การศึกษาการกลายพันธุ์ของยีน RET Proto-oncogene ของชุดครอบครัวคนไทยที่เป็นเนื้องอกมะเร็งของต่อมไร้ท่อหลายตำแหน่งชนิด 2A

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Abstract: Mutation Study of RET Proto-oncogene in Multiple Endocrine Neoplasia 2A of Three Thai Families

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Background: Multiple endocrine neoplasia type 2A (MEN 2A) is an inherited disease caused by germline mutations in the RET proto-oncogene, leading to the development of endocrine neoplasia. The prognosis depends on the appearance and spread of medullary thyroid carcinoma (MTC). Identifying at-risk relatives before the disease's clinical signs, or biochemical parameters become evident for precision medicine. Objective: To study RET mutation in MEN2A in three family members of Thai descent in Rajavithi Hospital. Method: Three families with MEN2A (22 samples) were examined. Peripheral blood DNA was amplified by polymerase chain reaction. Melting Curve analysis was performed to detect the mutation of RET proto-oncogene exon 11 by specific primer Real-time PCR technique. Molecular analysis was carried out in three index patients and 22 relatives of MEN2A patients. Result: Molecular investigations showed a mutation at codon 634 and exon 11 in all MEN 2A patients. In 3 MEN2A Families, 9 out of 22 relatives were C34R mutation (TCG->CGC) and C634Y mutation (TGC-> TAC). C634R mutation in a 14-year-old boy is essential evidence for prophylaxis thyroidectomy, confirmed by histopathology examination. Conclusion: The presence of RET C634R in the family member is essential evidence of thyroidectomy to prophylaxis MTC. RET mutation of this study is helpful for future screening and management of MEN2A families. The individual physician's decision-making or the wishes of the patient or the patient's family, according to 634 codon mutation, should be screening for pheochromocytoma and management following by stand treatment if they have biochemical evidence and following the screening of medullary thyroid carcinoma and parathyroid disease.

Keywords: MEN2A, RET mutation, Thai

บทคัดย่อ

ภูมิหลัง: เนื้องอกมะเร็งของต่อมไร้ท่อหลายตำแหน่งชนิด 2A เป็นโรคทางพันธุกรรมที่ถ่ายทอดด้วยเซลล์ต้นกำเนิดที่ชื่อว่า RET proto-oncogene โดยทำให้เกิดเนื้องอกที่อวัยวะของต่อมไร้ท่อหลายจุด แต่การพยากรณ์โรคนั้นจะขึ้นกับมะเร็งชนิด medullary ของไทรอยด์เป็นหลัก ฉะนั้น สมาชิกในครอบครัวที่มีความเสี่ยง ควรได้รับการตรวจทางห้องปฏิบัติการเพื่อหาการกลายพันธุ์ (gene mutation) และสามารถนำเข้าสู่กระบวนการรักษาแบบแม่นยำเฉพาะบุคคล (personalized precision medicine) ก่อนที่จะเกิดเนื้องอกมะเร็งของต่อมไทรอยด์ (thyroid carcinoma) **วัตถุประสงค์:** เพื่อศึกษาการกลายพันธุ์ของ RET ในเนื้องอกมะเร็งของต่อมไร้ท่อหลายตำแหน่ง ชนิด 2A ที่มีอยู่สามครอบครัวที่มารักษาที่โรงพยาบาลราชวิถี **วิธีการ:** สมาชิกของครอบครัวทั้ง 3 ครอบครัวจำนวน 22 คน ได้เข้าร่วมการศึกษาโดยการออกแบบและสังเคราะห์ SYBR Green Probe, Primer ที่จำเพาะ เพื่อทำการตรวจวิเคราะห์หา melting curve analysis สำหรับ gene mutation ตำแหน่ง codon 634 บน exon 11 ของ RET proto-oncogene ด้วยเทคนิค real-time PCR จากคนไข้ที่มารักษาที่ราชวิถี จำนวน 3 ราย **ผล:** สมาชิกของครอบครัวทั้ง 3 ครอบครัวพบการกลายพันธุ์ที่ตำแหน่ง 634 ของ exon 11 โดยพบว่ามีสมาชิกที่มีการกลายพันธุ์อยู่ 9 คนใน 22 คน ด้วยเทคนิค Real-time PCR **สรุป:** การพบการกลายพันธุ์ RETC634R มีความสำคัญต่อการวางแผนรักษาของผู้ป่วยที่เป็นเนื้องอกมะเร็งของต่อมไร้ท่อหลายตำแหน่ง ชนิด2A เป็นอย่างมาก เพื่อเตรียมการก่อนการผ่าตัดต่อมไทรอยด์ เพื่อป้องกันการกลายเป็นมะเร็งไทรอยด์ชนิด medullary และการศึกษาการกลายพันธุ์จะมีประโยชน์ต่อครอบครัวที่ต้องมีการวางแผนการรักษาในอนาคตเป็นอย่างมาก ควรพิจารณาการตัดสินใจการรักษาของแพทย์แต่ละคนหรือความปรารถนาของผู้ป่วยหรือครอบครัวของผู้ป่วยตามการกลายพันธุ์ 634 codonควรตรวจคัดกรอง pheochromocytoma และการจัดการตามด้วยการรักษาหากมีหลักฐานทางชีวเคมีและการตรวจคัดกรองมะเร็งต่อมไทรอยด์ชนิด medullary และโรคพาราไทรอยด์

คำสำคัญ: เนื้องอกมะเร็งของต่อมไร้ท่อหลายตำแหน่งชนิด2A, การกลายพันธุ์ RET, คนไทย

Introduction

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal dominantly inherited endocrine tumor syndrome characterized by tumor development in various endocrine organs, including the thyroid, adrenal medulla, and parathyroid. The estimated prevalence is 2.5 per 100,000 in the general population. MEN2 shows a high penetrance for medullary thyroid carcinoma (MTC), a rare calcitonin (Ct)-secreting tumor derived from thyroid parafollicular or C cells of the thyroid; in which they are derived from the neural crest.

Early identification of patients with MTC leads to a shift in disease presentation from a clinically evident tumor to a preclinical disease, resulting in a higher cure rate of the affected patients with much better prognosis[1]. Each variant of MEN2 results from a different mutation of the RET gene with good genotype-phenotype correlations.

There are rare families with features of classical MEN2A with no identifiable RET germline mutation. In this study, the diagnosis of classical MEN2A can be made if one or more first-degree relatives have characteristic clinical features of the entity collected from Rajavithi Hospital in Bangkok of Department of Medical Service (DMS) as a referral and excellent center of head and neck management.

Materials and Methods

The diagnostic criteria for MEN2 may have varied presentations. At least one of the following criteria had to be present for inclusion in the study.

- Combination of medullary thyroid carcinoma (MTC) and PHEO.
- Evidence of one lesion of MTC or pheochromocytoma (PHEO) with a family history of MEN2.
- Identification of pathological germline mutation in the *RET* gene.

The selected patients' data were deposited into the database. Possible inconsistencies and missing data were validated and corrected if appropriate. The ethics committee of Rajavithi Hospital approved this proposal.

The screening examinations analyze serum calcium, phosphorus, and intact parathyroid hormone (iPTH) for parathyroid tumors. Serum calcitonin is used for diagnosing medullary thyroid carcinoma. PHEO was based on measurements of urinary metanephrine, normetanephrine, epinephrine, norepinephrine, and dopamine per mg creatinine levels as a minimum.

Real time PCR for RET mutation

We used real-time PCR and melting curves on Light Cycler (Roche Diagnostics Mannhein, Germany) to analyze the RET mutation. PCR was performed in the total volume of 20 µl in the Light Cycler glass capillaries. The reaction mixture contained 2 µl of 10x Fast Start DNA

Master Hybprobe (Roche Diagnostics Mannhein, Germany), 1.6 µl of MgCl2 (25 mM), 1 µl of each primer (10 µM), 0.4 µl of each probe (10 µM), 11.6 µl of distilled water and 2 µl of DNA (100-500 ng). PCR conditions were followed by initial denaturation at 95 ºC for 10 min and 45 cycles of denaturation at 95 ºC for 10 s, annealing at 68 ºC for 10 s, and extension at 72 ºC for 10 s, respectively. After amplification, the melting analysis was performed by denaturation at 95 ºC for 20 s, annealing at 40 ºC for 20 s, and increasing the temperature to 85 ºC with a ramp rate of 0.2º C/s.

The amplification primers were RET Forward (5' – CCTCTGCGGTGCCAAGCCT – 3') and RET Reverse (5' – GCTGACCGGGAAGGTGGG – 3'). The wild-type probe stretched from codon 624 to codon 637. The sensor 3' fluorescence-labeled probe was 5'- ACCGTGCGGCACAGCTC – 3', and the sequence of another probe labeled 5' with LC-Red640 was 5' – TCGCACAGTGGATCTGTGGGTGG – 3'.

Study design

A cross-sectional study design with a study group was designated, in which Mutant *RET* patients had to provide clinical and genetic information they had been treated up to 2022.

Result

As shown in Table 1, twenty-two MEN2A family members were screened for RET 634 mutation from the germline with biochemical variables from serum and urine collected from their local hospital of MEN2A families (Table 2). C634R was mostly found (5 cases) in one family in Nan Province in the northern part of Thailand, and C634Y was found in two other families (4 cases) from Kampaengphet and Phetchabun Provinces, which were located between the Northern and central parts of Thailand. Table 2 shows biochemical measurements in family members of three families that were checked during the study, such as RET mutation, calcitonin for medullary thyroid carcinoma, calcium and parathyroid hormone for primary hyperparathyroidism, and urine metanephrine, epinephrine and nor-metanephrine for pheochromocytoma.

The pedigree of the three families shown in Figures B and C represent N Nan, K Kampaengphet, and P Phetchabun's family members.

Table 1 General characteristic and *RET* mutation finding

Characteristics	n = 22	Percent
Sex	Male:12	54.5
	Female:10	45.5
RET Mutation	Wild type:13	59.1
	C634R:5	22.7
	C634Y:4	18.2

Table2. *RET* mutation study and biochemical of associated tumor in MEN2A

Case	Mutation	Calcitonin (pg/mL)	Calcium (mg/dL)	iPTH (pg/ml)	Urine Metanephrin	Urine Epinephrin	Urine Normetanephrin
N1	C634R	1445	10.1	30	/	/	X
N2	WT	<2	9.7	50	X	X	X
N3	C634R	4462	9.1	17	/	/	X
N4	WT	7.3	15.4	43	X	X	X
N5	C634R	<2	8.6	11	X	X	X
N6	C634R	450	8.9	46	/	/	X
N7	WT	<2	9.2	29	X	X	X
N8	WT	<2	9.4	27	X	X	X
N9	WT	<2	9.3	25	X	X	X
N10	WT	<2	9.5	26	X	X	X
N11	WT	<2	9.0	36	X	X	X
N12	C634R	<2	8.6	23	X	X	X
P1	WT	<2	8.9	26	X	X	X
P2	C634Y	7025	9.8	15	/	X	X
P3	WT	<2	9.1	33	X	X	X
K1	WT	4.9	7.1	17	X	X	X
K2	C634Y	<2	10.7	39	X	X	X
K3	C634Y	1554	10.5	60	/	X	NA
K4	C634Y	670	8.8	45	/	X	NA
K5	WT	5.3	12.5	54	X	X	X
K6	WT	<2	8.2	23	X	X	X
K7	WT	<2	9.3	15	X	X	X

* N was Nan's family members, K was Kampaengphet's family members and P was Phetchabun's family members, / as confirmed the positive test, X confirm the negative test, NA non-access data

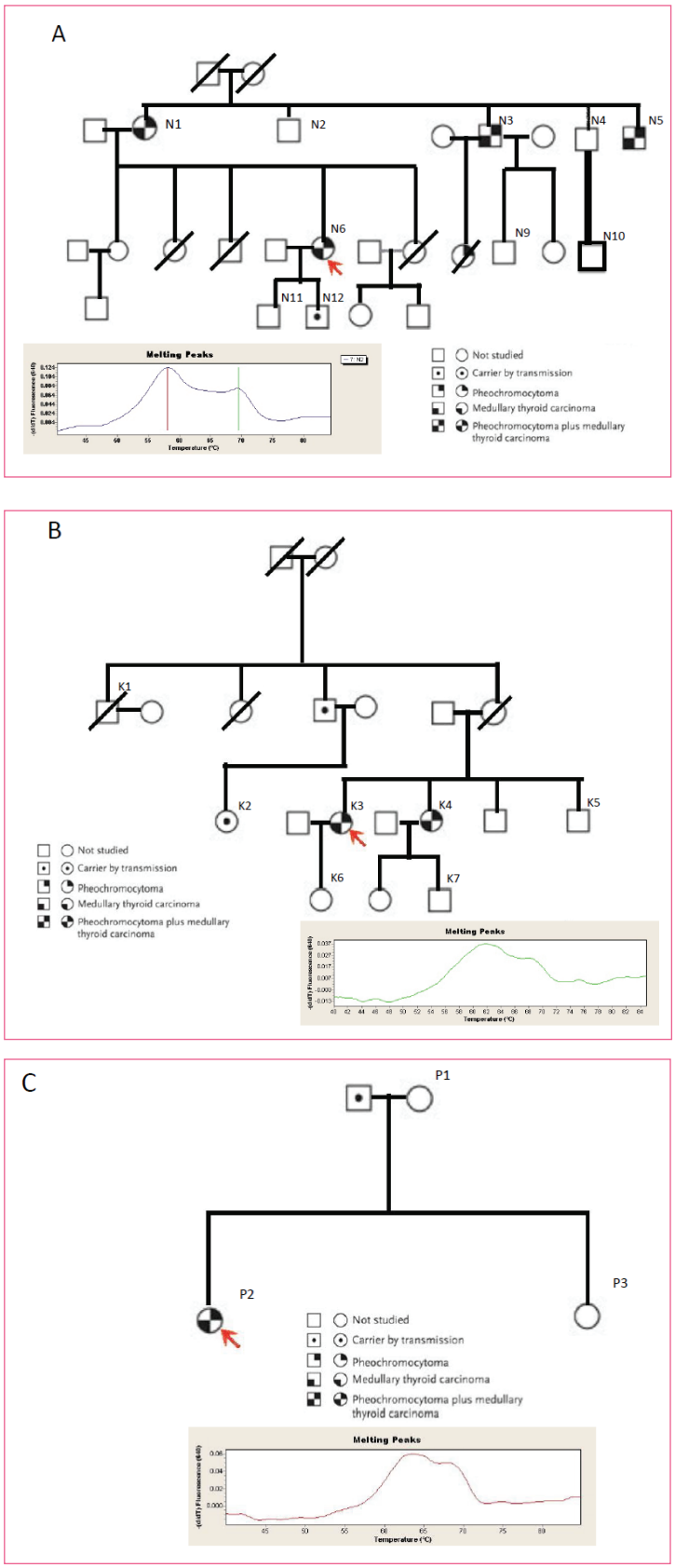

Figure 1 Pedigree of three families that harbor RET mutation. A: Nan family, B: Kampaengphet family, and C: Phetchabun family

Discussion

Classical MEN2A is the most common MEN2A variant and in 95% of patients, *RET* germline mutations occur in codons 609, 611, 618, or 620 of exon 10 or codon 634 of exon 11[2].

C634R was the most common mutation at codon 634 in this study, according to previously reported data[3, 4]. MEN2A patients had the potential to develop bilateral adrenal PHEO, and surgical management remains controversial. Prophylactic total adrenalectomy was never performed because it commits the patient to lifelong steroid hormone replacement and the risk of a life-threatening Addisonian crisis.

MTC was previously the primary cause of death in patients with MEN2; however, early thyroidectomy has lowered mortality from hereditary MTC[5]. It is also probable that improved management of PHEO has reduced the rate of premature mortality in patients with MEN2.

As demonstrated in the ATA-2015, the clinician should remind patients about both the benefit and potential risks of mutation, and genetic counseling about these reproductive options should be considered for all patients carrying the RET mutations who are of reproductive age, particularly those who have RET mutations in codon 634 and 918[6].

Primary hyperparathyroidism is often clinically occult and does not differ from that seen in patients with mild sporadic primary hyperparathyroidism. It has been reported in 10–25% of patients with MEN2A, but it is rarely the first manifestation of this syndrome[7, 8]. Collection and analysis of clinical information make it possible to understand rare diseases' clinical features and standardize their medical management. In patients with RET C634 codon mutations, the incidence of PHEO is 50% by the 5th decade and approaches 90% by the 8th decade[9]. In patients with RET codon mutations, other than C634, the incidence of PHEO ranges from 4% to 25%. However, in our study, the PHEO range incidence is 25-33.3% in different families in Table 2. Hyperparathyroidism (HPTH) occurs in about 30% of patients with MEN2A and RET 634 codon mutations and is less frequent in patients with mutations in other RET codons, but our study has not found a case of HPTH. HPTH is usually mild and asymptomatic, but surgical resection is indicated if patients develop symptoms or signs related to hypercalcemia. The surgical options are either subtotal 31/2 gland resection or total parathyroidectomy with a parathyroid autograft to a heterotopic site[10]. C634Y mutation in Spanish families occurred in 73% of cases[11], but in our study, it was found in 42.8% of all candidate carriers. C634Y mutation in Spanish families, specific for MTC and phaeochromocytoma, was around 80%, which was close to the 75% prevalence of our study.

Cutaneous lichen amyloidosis (CLA) is a rare disorder that usually occurs sporadically but may present in a hereditary pattern, either as a separate entity or in association with other diseases, one of which is MEN2A[12]. In one study, CLA, or regional pruritus without CLA, occurred in 36% of patients with the *RET* codon 634 mutation[13]. Our study found this lesion in Phetchabun family index case and her sister at the scapular region of the back corresponding to dermatomes T2–T6, such as in the previous study[14, 15].

For RET mutations in codon C634, the MTC has an earlier age onset and a more aggressive clinical presentation, in which the discovery of RET mutations is the cause of MEN2, which oncologists based the timing of thyroidectomy mainly on the presence of a specific RET codon mutation. There were also some problems with this strategy. There is substantial variability in the age at which MTC develops, not only among families with the same RET codon mutation but among individuals within the same family. Our Nan family member (N3 in Table 2) had a long-term clinical course of thyroid goiter and pheochromocytoma-like symptoms more than 40 years before enrolling in screening in our program. Later, he was admitted for bilateral adrenalectomy followed by total thyroidectomy in the same admission by the ENT department. *RET* codon 634 mutations are associated with high penetrance of PHEO, which in one study increased with age, being 25% by age 30 years, 52% by age 50 years, and 88% by age 77 years[12].

Prophylactic thyroidectomy, being extremely useful in youngsters, applies to a small number of patients with hereditary MTC, and no sporadic MTC. In families with MEN2A, all stages of the disease are found. In 75% of patients with clinically evident thyroid nodules, the MTC has already spread to regional lymph nodes, and 10% have metastases at distant sites[13]. Our study found that a young member in the NAN family (N12 in Table

2) had *RET* mutation, but all parameters were normal. Although we found a thyroid nodule by ultrasound, he refused to undergo surgery after counseling to treat *RET* mutation. Generally, in patients with MEN2A and a RET C634 codon mutation, the thyroid should be removed around five years of age[14].

In this regard, our study is a powerful tool for improving the future management of MEN2 in Thai patients. Although we are currently collecting only descriptive information, adding more data, such as details of symptoms, imaging studies, and biochemical assays, will further enhance the value of the database. There were some limitations in our study. Firstly, fewer participants were collected and tested for mutation, which was 22 from 34 candidate relatives (64.7%), despite the research team contacting all candidate relatives to test the mutation and test for biochemical markers involved in MEN2a spectrum neoplasm. Secondly, some mutation carriers have different medical treatment right, but they have been suspected of clinical manifestations in MEN2A. As a result, our team sent document data to them to follow in their treatment according to the medical treatment hospital to follow up, counseling, and management with individual risk findings. Thirdly, the limitation was a dynamic test in carriers such as calcium stimulation for test calcitonin in MCT that need close monitoring during the process of calcium gluconate loading. However, we had support from a local hospital. Patients' close family members controlled all safety protocols with physicians and co-ordination nurses of our team that need standard procedure with step by step of the test.

In the future, we would be working with other specialists such as urologists, otolaryngologists, radiologists, and endocrinologists to approach a multidisciplinary team to act on all processes from index case finding, candidate carriers finding, screening testing, pre-counseling, tumor operation, post-counseling until to family planning in carriers RET mutation with future plans for their offspring of these families.

Conclusion

This study was the large sample sizes of Thai families that had MEN2A from RET634 mutation. We need to further approach these families according to guideline recommendations and put RET mutation in precision medicine service in Rajavithi Hospital with a multidisciplinary approach in rare disease in cancer service.

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