Original Article

RET gene mutations identified by exome sequencing in a multiple endocrine neoplasia type 2A

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Received January 23, 2017; Accepted April 6, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: Objective: To demonstrate a suitable example of genetic diagnosis of multiple endocrine neoplasia type 2A (MEN 2A) using rearranged during transfection (RET) gene mutations identified by exome sequencing. Methods: We sequenced the whole exome of a large Chinese MEN 2A pedigree to identify the variants of the RET proto-oncogene and followed this by validation. Results: All the ten familial members were found the existence of TGC → TAC heterozygous missense mutation in the 634 locus of exon 11 of the RET proto-oncogene, namely p.C634Y mutation, among who 7 patients were diagnosed as MEN 2A, and the other 3 members were diagnosed as the mutation carrier. The pedigree analysis showed that gene mutation carriers existed in the III, IV and V generation, which was consistent with the transmission rules of single gene dominant inheritance. Conclusions: The results confirmed the successful clinical utility of whole exome sequencing, and our data suggested that RET gene sequencing in MEN 2A pedigree can be helpful for the early diagnosis so as to improve the prognosis. As for the asymptomatic RET gene mutation carriers, individualized prophylactic thyroidectomy or close follow-up observation should be performed according to their calcitonin levels.

Keywords: Multiple endocrine neoplasia type 2A, exome sequencing, RET proto-oncogene

Introduction

Multiple endocrine neoplasia type 2 (MEN2), also known as the Sipple syndrome, is firstly discovered by Sipple in 1961 [1], which is one autosomal dominant inheritance disease with the incidence rate as about 1/30000 [2]. According to the clinicopathologic features, MEN2 can be divided into two subtypes: MEN 2A and MEN 2B. MEN 2A accounts for 95% of MEN2 cases and has four variants: MEN 2A is characterized by medullary thyroid carcinoma (MTC), pheochromocytoma (PHEO), and hyperparathyroidism. Additionally, a small number of patients exhibit skin lichen amyloidosis or Hirschsprung's disease [3, 4]. MTC is often the first manifestation of this subtype, with an incidence of close to 100% [5] progressed to MTC or exhibit lymph node metastasis. MTC is the predominant cause of death in patients with MEN 2A [6], and it has previously been reported that the recurrence rate was 50% in patients with MTC [7]. Since Donis-keller reported the first rearranged during transfection (RET) mutation in MEN 2A patients in 1993 [8], a total of more than 70 mutations have been reported (www.arup.utah.edu/database/MEN2/MEN2_welcome.php). RET proto-oncogene is the only virulence gene found to be associated with the onset of MEN 2A so far, and mainly concentrate on 5, 8, 10, 11, 13, 14, 15 and 16 exons [8]. Currently, detecting the RET germline mutation has been used as an important diagnosis of MEN2. In this study, we retrospectively analyzed the clinical diagnosis and treatment data of a Chinese MEN 2A family, and RET gene identified by exome sequencing. This study is reported below.

Materials and methods

Study subject

A total of 23 individuals, the four generations of a Han MEN 2A pedigree (excluding four dead MEN 2A cases), were collected from the Depart-
RET mutations screening in MEN 2A by exome sequencing

Department of Surgical Oncology, Taizhou Cancer Hospital, from 1976 to 2013, including 15 males and 8 females, aging 10 to 65 years. This study was approved by the hospital ethics committee, and all the patients signed the informed consent.

Detection of RET proto-oncogene

5 mL of peripheral anticoagulated blood was extracted from the 23 family members, respectively, for DNA extraction, PCR amplification, identification, purification, and direct sequencing of RET; the primer sequences and experimental steps referred to the references [9-11].

Histopathological and clinical pathological staging

Histopathological classification and TNM staging referred to the reference [12]. Immunohistochemical staining used the MaxVision™ method (Maxim, USA), and the procedures were carried out according to the kit instructions.

Imaging and biochemical assay

The imaging inspections included ultrasound and computed tomography (CT) toward the thyroid, adrenal gland, and parathyroid at least. The detection of serum calcitonin and carcino-embryonic antigen (CEA) used the chemiluminescence immunoassay.

Results

Family survey results and general clinical data

The proband (III-9) was a 29-year-old woman who had been diagnosed as having MTC in 1976 with a year history of a palpable neck mass in the left thyroid (Figure 1). Biochemical examination disclosed increased CEA (35.71 ng/mL; normal range, 5 ng/mL), while ultrasound scanning disclosed multi-centric hypoechoic nodules with calcifications in left thyroid lobes (maximum diameter: 3.5 cm). Then left subtotal thyroidectomy and modified left cervical lymph node dissection was performed. The postoperative histopathological examination showed multi-centric left MTC with lymph node metastases. In 2013, ultrasound revealed a small amount residue below the left thyroid leaf while no clear nodule under the right thyroid, as well as no lymph node in the neck or mass in bilateral adrenal glands; serum calcitonin 890.00 ng/L (normal range: 0-8.40 ng/L for male, and 0-5.00 ng/L for female); CEA 10.06 ng/mL. Because the patient refused prophylactic total thyroidectomy, so she was performed strict follow-up.

Young brother of the proband (III 10) had onset at the age of 40 years. He was admitted and performed ultrasound in 1993 due to “left neck mass for half a year” which revealed left thyroid masses with the maximum diameter as 3.7 cm. The postoperative pathology report after left subtotal thyroidectomy + left cervical lymph node dissection revealed that: left MTC (pT2N0M0). In September 1, 2011, he was re-admitted due to “physical examination revealed bilateral adrenal masses for 10 days”. CT revealed multiple space occupation in bilateral adrenal glands, with the maximum right and left diameters as 5.5 cm and 8.0 cm, respectively; ultrasound showed the left thyroid had been removed, but the right thyroid exhibited masses with the maximum diameter as 3.7 cm; no lymph node swelling was seen in the neck; Serum calcitonin 635.00 ng/L and CEA 10.06 ng/mL; CT revealed no lung, liver, or bone metastasis. On Sep 17th, 2011, this patient was performed bilateral pheochromocytoma resection, and the postoperative pathological report confirmed bilateral pheochromocytoma. The patient refused further thyroid surgery. In

Figure 1. MEN2A pedigree of RET proto-oncogene p.C634Y mutation.
February 2013, ultrasound showed the left thyroid had been removed while the right thyroid exhibited masses with the maximum diameter as 1.4 cm; no lymph node swelling was found in the neck, and no bilateral adrenal mass was found; serum calcitonin 714.00 ng/L, and CEA 15.89 ng/ml. The patient still refused thyroid surgery (cT1N0M0), so he was closely followed up.

The cousin of the proband (III 6), male, had onset at the age of 45 years. He was checked ultrasound in other hospital due to “paroxysmal hypertension for 2 years” in 2012, and revealed space-occupying masses in the right adrenal gland with the maximum diameter as 8.0 cm. After the right pheochromocytoma resection, the postoperative pathological report confirmed pheochromocytoma. On April 2nd, 2012, the patient was re-admitted for “thyroid masses found in physical examination for one week”. Ultrasound showed substantial space occupation in the bilateral thyroid with the maximum right and left diameters as 2.5 cm and 0.6 cm, respectively; the right supraclavicle and neck exhibited multiple lymph nodes, and the left adrenal mass exhibited the maximum diameter as 3.0 cm; serum calcitonin 1820.00 ng/L; CEA89.83 ng/mL; CT revealed no lung, liver, or bone metastasis. On April 19th, 2012, the patient was performed left pheochromocytoma resection, and the postoperative pathological report revealed left pheochromocytoma with cystic degeneration. On April 30th, 2012, the patient was performed total thyroidectomy + bilateral cervical lymph node dissection, and the postoperative pathological report revealed bilateral MTC with lymph node metastasis (pT2N1bM0). In February 2013, ultrasound showed bilateral thyroid resection, no lymph node in the neck, and no bilateral adrenal mass; serum calcitonin <2.00 ng/L; CEA2.37 ng/mL. The patient complained certain discomfort, and was continued the follow-up.

The maternal nephew of the proband (IV1), male, had onset at the age of 32 years. She was admitted in 1992 due to “left neck mass for six months”. Ultrasound showed bilateral thyroid masses with the maximum right and left diameters as 0.6 cm and 3.1 cm, respectively. The postoperative pathological report after left lateral thyroid lobectomy + left cervical lymph node dissection revealed left MTC (pT2N0M0). In 1998, she was re-admitted due to “right neck mass for two months”. Ultrasound showed right thyroid masses with the maximum diameter as 2.4 cm. After right subtotal thyroidectomy + left cervical lymph node dissection, the postoperative pathological report revealed right MTC (pT2N0M0). In February 2013, ultrasound showed the left thyroid had been removed while the right thyroid exhibited masses with the maximum diameter as 1.3 cm; no neck lymph node or bilateral adrenal mass; serum calcitonin 7.10 ng/L; CEA1.69 ng/mL. The patient complained certain discomfort, and was continued the follow-up.

The nephew of the proband (IV12), male, had onset at the age of 29 years. He was admitted in 2002 due to “right neck mass for 1 day”. Ultrasound showed bilateral thyroid lumps with the maximum right and left diameters as 2.1 cm and 0.3 cm, respectively. After right lobe thyroid lobectomy + right cervical lymph node dissection, the postoperative pathological report revealed right MTC (T2NOM0). In
February 2013, ultrasound showed the right thyroid had been removed as well as heterogeneous changes in the left thyroid; no neck lymph node or bilateral adrenal mass was found; serum calcitonin <2.00 ng/L; CEA 1.78 ng/mL. The patient complained certain discomfort, and was continued the follow-up.

The nephew of the proband (IV14), male, had onset at the age of 24 years. He was admitted in 2002 due to “right neck mass for three months”. Ultrasound showed bilateral thyroid lumps with the maximum right and left diameters as 2.2 cm and 3.0 cm, respectively. After total thyroid lobectomy + bilateral cervical lymph node dissection, the postoperative pathological report revealed right MTC (T2N0M0). In February 2013, ultrasound revealed the right thyroid had been removed, but the left thyroid had certain residual masses with the maximum diameter as 1.5 cm; serum calcitonin 6.23 ng/L; CEA 15.20 ng/mL. The patient refused residual thyroidectomy, so he was strictly followed up.

The son (V5) of the nephew (IV14) of the proband, male, had onset at the age of 8 years. This patient was inspected ultrasound in September 2011 due to “positive results of RET gene mutation detection”, which showed bilateral thyroid masses with the maximum right and left diameters as 0.2 cm and 0.2 cm, respectively; serum calcitonin 8.46 ng/L. In February 2013, ultrasound showed right thyroid masses with the maximum diameter as 0.2 cm; serum calcitonin 4.19 ng/L; CEA 1.21 ng/mL. Because this patient refused total thyroidectomy, so he was strictly followed up.

At the same time, the son (V1, 15 years) of the maternal nephew (IV1) and the son (V, 12 years) of the nephew (IV14) of the proband were found positive results of RET gene mutation detection, so in September 2011, they were performed ultrasound which didn’t show clear space-occupying lesion in bilateral thyroid; the serum calcitonin levels in V1 and V4 were 8.98 ng/L and 23.70 ng/L, respectively, indicating that they were RET gene mutation carriers. In February 2013, ultrasound showed no clear space-occupying lesion in bilateral thyroid; the serum calcitonin levels in V1 and V4 were 9.51 ng/L and 14.50 ng/L, respectively. These two patients had been followed up for 17 months, and they both refused preventive total thyroidectomy, so they were strictly followed up.

All the patients were performed thyroid hormone replacement and/or prophylactic supplementation of calcium and vitamin D3 after bilateral thyroidectomy [13]; the thyroid and parathyroid functions were normal.

Detection of RET proto-oncogene

A heterozygous nucleotide substitution within exon 11 of RET, c.1901G>A, was verified in the proband (III-9) and another 9 members. This variant substitutes cysteine with tyrosine at codon 634 (p.C634Y) which targets to one of five cysteine residues in the extracellular domain of RET (Figure 2), including 8 MEN 2A patients and 2 asymptomatic p.C634Y carriers (V1 and V4); the remaining 13 family members had neither RET gene mutation nor clinical manifestations and radiography of MTC, nor abnormal calcitonin level. In addition, two patients had been diagnosed as MTC by previous surgery and pathology, and because they had been dead, their RET gene cannot be tested. The detection results of RET gene mutation were fully consistent with the clinical and pathological diagnosis.

Discussion

MEN 2A is a kind of autosomal dominant inherited single gene disease, and its penetrance
rate almost reaches 100%; it mainly appears as MTC, PHEO, and HPT. 98% MEN 2A is caused by the mutations of RET proto-oncogene, and no mutation other than RET has ever been identified so far [14, 15]. 93% to 98% of MEN 2A is caused by the single-base substitution mutation of codon 609, 611, 618, and 620 in exon 10 or codon 634 in exon 11 of RET proto-oncogene, among which 87% was caused by the mutation of codon 634 with the most common type as C634R (TGC → CGC), accounting for 52%, followed by C634Y (TGC → TAC), accounting for 25% [16]. Therefore, when screening MEN 2A pedigrees, these common codons can be firstly performed the RET gene detection. The patients in this pedigree had MTC and/or adrenal PHEO while did not exhibit bone, muscle, or nerve mucosal abnormalities; the follow-up have not revealed hyperparathyroidism so far, and RET gene detection showed p.C634Y, which was a common MEN 2A mutation type; and their clinical manifestations and genetic diagnosis all supported the diagnosis of MEN 2A.

MTC is the key toward the diagnosis of MEN 2A, and its main clinical manifestation is painless thyroid masses, which mostly exhibit such performance as bilateral, multi-center, possible serum calcitonin increasing or diarrhea, or soreness and weakness in limbs. Most patients have already progressed to MTC or accompanied by cervical lymph node metastasis on admission, so imaging studies, especially cervical and superior mediastinal ultrasound can help the diagnose; if the patient is suspected as local lymph node metastasis and/or calcitonin >400 ng/L, it’s necessary for further cervical, thoracic, and liver CT, magnetic resonance imaging (MRI), or emission computed tomography (ECT) should be continued; if no distant metastasis or only a slight distant metastasis exists, bilateral thyroidectomy + bilateral VI region or + at least metastatic side cervical lymph node dissection should be performed; if severe distant metastasis occurs, palliative cervical surgery or other palliative treatments can be performed so as to remit its impact on breathing or to relieve local pain. In this study, two MEN 2A patients (III 9 and IV 11) occurred postoperative thyroid tissue residual due to lacking the recognition of MTC induced insufficient resection range in early years, and IV 14 did not really achieve bilateral thyroidectomy; therefore, it can be speculated that possible minor thyroid tissue residual occurred postoperative compensatory increasing or the original thyroid tissue residual was large. The serum calcitonin level reviewed recently was significantly higher than 150 ng/L, so it can be considered as recurrence or metastasis [20]. Two cases (III 6 and IV 1) were performed normative total thyroidectomy + bi-cervical lymph node dissection and exhibited their short-term serum calcitonin levels within normal ranges. It can be concluded that if patients with MTC have thyroid tissue residual, it will be difficult to achieve postoperative complete remission and will lead to increased risks of reoperation. As for the MEN 2A patients with thyroid tissue residual, their serum calcitonin levels should be closely monitored, as well as cervical ultra-
sound and supplementary thyroidectomy as soon as possible, so as to prevent thyroid tissue residual caused recurrence and metastasis of MTC. If the serum calcitonin \( >150 \text{ ng/L} \), the benefits of second surgery will be significantly reduced.

Although the majority of MEN 2A patients are admitted due to discovering neck masses, the cousin (III 6) and the maternal nephew (IV 1) of the proband visited clinics firstly due to paroxysmal hypertension instead of neck mass, which may be caused by PHEO, so such symptoms as headache, dizziness, or palpitations need to cause patients’ attention. Although medullary thyroid lesions occur early, clinical symptoms are not obvious, so they might often be found later and mostly in physical inspections after visiting the doctors; it further indicates the importance of screening RET gene mutations and monitoring serum calcitonin in MEN2A family members in order to discover MTC early.

Surgical resection of tumor lesions is the preferred method in treating pheochromocytoma in MEN 2A patients, which especially must be removed before MTC surgery. Even pheochromocytoma is asymptomatic; it should also be treated first. If thyroid surgeries are performed first, it may lead to serious perioperative complications, or even threaten lives. Because more than 50% of pheochromocytoma is bilateral, bilateral adrenalectomy or adrenal gland-retaining adrenalectomy is usually used; however, the postoperative adrenal tumor recurrence rates of these two methods have no significant difference, but the latter can avoid hormone replacement and Addsw syndrome crisis [21, 22], so the latter is much more desirable. The patients combined with PHEO in this study were firstly performed adrenal pheochromocytoma surgery, and after effective preoperative antihypertension, full expansion, and avoiding squeezing the tumor as much as possible intraoperatively, the surgical risks were effectively controlled.

As for the RET gene mutation carriers while without relevant clinical manifestations, serum calcitonin detection, as well as simultaneous neck ultrasound, should be performed before prophylactic thyroidectomy. According to the guidelines of ATA in 2009 [20], if all the thyroid nodules are less than 5 mm in diameter, calcitonin \( <40 \text{ ng/L} \), and no cervical lymph node metastasis exists, and only total thyroidectomy can be performed. For example, the subject V5, although the 17-month follow-up showed no increase in his thyroid nodules, and his serum calcitonin had returned to the normal level, prophylactic total thyroidectomy should still be considered while no central lymph node dissection was necessary because when the serum calcitonin is \( <30 \text{ ng/L} \), the tumor is basically confined within the thyroid capsule while has no lymph node metastasis [20, 23, 24]. The follow-up of V1 and V4 revealed that their serum calcitonin levels were slightly higher than the normal, but their imaging results showed no clinical manifestation. According to the latest study by Elisei et al [24], V4 should be treated with prophylactic total thyroidectomy, but V1 should be considered carefully. We believe that pentagastrin stimulation test should be performed, and if calcitonin is \( >10 \text{ ng/L} \), prophylactic total thyroidectomy should be implemented immediately. At the same time, it also suggests that clinicians should implement individualized treatment programs to asymptomatic RET mutation carriers so as to avoid or reduce surgical complications.

RET gene detection has great significance toward the diagnosis and treatment of MEN 2A, and the patients with a number of endocrine glands involved and hormone levels increased, as well as the members with a positive family history of MEN 2A should be paid high attention and be suggested to screen mutant genes and analyze DNA sequences so as to improve the detection rate of MEN 2A. Gene mutation carriers should be performed prophylactic resection as early as possible, and MEN 2A patients should be assigned regular follow-up, hoping to detect the MTC recurrence as early as possible and to improve their quality of life.

Acknowledgements

This research project is supported by the Key discipline of Oncology of Wenling (No. 1401ky-42RET).

Disclosure of conflict of interest

None.

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