33 $\pm$ 13.6 (77.5%)		16 (69.6%)	17 (77.3%)		26 (70.3%)	7 (87.5%)		16 (72.7%)	15 (100%)		18 (72%)	12 (85.7%)	
GA		29 $\pm$ 13.5 (22.5%)		7 (30.4%)	5 (22.7%)		11 (29.7%)	1 (12.5%)		6 (27.3%)	n.o.		7 (28%)	2 (14.3%)	
AA		n.o.		n.o.	n.o.		n.o.	n.o.		n.o.	n.o.		n.o.	n.o.	
P-Value		$P=0.290$		$P=0.738$			$P=0.419$			$P=0.063$			$P=0.287$		
G		33 $\pm$ 13.6 (88.6%)		39 (84.8%)	39 (88.6%)		63 (85.1%)	15 (93.8%)		38 (86.4%)	30 (100%)		43 (86%)	26 (92.9%)	
A		30 $\pm$ 13.2 (11.4%)		7 (13.2%)	5 (11.4%)		11 (14.9%)	1 (6.2%)		6 (13.6%)	n.o.		7 (14%)	2 (7.1%)	
OR				OR=1.40			OR=2.62			OR=10.30			OR=2.12		
95% CI				CI=0.40–4.79			CI=0.31–21.89			CI=0.56–190.09			CI=0.41–10.97		
P-Value		$P=0.820$		$P=0.592$			$P=0.374$			$P=0.117$			$P=0.372$		

c.2307T>G

TT	32 ± 11.6 (60%)	13 (56.5%)	16 (72.7%)	22 (59.5%)	7 (87.5%)	12 (54.6%)	10 (66.7%)	14 (56%)	9 (64.3%)
TG	28 ± 17.3 (27.5%)	6 (26.1%)	5 (22.7%)	10 (27%)	1 (12.5%)	6 (27.3%)	4 (26.7%)	7 (28%)	4 (28.6%)
GG	44 ± 10.8 (12.5%)	4 (17.4%)	1 (4.6%)	5 (13.5%)	n.o.	4 (18.2%)	1 (6.6%)	4 (16%)	1 (7.1%)
P-Value	<i>P</i> = 0.088	<i>P</i> = 0.336	<i>P</i> = 0.293	<i>P</i> = 0.293		<i>P</i> = 0.578		<i>P</i> = 0.806	
T	32 ± 13.3 (73.8%)	32 (69.6%)	37 (84.1%)	54 (73%)	15 (93.8%)	30 (68.2%)	24 (80%)	35 (70%)	22 (78.6%)
G	34 ± 17.8 (26.3%)	14 (30.4%)	7 (13.9%)	20 (27%)	1 (6.2%)	14 (31.8%)	6 (20%)	15 (30%)	6 (21.4%)
OR		OR = 2.31	OR = 5.56	OR = 5.56		OR = 1.87		OR = 1.57	
95% CI		CI = 0.83–6.43	CI = 0.69–44.84	CI = 0.69–44.84		CI = 0.62–5.59		CI = 0.53–4.66	
P-Value	<i>P</i> = 0.156	<i>P</i> = 0.108	<i>P</i> = 0.107	<i>P</i> = 0.107		<i>P</i> = 0.265		<i>P</i> = 0.415	

c.2508C>T

CC	34 ± 13.8 (100%)	23 (100%)	22 (100%)	37 (100%)	8 (100%)	22 (100%)	15 (100%)	25 (100%)	14 (100%)
CT	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
TT	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
P-Value	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
C	34 ± 13.8 (100%)	46 (100%)	44 (100%)	74 (100%)	16 (100%)	44 (100%)	30 (100%)	50 (100%)	28 (100%)
T	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
OR	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

c.2712C>G

CC	33 ± 13.8 (80%)	17 (73.9%)	17 (77.3%)	27 (73%)	7 (87.5%)	18 (81.8%)	14 (93.3%)	20 (80%)	12 (85.7%)
GC	30 ± 13.3 (20%)	6 (26.1%)	5 (22.7%)	10 (27%)	1 (12.5%)	4 (18.2%)	1 (6.7%)	5 (20%)	2 (14.3%)
GG	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
P-Value	<i>P</i> = 0.527	<i>P</i> = 1.000	<i>P</i> = 0.657	<i>P</i> = 0.657		<i>P</i> = 0.629		<i>P</i> = 0.458	
C	32 ± 13.8 (50%)	40 (87%)	39 (88.6%)	64 (86.5%)	15 (93.8%)	40 (90.9%)	29 (96.7%)	45 (90%)	26 (92.6%)
G	30 ± 13.3 (50%)	6 (13%)	5 (11.4%)	10 (13.5%)	1 (6.2%)	4 (9.1%)	1 (5.3%)	5 (10%)	2 (7.4%)
OR		OR = 1.17	OR = 2.34	OR = 2.34		OR = 2.90		OR = 1.44	
95% CI		CI = 0.33–4.15	CI = 0.28–19.75	CI = 0.28–19.75		CI = 0.31–27.32		CI = 0.26–7.98	
P-Value	<i>P</i> = 0.907	<i>P</i> = 0.808	<i>P</i> = 0.433	<i>P</i> = 0.433		<i>P</i> = 0.352		<i>P</i> = 0.673	

In bold were marked statistically significant (*P*-value < 0.05) and borderline significant results. We have made no corrections for the multiple statistical testing.*Clinical data were not available for all patients; the patients diagnosed before symptoms occurred were not included either.
n.a., not analyzed; n.o., not observed.

Table 4 Correlation of RET polymorphisms genotypes and the clinical picture in patients with sporadic MTC.

Genotype/Allele		Age at diagnosis: mean ± s.d. (n (%))	Progression of the disease (TNM) (n (%))						Multifocality (n (%))		Other malignancies (n (%))	
n*		88	T1 + T2	T3 + T4	N0	N1	M0	M1	No	Yes	No	Yes
c.73+g277T>C												
CC	51 ± 15.5 (51.1%)		21 (40.4%)	19 (67.9%)	22 (40.0%)	18 (62.9%)	34 (47.2%)	6 (75%)	41 (50.6%)	5 (50%)	43 (51.2%)	3 (42.9%)
CT	56 ± 15.3 (46.6%)		29 (55.8%)	9 (32.1%)	25 (55.6%)	13 (37.1%)	36 (50%)	2 (25%)	39 (48.2%)	4 (40%)	39 (46.4%)	4 (57.1%)
TT	40 ± 9.2 (2.3%)		2 (3.8%)	n.o.	2 (4.4%)	n.o.	2 (2.8%)	n.o.	1 (1.2%)	1 (10%)	2 (2.4%)	n.o.
P-Value	P=0.148		P=0.051		P=0.056		P=0.320		P=0.198		P=0.814	
C	53 ± 15.5 (74.4%)		71 (68.3%)	47 (83.9%)	69 (70.4%)	49 (79%)	104 (72.2%)	14 (87.5%)	121 (74.7%)	14 (70%)	125 (74.4%)	10 (71.5%)
T	55 ± 15.3 (25.6%)		33 (31.7%)	9 (16.1%)	29 (29.6%)	13 (21%)	40 (27.8%)	2 (16.5%)	41 (25.3%)	6 (30%)	43 (25.6%)	4 (28.6%)
OR			OR=2.43		OR=1.58		OR=2.00		OR=0.79		OR=0.86	
95% CI			CI=1.07–5.53		CI=0.75–2.65		CI=0.44–9.17		CI=0.29–2.19		CI=0.26–2.88	
P-Value	P=0.455		P=0.035		P=0.229		P=0.372		P=0.652		P=0.807	
c.135G>A												
GG	51 ± 15.7 (52.3%)		22 (43.1%)	18 (62.1%)	22 (45.2%)	18 (52.8%)	34 (47.2%)	5 (62.5%)	42 (50.6%)	4 (40%)	43 (51.2%)	3 (42.9%)
GA	56 ± 15.3 (45.4%)		27 (52.9%)	11 (37.9%)	23 (54.8%)	15 (41.7%)	36 (50%)	3 (37.5%)	38 (48.2%)	5 (50%)	39 (46.4%)	4 (57.1%)
AA	40 ± 9.2 (2.3%)		2 (3.9%)	n.o.	2 (5.5%)	n.o.	2 (2.8%)	n.o.	1 (1.2%)	1 (10%)	2 (2.4%)	n.o.
P-Value	P=0.145		P=0.107		P=0.198		P=0.646		P=0.118		P=0.814	
G	53 ± 15.7 (75%)		71 (69.6%)	47 (81%)	67 (71.3%)	51 (77.3%)	104 (71.9%)	13 (81.3%)	121 (75.6%)	13 (66.7%)	125 (74.4%)	10 (71.4%)
A	55 ± 15.3 (25%)		31 (30.4%)	11 (19%)	27 (28.7%)	15 (22.7%)	40 (28.1%)	3 (28.7%)	41 (24.4%)	7 (33.3%)	43 (25.6%)	4 (28.6%)
OR			OR=1.87		OR=1.37		OR=1.67		OR=0.63		OR=0.86	
95% CI			CI=0.86–4.07		CI=0.66–2.84		CI=0.45–6.16		CI=0.24–1.69		CI=0.26–2.88	
P-Value	P=0.463		P=0.117		P=0.397		P=0.444		P=0.357		P=0.807	
c.1296A>G												
GG	53 ± 14.8 (60.2%)		29 (55.8%)	19 (67.9%)	25 (58.1%)	23 (62.2%)	43 (59.7%)	5 (62.5%)	47 (58%)	8 (80%)	53 (63.1%)	2 (28.6%)
GA	55 ± 16.3 (27.3%)		16 (30.8%)	6 (21.4%)	11 (25.6%)	11 (29.7%)	19 (26.4%)	3 (37.5%)	22 (27.2%)	2 (20%)	20 (23.8%)	4 (57.1%)
AA	47 ± 17.0 (12.5%)		7 (13.4%)	3 (10.7%)	7 (16.3%)	3 (8.11%)	10 (13.9%)	n.o.	12 (14.8%)	n.o.	11 (13.1%)	1 (14.3%)
P-Value	P=0.397		P=0.568		P=0.538		P=0.486		P=0.306		P=0.135	
G	54 ± 15.2 (73.9%)		74 (71.2%)	44 (78.6%)	61 (70.9%)	57 (77%)	105 (72.9%)	13 (81.3%)	116 (71.6%)	18 (90%)	126 (85.1%)	8 (57.1%)
A	53 ± 16.6 (26.1%)		30 (28.8%)	12 (21.4%)	25 (29.1%)	17 (23%)	39 (27.1%)	3 (18.7%)	46 (28.4%)	2 (10%)	42 (14.9%)	6 (42.9%)
OR			OR=1.49		OR=1.37		OR=1.61		OR=3.57		OR=0.44	
95% CI			CI=0.67–3.20		CI=0.67–2.81		CI=0.44–5.95		CI=0.80–16.00		CI=0.15–1.36	
P-Value	P=0.708		P=0.311		P=0.383		P=0.476		P=0.097		P=0.154	
c.2071G>A												
GG	52 ± 14.2 (59.1%)		33 (63.5%)	14 (50%)	26 (60.5%)	21 (56.8%)	43 (59.7%)	4 (50%)	48 (59.3%)	6 (60%)	50 (59.5%)	4 (57.1%)
GA	54 ± 17.0 (38.6%)		17 (32.7%)	14 (50%)	15 (34.9%)	16 (43.2%)	27 (37.5%)	4 (50%)	30 (37.5%)	4 (40%)	31 (36.9%)	3 (42.9%)
AA	46 ± 31.1 (2.3%)		2 (3.9%)	n.o.	2 (4.7%)	n.o.	2 (2.8%)	n.o.	3 (3.7%)	n.o.	3 (3.6%)	n.o.
P-Value	P=0.714		P=0.218		P=0.345		P=0.730		P=0.826		P=0.536	
G	53 ± 15.3 (78.4%)		83 (79.8%)	42 (75%)	67 (77.9%)	58 (78.4%)	113 (78.5%)	12 (75%)	126 (77.8%)	16 (80%)	131 (78%)	11 (78.6%)
A	54 ± 15.4 (21.6%)		21 (20.2%)	14 (25%)	19 (22.1%)	16 (21.6%)	31 (21.5%)	4 (25%)	36 (22.2%)	4 (20%)	37 (22%)	3 (21.4%)
OR			OR=0.76		OR=1.03		OR=0.82		OR=1.14		OR=1.04	
95% CI			CI=0.35–1.64		CI=0.48–2.18		CI=0.25–2.73		CI=0.36–3.63		CI=0.27–3.91	
P-Value	P=0.722		P=0.484		P=0.943		P=0.750		P=0.821		P=0.956	

c.2307T>G

TT	51 ± 16.3 (52.3%)	24 (42.2%)	16 (57.1%)	18 (41.9%)	22 (59.5%)	34 (47.2%)	6 (75%)	41 (50.6%)	6 (60%)	43 (51.2%)	4 (57.1%)
TG	55 ± 14.8 (45.4%)	26 (50%)	12 (42.9%)	23 (53.5%)	15 (40.5%)	36 (50%)	2 (25%)	38 (46.9%)	4 (40%)	39 (46.4%)	3 (42.9%)
GG	54 ± 0.0 (2.3%)	2 (3.8%)	n.o.	2 (4.6%)	n.o.	2 (2.8%)	n.o.	2 (2.5%)	n.o.	2 (2.4%)	n.o.
P-Value	P=0.549	P=0.425	n.o.	P=0.161	n.o.	P=0.320	n.o.	P=0.782	n.o.	P=0.891	n.o.
T	53 ± 15.6 (75%)	74 (71.2%)	44 (78.6%)	59 (68.6%)	59 (79.7%)	104 (72.2%)	14 (87.5%)	120 (74.1%)	16 (80%)	125 (74.4%)	11 (78.6%)
G	55 ± 14.5 (25%)	30 (28.8%)	12 (21.4%)	27 (31.4%)	15 (20.3%)	40 (27.8%)	2 (12.5%)	42 (25.9%)	4 (20%)	43 (25.6%)	3 (21.4%)
OR	OR=1.49	OR=1.49	n.o.	OR=1.80	n.o.	OR=2.69	n.o.	OR=1.40	n.o.	OR=1.26	n.o.
95% CI	CI=0.69–3.20	CI=0.69–3.20	n.o.	CI=0.87–3.72	n.o.	CI=0.59–12.38	n.o.	CI=0.44–4.42	n.o.	CI=0.35–4.74	n.o.
P-Value	P=0.455	P=0.311	n.o.	P=0.113	n.o.	P=0.203	n.o.	P=0.567	n.o.	P=0.731	n.o.

c.2508C>T

CC	53 ± 15.6 (90.9%)	49 (63.5%)	24 (50%)	39 (90.7%)	34 (91.9%)	66 (91.7%)	7 (87.5%)	74 (91.6%)	9 (90%)	76 (90.5%)	7 (100%)
CT	50 ± 14.8 (9.1%)	3 (3.27%)	4 (50%)	4 (9.3%)	3 (8.1%)	6 (8.3%)	1 (12.5%)	7 (8.6%)	1 (10%)	8 (9.5%)	n.o.
TT	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
P-Value	P=0.714	P=0.232	n.o.	P=0.850	n.o.	P=0.536	n.o.	P=0.886	n.o.	P=0.513	n.o.
C	53 ± 15.5 (95.5%)	101 (97.1%)	52 (92.8%)	82 (95.3%)	71 (95.9%)	138 (95.8%)	15 (93.7%)	155 (95.7%)	19 (95%)	160 (95.2%)	14 (100%)
T	50 ± 14.8 (4.5%)	3 (2.9%)	4 (7.2%)	4 (4.7%)	3 (4.1%)	6 (4.2%)	1 (6.3%)	7 (4.3%)	1 (5%)	8 (4.8%)	n.o.
OR	OR=0.39	OR=0.39	n.o.	OR=1.15	n.o.	OR=0.65	n.o.	OR=0.86	n.o.	OR=1.54**	n.o.
95% CI	CI=0.08–1.79	CI=0.08–1.79	n.o.	CI=0.25–5.33	n.o.	CI=0.07–5.79	n.o.	CI=0.10–7.36	n.o.	CI=0.08–27.98	n.o.
P-Value	P=0.593	P=0.224	n.o.	P=0.854	n.o.	P=0.701	n.o.	P=0.889	n.o.	P=0.772	n.o.

c.2712C>G

CC	52 ± 14.2 (59.1%)	17 (32.7%)	14 (50%)	15 (34.9%)	16 (43.2%)	27 (37.5%)	4 (50%)	31 (38.3%)	4 (40%)	31 (36.9%)	4 (57.1%)
GC	54 ± 17.0 (38.6%)	33 (63.5%)	14 (50%)	26 (60.5%)	21 (56.8%)	43 (59.7%)	4 (50%)	47 (58%)	6 (60%)	50 (59.5%)	3 (42.9%)
GG	46 ± 31.1 (2.3%)	2 (3.8%)	n.o.	2 (4.6%)	n.o.	2 (2.8%)	n.o.	3 (3.7%)	n.o.	3 (3.6%)	n.o.
P-Value	P=0.390	P=0.218	n.o.	P=0.345	n.o.	P=0.320	n.o.	P=0.825	n.o.	P=0.536	n.o.
C	53 ± 15.3 (78.4%)	67 (42.2%)	42 (75%)	56 (65.1%)	53 (71.6%)	97 (67.4%)	12 (75%)	109 (67.3%)	14 (70%)	112 (66.7%)	11 (78.6%)
G	53 ± 17.4 (21.6%)	37 (50%)	14 (25%)	30 (34.9%)	21 (28.4%)	47 (32.6%)	4 (25%)	53 (32.7%)	6 (30%)	56 (33.3%)	3 (21.4%)
OR	OR=1.66	OR=1.66	n.o.	OR=1.35	n.o.	OR=1.45	n.o.	OR=1.14	n.o.	OR=1.83	n.o.
95% CI	CI=0.80–3.42	CI=0.80–3.42	n.o.	CI=0.69–2.65	n.o.	CI=0.45–4.75	n.o.	CI=0.41–3.12	n.o.	CI=0.49–6.84	n.o.
P-Value	P=1.000	P=0.325	n.o.	P=0.379	n.o.	P=0.536	n.o.	P=0.807	n.o.	P=0.367	n.o.

In bold were marked statistically significant (P -value <0.05) and borderline significant results. We have made no corrections for the multiple statistical testing.*Clinical data were not available for all patients; **OR calculation was performed introducing the amendment according to Deeks & Higgins (2010).
n.o., not observed.

Figure 2

Linkage disequilibrium (LD) plots; (A) patients with hereditary MTC, (B) patients with sporadic MTC. The LD plots were constructed with *Haploview v.4.2* software (Barrett *et al.* 2005). Numbers in squares represent the D' statistic $\times 100$ for each pairwise comparison, if $D' = 1$ the squares are shaded only. Gray color intensity indicates the intensity of r^2 ($r^2 = 0$: white, $0 < r^2 < 1$: shades of gray, $r^2 = 1$: black). Haplotypes frequencies are shown on the right.

an earlier onset of the disease that was reported by other researchers (Robledo *et al.* 2003, Cardot-Bauters *et al.* 2008).

Other studies indicated a strong linkage disequilibrium (LD) between the c.2071G>A and c.2712C>G (p.S904S, rs1800863) polymorphisms in exon 15 (Robledo *et al.* 2003, Elisei *et al.* 2004, Cebrian *et al.* 2005, Fernandez *et al.* 2006b, Fugazolla *et al.* 2008, Siqueira *et al.* 2010, Machens *et al.* 2012, Lantieri *et al.* 2013). The analysis using the *Haploview* software we conducted in this study has also confirmed strong LD in patients with the hereditary form of MTC ($r^2 = 0.770$; $D' = 0.914$) and complete linkage disequilibrium in sMTC patients ($r^2 = 0.969$; $D' = 1$). Therefore, we conclude that the genotype distribution and the allele frequencies of the c.2712C>G were similar to c.2071G>A. These results are in accordance with those, reported by Fugazzolla *et al.* (2008) and Machens *et al.* (2012), who found any association between the G691S/S904S variants and an increased risk or the clinical course of sMTC form.

In contrast, we observed an incomplete linkage equilibrium between c.73+9277T>C and c.135G>A polymorphisms localized in the 5' region of the *RET* proto-oncogene and c.2307T>G (p.L769L, rs1800861) variant localized in exon 13 of the *RET* gene. We have observed a lower prevalence of rare homozygotes among patients with sporadic MTC as compared to hereditary cases and to the controls. Moreover, we found statistically significant differences in genotype distribution between sMTC patients and controls, with the heterozygous TG genotype of c.2307T>G polymorphic variant being more frequent in sporadic patients (46.2%) than in hMTC patients (30.0%)

and in controls (40.4%). This was in accordance with the results reported for patients from central Poland in whom the heterozygous c.2703T>G variant was found in 48.3% cases as compared to 39.5% in healthy individuals. The authors also reported that this variant predisposes to sporadic MTC. Their computer simulations also showed that the c.2703G allele may influence mRNA stability by reducing its minimal free energy (MFE) by 17% for the wild-type, 7% for the p.Y791F mutant and less than 5% for the c.2712C>G and c.2508C>T variants as well as for the most frequent p.C634R mutation (Sromek *et al.* 2010). We did not observed an association between the c.2703G allele with a younger age at diagnosis, which was suggested by Sromek *et al.* (2010), nor with pheochromocytoma or PHPT. On the contrary, this allele seemed to have a protective effect: it was present in only one PHPT patient (6.2% vs 27% among other patients) and in six out of twenty-two pheochromocytoma patients (13.9% vs 30.4% in other patients), but these results were not statistically significant. Any significant associations in sporadic MTC cohort were found either. Nonetheless, we observed a slightly lower frequency of the c.2703G allele in patients with more advanced disease (T3 and T4) and metastases (N1 and M1) present at the time of diagnosis. Our results do not allow to draw univocal conclusions so as to the influence of the c.2307T>G polymorphism on the MTC course. Haplotype analysis in sMTC patients has shown an incomplete linkage equilibrium of the c.2307T>G variant with c.2508C>T ($D' = 1.000$ but $r^2 = 0.135$).

Several studies have pointed out to c.2508C>T (p.S836S, rs1800862), a synonymous variant in exon 14,

Table 5 The *RET* gene selected haplotypes and their relation to clinical picture of MTC.

Alleles/haplotypes		Age of diagnosis		PHEO		PHPT		Multifocality		N1		M1	
		OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value
C.73+9277T>C	C	1.96 (1.01–3.87)	0.049	3.41 (1.57–7.40)	0.002	4.37 (1.56–12.28)	0.005	3.17 (1.03–10.26)	0.044	0.60 (0.27–1.35)	0.216	0.63 (0.13–3.11)	0.573
C.1296A>G	C	1.96 (1.01–3.87)	0.049	3.41 (1.57–7.40)	0.002	4.37 (1.56–12.28)	0.005	3.17 (1.03–10.26)	0.044	0.60 (0.27–1.35)	0.216	0.63 (0.13–3.11)	0.573
C.2071G>A	C	2.08 (1.06–4.07)	0.034	3.27 (1.51–7.10)	0.003	5.62 (1.60–19.92)	0.007	1.98 (0.88–4.46)	0.101	0.60 (0.27–1.35)	0.216	0.63 (0.13–3.11)	0.573
C.2307T>G	C	2.02 (1.09–3.75)	0.025	3.10 (1.19–9.60)	0.029	3.10 (1.12–8.60)	0.028	1.49 (0.67–3.30)	0.327	0.87 (0.41–1.86)	0.725	0.63 (0.13–3.11)	0.573
C.135G>A	C	2.06 (1.06–3.99)	0.032	3.39 (1.60–7.21)	0.002	3.73 (1.35–10.27)	0.011	1.95 (0.87–4.40)	0.107	0.56 (0.25–1.24)	0.150	0.58 (0.12–2.84)	0.502
C.2712C>G	C	1.84 (1.01–3.34)	0.047	2.10 (1.02–4.32)	0.045	2.48 (0.91–6.73)	0.075	1.30 (0.59–2.86)	0.514	0.71 (0.34–1.48)	0.359	0.50 (0.10–2.40)	0.383
C.2508C>T	C	1.68 (0.99–2.87)	0.057	4.31 (1.02–19.19)	0.048	4.34 (1.03–19.30)	0.048	1.34 (0.66–2.71)	0.421	1.35 (0.70–2.62)	0.371	3.64 (0.77–17.25)	0.104
C.2307T>G	C	1.94 (1.01–3.73)	0.046	3.26 (1.54–6.91)	0.002	3.61 (1.31–9.92)	0.013	2.37 (1.06–5.26)	0.035	0.71 (0.32–1.59)	0.405	0.64 (0.13–3.15)	0.586
C.2071G>A	C	1.84 (1.01–3.34)	0.047	2.10 (1.02–4.32)	0.045	2.55 (0.94–6.91)	0.067	1.78 (0.83–3.77)	0.138	0.95 (0.45–2.00)	0.893	0.58 (0.12–2.84)	0.502
C.1296A>G	C	0.94 (0.58–1.55)	0.819	0.92 (0.47–1.77)	0.793	1.06 (0.40–2.77)	0.909	0.88 (0.46–1.70)	0.711	1.61 (0.87–2.97)	0.127	3.90 (1.03–14.80)	0.046
C.2712C>G	C	1.40 (0.83–2.36)	0.187	2.93 (1.09–7.88)	0.033	3.12 (1.38–11.20)	0.017	1.86 (0.92–3.77)	0.077	1.03 (0.55–1.93)	0.928	1.75 (0.52–5.95)	0.370
C.2508C>T	C	1.03 (0.52–2.02)	0.937	0.85 (0.16–4.48)	0.844	0.29 (0.04–2.27)	0.241	0.37 (0.11–1.25)	0.109	2.58 (0.96–6.90)	0.059	2.28 (0.52–9.96)	0.275
C.2307T>G	C	1.34 (0.80–2.23)	0.264	2.89 (1.32–6.32)	0.008	3.02 (1.35–10.74)	0.018	1.75 (0.88–3.50)	0.110	1.03 (0.55–1.93)	0.928	1.75 (0.51–5.95)	0.370
C.135G>A	C	1.45 (0.85–2.50)	0.175	1.61 (0.75–3.46)	0.221	4.08 (0.92–18.16)	0.065	1.43 (0.70–2.95)	0.329	1.43 (0.73–2.78)	0.293	3.71 (0.78–17.61)	0.084
C.2712C>G	C	1.58 (0.87–2.87)	0.135	2.12 (0.85–5.31)	0.107	5.92 (0.77–45.42)	0.087	1.45 (0.66–3.21)	0.358	1.85 (0.90–3.80)	0.096	5.73 (0.91–46.11)	0.068
C.2508C>T	C	0.52 (0.26–1.04)	0.066	1.03 (0.29–3.72)	0.960	0.20 (0.03–1.21)	0.084	0.82 (0.36–1.89)	0.638	0.49 (0.22–1.02)	0.071	0.22 (0.03–1.74)	0.150
C.2307T>G	C	0.63 (0.34–1.18)	0.148	0.19 (0.03–1.18)	0.079	0.19 (0.03–1.49)	0.114	0.70 (0.30–1.59)	0.390	0.62 (0.29–1.30)	0.203	0.22 (0.03–1.74)	0.150
C.1296A>G	C	0.68 (0.36–1.26)	0.218	0.58 (0.23–1.44)	0.239	0.20 (0.03–32.73)	0.125	0.71 (0.33–1.63)	0.423	0.46 (0.22–0.98)	0.044	0.18 (0.02–1.41)	0.082

Statistically significant results are marked in bold (*P*-value < 0.05).

Table 6 Mutations in the *RET* gene against the haplotype background in hereditary medullary thyroid carcinoma patients.

Haplotypes			Mutations at codon 634		p.C634R mutation		Mutations at codons 768, 791, 804 and 844	
c.73+9277T>C	c.135G>A	c.2307T>G	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value
C	G	T	4.58 (1.75–11.90)	0.002	5.65 (2.27–14.04)	<0.001	0.16 (0.06–0.47)	<0.001
T	A	G	0.18 (0.06–0.51)	0.001	0.11 (0.03–0.04)	<0.001	4.50 (1.54–13.18)	0.006

CI, confidence interval; OR, odds ratio.

as being associated with an increased risk of sporadic MTC (Fugazzola *et al.* 2008, Siqueira *et al.* 2010). In a Spanish and German population, this variant has been shown to be concomitant with a nearly threefold increase in the risk of sMTC, and it was most frequently detected in patients with the p.M918T somatic mutation (Gimm *et al.* 1999, Ruiz *et al.* 2001). In contrast, Machens *et al.* (2012) did not report a significant association of c.2508C>T variant with an increased risk of sMTC in their group of German patients.

In this study, we have not found the c.2508T allele among hereditary MTC patients admitting that patient cohort is relatively small for studying a rare polymorphic variant. Among sporadic cases, the c.2508T allele was present at a 4.4% frequency, implying its role as a risk factor for sMTC, but the frequency in controls was similar (3.4%), so it does not seem to be the case.

Ceolin *et al.* (2012) found that c.2508C>T and c.2307T>G polymorphic variants are associated with an increased risk of sMTC in the Brazilian population. The adjusted OR for individuals with haplotypes including three or more polymorphic alleles was 3.79. No significant associations between the observed *RET* gene variants and disease onset or tumor size were noticed. In contrast, patients carrying haplotypes with three or four risk alleles had a nearly sixfold (OR=5.84) higher risk of lymph node involvement and distant metastases at the time of diagnosis. The results of these studies showed an additive effect of the described polymorphic variants on the risk of developing an aggressive MTC. In their last report, Ceolin *et al.* (2016) have shown that the c.2508C>T variant is in a strong LD with *RET* 3'UTR variants. They also performed an *in silico* analysis, which indicated that 3'UTR variants may affect the secondary structure of *RET* mRNA and posttranscriptional processing. In the same line, polymorphisms closer to the 3' region of the *RET* gene and metastases appeared to be associated in our study, but this result was borderline significant.

Our haplotype analysis has shown that the C-G-A-G-T-(C)-C (c.73+9277T>C – c.135G>A – c.1296A>G – c.2071G>A – c.2307T>G – (c.2508C>T) – c.2712C>G) allele

combination predisposes to pheochromocytoma. Among carriers of this haplotype, twice as many individuals developed pheochromocytoma by the age of 40 years.

This haplotype was also three times more frequent in patients with multifocal MTC and its carriers had PHPT four times more often than non-carriers. This may indicate that this haplotype is associated with the MEN2A syndrome, and identifying it in young patients may be a predictive marker for early adrenal and parathyroid glands diagnostics. This is of great importance because pheochromocytoma and PHPT when untreated are a real threat to patients' lives, greater than the MTC itself.

This study is the first one to report on *RET* mutations in association with the haplotypes. Our results indicate a strong relationship between mutations in codon 634 and the C-G-T (c.73+9277T>C – c.135G>A – c.2307T>G) allele arrangement. For the most frequent mutation: p.C634R, this association has been even stronger, whereas mutations related to isolated MTC (p.E768D, p.Y791F, p.V804M, p.R844Q) co-existed rarely with the C-G-T haplotype block and frequently with the alternative T-A-G combination. The co-occurrence of the p.E768D, p.Y791F, p.V804M or p.R844Q *RET* mutations with the c.73+9277T allele, which lowers the *RET* gene expression may be associated with a further attenuation of those weaker activating mutations and milder clinical picture of the disease.

In conclusion, it seems that the c.73+9277T>C variant associated with alteration in activity of the *RET* enhancer, the c.73+9277T>C – c.135G>A – c.2307T>G alleles combination, or another sequence variant in LD with these polymorphisms may modify the clinical course of the MTC and the MEN2A syndrome. From the clinical point of view, determining this haplotype could help to predict the disease course and define the optimal age for prophylactic thyroidectomy in patients with *RET* mutations defined as moderate risk ('MOD') by the 2015 American Thyroid Association (ATA) recommendations (Wells 2015). Haplotype defining may also become a powerful diagnostic tool for the early presence of pheochromocytoma and primary hyperparathyroidism in MEN2A patients.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-17-0452>.

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.

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Author contribution statement

Marta Kaczmarek-Ryś designed the study and she is guarantor. Marta Kaczmarek-Ryś, Katarzyna Ziemnicka and Andrzej Pławski prepared the paper. Marta Kaczmarek-Ryś, Bartłomiej Budny, Szymon Hryhorowicz, Paweł Boruń, Justyna Hoppe-Gołębiowska and Maria Sromek contributed to the experimental and laboratory work. Katarzyna Ziemnicka, Monika Gołąb and Małgorzata Czetwertyńska were responsible for patients' clinical examination and qualification to the study. Marta Kaczmarek-Ryś and Marlena Szalata performed statistical calculations and Michał Michalak consulted and supervised statistical analysis of the data. Marek Ruchała and Ryszard Słomski coordinated the whole research. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

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