Review Article on Asian and Western Practice in Thyroid Pathology: Similarities and Differences

Updates in the advances of sporadic medullary thyroid carcinoma: from the molecules to the clinic

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Abstract: Medullary thyroid carcinoma (MTC) is a rare neuroendocrine malignancy that originates in parafollicular cells. It is well-known that a quarter of MTC are involved in hereditary multiple endocrine neoplasia type 2 syndromes, whereas most MTC are sporadic. Unlike the commonly encountered gastrointestinal or pulmonary neuroendocrine tumors, most sporadic MTCs have distinct genetic alterations featured by somatic changes of either Rearranged during Transfection (*RET*) or *RAS* point mutation. The increasing application of next-generation sequencing, whole-exome sequencing, and other molecular detection techniques enables us to understand MTC comprehensively concerning its detailed molecular changes and their clinical correlations. This article reviews the advances in genetic alterations and their prognostic impact in sporadic MTC among different populations and discusses the associated tumor immune microenvironments and the potential role of immunotherapy targeting PD-L1/PD-1 in treating MTC. Furthermore, the current multikinase inhibitor targeting therapy for sporadic MTC has been summarized here and its efficacy and drug toxicity are discussed. Updates in advance of the role of calcitonin/procalcitonin/calcitonin-related polypeptide alpha (CALCA) gene transcripts in diagnosing and handling MTC are also mentioned. The treatment of advanced MTC is still challenging and might require a combination of several modalities.

Keywords: Sporadic medullary thyroid carcinoma (MTC); genetic alteration; Rearranged during Transfection (RET); RAS; immune microenvironment; immunotherapy; targeting therapy; multikinase inhibitor

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Introduction

Medullary thyroid carcinoma (MTC) arises from the parafollicular cells (C cells), and accounts for less than 5% of thyroid malignancies (1,2). As a neuroendocrine tumor, MTC secretes calcitonin, which leads to the elevation of serum calcitonin. Therefore, serum calcitonin becomes a vital marker to monitor the occurrence or recurrence of MTC (3). Histologically, the frequent amyloid deposits and occasional psammoma bodies in the stroma differentiate MTC from other extrathyroid-originated neuroendocrine tumors. Unlike other neuroendocrine tumors, about a quarter of MTC appears as a predominant part of hereditary multiple endocrine neoplasia type 2 (MEN2) syndromes. With the involvement of other synchronous and metachronous lesions (including

pheochromocytoma, hyperparathyroidism, and mucosal neuroma), MEN2 is divided into three subtypes: MEN2A, MEN2B, and familial MTC (4). Most patients with MEN2A have germline mutations in the Rearranged during Transfection (*RET*) C634, whereas patients with MEN2B and FMTC have germline mutations in *RET* M918T (5,6). Somatic *RET* mutation is identified in a considerable proportion of sporadic MTCs, and *RET* M918T is the most frequent genetic change (7-19). *RAS* point mutations are usually mutually exclusive with *RET* mutations, and occur in 0–81% of *RET*-negative MTCs (12,15,17,20-25). Several independent studies have shown that *RET* and *RAS* mutations have prognostic significance (7,11,17,25-29). Hence, the prevalence of *RET* and *RAS* mutations in different populations might partly explain the difference in prognosis among different populations with sporadic MTC. Many genetic changes are found in the *RET* and *RAS*-negative cases, although their molecular mechanism and clinic significance are yet to be determined.

Immune checkpoint inhibitors targeting PD-L1/PD-1 are regarded as one of the most significant medical breakthroughs in the 21st century (30-32). Previous studies have revealed poor tumor-infiltrating lymphocytes, low expression of PD-L1 and low mutational burden in MTCs (24,33,34), which disfavor the application of immunotherapy. However, several reports from China have proved that PD-L1 expression is related to advanced disease. The positive correlation between PD-L1 expression and advanced disease may bring the dawn of immunotherapy in advanced MTC, and relevant clinical trials are expected (35,36). The American FDA has approved two multikinase inhibitor (MKI) drugs, vandetanib and cabozantinib, in the treatment of advanced MTC. A more specific RET-targeting tyrosine kinase inhibitor (TKI) is currently underdeveloped. The combination of TKI therapy and immunotherapy might represent a novel therapeutic option in the treatment of advanced MTC (1).

This article focuses on the advances in genetic alterations and their prognostic impact in sporadic MTC among different populations and discusses the potential role of immunotherapy targeting PD-L1/PD-1. Furthermore, the current multikinase inhibitor target therapy for sporadic MTC is summarized. Updates in advance of the role of calcitonin/procalcitonin/calcitonin-related polypeptide alpha (CALCA) gene transcripts in diagnosing and handling MTC are also mentioned.

RET mutations

The *RET* gene was firstly described in 1985, as a transforming gene encoding a receptor tyrosine kinase (37,38). *RET* is localized on 10q11.2, and consists of 21 exons (38,39). RET is a single-pass transmembrane protein, which contains the following functional domains: an extracellular domain with four repeated cadherin-like regions, a cysteine-rich region, a transmembrane domain, a broad intracellular tyrosine kinase domain, and a carboxy-terminal domain (allowing for three isoforms, RET 9, 43, and 51) (*Figure 1*) (40-42). The intracellular domain encompasses two tyrosine kinase subdomains (TKS1 and TKS2) that are involved in the activation of many intracellular signal transduction pathways (40-42).

As a receptor tyrosine kinase, RET plays a crucial role in cell signal transduction, and the germline mutation of *RET* leads to the destruction of cell proliferation and differentiation in tissues derived from neural crest cells, including parafollicular thyroid cells, parathyroid gland, adrenal medulla, and intestinal autonomic nerve plexus (5,40,43). The molecular mechanism of somatic *RET* mutation is supposed to be like germline mutation (44). RET protein performs its pivotal role in regulating cellular transformation, survival, differentiation, proliferation, and migration. The transformation is done through the binding of its ligand GFRa complex, triggering its homodimerization, phosphorylation of tyrosine residues, and initiation of several intracellular signaling cascades, including MAPK and PI3K pathways (*Figure 1*) (2,41,42,45,46).

Sporadic MTCs have various somatic gene point mutations and deletions. The prevalence of somatic *RET* mutations in sporadic MTCs varies from 19.4–88.9% among different populations (4,7-19,27). The lowest mutation rate of somatic *RET* is from a Chinese population, while the highest from the US population. There is a general agreement that *RET* T918M, a point mutation in the methionine residue in exon 16 corresponding to the intracellular tyrosine kinase receptor domain, is the most frequent somatic *RET* mutation, the germline mutation of which was originally known as the activating mutation in MEN 2B. Other mutations in the *RET* gene in sporadic MTC have been recognized at codons 609, 611, 618, 620, 630, 631, 632, 634, 636, 639, 641, 748, 766, 768, 876, 883, 884, 901, 908, 919, 922, and 930 at exons 10, 11, 13, 14, 15 and 16 (4,8,15,40,47-50).

There are many pieces of evidence that patients with

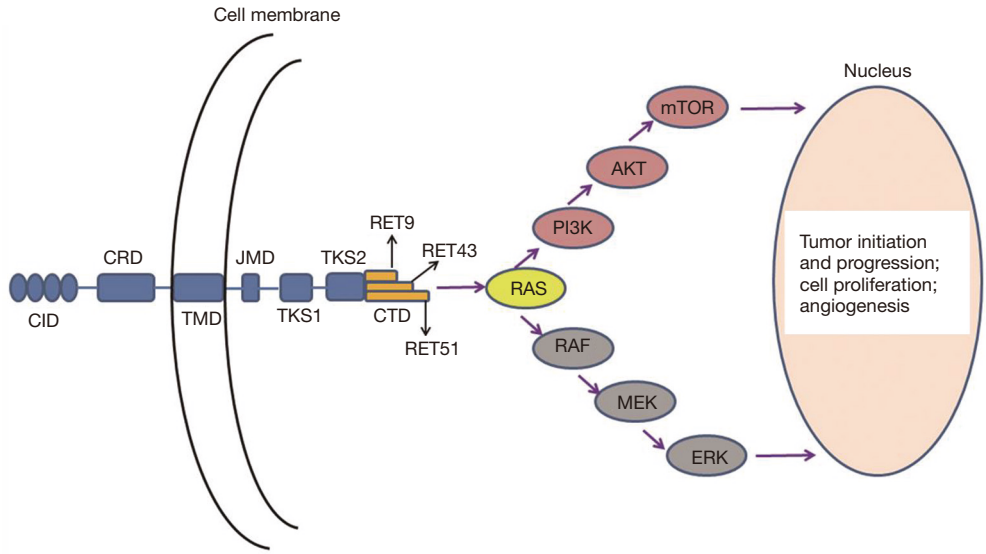

Figure 1 The structural scheme of RET protein and the principle activates downstream signal pathways. CID, cadherin-like domain; CRD, cysteine-rich domain; TMD, transmembrane domain; JMD, juxta-membrane domain; TKS, tyrosine kinase subdomain; CTD, carboxy-terminal domain.

sporadic MTC harboring *RET* mutations have a poorer prognosis than those harboring non-*RET* mutations (7,11,17,25-28,51). A statistically significant correlation was reached between *RET* mutations and the advanced tumor stage, higher T category, and lymph nodes or distant metastases and a worse patient outcome (17). In a recent meta-analysis involving 23 studies with 964 patients with MTC, *RET* mutation was determined to be associated with an elevated risk for lymph node metastasis, distant metastasis, advanced tumor stage, tumor recurrence, and patient mortality (28). Furthermore, tumors with somatic codon 918 mutations appear more aggressive than tumors with other *RET* mutations (17). Interestingly, *RET* mutations in sporadic MTCs may not necessarily lead to tumorigenesis but are essential for disease progression. This behavior is supported by the fact that the incidence of *RET* mutations in sporadic small MTC (smaller than 1 cm: microMTC) is lower than in larger tumors, and that *RET* mutations often exhibit mutational heterogeneity even in the advanced disease when present (38,52). Romei *et al.* hypothesized that either other oncogenes are responsible for most microMTCs, thereby identifying a tumor subpopulation or that *RET* mutation might or might not occur later during tumor progression (52).

Other somatic changes of *RET*, including copy number alteration and retrocopy, codon deletions, and rearrangements, were likely to be involved in the pathogenesis and progression of MTCs (17,47,53-55). Bim *et al.* identified copy number alteration and retrocopy in the *RET* gene; determined a recurrent novel point mutation (G548V) exclusively in the somatic retrocopy of *RET* in both sporadic and hereditary MTCs and MTC cell lines, and identified retrocopies produced in somatic cells might play a role in the pathogenesis and progression of MTCs (53). A rare *RET* fusion, *MYH13* exon 35 with *RET* exon 12, was reported in a patient who died of aggressive sporadic MTC with the survival of fewer than ten months after diagnosis, which might be a novel driver genetic change, and a *RET* fusion also provides a possible target for the treatment of RET TKI (54).

RAS mutations

The RAS (P21) protein is located on the inside of the cell membrane and plays a vital role in the transduction of cell growth and differentiation signals (56). It belongs to the guanosine triphosphate (GTP) binding protein (a coupling factor of cell information transmission), which regulates information transduction through the transformation between GTP and guanosine diphosphate (GDP) (56,57). There are three members of the *RAS* gene family: *HRAS*, *KRAS*, and *NRAS* (58). The various *RAS* genes are found

on different chromosomes but have similar structures, all composed of four exons. RAS takes part in both MAPK and PI3K signal pathways. *RAS* point mutations in MTC occur in *HRAS* and *KRAS* within hot spots in exons 2, 3, and 4, and rarely involve *NRAS*. They are usually mutually exclusive with *RET* mutations and are present in approximately 0–81% of *RET* wild-type sporadic MTCs (15,17,20-25), suggesting that *RET* and *RAS* somatic mutations are likely mutually exclusive in MTC tumorigenesis and development. Patients harboring *RAS* mutations (*RET* negative) showed a better prognosis than those harboring *RET* mutations or presenting no mutations (17,29).

However, the outcome of patients with a somatic *RET* mutation was significantly worse than both *RAS* positive/*RET* negative and *RAS* negative/*RET* negative cases (13,17). *RAS* mutations were reported to be significantly associated with higher intensity of p-S6 expression, suggesting that the mTOR pathway was activated in such MTCs (25). These findings suggest *RAS* mutations could play a role in tumor development, which leads to a less aggressive tumor phenotype, whereas *RET* mutations could be responsible for a more aggressive phenotype associated with a worse prognosis (22,27).

Other genetic alterations

Other somatic mutations were found in a subset of patients with MTC, especially patients without *RET* and *RAS* mutations. Ciampi *et al.* identified somatic mutations in the genes of *MET*, *TP53*, *TSH* receptor, *EIF1AX*, *CHK2*, *PPM1D* (29). In a study of MTC in Taiwan's population, they identified ten novels MTC somatic mutations: *BICD2*, *DLG1*, *FSD2*, *IL17RD*, *KLHL25*, *PAPPA2*, *PRDM2*, *PSEN1*, *SCRN1*, and *TTC1*, using whole-exome sequencing (33). They further analyzed 1,152 MTCs from COSMIC data and found that most of the variants were involved in pathways of PI3k-Akt, ErbB, MAPK, mTOR, and VEGF signaling pathways, and some were included in the pathogenesis of thyroid cancer, central carbon metabolism, and microRNAs in cancer (33). *FAT1* and *FAT4*, two members of the *FAT* gene family, both located at chromosome 4q, were identified as the two most commonly mutated genes in addition to *RET* in a small cohort of 18 cases of sporadic MTCs from China by Qu *et al.* They further showed that *FAT1* and *FAT4* knockdowns could promote MTC cell proliferation, using TT and MZ-CRC-1 cell lines (15). It is a novel finding that, on the gene expression profile, MTCs could be clustered into two molecular subtypes: the mesenchymal-like subtype, characterized by the epithelial-mesenchymal transition, and the proliferative-like subtype, associated with enrichment of cell cycle pathways. Most events of structural recurrence occur in the latter (15).

Copy-number losses in chromosome 4q and 1p cause frequent somatic changes for patients with sporadic MTC (15). A study in MD Anderson Cancer Center reported that 19% of patients with sporadic MTC had an aberrant loss of the cyclin-dependent-kinase inhibitors 2C (*CDKN2C*), found in the 1p32 chromosomal region. The aberrant *CDKN2C* loss was associated with distant metastasis at presentation and an unfavorable overall survival (OS). These findings were explained by showing that aberrant loss of *CDKNs* could lead to unrestrained phosphorylation of retinoblastoma protein and unregulated progression through the S phase of the cell cycle, thus resulting in the development of cancers. They also demonstrated the clinical impact of aberrant *CDKN2C* loss was enhanced by concomitant *RET* M918 mutation (59). Anaplastic lymphoma kinase (*ALK*) translocation, a frequent genetic alteration in anaplastic large cell lymphoma and lung adenocarcinoma, was identified in two cases by Ji *et al.* from screening 98 cases of MTC, with the partner genes of *EML4* and *GFPT1*, respectively (60). This finding may show that a rare subset of patients with MTC with *ALK* fusion might receive help from ALK target therapy. No *NTRK* translocation has been reported in MTC, although *NTRK* mutation has been reported in MTC (61).

Tumor microenvironment and immunotherapy

In the past decade, people have achieved gratifying results through using immunotherapy in treating many solid cancers, which was an essential milestone in the history of medical development (62). The successful findings in tumor immunotherapy are due to the discovery of immune checkpoint molecules, which are especially important in the regulation of tumor microenvironments. The tumor microenvironment is the internal environment in which tumor cells are produced and live. It consists of not only tumor cells but also fibroblasts, immune and inflammatory cells, and other cells in close contact with tumor cells, as well as adjacent cell stroma, microvessels, and infiltrated biological molecules (42,63-65). Malignant cells actively take part in the reconstruction of a pre-existing matrix, creating a new microenvironment that can be characterized

by inflammation or desmoplastic characteristics. Tumor microenvironment and cancer cells interact and influence each other. They are thereby promoting tumor progression and metastasis (42,63-65). It has been proposed to classify tumor microenvironments into four groups on their status of PD-L1 and tumor-infiltrating lymphocytes to streamline immunotherapy (31).

MTCs often manifest as few tumor-infiltrating immune cells with low expression of PD-L1 both in the tumor and in the stroma cells (34,35), and a low mutational rate (24). However, there is also evidence showing a positive immune reactivity of MTC. In a large cohort of 201 consecutive Chinese patients with MTC, Shi *et al.* demonstrated PD-L1 positivity was associated with aggressive clinicopathologic features (a larger tumor size, lymph node metastases, and advanced TNM staging) and is independently predictive of structural recurrence, and biochemical recurrence/persistent disease (36). Furthermore, they revealed a higher rate of PD-L1 expression in patients with incurable recurrence (36). Bi *et al.* have reported similar findings (35). They detected the expression of PD-L1 and PD-1 in 87 cases of MTC and found PD-L1 positivity was significantly correlated with distant metastases at the surgery. That co-expression of PD-1 and PD-L1 in MTC was correlated with advanced pathologic TNM stage III/IV and distant metastases at the surgery. The positive correlation between PD-1/PD-L1 and prognosis suggests that immunotherapy targeting PD-L1/PD-1 might be effective in treating advanced MTC. Several ongoing trials of immunotherapy in treating advanced MTC are under study, but no definite result has been reported. Although the FDA has approved several anti-angiogenic multikinase inhibitors (aaMKIs) in treating advanced MTC (discussed below), novel methods are still required to treat patients who fail to respond to aaMKI. Immunotherapy might be a choice in the future (66).

Targeting therapy in MTC

By far, surgery is still to be the only therapeutic method in treating most MTCs. Although radioactive iodine and TSH-suppressive therapies can treat differentiated thyroid carcinoma, they have no role in the management of MTC since the neuroendocrine-derived tumor cells do not concentrate iodine or respond to TSH in the same manner that follicular cells do. With the development of targeted therapy in treating advanced or unresectable MTC two aaMKIs, vandetanib, and the American FDA approved cabozantinib for the treatment of advanced MTC in 2011 and 2012, respectively. In the phase III trials of both drugs, patients with metastatic MTC had a significantly longer median progression-free survival (PFS) in the treatment group than in the placebo group (67-69). Both of the drugs did not show their efficacy in improving the OS of patients (67-70). Nevertheless, both studies met their pre-specified primary endpoints for improving PFS and were thus approved by the FDA as the first effective drugs for the treatment of advanced MTC.

Further, evidence showed that cabozantinib provided the most significant clinical benefit to patients with *RET* M918T or *RAS* mutation (71). The most common adverse drug reactions for vandetanib and cabozantinib were diarrhea, rashes, nausea, hypertension, and headaches (67,69,72,73). It is worth noting QT prolongation represented a rare but critical adverse event in the therapy of vandetanib, which potentially evolved into torsade de pointes and sudden death (67,73). Hence, the drug can only be prescribed by qualified physicians and should be used under close surveillance. Despite the advances in the management of metastatic MTC, aaMKIs show significant off-target toxicity and limited efficacy (67,69). LOXO-292 and BLU-667, a new generation of small-molecule TKIs, are highly selective RET inhibitors, and both of them showed better therapeutic efficacy than approved MKIs in the preliminary studies (74-77). At present, clinical trials of both are currently under evaluation (76).

The role of calcitonin/procalcitonin/CALCA gene transcripts in diagnosing and handling MTC

Calcitonin is a hormone that regulates the level of serum calcium, secreted by the C cells of the thyroid and metabolized by the kidneys (78). Calcitonin is regarded as a sensitive marker for diagnosing MTC, and is used for postoperative monitoring of MTC patients (3,79-81). However, the role of routine calcitonin screening in patients with thyroid nodules is still questionable, since the incidence of MTC is low and other non-neoplastic and neoplastic conditions may cause the increase of serum calcitonin (79,82,83). Calcitonin measurement in aspiration needle washout of thyroid nodules and neck lesions is reported to have a higher sensitivity than traditional cytology in diagnosing MTC. It may represent an ancillary tool utilized in patients with high serum calcitonin to avoid false-negative MTC by cytology (83,84). A large-scale retrospective analysis shows that postoperative biochemical remission of serum calcitonin is significantly correlated with

a decrease of the 5-year recurrence rate, but not with the improved 5-year survival rate (81).

Procalcitonin is the precursor of calcitonin. It is widely used as an indicator of severe infection, sepsis, and multiple organ failure (85-87). In recent years, a growing body of research has shown that procalcitonin presents an equivalent or even superior alternative of calcitonin in the management of MTC (88-92). Camacho *et al.* show that the expression of CALCA mRNA from the peripheral blood presents higher sensitivity, specificity, together with higher positive predictive value, and negative predictive value than did serum calcitonin (93). Thus, the detection of CALCA mRNA expression could serve as an alternative to the calcitonin-stimulation test since the former is more convenient to perform (93).

Conclusions

Advanced MTC is still one of the most challenging malignancies for clinicians. With the increasing development of molecular detection techniques, many genetic alterations have been discovered, while *RET* and *RAS* point mutations are still the most frequent and most significant changes. There is growing evidence that genetic changes might be used to predict the clinical outcome of patients with sporadic MTC, on different *RET* point mutations and *RAS* mutations. The in-depth understanding of molecular mechanisms and immune microenvironments of sporadic MTC has prompted the development of targeted therapy and immunotherapy. It is promising that the combination of TKI-targeted therapy and immune checkpoint inhibitors might be a novel therapeutic approach for treating patients with advanced MTC according to the individual tumor mutation profile and tumor microenvironment.

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