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The prevalence of somatic *RAS* mutations in medullary thyroid cancer — a Polish population study

Częstość występowania mutacji somatycznych *RAS* w raku rdzeniastym tarczycy — analiza populacji polskiej

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Abstract

Introduction: Somatic *RET* mutations are detectable in two-thirds of sporadic cases of medullary thyroid cancer (MTC). Recent studies reported a high proportion of *RAS* somatic mutations in *RET* negative tumours, which may indicate *RAS* mutation as a possible alternative genetic event in sporadic MTC tumorigenesis. Thus, the aim of the study was to evaluate the frequency of somatic *RAS* mutations in sporadic medullary thyroid cancer in the Polish population and to relate the obtained data to the presence of somatic *RET* mutations.
Material and methods: Somatic mutations (*RET*, *RAS* genes) were evaluated in 78 snap-frozen MTC samples (57 sporadic and 21 hereditary) by direct sequencing. Next, three randomly selected *RET*-negative MTC samples were analysed by the next generation sequencing.
Results: *RAS* mutation was detected in 26.5% of 49 sporadic MTC tumours. None of all the analysed samples showed *N-RAS* mutation. When only *RET*-negative samples were considered, the prevalence of *RAS* mutation was 68.7%, compared to 6% observed in RET-positive samples. Most of these mutations were located in *H-RAS* codon 61 (72%). None of 21 hereditary MTC samples showed any *RAS* mutations.
Conclusions: *RAS* mutations constitute a frequent molecular event in RET-negative sporadic medullary thyroid carcinoma in Polish patients. However, their role in MTC tumorigenesis remains unclear. **(Endokrynol Pol 2015; 66 (2): 121–125)**

Key words: *medullary thyroid cancer; RET; RAS; driver mutation*

Streszczenie

Wstęp: Somatyczne mutacje proto-onkogenu *RET* wykrywane są w trzech czwartych wszystkich sporadycznych raków rdzeniastych tarczycy (MTC). Ostatnie badania wykazały, że mutacja genu *RAS* jest również częstym wydarzeniem w sporadycznych guzach MTC, co może oznaczać, że mutacje genów z rodziny RAS są alternatywnym wydarzeniem molekularnym w kancerogezie sporadycznej postaci tego raka.
Z tego względu celem niniejszej pracy było oszacowanie częstości występowania mutacji genów *RAS* w sporadycznym raku rdzeniastym tarczycy w populacji polskiej i odniesieniu częstości ich występowania do obecności mutacji somatycznych proto-onkogenu *RET.*
Materiał i metody: Materiał do badań stanowiło 78 fragmentów guza raka rdzeniastego tarczycy (57 próbek postaci sporadycznej i 21 dziedzicznej MTC). Analizowano mutacje genu *RET*, *H-RAS*, *K-RAS* i *N-RAS* metodą bezpośredniego sekwencjonowania a także 3 próbki raka sporadycznego, wybrane losowo, zostały zeskwencjonowane metodą głębokiego sekwencjonowania (Illumina).
Wyniki: Mutację genów *RAS* wykryto w 26,5% z 49 przeanalizowanych guzów sporadycznej postaci MTC. Natomiast, gdy tylko brano pod uwagę próbki *RET*-negatywne, częstość występowania mutacji genów RAS wynosiła 68,7% w porównaniu z 6% obserwowanych w guzach *RET*-pozytywnych. Nie wykryto, w żadnej z próbek, mutacji genu *N-RAS*. Najczęściej wykrywaną mutacją była zmiana w kodonie 61 genu *H-RAS* (72%). Nie wykryto mutacji genów *RAS* w żadnej z próbek dziedzicznego guza raka tarczycy.
Wnioski: Mutacje somatyczne genów RAS są częstym wydarzeniem obserwowanym w RET-negatywnych sporadycznych rakach rdzeniastych tarczycy w populacji polskiej. Jednakże rola tych mutacji w rozwoju rdzeniastego raka tarczycy nie jest do końca poznana. **(Endokrynol Pol 2015; 66 (2): 121–125)**

Słowa kluczowe: *rak rdzeniasty tarczycy; RET; RAS; mutacja inicjujaca*

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Introduction

Medullary thyroid cancer (MTC) arises from calcitonin secreting parafollicular C cells of thyroid and accounts for 3–5% of all thyroid cancers [1–3]. The disease in ultrasound imaging does not differ from other type of tumour [4]. Medullary thyroid cancer (MTC) occurs as both hereditary and sporadic form. The hereditary type of MTC is secondary to the *RET* (Rearranged during Transfection) proto-oncogene germline mutations that are associated with multiple endocrine neoplasia type 2 (MEN2a, MEN2b) and familial medullary thyroid cancer (FMTC).

The *RET* gene is located on chromosome 10q 11.2, contains 21 exons, and encodes a receptor tyrosine kinase, which is a transmembrane protein comprising of extracellular, transmembrane, and intracellular (cytoplasmic) domains. The *RET* gene plays an important role during morphogenesis and is normally activated by the ligand belonging to glial cell-derived neurotropic factor (GDNF) family and co-receptor GFRα. Mutations of the *RET* proto-oncogene lead to its autophosphorylation and to a gain of function resulting in constitutive activation of RET receptor [5–7].

The global risk of the detection of a germinal *RET* mutation in the Polish MTC population is about 10%, even if there is no positive family history and no features of MEN2a syndrome are present [8]. Simultaneously, the risk of MTC development in *RET* germline mutation carriers is nearly 100%. Therefore, molecular diagnostics is obligatory in all MTC subjects. These germline mutations concern the following *RET* gene exons 5, 8, 10, 11, 13, 14, 15, and 16. A germline mutation with a higher transforming activity, located in *RET* exon 16 (M918T), results in MEN2b syndrome (medullary thyroid cancer, pheochromocytoma, and typical phenotype features). The most characteristic mutation for MEN2a syndrome, located in *RET* exon 11 (codon 634), also related with the highest probability of pheochromocytoma and parathyroid hyperplasia, is observed in up to 20% of MEN2a subjects [9, 10].

Somatic *RET* mutations are also detectable in two-thirds of sporadic MTC cases. Alteration in *RET* exon 16 (M918T) is the most frequent one, accounting for nearly 80% of all detected *RET* somatic mutations. It is associated with more aggressive course of the disease [11–13]. Recent studies suggest a high proportion of *RAS* somatic mutations in *RET*-negative tumours [14–16]. Thus, *RAS* gene mutations could be considered as an alternative genetic event in sporadic MTC tumorigenesis. These mutations are present in three RAS proteins: K-RAS, H-RAS, and N-RAS — small GTPases that play a role in cellular growth, differentiation, adhesion, and migration. Normally, *RAS* is activated by external signals from tyrosine kinases receptor (Fig. 1). Mutations of this gene lead to constitutive activation of the RAS protein. Most *RAS* mutations are limited to hot spot codons located in exons 2 and 3. A mutation in exon 3 of *N-RAS* (codon 61) is the most frequent in poorly differentiated thyroid cancers [17, 18] (Fig. 1).

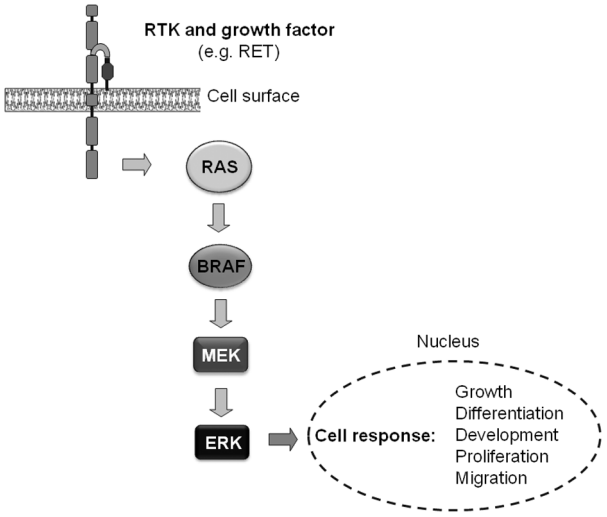

Figure 1. *MAP Kinase signalling pathway. RTK — receptor tyrosine kinase*

Rycina 1. *Ścieżka sygnałowa MAPK. RTK — receptor kinazy tyrozynowej*

The aim of our study was to evaluate the frequency of somatic *RAS* mutations in sporadic medullary thyroid cancer in the Polish population and to relate the obtained data to the somatic *RET* mutations.

Material and methods

Samples

Seventy-eight MTC samples were analysed (57 sporadic and 21 hereditary). Tumour samples were collected from the biobank of the Department of Nuclear Medicine and Endocrine Oncology in M. Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch. *RET* mutation status was evaluated in all 57 sporadic MTC tumour samples, whereas *RAS* mutation status was assessed in 49/57 sporadic MTC samples and in all 21 hereditary MTC tumours.

RET and RAS analysis method

DNA for *RET* germline mutations was extracted from peripheral blood by the desalting method and Genomic Maxi AX Kit (A&A Biotechnology). Mutation screening was performed according to a standard algorithm approved by the American and European MTC Management Guidelines [9, 10], which assumes analysis of exons

10, 11, 13, 14, 15, and 16. These exons were sequenced directly using Big Dye 1.1 reagent and a 3130xl Genome Analyser (Life Technologies). For *RET* and *RAS* somatic mutations, DNA was extracted from snap-frozen tissue samples by the DNeasy Blood and Tissue Kit (Qiagen). Twenty-one exons of the *RET* gene and exon 2 and 3 of *H-RAS*, *K-RAS*, and *N-RAS* (codons 12, 13, and 61) were sequenced directly as described above.

Deep sequencing

Next generation sequencing of RNA was performed in three randomly selected *RET*-negative MTC samples using TruSeq Sample Preparation Kit (Illumina) and TruSeq SR Cluster Kit (Illumina) according to the manufacturer's protocol. Single-end 76 bp sequencing on Genome Analyzer IIx (Illumina) was carried out. Low quality reads were filtered out with FASTX_Toolkit 0.0.13. Next, good quality reads were aligned to the human genome hg19 using TopHat 2.0.11 [19]. Duplicate reads were removed with PicardTools 1–56. Variants (single nucleotide variants, small insertions, and deletions) were identified using VarScan 2.3.6 [20]. The annotation of variants was performed with Annovar [21].

Table I. ***Somatic* RET *mutations were analysed in all 57 sporadic MTC tumours. The most frequent one was* RET *M918T mutation, comprising nearly 50% of all* RET*-positive sporadic MTC samples***

Tabela I. ***Mutacje somatyczne proto-onkogenu* RET *oznaczono we wszystkich 57 próbkach sporadycznego MTC. Najczęściej wykrywaną mutacją, stanowiącą prawie 50% wszystkich* RET*-dodatnich próbek, była mutacja M918T***

Number of samples	Type of *RET* mutation	Exon
16	M918T (Met>Thr)	16
1	A883F (Ala>Phe)	15
5	C634R (Cys>Arg)	11
1	C634W (Cys>Trp)	11
1	C630R (Cys>Arg)	11
1	C630G (Gys>Gly)	11
1	C618S (Cys>Ser)	1
1	H568N (His>Asn)	8
2	Del 7 bp	11
1	Del 6 bp	11
1	Del 19 bp	11
2	Del/ins	11
Total: 33 (57.9%)		

Results

Somatic RET mutational screening in sporadic MTC

Among 57 sporadic MTC samples somatic *RET* point mutations were found in 33 cases (57.8%). The most frequent *RET* mutation was localised in codon M918T, 16/33 (48.5%). The other *RET* mutations were detected in codon C634, 6/33 (18%), codon C 630, 2/33 (6%), and one in codon C618S (3%), codon A883F (3%), and H568N (3%). Deletions of 6 to 9 nucleotides in *RET* exon 11 were found in 4 sporadic MTC tumours and one deletion with insertion in exon 11. Detailed results are presented in Table I.

Somatic RAS mutational screening in sporadic MTC

The status of three *RAS* genes was investigated in 28 somatic *RET*-positive MTC samples and 16 somatic *RET*-negative samples. A *RAS* mutation was found in 2/33 of sporadic MTC samples with *RET* somatic mutation (6%) and in 11/16 (68.8%) of *RET* negative MTC tumours. Among sporadic *RET*-positive samples, one tumour harboured *H-RAS* codon 61 mutation, whereas *K-RAS* codon 13 mutation was found in the second sample. Among *RET*-negative samples, 8 *H-RAS* mutations were detected and 3 *K-RAS*. The details are reported in Table II.

Table II. ***The presence of* RAS *mutation was evaluated in 49 sporadic MTC samples (33* RET*-positive and 16* RET*-negative).* RAS *mutation was detected in 11/16* RET*-negative tumours and in 2/33* RET*-positive samples***

Tabela II. ***Status mutacji* RAS *oceniono w 49 próbkach sporadycznego MTC (33* RET*-dodatnich i 16* RET*-ujemnych). Mutacje* RAS *wykryto w 11/16 RET –ujemnych guzach i w 2/33* RET*-dodatnich próbkach***

Number of samples with a particular mutation	*H-RAS* mutations	*K-RAS* mutations	*RET* mutations
4	Q61K (Gln>Lys)		Negative
4	Q61R (Gln>Arg)		Negative
2		G12R (Gly>Arg)	Negative
1		Q61R (Gln>Arg)	Negative
1	Q61R (Gln>Arg)		H568N
1		G13S (Gly>Ser)	M918T
Total: 13 (26.5%)			

None of 21 hereditary MTC samples showed any *RAS* mutations. Moreover, none of all analysed samples (21 germline *RET*-positive, 28 somatic *RET*-positive, and 16 somatic and germline *RET*-negative samples) showed *N-RAS* mutation.

Next generation sequencing

The NGS was performed in three randomly selected *RET*-negative MTC samples. In RNA-seq, 22 million good quality reads were obtained for MTC10, 24 million for MTC20, and 28 million for MTC22 to detect single nucleotide variants, insertions, and deletions. Among identified variants, two mutations were present in *HRAS* exon 3 in two distinct MTC samples. They were p.Q61K (c.C181A) *HRAS* mutation in MTC20 and p.Q61R (c.A182G) *HRAS* mutation in MTC22. No other putative driver mutations were found.

Discussion

Somatic mutations of three *RAS* genes have been reported with reference to several tumours [22], among them differentiated thyroid cancer [23–25]. From the clinical point of view, screening for *RAS* mutations is important in patients with colorectal carcinoma where the presence of *K-RAS* mutation is associated with poor response to anti-EGFR therapies [26–28]. However, in differentiated thyroid cancer, the detection of *RAS* mutation has not thus far determined any treatment decision. Interestingly, all mutations that play role in thyroid cancer despite its origin (follicular or parafollicular cells) are involved in MAPK kinase pathway.

The prevalence of *RET* somatic mutations (57,8%) in our analysed group is consistent with previous reports [15, 29–31], where the frequency of somatic *RET* mutation ranges between 38% [29] and 71% [15]. Moreover, similarly to other data [29, 30], the most frequent somatic *RET* mutation in our group was M918T, which constituted nearly 50% of all *RET* somatic mutations. However, other somatic mutations in exons 10–11 were also found in our material.

In the present study, a mutational analysis of *H-RAS*, *K-RAS*, and *N-RAS* in a series of 65 MTC samples (49 sporadic and 21 hereditary), representative for the Polish population, was performed. *RAS* mutation was detected in 26.5% of all sporadic MTC tumours, but when only *RET*-negative samples were considered, the prevalence of *RAS* mutation was 68.7%. A higher proportion of *H-RAS* mutation that constituted 72% of all detected *RAS* mutations was observed, which is consistent with all reported results [15, 16, 32] and comparable with studies published by Moura et al. and Boichard et al. that reported RAS mutations present in 68% and 81% of RET-negative sporadic MTC samples, respectively [14, 16]. The results of different analyses of *RAS* mutations in MTC are controversial with reference to their different frequency reported by different studies. Ciampi et al. suggest that *RAS* mutations are extremely rare in MTC and stand for ~11% of all sporadic MTC cases and 17.6% when only *RET*-negative MTC samples were evaluated [33]. Schlumberger also reported such a low prevalence of *RAS* mutation in MTC, only in 8% of all sporadic MTC [34]. Some studies did not show any *RAS* mutation in MTC [35] or confirmed its very low frequency, like in the Shulten et al. study [36], where the only one sample among 15 MTC tumours analysed demonstrated RAS mutation, while in our study RAS mutation was diagnosed in 26.5% of all cases.

The reason of variability in the prevalence of RAS mutations in MTC is unknown. One explanation could be related to differences between analysed populations. However, when we look at Italian results only, some differences may also be noticed. Ciampi et al. reported a very low prevalence of RAS mutation in RET-negative sporadic MTC (17.6%) [32] while in the Scarpa and Fugazzola group the frequency of RAS mutation was 57% [31]. This percentage was even higher in our group — 68.7 % of *RET*-negative tumours showed *RAS* mutation. However, of note, the Elisei group involved the largest series of analysed MTC samples from different Italian centres [32]. The second explanation could be the different methodologies used for mutational RAS screening and their sensitivity. This is also visible in the Italian results, where Ciampi et al. used PCR and direct sequencing [32], while Simbolo et al. applied next generation sequencing [31]. Nonetheless, these differences do not explain all discrepancies because PCR and direct sequencing were used in many studies. The third explanation is related to the material used for analysis. Some studies comparing the results achieved from formalin-fixed paraffin-embedded (FFPE) samples and frozen tissues demonstrated a higher rate of RAS mutations in snap-frozen tissues than in FFPE samples [37]. Another important issue related to FFPE samples is the poor quality of the material leading to false-negative results [38]. This could explain the low rate of RAS mutation in the Schlumberger et al. and Rapa et al. studies as both groups used FFPE samples for RAS mutational screening, contrary to data from Ciampi et al. performed on snap-frozen samples [32, 34, 35]. We underline that we also used snap-frozen MTC samples.

Interestingly, in our set of *RET*-positive sporadic MTC samples, *RAS* mutations were also found (6%). Until now *RET* and *RAS* mutations were reported as mutually exclusive [15, 16, 32], and only in one study was *RAS* mutation detected in *RET*-positive MTC [14]. The coexistence of two oncogenic mutations is rare. In some papillary thyroid cancer cases RET rearrange-

ments coexist with *B-RAF* or *RAS* mutation [39–41] or *BRAF* and *K-RAS* mutations [42]. A similar situation is observed in lung adenocarcinoma, where *PIK3CA* mutations are accompanied by with *K-RAS* or *EGFR* mutations [43, 44]. These data may suggest that *RAS* could be a secondary event, not a driver mutation. However, to date there is no published transforming assays demonstrating that *RAS* mutations play an important role in MTC tumorigenesis.

Summary

The analysis of three *RAS* genes (*H-RAS, K-RAS, N-RAS*) revealed that 29% of sporadic MTCs harbour *RAS* mutations. This rate was much higher when only *RET*-negative sporadic MTC were analysed — 69%. Most of these mutations were located in *H-RAS* codon 61 (72%). 3% of *RAS* mutations were detected in *RET*-positive sporadic MTC. No *N-RAS*-positive samples were found.

The prevalence of *RET* somatic mutations in the Polish population is high and is observed in nearly 58% of all sporadic MTC tumours. Among them, M918T RET mutations constitute 50%.

Conclusions

RAS mutations constitute a frequent molecular event in RET-negative sporadic medullary thyroid carcinoma in Polish patients. However, their role in MTC tumorigenesis remains unclear.

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