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ORIGINAL ARTICLE

RET Proto-Oncogene Germline Mutation in *Pheochromocytoma* Patients — Incidence and Clinical Consequences

Częstość występowania i znaczenie kliniczne mutacji protoonkogenu RET u chorych z guzem chromochłonnym

Streszczenie

Wstęp Dotychczasowe badania wskazują, że częstość zespołu mnogiej gruczolakowatości (MEN 2), w skład którego wchodzi guz chromochłonny, jest większa niż dotychczas sądzono. Zespół ten dziedziczony jest w sposób autosomalny dominujący i wywołuje go mutacja protoonkogenu RET.

Celem pracy jest ocena częstości występowania oraz kliniczne znaczenie mutacji protoonkogenu RET u chorych z guzem chromochłonnym.

Materiał i metody Badania genetyczne w kierunku mutacji protoonkogenu RET przeprowadzono u 106 chorych (średni wiek: 49 ± 14,1 roku, 26M, 80K) z rozpoznanym i potwierdzonym histopatologicznie guzem chromochłonnym.

Pacjenci ci byli uprzednio hospitalizowani i leczeni w Klinice Chorób Wewnętrznych i Nadciśnienia Tętniczego Akademii Medycznej w Warszawie w latach 1957–1998 oraz w Klinice Nadciśnienia Tętniczego Instytutu Kardiologii w Warszawie od roku 1980 do 2001. Oceniano również stężenie kalcytoniny (CT), zarówno w warunkach podstawowych, jak i po stymulacji pentagastryną, oraz stężenie parathormonu.

Wyniki Obecność mutacji protoonkogenu RET wykazano u 8 chorych (7,4%) — w eksonie 11, w kodonie 634, TGC na CGC u 5 chorych, u pozostałych 3 odpowiednio — w eksonie 11, kodonie 634, TGC na GGC, w eksonie 11, kodonie 634, TGC na TGG oraz w eksonie 13, kodonie 791, TAT na CGC. Nadczynność komórek C potwierdzoną dodatnim testem pentagastrynowym stwierdzono u 5 nosicieli, u 2 chorych wynik testu był wątpliwy, jedynie u 1 chorego stężenie kalcytoniny było prawidłowe. Prawidłowe stężenie CT obserwowano u chorego z mutacją w eksonie 13, kodonie 791, TAT na CGC. U 4 nosicieli potwierdzono histopatologicznie obecność raka rdzeniastego tarczycy (biopsja cienkoigłowa). U 3 chorych wykonano totalną tyreidektomię, dwóch nie wyraziło zgody na dalsze leczenie (w tym jeden z pozytywnym wynikiem biopsji). Pozostali chorzy zostali poinformowani o konieczności totalnej tyroidektomii. U żadnego nosiciela nie stwierdzono nadczynności przytarczyc.

Wnioski Wyniki badań autorów potwierdzają doniesienia o konieczności poddawania przesiewowym badaniom genetycznym oceniającym obecność mutacji protoonkogenu RET pacjentów z guzem chromochłonnym. Potwierdzenie

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nosicielstwa tej mutacji stanowi wskazanie do wykonania totalnej tyroidektomii.
Genetyczne badania przesiewowe mogą mieć również znaczenie w wykrywaniu zespołu MEN 2 oraz ustaleniu dalszego postępowania u członków rodzin, u których stwierdza się mutację protoonkogenu RET.

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Introduction

Pheochromocytoma represents one of the forms of secondary hypertension and accounts for 0,1–0,9% of all cases of hypertension. The large variability of dramatic symptoms and signs is mainly related to the effects of high circulating levels of norepinephrine and epinephrine secreted by the chromaffin tumour [1–3].

Recent studies have shown that the proportion of sporadic *pheochromocytoma* is dramatically decreasing. The recent study of Neumann *et al.*, based on a large, unselected series of a registry of *pheochromocytoma*, indicates that about 25% of apparently sporadic *pheochromocytoma* patients may be carriers of mutations [4]. When hereditary, *pheochromocytoma* can be a component of multiple endocrine neoplasia type 2 (MEN 2), caused by mutations of RET gene, von Hippel-Lindau disease, caused by mutations of VHL gene; and rarely, neurofibromatosis type 1 [1–3]. Recently, mutations of the gene for succinate dehydrogenase subunit D and subunit B were identified in another related neuroendocrine disease with coexistence of *pheochromocytoma* and paragangliomas of the neck or glomus tumour [4].

MEN 2 is an autosomal dominant syndrome identified to date in 500–1000 kindred. MEN2A accounts for over 75% of MEN 2.

It has been reported that patients with MEN 2A are predisposed to have *pheochromocytoma* in about 50% of affected individuals and medullary thyroid cancer (MTC) and/or C-cell hyperplasia [CCH] in about 95% of cases [5–7]. In patients with MEN 2A approximately 20% may be characterised by hyperparathyroidism in addition to MTC and/or CCH [8, 9]. MEN 2B is characterised by the major neoplasms of MEN 2A (MTC and *pheochromocytoma*), plus decreased upper/lower body ratio, a marfanoid habitus, and mucosal and intestinal ganglioneuromatosis, but not hyperparathyroidism.

The susceptibility gene for MEN 2, is the RET proto-oncogene (21 exons), located on chromosome 10 (10q11.2). Most mutations affect codon 634 [5, 7–10].

Mutations of the RET proto-oncogene, which have been identified in all families with MEN 2, can be used to confirm the clinical diagnosis and identify asymptomatic family members with this syndrome.

Taking into account the current knowledge suggesting that almost 25% patients with *pheochromocytoma* have a hereditary disease, it became apparent that all patients with *pheochromocytoma* should be screened for MEN 2, von Hippel-Lindau disease or one of the syndromes associated with *pheochromocytoma* and paragangliomas. In the case of RET proto-oncogene mutation, genetic screening should also be performed in members of carrier families to prevent further morbidity and mortality in patients and their families [5, 7, 11, 12].

Therefore we evaluated the incidence of RET proto-oncogene germline mutations in 106 patients with *pheochromocytoma*.

Material and methods

Study design and subjects

In our study we screened for MEN 2 and clinically evaluated unrelated 106 patients with *pheochromocytoma* (mean age: 49 ± 14,1 years, 26 male, 80 female) hospitalised and surgically treated in the Department of Internal Medicine and Hypertension, Warsaw School of Medicine (years 1957–1998), and in the Department of Hypertension Institute of Cardiology in Warsaw (years 1980/81–2001). Diagnosis of *pheochromocytoma* was verified in all cases by histological examination of the tumour removed during surgery [3, 5, 13]. In patients with RET proto-oncogene mutation, serum calcitonin (CT) (basal and after pentagastrin stimulation) and serum parathormone (PTH) evaluations were performed.

The protocol was accepted by the Ethical Committees of the Medical University of Warsaw and the Institute of Cardiology in Warsaw.

Molecular genetic analysis for RET gene

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. Single-strand conformation polymorphism (SSCP) analysis was used to find point mutations, small deletions or insertions. For each exon (exon 10, 11, 13 14, 15, 16) a separate set of primers was used. PCR — amplified

fragments (20 μl) were denatured by adding 30 μl denaturing solution (containing 95% formamide, 10 mM NaOH, 0,25% xylene cyanol, 0,25% bromophenol blue) and heating to 95% C for three minutes before chilling on ice. Denatured fragments were separated on a polyacrylamide gel (GeneGel Excel 12,5/24 Kit, Amersham Biosciences). After separation at 5 or 6 W for 1,5 h the fragments were stained with silver as described elsewhere. In case of aberrant bands the probes were amplified anew for sequencing. In specially difficult cases aberrant bands were cut out of the gel, dissolved in 10 mM Tris (ph 8,0) and reamplified for sequencing. All mutations were confirmed by sequencing. Mixtures of 20 μl contained approx. 100 ng genomic DNA, 0,2 mM of each dNTP, 2 μM of each primer, 1,5 mM $MgCl_2$ and 1 U Taq DNA polymerase (Amersham/Pharmacia). PCR conditions and sets of primers have been previously described [6, 8, 13].

Blood analysis

Parathormone was measured using an RIA of the intact human parathyroid hormone (N-TACT from Incstar). Calcitonin was measured using the RIA mat-RIA Calcitonin I (from Mallinckrodt). Samples of the pentagastrin test were taken at times 0, 2 and 5 min after injection of 0,5 μg/kg bw [12]. Plasma concentration of catecholamines was determined by radioenzymatic procedure using a commercial kit. Urinary excretion of catecholamines and metabolites was determined by fluorometric method and later by HPLC method [11, 12].

Results

In the series of 106 patients with *pheochromocytoma* genetic testing revealed germline mutations in the RET proto-oncogene in 8 cases (7,4%). These results have been included in a previously published study [5]. Five carriers had mutations of RET proto-oncogene in exon 11, codon 634, TGC to CGC, the following three — in exon 11, codon 634, TGC to GGC, exon 11, codon 634, TGC to TGG and in exon 13, codon 791, TAT to TTT, respectively.

The age at the onset of symptoms was younger in RET proto-oncogene mutation carriers (mean — 43,0 ± 6,9 years *vs.* 50,0 ± 14,4 years), but the difference was not statistically significant (p = 0,06). All of them had a unilateral, single adrenal *pheochromocytoma* tumour. Of all patients positive for *RET* mutations, none had clinical evidence of MTC at presentation. Hyperactivity of thyroid C-cells, suggesting MTC or C-cells hyperplasia, was found in 5 carriers, borderline values of basal and after pentagastrin calcitonin (CT) level were found in 2 carriers and CT concentration was normal in only one patient. Normal values of CT were found in patients with mutation in codon 791 (TAT to TTT). None of the RET gene mutations carrier manifested hyperparathyroidism.

In four patients with RET proto-oncogene mutations MTC was confirmed histopathologically in fine-needle biopsy. In three of them total thyroidectomy was performed. Two patients refused to be surgically treated (one with positive result of biopsy); the next three RET proto-oncogene germline mutation carriers have been informed that prophylactic total thyroidectomy should be considered [13]. Characteristics of *pheochromocytoma* patients with RET proto-oncogene mutations are presented in table I.

In the family of a 42-year-old female index case patient with *pheochromocytoma*, newly diagnosed MTC and RET proto-oncogene mutation, RET proto-oncogene germline mutations have been detected in 10 members. Four members agreed to be admitted and diagnosed in the Department of Internal Medicine and Hypertension for MEN 2. In one of them asymptomatic *pheochromocytoma* and MTC were diagnosed and surgically treated, in a further three family members MTC was confirmed and surgically treated. Other affected members of the family refused further diagnostic procedures.

Discussion

We are presenting a cohort of 106 *pheochromocytoma* patients being screened for RET proto-oncogene germline mutations. RET proto-oncogene mutation occurred in 8 patients (7,4%). Every carrier of the RET proto-oncogene mutation has been informed about the necessity of prophylactic total thyroidectomy. In our own material MTC was histopathologically confirmed by fine-needle biopsy in four cases. Three patients were surgically treated, two carriers refused surgery (one with positive result of the biopsy), in three cases we are waiting for the decision, so up to now we cannot determine the frequency of MTC in our group of patients with RET proto-oncogene germline mutations. However, the current knowledge indicates that the penetration of the neoplasia in patients with RET proto-oncogene mutations is virtually 100%, so there is an agreement that *RET* testing should replace calcitonin testing to diagnose the MEN 2A carrier and should be the only indication for total thyroidectomy. Mortality in MEN 2A is greater from MTC than from *pheochromocytoma*, so thyroidectomy is the goal therapy in all MEN 2A

Table I. Characteristic of RET proto-oncogene mutation carriers (data collected before thyroidectomy)

Tabela I. Charakterystyka kliniczna nosicieli mutacji protoonkogenu RET (dane uzyskane przed wykonaniem tyroidektomii)

Patient	Sex	MTC confirmed	Age (years)	RET mutation	PTH [pg/ml]	Calcitonin [pg/ml] 0 min	2 min	5 min
GB	F		42	634 TGC/CGC	30	66	> 1000	> 1000
KK	F	+	47	634 TGC/GGC	54	> 1000	> 1000	> 1000
ŁL	F		31	634 TGC/CGC	24	90	> 1000	> 1000
KP	F	+	39	634 TGC/CGC	56	15	77	89
JS	F	+	54	634 TGC/CGC	24	60	> 1000	> 1000
WP	M	+	42	634 TGC/CGC	18	> 1000	> 1000	> 1000
MS	F		47	634 TGC/TGG	18	7	80	60
PG	M		39	791 TAT/TTT	25	5	27	18

Range of the serum calcitonin level: normal basal values — ≤ 30 pg/ml
After pentagastrin administration: 30–100 pg/ml — borderline result, above 100 pg/ml — pathological result

carriers to prevent or cure MTC. Therefore, there is a consensus that all RET proto-oncogene mutation carriers should undergo total thyroidectomy [13].

The primary secretory product of MTC is CT, which is important only as an excellent tumour marker. CT values (basal or stimulated by pentagastrin) are very often but not always elevated in MTC. Definitely positive pentagastrin test was found in five carriers, borderline in two of them, normal values of CT were observed only in one patient. It is worth noting that borderline pentagastrin test result was observed in a patient with diagnosed MTC, which confirmed, the mentioned above, limited significance of CT estimation as a qualification for surgery [13].

Several mutations in RET proto-oncogene have been found in patients with MEN 2A. Approximately 93–98% of the MEN 2A families have mutations of one of the five cysteine conserved residues in exon 10 (codon 609, 611, 618, and 620) or exon 11 (codon 634) in the extracellular domain of the RET proto-oncogene. In addition to the common mutations, which affect cysteine residues, some rare non-cysteine mutations have been described within exon 13, 14, 15. More than 20 missense mutations associated with MEN 2A and other neoplastic diseases such as MEN 2B, familial medullary thyroid carcinoma (FMTC), sporadic medullary thyroid carcinoma were found in the RET proto-oncogene [9, 14, 15]. The aggressiveness of MTC correlates with the mutated *RET* codon [13]. Patients with MEN 2B and/or *RET* codon 883, 918, or 922 are classified as having the highest risk from aggressiveness of MTC (level 3), those with 611, 618, 620, or 634 of *RET* codon mutation — as having a high risk for MTC (level 2), and those carriers with *RET* codon 609, 768, 790, 791, 804, and 891 mutations are classified as having the lowest risk among the three *RET* codon mutation stratification categories (level 1) [13]. In our material, the mutation in codon 634 occurred in 7 carriers; the most common was missense mutation TGC to CGC (5 carriers). It has been suggested that patients with mutation in codon 634 TGC to CGC had a greater risk of developing parathyroid hyperactivity [15]. We found no signs of hyperparathyroidism in any of the RET mutation carriers.

The normal values of CT level (basal and after pentagastrin stimulation) were found only in one patient with mutation in codon 791 of RET proto-oncogene (level 1 of the scale of MTC aggressiveness). Recent study of Niccoli-Sire *et al.* indicates that the phenotype of patients with non-cysteine *RET* mutations is characterised by a late onset of the disease, suggesting a delayed appearance of C-cell disease rather than a less aggressive form [16]. Therefore, in the carriers with the lowest risk of MTC, total thyroidectomy is also recommended independently of CT values.

Based on the European Study data from 300 MEN 2 patients with *pheochromocytoma*, a variable chronology between diagnosis of MTC and *pheochromocytoma* can be observed. *Pheochromocytoma* was revealed first in 25% of cases, after MTC in 40% and in 35% of cases MTC and *pheochromocytoma* were diagnosed at the same time [17]. In our group, the first manifestation of the disease was *pheochromocytoma* in all RET proto-oncogene mutation carriers.

It should also be noted that RET proto-oncogene germline mutations vary in series of patients with apparently sporadic *pheochromocytoma* (from 0 up to 20%) [18–22]. In our study *RET* mutations were found in 8 from 106 patients with apparently sporadic *pheochromocytoma* (7,4%). This indicates that genetic testing in *pheochromocytoma* patients may be very useful in diagnosis of MEN 2 syndrome.

Recent data have shown that mutations of *RET* can also be used to confirm the clinical diagnosis and identify asymptomatic family members with MEN 2 syndrome. In our study, in a family known to have MEN 2, first-degree relatives diagnosed as gene mutation carriers were further screened for MTC and *pheochromocytoma*. Based on the results of genetic testing in this family, our study confirmed the necessity for predictive DNA testing of at-risk family members in kindred with MEN 2.

Taken together, presymptomatic identification of gene carriers by mutation analysis in the RET proto-oncogene and the option of prophylactic thyreoidectomy have had a great impact on the diagnosis and management of MEN 2A patients. In all families with an identified mutation in the RET proto-oncogene, it is necessary to perform thyroidectomy before medullary thyroid carcinoma occurs. Therefore, genetic screening has become a routine procedure for these patients.

Summary

Background In patients with *pheochromocytoma* there may exist more often than expected the autosomal dominant cancer syndrome — multiple endocrine neoplasia type 2 (MEN 2). The susceptibility gene for MEN 2 is the RET proto-oncogene. Germline mutations can be identified by analysis of exons 10, 11, 13–16 of the RET gene.

The aim of the study was to evaluate the frequency of these mutations in patients with *pheochromocytoma* and to report on the conclusions which patients and physicians have drawn.

Material and methods We screened for germline mutations in the RET proto-oncogene and clinically evaluated 106 unselected patients with *pheochromocytoma* (mean age: 49 ± 14,1 years, 26 male, 80 female) histopathologically confirmed, diagnosed and treated in the years 1957–1998 in the Department of Internal Medicine and Hypertension, Warsaw School of Medicine and in the years 1980/81–2001 in the Department of Hypertension, Institute of Cardiology, Warsaw. Determination of calcitonin concentration (CT) was performed in basal conditions and after pentagastrin stimulation; parathormone level was also determined.

Results Genetic testing revealed germline mutations in the RET proto-oncogene in 8 patients (7,4%). Carriers had mutation of exon 11, codon 634: TGC to CGC (5 patients), exon 11, codon 634: TGC to GGC (1 patient), exon 11, codon 634: TGC to TGG (1 patient) and in exon 13, codon 791: TAT to TTT (1 patient). Hyperactivity of thyroid C-cells was found in 5 carriers, borderline values of basal and after pentagastrin CT were found in 2 carriers and in only one patient CT concentration was normal. In four patients with RET proto-oncogene mutations, MTC was confirmed histopathologically in fine-needle biopsy. In three of them total thyroidectomy was performed. Two patients refused to be surgically treated (one with positive result of biopsy); the next three RET proto-oncogene germline mutation carriers have been informed that prophylactic total thyroidectomy should be considered. In none of the carriers hyperparathyroidism was observed.

Conclusions Our study indicates that patients with *pheochromocytoma* should be genetically screened for mutations of the RET proto-oncogene. The carriers of these mutations should undergo thyroidectomy. In addition, genetic studies can be useful for the screening of the carriers families.

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