DE GRUYTER OPEN

ПРИЛОЗИ, Одд. мед. науки, XXXVI 1, 2015 **МАНУ**
CONTRIBUTIONS, Sec. Med. Sci., XXXVI 1, 2015 **MASA**

10.1515/prilozi-2015-0034 ISSN 1857-9345
UDC: 616.441-006.6:575.224.23

NOVEL RET MUTATIONS IN MACEDONIAN PATIENTS WITH MEDULLARY THYROID CARCINOMA: GENOTYPE-PHENOTYPE CORRELATIONS

Rubens Jovanovic[1], Slavica Kostadinova-Kunovska[1], Vesna Janevska[1], Biljana Bogoeva[1], Liljana Spasevska[1], Daniela Miladinova[2], Ana Ugrinska[2], Marina Zdraveska-Kochovska[2], Dejan Trajkov[3], Gordana Petrusevska[1]

[1] Institute of Pathology, Medical Faculty, Skopje, R. Macedonia
[2] Institute of Pathophysiology and Nuclear Medicine, Medical Faculty, Skopje, R. Macedonia
[3] Institute of Immunobiology and Human Genetics, Medical Faculty, Skopje, R. Macedonia

Correspondending Author: Rubens Jovanovic MD, MSci, Institute of Pathology, Medical Faculty, 50 Divizija 6b, 1000 Skopje, R. Macedonia; E-mail: rubensjovanovic@medf.ukim.edu.mk

Abstract

Medullary thyroid carcinomas (MTCs) are rare neoplasms comprising 2–10% of all thyroid malignancies. More than 75% are sporadic tumors and the remainder is familial and MEN2 related. Both sporadic and syndromic MTCs frequently show mutations in the RET proto-oncogene. It has been noted that some MTC cases present an indolent, and some an aggressive clinical course. Ki-67 expression is generally low, with documented exceptions, whereas high expression of Bcl-2 has been reported in majority of the cases. Some studies have shown that Ki-67 and Bcl-2 expressions have prognostic value, as well as RET mutational status. We analyzed 20 unrelated MTC cases for Ki-67, Bcl-2 expression and RET mutations and tested their intercorrelations, correlations to the morphologic features and stage of the tumors, as well as their influence on survival. In 13 of the 20 analyzed cases we found 23 sequence changes distributed in exons 8, 10–13 and 16. There were 11 different missense mutations, single nucleotide deletion with frameshift, and 8 different synonymous mutations. Only 4 of the sequence changes have been previously published. Twelve patients (60%) had tumors expressing one or more missense mutations or single nucleotide deletion and 7 of them (35%) had at least one damaging or possibly damaging RET mutation. Most of the tumors had low Ki-67 expression (mean 6.48% of cells) and high Bcl-2 expression (mean 68.3%). Significantly better survival was observed in cases with low Ki-67 ($< 6.5\%$; $p < 0.05$), high Bcl-2 expression ($> 68.3\%$; $p < 0.01$) and younger age at diagnosis (< 51 years; $p < 0.05$).

Key words: RET mutations, Medullary thyroid carcinoma, Ki-67, Bcl-2.

Introduction

Medullary thyroid carcinomas (MTCs) are rare neoplasms comprising 2–10% of all thyroid malignancies [1, 2]. Most of them appear as sporadic (75–80%), and the reminder as familial and MEN2 related cases [1, 3, 4]. According to the US National Cancer Institute (NCI) the incidence of MTCs in the US ranges between 500–1000 cases per year (in a population of 300 mil.), or 2–3% of all thyroid cancers which incidence is 37300 cases per year. According to the statistics from France and USA 43% and 44% of MTCs, respectively, are familial cases, and frequently associated with MEN2 syndrome, while in Sweden, this percentage is much lower – around 26%. According to the NCI estimation, the same percentage should be applicable to Europe in general [2]. Having in mind the aforementioned proportions, data from the Macedonian Institute for Public Health, the Institute of Pathology and the Institute for Radiotherapy and Oncology in Skopje, there should be 2–3 newly diagnosed cases of MTC in Macedonia (population of approx. 2 mil.) per year.

However, in the last 15 years we have diagnosed only 22 cases and none of them was MEN2 related, neither a case of Familial Medullary Thyroid Cancer (FMTC).

It has been noted that some MTC cases present an indolent, and some an aggressive clinical course [1, 4, 5].

In general, addressing the issue of Ki-67 there are reports about very low Ki-67 expression in all MTCs [6], with documented exceptions [7], whereas for the Bcl-2, high expressions have been reported in majority of the cases, and some studies have shown that Bcl-2 expression has a positive prognostic value [8].

RET proto-oncogene mutations are frequently found in MTC, as well as in pheochromocytoma and paraganglioma, especially in familial and multiple endocrine neoplasia 2 (MEN2) – related cases [1, 3, 10–14]. RET mutations are also associated with variety of other pathologic conditions in humans, including Hirschsprung's disease and malformations of the urinary tract (3, 10, 15–19). More than 400 different single nucleotide polymorphisms and mutations in the coding sequence of the ret proto-oncogene have been published [20, 21]. Some of them are more frequent in specific disorders, and some of them have impact on the clinical expression of the disease [5]. In general gain-of-function mutations, frequently encountered in MEN2 and Familial MTC, are usually located in one of the six highly conserved codons including codons 609, 611, 618, 620 in exon 10, and codons 630 and 634 in exon 11 that code for cysteine [15]. Most of the clinically relevant mutations published up to date are located in "risk exons" 8, 10, 11, 13, 14, 15 and 16 [1, 3, 5, 13, 22–27]. RET mutations have been found in sporadic MTC also, with a frequency from 23% to 70 % in different series [28–34]. In most such cases the mutation involved is a MEN2B-like (M918T), although other codons may be involved as well [28, 33]. Cases with somatic deletions have been descrybed as well [30, 31]. Several studies have shown correspondence between specific RET mutations and the disease phenotype, as well as the aggressiveness of the MTC [30, 35, 36]. The mutation M981T has been recognized as an aggressive one and majority of patients with this mutation in germline sate usually die at young age. Opposite to this, mutations like Y791F or A883T have not been associated with MTC at all, at least not in heterozygous state [36, 37].

Aim

The aim of our study was to determine the incidence and spectrum of RET mutations in Macedonian patients with apparently sporadic MTC, and explore possible correlations between the presence of RET mutations and the immunohistochemical expression of Ki-67, Bcl-2 and disease outcome.

Patients, material and methods

We retrospectively analyzed group of 20 unrelated MTC cases (7 males and 13 females) diagnosed in 15 years period at the Laboratory for pathohistology and clinical cytology from the Institute for Radiotherapy and Oncology and the Institute of Pathology, Medical Faculty, Skopje. Besides the standard histopathological analysis including the routine immunohistochemistry, for all of the cases we had immunostainings for the Ki-67 proliferation marker and the anti-apoptotic marker Bcl-2 previously done. Immunostainings (EnVision Flex visualization system and PT link pretreatment, DAKO, Denmark) for Ki-67 (clone MIB-1, dilution 1 : 25, DAKO) and Bcl-2 (clone 124, dilution 1 : 50, DAKO) have been quantitatively re-evaluated by two pathologists independently using the Image Analysis Software LuciaM. Positive signals were counted in 5 sets of 1000 cells, and results were recorded as percents. In our analysis we also included the following parameters: the greatest tumor diameter, presence of lymph node metastases at the time of operation, the stage of the disease, as well as the time from the primary operation until the eventual recurrence and/or appearance of lymph node or distant metastases, and survival of the patients. We incorporated the clinical data from the University Clinic for Thoracovascular Surgery and from the Institute for Pathophysiology and Nuclear Medicine in Skopje. All tumor samples were tested for RET proto-oncogene mutations in exons 1–19. In cases where mutations were found, after obtaining an informed consent from the patients willing to undergo genetic testing in accordance with institutional and national regulations, we tested patients' DNA for germline mutations.

Tumor and genomic DNA

Five micron – thick sections from each of the selected paraffin blocks were deparaffinized on glass slides by heating at 65^{o}C for 1 hour, followed by immersion in xylene for 2 minutes, absolute ethanol, 70% ethanol, and distilled water for 2 minutes each. After deparaffination

each sample was transferred into separate sterile test tube for further DNA extraction using Dynabeads® DNA DIRECT™ Universal kit (Life Technologies – Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. Genomic DNA was extracted from peripheral blood or buccal swab using the same kit.

PCR and post-PCR analysis

Exons 1–19 of the Ret proto-oncogene were amplified in separate hot-start touch-down PCRs (Tab. 1) on Auto-Q Server Thermal cycler (Quanta Biotech Ltd, UK).

Table 1

Primer sequences and PCR parameters

Exon	Primer sequences	Touch-down annealing range (1°C /cycle)	Annealing (°C)	No. of cycles	Mg^{2+} m/mol
1	F: CGCTTACCTCGCTTCAGTC R: CGGCAGAACTCACCTTTG	62.4–58.4	58.4	35	1.5
2	F: TCACTCACTTCCCTACTTCC R: CCTTACTGCGGACACTGAG	59.1–55.1	55.6	35	1.5
3	F: GCTGTGCCCGCGTATACTTC R: CTGTAGGCCACGCTGATGTTG	63.6–59.6	58.5	35	1.5
4	F: AGCAGCGGGAGAAGTACGAG R: AACTGTGGCCGGAGACAGAC	67.4–63.4	64.4	35	1.5
5	F:TCTCGCCTGCACTGACCAAC R:TGAAGAGCGAGCACCTCATTTC	68.8–64.8	63.8	35	1.5
6	F: TCTCAACCGGAACCTCTC R: GACAGGCAATAGGTATGG	62.8–58.8	57.8	35	1.5
7	F: ATCGGGAAAGTCTGTGTG R: AGCAGGCACTCACATGAC	61.8–57.8	57.8	35	1.5
8	F: TGACCCTGCTTGTCTGCCAC R: CGTTTCCAGGGCTTACCTTTG	68.6–63.6	63.6	35	1.5
9	F: AGGGATCACCAGGAACTTC R: TTAAACCCTGCTTACGGAG	61.6–57.6	58.6	35	1.5
10	F: GGACACTGCCCTGGAAATATG R: CCACTCACCCTGGATGTCTTC	63.2–59.2	59.2	35	1.5
11	F: AGATCCACTGTGCGACGAG R: CCCTCACCAGGATCTTGAAG	61.3–57.3	58.3	35	1.5
12	F: CTTCCCTCATTTCCAACATAG R: GTACCTTTCAGCATCTTCAC	62.6–58.6	57.6	35	1.5
13	F: AACCTGCTCTGTGCTGCATTTC R: CTGCAGCTGGCCTTACCATC	68.8–63.8	63.8	35	1.5
14	F: AAGACCCAAGCTGCCTGAC R: CATATGCACGCACCTTCATC	59.6–55.6	55.6	35	1.5
15	F: GCTCGTTCATCGGGACTTG R: ACCTGGCTCCTCTTCACGTAG	62.1–58.1	58.1	35	1.5
16	F: CTTTAGGGTCGGATTCCAG R: GAGAGCAACACCCACACTTAC	55.1–50.1	50.1	35	1.5
17	F: ACTGGTCCTTTCACTCTCTG R: ATCTCCTCGCTGCAGTTG	56.1–52.1	51.1	35	1.5
18	F: CTGTCTGCTCTTCCCACCAG R: ATTGGACCCAGGCACTCAC	67.1–63.1	63.1	35	1.5
19	F: CTGTCTTCCAGGACTACTTG R: TGGGAAATTCTACCATAGAG	63.3–59.3	58.3	35	1.5

Amplicons were purified with PureLink™ PCR purification kit (Life Technologies – Invitrogen, Darmstadt, Germany), and screened for polymorphisms/mutations with a Single Strand Conformation Polymorphism (SSCP) analysis. Twelve percent precast polyacrylamide gels (GE Healthcare, Biosciences, and Uppsala, Sweden) were used for the SSCP analysis. The electrophoresis was performed on Multiphor II electrophoresis system (GE Healthcare, Amersham Biosciences, and Uppsala, Sweden). Gels were stained according to the silver staining protocol [38–39]. For comparison of the banding patterns we used amplicons from previously analyzed cases without RET mutations. Positive samples were sequenced using Big Dye Terminator v1.1 according to the manufacturer's protocol on ABI Prism 310 Genetic Analyzer (Foster City, USA). Forward and reverse strand sequencing was performed. Sequencing reactions were purified with Centri-sep columns (Princeton separations, Adelphia, NJ, USA) before sequencing, according to the manufacturer's protocol. Previously unpublished missense mutations were run through SIFT and PROVEAN algorithms for prediction of the impact of missense mutations on protein function [40–41]. Hundred and five control DNA samples from healthy individuals were screened for the newly discovered mutations which were previously unpublished in order to establish their incidence in general population. Eighty-seven DNA samples from healthy individuals were provided by the Macedonian Human DNA Bank at the Institute for Immunobiology and Human Genetics, and 18 from the Institute of Pathology, Medical Faculty, Skopje.

All data were analyzed with "Statistica" software [42], using both descriptive and analytical statistics (parametric and non-parametric correlations, t-test, Chi-square, Kruscal-Wallis ANOVA and median test, Mann-Whitney U test and survival analyses).

Results

The mean age of the patients at the time of diagnosis was 51.2 years (Min. 19; Max. 72; St. dev. 14,26), and the mean follow up time was 73 months (Min. 6; Max. 184; St. dev. 54,18). Sixty-five percent of the patients were women (13 of 20). The mean value of the greatest tumor diameters was 4.13 cm (min. 2.5 cm; max. 9 cm; St. dev. 1.72). Frequencies for the rest of the parameters are presented in figures 1–2.

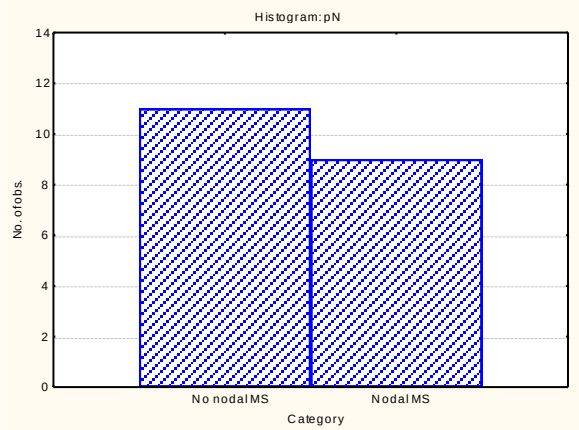


Figure 1 – Presence of lymph node metastases at the time of operation (frequency)

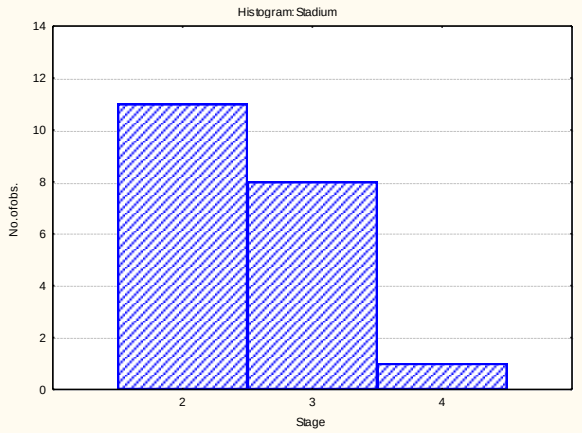


Figure 2 – Distribution of the patients according to the disease stage

Ten of the patients died during the follow up (3 males and 7 females). One of the Stage II patients (MTC18) developed multiple nodal metastases 50 months after the primary operation and died at 128 months after the initial diagnosis. One of the Stage III patients developed multiple lymph node metastases 19 months after the primary operation and died at 37 months after the initial diagnosis, while another one (MTC12) developed distant metastases (rib and liver) 51 months after the primary operation, and was still alive at 139 months after the initial diagnosis. Table 2 lists the clinical data and morphologic features of the cases with RET mutations.

Table 2

Clinical data and tumor features for each of the cases with detected RET mutations or polymorphisms

Patient	Gender	Age at Dg.	Tumor size (cm)	pT	pN	Stage	Ki-67 (%Cells)	Bcl-2 (%Cells)	Follow up (months)
MTC2	m	60	3	2	1a	3	1	90	116
MTC3	m	41	2.5	2	1a	3	0.5	90	29
MTC4	f	64	4	2	0	2	2	80	26
MTC5	m	62	2.5	2	1a	3	1	80	37
MTC8	f	51	3.5	2	0	2	30	5	8 (dead)
MTC10	f	71	4	2	1a	3	20	0.5	6 (dead)
MTC11	f	72	3.5	3	1a	3	5	95	10(dead)
MTC13	f	28	3	2	0	2	< 0.5	90	184
MTC14	m	66	6	3	0	2	4	20	73
MTC15	f	46	4	2	0	2	5	95	41
MTC16	f	42	3	2	0	2	1	90	66
MTC17	f	39	4	2	0	2	5	80	86
MTC18	f	54	2.5	2	0	2	20	70	128

Dg. = diagnosis; pT = T parameter of pTNM; pN = lymph node status from pTNM

RET Mutations

In 13 of the 20 analyzed tumor samples from 20 different and unrelated patients we found total of 23 sequence changes distributed in 6 exons (exons 8, 10–13 and 16). Among these 23 sequence changes there were 11 different missense changes, a single nucleotide deletion with frameshift, and 8 different synonymous sequence changes (Tab. 3). Eight of the total sequence changes were located in exon 12 (5 different missense mutations – one of them in 2 patients; and 3 different synonymous mutations), 5 were in exon 11 (1 single nucleotide deletion with frameshift, 2 different missense changes – one of them in 2 cases; and 2 different synonymous mutations), 4 in exon 16 (3 different missense and 1 synonymous mutation), 2 in exon 13 (1 missense mutation and 1 synonymous polymorphism in 2 cases), 1 missense mutation in exon 10, and 1 synonymous mutation in exon 8 (Tab. 3).

Table 3

Mutations and the prediction scores according to SIFT and PROVEAN algorithms for prediction of the impact of missense mutations on protein function

Patient	Exon	Nucleotide	Codon	Mutation	SIFT[a]	PROVEAN[b]
MTC2	16	c.2771 T>C	924 TTT>TCT	F924S	0.356 (tolerated)	-2,42 (neutral)
MTC3	12	c.2206 G>A	736GGA>AGA	G736R (homozyg.)	0.000 (damag.)	– 7.719 (deleter.)
	13	c.2296CC>AT	766CCG>ATG	P766M	0.028 (damag.)	-1.96 (neutral)
		c.2307G>T	769CTG>CTT	L769L germ. (homozyg.) (rs1800861)	/	/
MTC4	11	c. 2040delC	680A/-	frameshift; stop signal at codon 730	/	/
		c.2038G>A	680GCC>ACC	A680T (rs184498773)	0.006 (damag.)	-1.49 (neutral) Polyphen - benign
		c. 2037C>T	679CCC>CCT	P679P	/	/
MTC5	12	c.2180G>A	727GGA>GAA	G727E	0.505 (tolerated)	-1.785 (neutral)
		c.2206 G>A	736GGA>AGA	G736R	0.000 (damag.)	-7.719 (deleter.)

MTC8	10	c.1804°>G	602ATT>GTT	I602V	0.003 (damag.)	-0.759 (deleter.)
MTC10	11	c.2071G>A	691GGT>AGT	G691S germ. (rs1799939)	0.178 (tolerated)	-0.95 (neutral)
MTC11	16	c.2753T>C	918ATG>ACG	M918T (homozyg.) (rs74799832)	0.000 (damag.)	-5.47 (deleter.)
MTC13	13 16	c.2307G>T c.2752A>C	769CTG>CTT 918ATG>CTG	L769L germ. (rs1800861) M918L	/ 0.000 (damag.)	/ -2.62 (deleter.)
MTC14	12	c. 2256C>T	752TAC>TAT	Y752Y	/	/
MTC15	12	c.2249C>G c.2253G>C	750GCA>GGA 751GGG>GGC	A750G G751G	0.180 (tolerated) /	-2.381 (neutral) /
MTC16	11 16	c.1983C>T c.2733T>G	661CAC>CAT 911GGT>GGG	H661H G911G (splice var.)	/ /	/ /
MTC17	8 11	c.1602G>T c.2038G>A	534CTG>CTT 680GCC>ACC	L534L germ. A680T (rs184498773)	/ 0006 (damag.)	/ -1.49 (neutral) [c]Polyphen - benign
MTC18	12	c.2137G>A c.2214G>A	713GAG>AAG 738GTG>GTA	E713K (splice var.) V738V	0.027 (damag.) /	-2.159 (neutral) /

[a] SIFT Prediction Score cut off: 0.05

[b] Provean Prediction Score cut off: -2.5

[c] Polyphen – according to Polyphen algorithm for prediction of the consequences of missense mutations this mutation has been scored as benign in the Ensemble database.

germ. = germline mutation; homozyg. = homozygous mutation; splice var. = splice region variation;
"rs" codes in brackets denote the reference codes of the previously published sequence changes.

Germline sequence changes were detected in 4 of the patients:

Polymorphism L769L (rs1800861) homozygous in patient MTC3 and heterozygous in patient MTC13; polymorphism G691S (rs1799939) in heterozygous state in patient MTC10; and L534L heterozygous (previously unpublished to the best of our knowledge) in patient MTC17.

Only 4 of the sequence changes have been previously published:

– 3 of the missense polymorphism and mutations: G691S (rs1799939), M918T (rs74799832) and A680T (rs184498773); and

– 1 of the synonymous polymorphisms: L769L (rs 1800861)

There were 12 patients (60%) with tumors expressing one or more missense mutations/ polymorphisms or the single nucleotide deletion (c.2040delC). Seven of the patients (35%) had tumors with at least one damaging or possibly damaging RET mutation including the two splicing variants (discussed further). None of the damaging missense mutations was germline, neither was the single nucleotide deletion. Likewise, none of the novel (previously unpublished) missense mutations, neither the splicing variants were detected in the control DNA samples from healthy individuals.

Electropherograms of some of the mutations are shown in Figure 3.

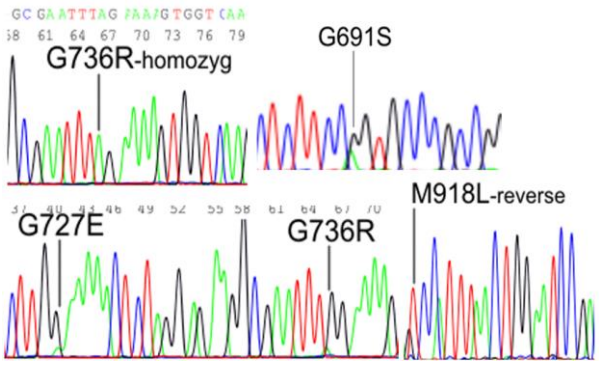


Figure 3 – Electropherograms of some of the mutations

Immunostainings and other investigated parameters

Most of the tumors had low Ki-67 expression (Fig. 4) with mean value 6.48% of tu-

mor cells (min. < 0.5%; max. 30%; St. dev. 9.91). Opposite to Ki-67, majority of the tumors had high Bcl-2 expression (mean 68.3%; min. 0.5%; max. 95%; St. dev. 33.8). There was significantly higher Bcl-2 expression compared to Ki-67 ($p < 0.05$) (Fig. 5).

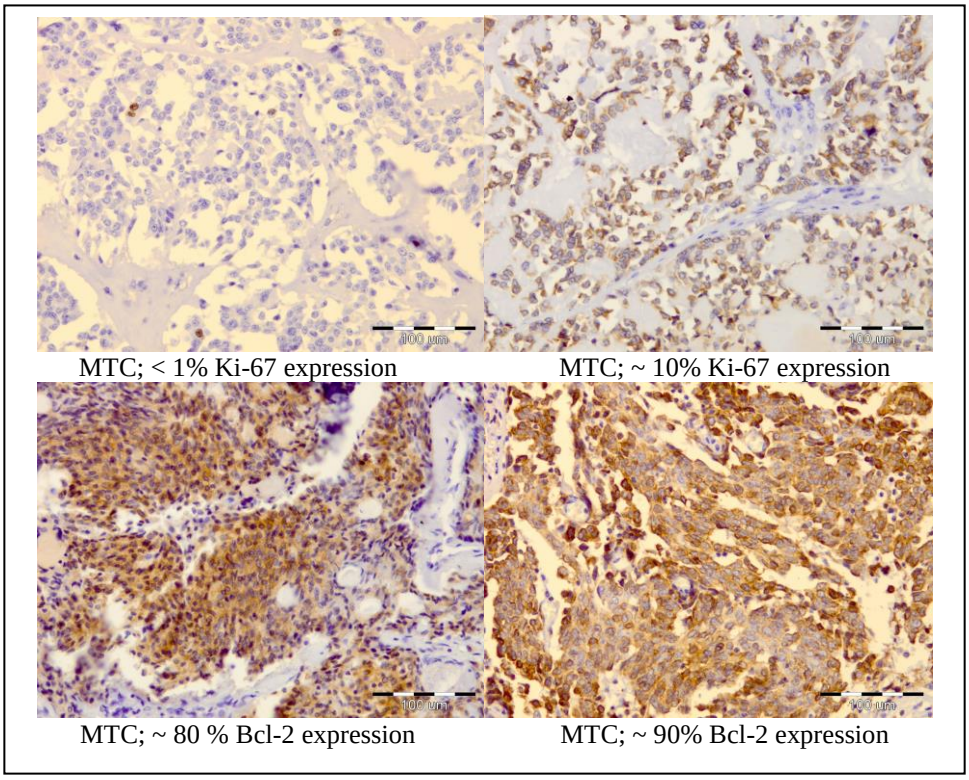


Figure 4 – Examples of immunohistochemical expressions for Ki-67 and Bcl-2.

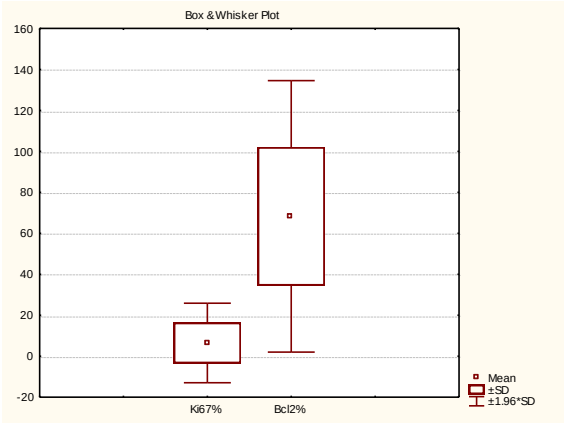
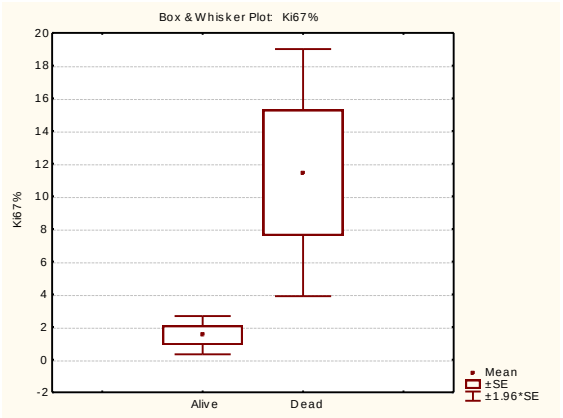


Figure 5 – Difference between mean Ki-67 and Bcl-2 expressions (% positive tumor cells)

We found a significant difference between the tumors from patients who died during the follow up in comparison to the tumors from the patients who were still alive at the end of the follow up regarding the Ki-67 and Bcl-2 expression. Ki-67 was significantly higher in patients who died (mean 11. 45; St. dev. 12.2) in comparison to those who remained alive (mean 1.5; St. dev. 1.9) ($p < 0.05$) (Fig. 6). Since the data were not normally distributed, and the Levene's test for homogeneity of variances was significant, we used the non-parametric Mann-Whitney U test to evaluate the significance of the differences.

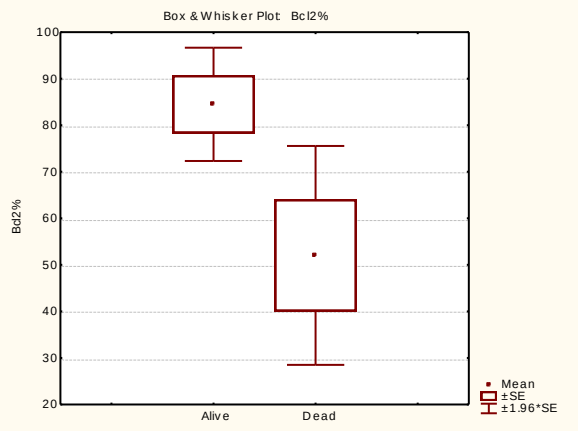


T-value = -2.55; df = 18; $p < 0.05$
Mann-Whitney U test: Z = -2.42; $p < 0.05$

Figure 6 – Ki-67 expression in tumors from patients who survived the follow up period vs. those who died

Opposite to Ki-67, Bcl-2 expression was higher in patients who survived during the follow up (mean 84.5%; St. dev. 19.64) in com-

parison to those who died (mean 52.1; St. dev. 37.9) ($p < 0.01$) (Fig. 7)



T-value = -2.4; df = 18; $p < 0.05$
Mann-Whitney U test: Z = 2.61; $p < 0.01$

Figure 7 – Bcl-2 expression in tumors from patients who survived the follow up period vs. those who died

Significant correlations between the analyzed parameters are summarized in Table 4.

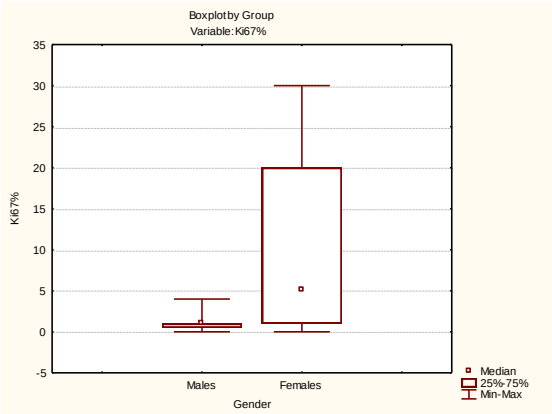
Table 4

Significant correlations between the analyzed parameters Spearman Rank Order Correlations

	Valid N	Spearman R	t(N-2)	p-level
Age & Alive/Dead	20	0.685	3.99	0.001
Age & MultipleEx	20	-0.542	-2.74	0.014
Gender & Ki67%	20	0.497	2.43	0.026
pT & Mutation	20	-0.56	-2.87	0.010
pT & Missense mut.	20	-0.60	-3.19	0.005
Tu. diam. (cm) & Damag. mut.	20	-0.603	-3.20	0.004
Alive/Dead & Ki67%	20	0.562	2.88	0.0099
Alive/Dead & Bcl2%	20	-0.61	-3.27	0.004
Alive/Dead & MultipleEx	20	-0.50	-2.45	0.0248
Stage&pN[1]	20	0.98	22.25	0.0000

[1] This correlation has not been elaborated further since the pN parameter is already incorporated in the Stage; pT = T parameter from pTNM; pN = Lymph node status from pTNM; Mutation = any sequence change; Damag. mut. = damaging mutation; Multiple Ex. = sequence changes in more than 1 exon

Age of the patients and Ki-67 expression were in positive correlation to the disease outcome ($p < 0.01$ and $p < 0.05$ respectively), while Bcl-2 expression and presence of mutations (sequence changes in general) in multiple RET exons were in negative correlation to the disease outcome ($p < 0.01$ and $p < 0.05$ respectively). There was also a negative correlation between the age of the patients and presence of mutations and polymorphism in multiple RET exons ($R = -0.54$; $p < 0.05$). It must be emphasized that when speaking of "presence of mutations in multiple RET exons", included in this category are all of the detected mutations including polymorphisms and tolerant/neutral, as well as damaging RET mutations according to SIFT and PROVEAN algorithms. The same applies for the category "mutation" (Tab. 4) which was negatively correlated to the pT parameter of the tumors. A rather unexpected finding was the negative correlation between the greatest tumor diameter and the presence of damaging RET mutations, meaning that damaging mutations were more frequent in smaller tumors. This correlation must be evaluated carefully, since the severity of the novel RET mutations was calculated by available on-line algorithms and not actually observed previously in clinical cases. A rather peculiar correlation was the one between the gender and Ki-67 expression ($R = 0.497$; $p < 0.05$). Intergroup difference was significant at level $p < 0.05$ (Fig. 8).

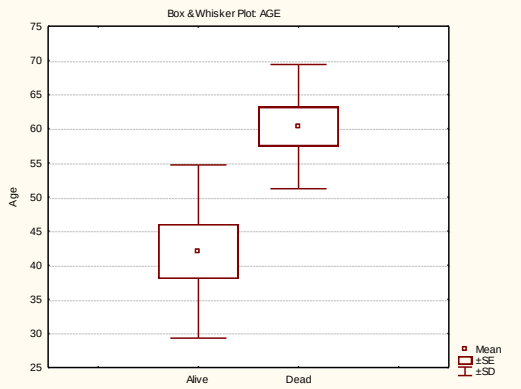


Mann-Whitney U test: U=18.5; Z = - 2.14; $p < 0.05$

Figure 8 – Ki-67 expression difference in tumors from male vs. female patients

Male patients had significantly lower mean Ki-67 expression (mean 1.14%; St. dev. 1.31) in comparison to females (mean 9.35; St. dev. 11.36) ($p < 0.05$).

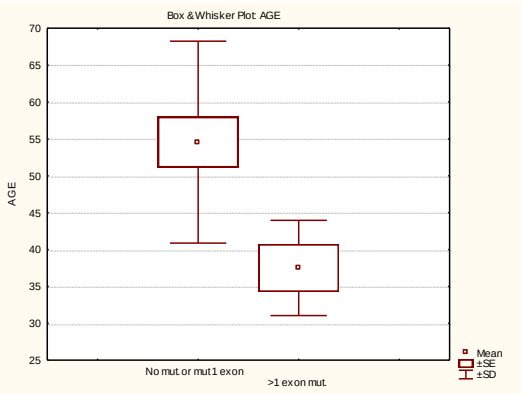
Statistical analysis of intergroup differences revealed significantly younger mean age of the patients who survived during the follow up (mean 42 years; St. dev. 12.68) in comparison to those who died (mean age 60.3; St. dev. 9.1) ($p < 0.01$), (Fig. 9).



T-value: -3.71; df = 18; p < 0.01

Figure 9 – Age at diagnosis for patients who survived vs. those who died during the follow up

Regarding the age and presence of multiple exon sequence changes, there was a significant age difference between cases with sequence changes in more than 1 exon (mean 37.5 years; St. dev. 6.45) in comparison to those with no RET mutations or mutation in one exon only (mean 54.6 years; St. dev. 13.68) (p < 0.05) (Fig. 10).



T-value: 2.39; df = 18; p < 0.05

Figure 10 – Age of the patients at diagnosis in cases with no mutation and mutation in 1 exon only vs. cases with mutations in more than 1 exon

None of the patients who died during the follow up had sequence changes in more than 1 exon Table 5).

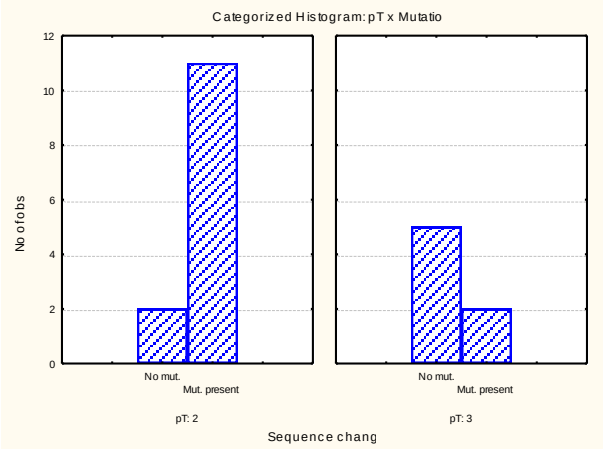
Table 5

Frequency of cases with sequence changes in more than 1 exon vs. the reset of the cases in respect to the survival of the patients

	No multiple Ex. sequence changes	Multiple Ex sequence changes.	Row
Alive	6	4	10
Dead	10	0	10
Totals	16	4	20

Chi-square (df = 1): 5; p < 0.05; V-square (df = 1): 4.75; p < 0,05; Yates corrected Chi-square: 2.81; p < 0.05

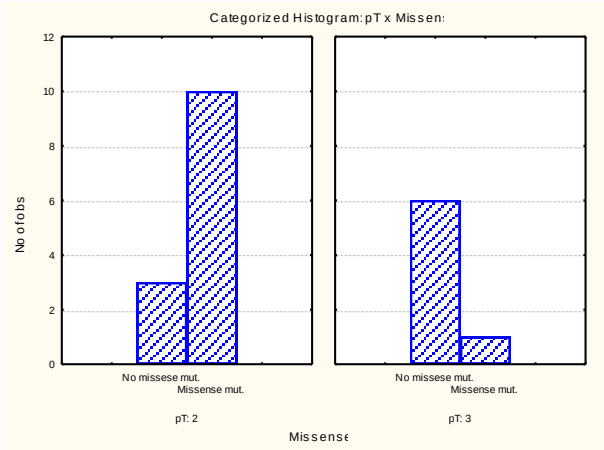
Tumors with pT2 parameter had more frequently some kind of sequence change in the RET proto-oncogene (11 of 13) in comparison to tumors with pT3 parameter (2 of 7) (Fig. 11).



Chi-square 6.28; p < 0.05; V-square 5.97; p < 0.05; Yates corrected Chi-square 4.06; p < 0.05

Figure 11 – Frequency of sequence changes according to the pT parameter of the tumors

Similar frequencies were observed for the presence of missense mutations (Fig. 12).

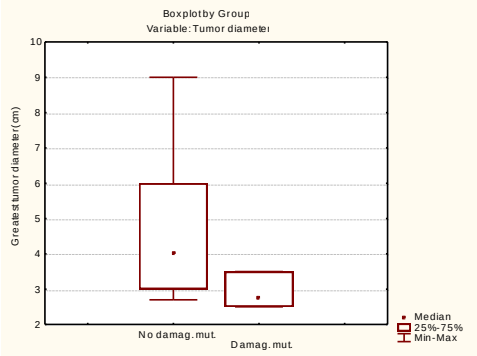


Chi-square 7.21; p < 0.01; V-square 6.85; p < 0.01; Yates corrected Chi-square 4.90; p < 0.05

Figure 12 – Frequency of missense mutations according to the pT parameter of the tumors

Tumors with pT2 had more frequently at least one missense sequence change (10 of 13 cases) in comparison to pT3 tumors (1 of 7).

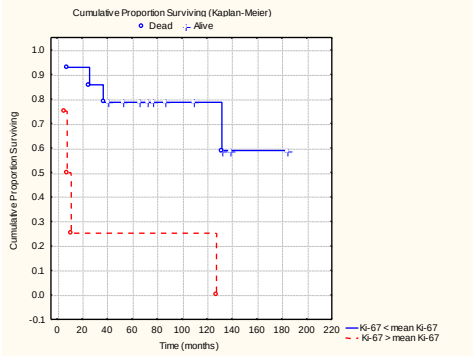
Tumors with mutations that were predicted to be damaging had also significantly smaller mean diameter (mean 2.92 cm; St. dev. 0.49) in comparison to the other tumors (mean 4.64 cm; St. dev. 1.8) (p < 0.01) (Fig. 13).



Mann-Whitney U test: U = 10,5; Z = 2.598; p < 0.01

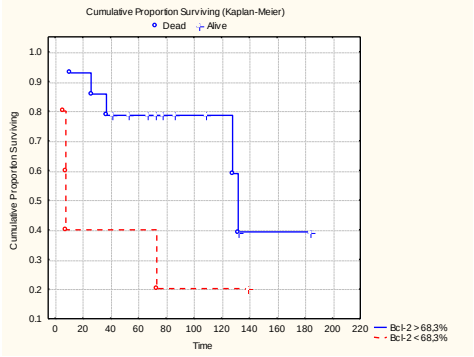
Figure 13 – Greatest diameter of the tumors with damaging mutation vs. the rest of the tumors

Survival analysis showed significantly better survival of the patients whose tumors expressed lower Ki-67 than the mean Ki-67 expression of the whole group (6.48%) in comparison to those with tumors expressing Ki-67 in more than 6.48% of cells (p < 0.01), as well as better survival for the patients whose tumors expressed higher Bcl-2 than the mean value of the whole group (68.3%) compared to those with lower Bcl-2 expression (p < 0.05) (Figures 14, 15).



Cox's F-Test: T1 = 8.080039; T2 = .9199612; p = .00296

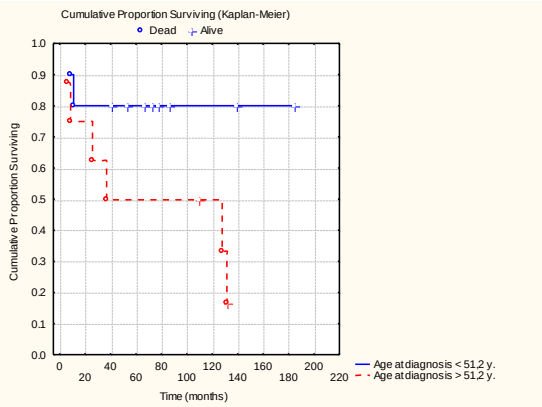
Figure 14 – Survival of the patients with tumors expressing Ki-67 in less than 6.5% of cells vs. those with tumors expressing Ki-67 in more than 6.5% of cells



Cox's F-Test: T1 = 8.508870; T2 = 1.491130; p = .02092

Figure 15 – Survival of the patients with tumors expressing Bcl-2 in more than 68.3% of cells vs. those with tumors expressing Bcl-2 in less than 68.3% of cells

Similarly, survival analysis showed significantly better survival of the patients who were diagnosed at age younger than the mean age of the whole group (51,6 years) in comparison to those who were older than 51,6 years at the time of diagnosis (p < 0,05) (Fig. 16).



Cox's F-Test: T1 = 5.718294; T2 = 3.281707; p = .01131

Figure 16 – Survival of the patients diagnosed at age younger than 51.6 years vs. those diagnosed at age older than 51.6 years

Discussion

The distribution of the patients according to age and gender is in accordance to other previously published studies [4, 6] who report slight female predominance, which in our case was higher than usual (1:1.85 male to female ratio), and 50 years mean age, similar to our mean age of 51.2 years. Most of the patients (n = 11) were in stage II by the time of operation, and the mean tumor diameter at operation was 4.13 cm (min 2.5 cm; max. 9.0 cm; med. 3.8 cm; St. dev. 1.72), which we consider a rather large tumor, although comparable to other studies [6]. However, it is quite unsatisfactory that none of the patients had been operated in stage I, although this could be easily explained by the fact that none of the patients was actually aware of being at an increased risk for MTC (i.e. positive familial history), which is on the other hand closely dependent on our ability to "recognize" such families. This implies a need for improvement of the screening process of patients/families at risk, and raising the awareness in the general population for the importance of thyroid gland screening. We can speculate that the missing cases in our series (up to the 30 predicted cases for 15 years period based upon data from European and US statistics) could be exactly those early stage cases that have re-

mained undiagnosed. This possibility goes along with the fact that at least 20% of MTCs in Europe are expected to be familial cases (FMTC), and those are usually with milder disease course and affecting younger individuals, approximately 10 years earlier than the sporadic MTC [1–4, 6]. From the 23 detected sequence changes in the RET proto-oncogene, 6 of the missense mutations including one of the splice variants (p.G736R and p.P706M in MTC3, p.G736R in MTC5, p.I602V in MTC8, p.M918T in MTC11, p.M918L in MTC13 and p.E713K splice var. in MTC18) detected in 6 of the 20 cases (30%) are probably damaging and could have possibly influenced the ontogenesis and/or tumor progression. The splice region variant p.E713K induces possible use of 2 cryptic splice sites which could lead to alternative transcripts with loss of 3 or 12 bases from exon 12, respectively. According to the splicing variant predicting software [43], alternative splicing could result in breakdown of 5 usual enhancer sites and introduction of 2 novel enhancers, while at the same time providing 2 new silencers instead of the disrupted 3 usual silencing sites with overall unknown signifycance regarding the splicing process. However, in case usual splicing takes place, this mutation is predicted to be a damaging one. The other splicing site variation (c.2733T>G; p.G911Gsplicing var.) has been already described with a frequency of 0.2% in series of more than 500 large intestine tumors (COSM1560792; Ensemble) [20]. According to the splicing variant predicting software [43] this variation could result in possible use of 2 alternative splicing sites. In the first case 70 bases (of 71) of exon 16 would be skipped resulting in shorter and probably non-functional or product with diminished function. However, in the second case the splicing would result with a product missing 13 bases and consequent frameshift. In this case there is no appearance of the stop codon, but instead the product would present a new sequence containing a Protein kinase C phosphorylation site (motif: [ST] x [RK]) thus leading to alteration of the second (downstream) intracellular tyrosin kinase domain of the RET protein. The c.2040delC is a frameshift single nucleotide deletion inducing a stop signal at codon 730 that could possibly result in non-functional protein or transcript degradation. The rest of the missense mutations were predicted to be neutral or tolerable regarding the protein function. In this series all of the sequence changes were located in 6 exons (exons 8, 10, 11, 12, 13 and 16), which is in concordance with other studies reporting that these are among the most frequently affected exons in sporadic and familial MTC, as well as in MEN syndromes altogether [1, 3, 5, 13, 22–27].

Only 4 of the 23 sequence changes that were found in our series were previously published (p.A680T – rs184498773, p.L769L – rs1800861, p.M918T – rs74799832 and p.G691S – rs1799939). For the rest of the mutations and the single nucleotide deletion we were not able to find any records neither in the publicly available databases, nor in the available literature.

Thirteen of our patients (65%) had some kind of sequence change in the RET proto-oncogene. If patients whose tumors had only synonymous mutations and known polymerphisms were subtracted, 9 cases remain (45%) with probably significant mutations, and at least 6 cases (30%) with damaging RET mutation. Several other studies have confirmed somatic RET mutations in sporadic MTC with frequencies ranging from 23–70% [29–34]. Regarding the germline mutations in apparently sporadic MTC, several studies have reported frequencies ranging from 1.5–22.7% in different series [13, 33]. In our series there were 4 patients (20%) with germline sequence changes: p.L769L (rs1800861) polymorphism homozygous in patient MTC3 and heterozygous in patient MTC13; G691S (rs1799939) polymorphism in heterozygous state in patient MTC10; and L534L heterozygous mutation (previously unpublished) in patient MTC17 – both the first and last of them synonymous and with no impact on the protein structure itself. The p.G691S was not predicted to be a damaging (it is in fact polymorphism) with the algorithms we used, although Yang et al. have reported it to be associated with vesico-ureteral reflux in French-Canadian population from Quebec [19], which was not the case with our patient. There were no significant germline mutations in our series confirming the sporadic nature of the tumors. Presence of damaging RET mutation was not correlated to the disease outcome during the follow up, and there

was no significant survival difference between the patients whose tumor had damaging mutation in comparison to the others., which is opposite to several other studies [27, 31, 44]. For example, Romei et al. [31] found somatic RET mutations in 8 of 18 cases of sporadic MTC. In 5 of the cases the mutation affected exon 16 (M918T) and in 3 cases exon 11 (C634A in 1 case and C634T in 2 cases). Six of those patients (75%) presented tumor recurrence or increased calcitonin levels during the postsurgical follow up in comparison to only 10% of the patients without RET mutation in tumor specimens ($p < 0.05$). This study setup clearly differs from ours, since we analyzed the overall survival of the patients and not the biochemical signs of recurrence. Elisei et al. [27] found that MTC patients with somatic RET mutation and advanced stage had significantly worse outcome and lower percentage of survival. However in these studies the age of the patients at diagnosis was not commented as a significant factor influencing the survival, although there are studies confirming that the age at diagnosis is the only independent prognostic parameter besides pTNM Stage correlated to the survival [6, 45]. We speculate that the influence of RET mutations on the survival was mitigated by the younger age of the patients, lower stage and smaller diameters of the tumors in which mutations were discovered (table 4). In our series the age at diagnosis was significantly correlated to the disease outcome, with younger patients having significantly better survival curve (Table 4, Figure 16). Another factor contributing for such results could be the relatively low frequency of mutations at codon 918 (only 10%) in our series in comparison to the others reporting frequencies of 20–80% [6]. This mutation is known as one of the most severe ones, and its lower frequency in our series might be influencing the loss of correlation between the presence of damaging (other than codon 918) RET mutations and survival of the patients. The Ki-67 expression was low, on average (6.48% of tumor cells), as expected and as published in other studies [6]. However in our series we encountered a rather odd phenomenon of significantly higher Ki-67 expression in women (mean 9.35% of tumor cells) compared to men (mean 1.14% of tumor cells) ($p < 0.05$). We have no plausible explanation for this phenomenon, and we haven't found similar published results in the reviewed literature. However, the gender itself had no influence on the disease outcome neither was correlated to any other of the investigated parameters. Ki-67 was correlated to the disease outcome ($R = 0.56$; $p < 0.01$), and patients with Ki-67 higher than the mean Ki-67 expression of the whole group (5.6%) presented significantly worse survival curve than those with lower Ki-67 expression (Figure 14). Opposite to the Ki-67 expression, we found high Bcl-2 expression averaging 68.3% of tumor cells. Our data generally correlate with the results from Cobanoglu et al. and Viale et al. [7, 8] who also found high Bcl-2 expression. However, in our series the average Bcl-2 expression (68.3%) is lower than that observed by Cobanoglu et al. who reported up to 90% positivity. This discrepancy has emerged because we had 4 patients with (extremely) low Bcl-2 expression (0.5%, 5%, 10%, and 20% respectively). Viale et al. found that 26 out of 33 MTC cases had Bcl-2 expression in more than 25% of the cells. They have reported longer survival in Bcl-2 positive cases. Likewise, in our study Bcl-2 was significantly higher in tumors from the patients who survived the follow up (mean 84.5%; St. dev. 19.64) in comparison to those who died (mean 52.1%; St. dev. 37.9) ($p < 0.01$). The patients whose tumors expressed Bcl-2 in more than 68% of the cells (the mean value for the whole group) presented significantly better survival than those with lower Bcl-2 expression.

In summary, our study is the first of this kind conducted on Macedonian patients with MTC to the best of our knowledge. Our results suggest that this population has significantly lower prevalence of somatic and germline mutations affecting codon 918 than reported in other populations. Ki-67 and Bcl-2 immunostainings for MTC specimens should be routinely performed since both markers show significant correlation to the disease outcome. Although RET mutational status showed no direct correlation to the disease outcome and overall survival in this series, we firmly stand behind the conclusions of other authors who favorite RET screening in patients with MTC at least for two major reasons: firstly, the notion that we miss at least 8 patients from the

calculated incidence of MTC for the past 15 years, especially MEN2 and FMTC related cases, suggest possible flaws in the current screening and diagnostic protocols that could be overcome by implementation of molecular diagnostic on routine basis for all cases of MTC; and secondly, in near future patients with detected RET mutations could benefit from application of specific tyrosine kinase inhibitor based therapy.

Acknowledgments

We thank Prof. Aleksandar Dimovski from the Center for Biomolecular Pharmaceutical Analyses, Faculty of Pharmacy, University "Ss Cyril and Methodius" in Skopje for his support and expertise in interpretation of the sequencing electropherograms.

We also thank Prof. Neli Basheska from the laboratory for pathohistology and clinical cytology from the Institute for Radiotherapy and Oncology at the Medical Faculty in Skopje, for providing most of the operative specimens.

REFERENCES

1. Jandrichova S, Vcelak J, Vlcek P, Neradilova M, Nemec J, Bendlova B. Screening of six risk exons of the ret proto-oncogene in families with medullary thyroid carcinoma in the Czech Republic. Journal of Endocrinology. 2004; 183: 257–265.
2. National Cancer Institute. MedullaryThyroid Cancer. available at: http://www.cancer.gov/cancertopics/pdq/genetics/medullarythyroid/HealthProfessional/page2/print
3. Eng Charis. Ret proto-oncogene in the development of human cancer. J Clin Oncol. 1999; 17(1): 380–393.
4. Maitra A, Abbas A. K. The endocrine system. In: Robbins and Cotran Pathologic basis of Disease. 7th edition, Philadelphia (Pennsylvania): Elsevier Saunders; 2005. 1155–1226.
5. Eng C, Clayton D, Schuffenecker I, Lenoir G, Cote G, Gangel R. F, et al. The relationship between specific ret proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. JAMA. 1996; 276: 1575–1579.
6. Matias-Guiu X, DeLellis R, Moley J. F, Gagel R. F, Slbores-Saavedra J, Bussolati G. et al. Medullary thyroid carcinoma. In: Pathology and genetics of tumors of endocrine system. World Health Organization Classification of Tumors, IARC, Lyon: IARC Press. 2001; 86–91.
7. Cobanoglu B, Ozercan M.R. Staining characteristics of BCL-2, BAX, p53, p21, Ki-67 andC-erbB2 in thyroid carcinomas. Erciyes Medical Journal 2007; 29 (3): 201–209.
8. Viale G, Roncalli M, Grimelius L, Graziani D, Wilander E, Johansson H, et al.: Prognostic value of bcl-2 immunoreactivity in medullary thyroid carcinoma. Hum Pathol. 1995; 26(9): 945–50.
9. Gerasimovski D. Census of population, households and dwellings in the Republic of Macedonia, 2002 Final data. Republic of Macedonia State Statistical Office. Book X. 2002; 10: 18–197.
10. Mograbi B, Bocciardi R, Bourget I, Juhel T, Farahi-Far D, Romeo G, et al. The sensitivity of activated Cys Ret mutants to glial cell line-derived neurotrophic factor is mandatory to rescue neuroectodermal cells from apoptosis. Mol Cell Biol. 2001; 21(20): 6719–6730.
11. Komminoth P. The role of ret proto-oncogene mutation analysis in the diagnosis of multiple endocrine neoplasia type 2 (MEN2) gene carriers and the discrimination of sporadic and familial medullary thyroid carcinomas and pheochromocytomas. Verh Dtsch Ges Pathol. 1995; 79: 50–55.
12. Quayle F. J, Fialkowski E. A, Benveniste R, Moley J. F. Pheochromocytoma penetrance varies by Ret mutation in MEN2A. Surgery. 2007; 142(6): 800–805.
13. Shirahama S, Ogura K, Takami H, Ito K, Tohsen T, Myiauchi A, et al. Mutational analysis of the ret proto-oncogene in 71 Japanese patients with medullary thyroid carcinoma. J Hum Genet.1998; 43(2): 101–106.
14. Linwah Y, Cote G.. J, Shapiro S. E, Ayers G. D, Herzog C. E, Sellin R. V, et al. Multiple endocrine neoplasia type 2. Evaluation of genotype-phenotype relationship. Arch Surg. 2003; 138: 409–416.
15. Arighi E, Popsueva A, Degl'Innocenti D, Borrello M. G, Carniti C, Perala N. M, et al. Biological effects of dual phenotypic Janus mutation of ret cosegregating with both multiple endocrine neoplasia type 2 and Hirschprung's disease. Mol Endocrinol. 2004; 18(4): 1004–1017.
16. Skinner M. A, Safford S. D, Reeves J. G, Jackson M. E, Freemerman A. J. Renal aplasia in humans is associated with Ret mutations. Am J Hum Genet. 2008; 82(2): 344–51.
17. Sanjay J, Encinas M, Johnson E. M Jr, Milbrandt J. Critical and distinct roles for key Ret tyrosine docking sites in renal development. Genes & Development. 2006; 20: 321–333.
18. Yu O. H, Murawski I. J, Myburgh D. B, Gupta I. R. Overexpression of Ret leads to vesicoureteric reflux in mice. Am J Physiol Renal Physiol. 2004; 287: 1123–1130.
19. Yang Y, Houle A-M, Letendre J, Richter A. Ret Gly691Ser mutation is associated with primary vesicoureteral reflux in the French-Canadian population from Quebec. Human Mutation. 2008; 29(5): 695–702.
20. Ensembl. Human gene view. available at: http://www.ensembl.org/Homo_sapiens/geneview?gene=OTTHUMG00000018024;db=vega

21. Gene Cards. RET gene card. available at: http://www.genecards.org/cgi-bin/carddisp.pl?gene=RET
22. Berndt I, Reuter M, Saller B, Frank-Raue K, Groth P, Grusendorf M, et al. A new hot spot for mutations in the ret proto-oncogene causing familial medullary thyroid carcinoma and multiple endocrine neoplasia type 2A. J Clin Endocrinol Metab. 1998; 83(3): 770–774.
23. Bolino A, Schuffenecker I, Luo Y, Seri M, Silengo M, Tocco T, et al. Ret mutations in exons 13 and 14 of FMTC patients. Oncogene. 1995; 10: 2415–2419.
24. Eng C, Mulligan L. M, Smith D. P, Healey C. S, Frilling A, Raue F, et al. Mutation of the ret proto-oncogene in sporadic medullary thyroid carcinoma. Genes, Chromosomes and Cancer. 1995; 12: 209–212.
25. Fattoruso O, Quadro L, Libroia A, Verga U, Lupoli G, Cascone E, et al. A GTG to ATG novel point mutation at codon 804 in exon 14 of the ret proto-oncogene in two families affected by familial medullary thyroid carcinoma. Human Mutation. 1998; 1: 167–171.
26. Da Silva A. M, Maciel R. M, Da Silva M. R, Toledo S. R, De Carvalho M. B, Cerutti J. M. A novel germline point mutation in ret exon 8 (Gly533Cys) in large kindred with familial medullary thyroid carcinoma. J Clin Endocrinol Metab. 2003; 88: 5438–5443.
27. Elisei R, Cosci B, Romei C, Bottici V, Renzini G, Molinaro E, et al. Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. J Clin Endocrinol Metab. 2008; 93(3): 682–687.
28. Eng C, Smith D. P, Mulligan L. M, Nagai M. A, Healey C. S, Ponder M. A et al. Point mutation within the thyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumors. Hum Mol Genet. 2003; 3(2): 237–241.
29. Hofstra R. M, Landsvater R. M, Ceccherini I, Stulp R. P, Stelwagen T, Luo Y, et al. A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature. 1994; 367(6461): 375–376.
30. Elisei R, Romei C, Cosci B, Agate L, Bottici V, Molinaro E, et al. RET genetic screening in patients with medullary thyroid cancer and their relatives: experience with 807 individuals at one center. J Clin endocrinol Metab. 2007; 92(12): 4725–9.
31. Romei C, Elisei R, Pinchera A, Ceccherini I, Molinaro E, Mancusi F et al. Somatic mutations of the ret protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. J Clin Endocrinol Metab. 1996; 81(4): 1619–22.
32. Zedenius J, Wallin G, Hamberger B, Nordenskjold M, Weber G, Larsson C. Hum mol Genet. 1994; 3(8): 1259–62.
33. Eng C, Mulligan L. M, Smith D. P, Healey C. S, Frilling A, Raue F, et al. Low frequency of germline mutations in the RET proto-oncogene in patients with apparently sporadic medullary thyroid carcinoma. Clin Endocrinol (Oxf). 1995; 43(1): 123–7.
34. Blaugrund J. E, Johns M. M Jr, Eby Y. J, Ball D. W, Baylin S. B, Hruban R. H, Sidransky D. Hum Mol Genet. 1994; 3(10): 1895–7.
35. Machens A, Dralle H. Genotype-phenotype based surgical concept of hereditary medullary thyroid carcinoma. World J Surg. 2007; 31(5): 957–68.
36. Frank-Raue K, Machens A, Scheuba C, Niederle B, Dralle H, Raue F, the German MEN2 Study Group. Difference in development of medullary thyroid carcinoma among carriers of RET mutations in codons 790 and 791. Clin endocrinol (Oxf). 2008; Epub ahead of print. PMID 18248648.
37. Elisei R, Cosici B, Romei C, Agate L, Piampiani P, Miccoli P, et al. Identification of novel point mutation in the RET gene (Ala883Thr), which is associated with medullary thyroid carcinoma phenotype only in homozygous condition. J Clin Endocrinol Metab. 2004; 89(11): 5823–7.
38. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphism of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA. 1989; 86: 2766–2770.
39. Glavac D, Dean M. Optimization of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations. Hum Mutat. 1993; 2(5): 404–414.
40. Kumar P, Henikoff S, Ng P. C. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009; 4(7): 1073–1081.
41. Choi Y, Sims G. E, Murphy S, Miller J. R, Chan A. P (2012) Predicting the Functional Effect of Amino Acid Substitutions and Indels. PLoS ONE 7(10): e46688. Available at: http://provean.jcvi.org/index.php
42. StatSoft, Inc. (2001). STATISTICA (data analysis software system), version 6. www.statsoft.com.
43. Desmet F. O, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acid Research, 2009. available at: http://www.umd.be/HSF/
44. Schilling T, Burck J, Sinn H. P, Clemens A, Otto H. F, Hoppner W, et al. Prognostic value of codon 918(ATG>ACG) RET proto-oncogene mutations in sporadic medullary thyroid carcinoma. Int J Cancer. 2001; 95(1): 62–6.
45. Leboulleux S, Baudin E, Travagli J. P, Schlumberger M. Medullary thyroid carcinoma. Clinical Endocrinology. 2004; 61: 299–310. doi: 10.1111/j.1365-2265.2004.02037.x

Р е з и м е

НОВИ RET-МУТАЦИИ КАЈ МАКЕДОНСКИ ПАЦИЕНТИ СО МЕДУЛАРЕН КАРЦИНОМ НА ТИРОИДНАТА ЖЛЕЗДА: ГЕНОТИПСКО-ФЕНОТИПСКИ КОРЕЛАЦИИ

Рубенс Јовановиќ[1], Славица Костадинова-Куновска[1], Весна Јаневска[1], Билјана Богоева[1], Лилјана Спасевска[1], Даниела Миладинова[2], Ана Угринска[2], Марина Здравеска-Кочовска[2], Дејан Трајков[3], Гордана Петрушевска[1]

[1] Институт за патологија, Медицински факултет, Скопје, Р. Македонија
[2] Институт за патофизиологија и нуклеарна медицина, Медицински факултет, Скопје, Р. Македонија
[3] Институт за имунобиологија и хумана генетика, Медицински факултет, Скопје, Р. Македонија

Медуларните карциноми на тироидната жлезда (МТС) се ретки неоплазми што сочинуваат 2–10% од сите малигни тумори на тироидната жлезда. Повеќе од 75% се јавуваат како спорадични тумори, а останатите се јавуваат во склоп на MEN2 синдром или како фамилијарни случаи. Како фамилијарните/синдромските, така и спорадичните тумори често покажуваат мутации во RET-протоонкогенот. Забележано е дека некои од туморите покажуваат индолентен клинички тек, додека други се агресивни. Експресијата на Ki-67, главно, е ниска, со документирани исклучоци. Наспроти тоа, во повеќето тумори се наоѓа висока експресија на Bcl-2. Неколку студии покажаа дека нивото на експресија на Ki-67 и Bcl-2 има прогностичко значење, како што има и мутациониот статус на RET-протоонкогенот. Во оваа студија ја анализиравме експресијата Ki-67 и Bcl-2 и присуството на RET-мутации во 20 несродни случаи на МТС, нивните меѓусебни корелации и корелациите со морфолошките особини на туморите и стадиумот на болеста, како и преживувањето на пациентите. Во 13 од анализираните примероци најдовме вкупно 23 промени во секвенцата на RET дистрибуирани во егзоните 8, 10–13 и 16. Најдовме 11 различни missense-мутации, еднонуклеотидна делеција и 8 различни синонимни мутации. Само 4 од овие промени се претходно објавени, останатите се новооткриени. Дванаесет пациенти (60%) имаа тумори со една или повеќе missense-мутации или еднонуклеотидна делеција, а седуммина од нив (35%) имаа барем една оштетувачка или веројатно оштетувачка мутација во RET-протоонкогенот. Повеќето тумори имаа ниска експресија на Ki-67 (средна вредност 6,48% од клетките) и висока експресија на Bcl-2 (средна вредност 68,3%). Воочивме значително подобро преживување на пациентите чии тумори имаа пониска експресија (< 6,5%) на Ki-67 ($p < 0,05$), повисока експресија (> 68,3%) на Bcl-2 ($p < 0,01$) и помлада возраст (< 51 години) при иницијалната дијагноза ($p < 0,05$).

Клучни зборови: RET-мутации, медуларен карцином на тироидна жлезда, Ki-67, Bcl-2.