THEMATIC REVIEW

HEREDITARY ENDOCRINE TUMOURS: CURRENT STATE-OF-THE-ART
AND RESEARCH OPPORTUNITIES**The state of science in medullary thyroid
carcinoma: current challenges and unmet needs**

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This paper is part of a thematic section on current knowledge and future research opportunities in hereditary endocrine tumours, as discussed at MEN2019: 16th International Workshop on Multiple Endocrine Neoplasia, 27–29 March 2019, Houston, TX, USA. This meeting was sponsored by *Endocrine-Related Cancer*

Abstract

The 16th International Multiple Endocrine Neoplasia Workshop (MEN2019) held in Houston, TX, USA, focused on emerging topics in the pathogenesis and therapy of malignant endocrine tumors associated with MEN syndromes. With MEN-2 syndromes, the most common malignancy is medullary thyroid carcinoma (MTC). In the spirit of the original MEN meeting workshop model, the conference included didactic lectures and interactive working groups of clinicians and researchers focused on the state of science in MTC and ongoing challenges or unmet needs in the understanding of MTC and to develop strategies to address these issues.

Key Words:

- ▶ multiple endocrine neoplasias
- ▶ medullary thyroid cancer
- ▶ treatment
- ▶ pathogenesis

Endocrine-Related Cancer
(2020) **27**, T27–T39

Introduction

Medullary thyroid cancer (MTC) is a rare malignancy of thyroid C cells derived from neural crest cells. In approximately 25% of cases, the etiology is an activating germline mutation of the *RET* proto-oncogene via autosomal dominant inheritance associated with the multiple endocrine neoplasia type 2 (MEN2) syndrome. Thyroidectomy offers the greatest chance for cure if the disease is detected early in its development or can effectively prevent the onset of disease in patients identified with MEN2 through genetic screening.

However, many patients may already have evidence of cervical lymph node metastases or distant metastases at diagnosis. The disease course can be highly variable, although it is usually indolent and can remain so even in the setting of metastatic disease. Although factors such as specific *RET* codon mutation in hereditary MTC, somatic mutations, and calcitonin and carcinoembryonic antigen (CEA) doubling times have prognostic value, the heterogeneity of clinical behavior of MTC is not fully explained by these factors alone (Elisei *et al.* 2008,

Meijer *et al.* 2010, Wells *et al.* 2015, Cote *et al.* 2017). A treatment with curative intent for patients with metastatic MTC still remains elusive. However, with increased understanding of pathogenic mechanisms over the last 15 years and development of novel agents targeting drivers of disease, the goal of identifying a cure is possible. During the 16th International Multiple Endocrine Neoplasia Workshop (MEN2019), held in Houston, TX, USA, the objectives of the MTC Working Group were to explore the challenges or unmet needs in the understanding of this malignancy and to develop strategies to address those needs.

State of science in medullary thyroid carcinoma

Available therapies for advanced, metastatic medullary thyroid carcinoma

Aberrant activation of the RET receptor (in almost 100% cases of hereditary MTC and 45% of sporadic MTC) and overexpression of other receptor tyrosine kinases upregulate intracellular signaling pathways mediating tumorigenesis and angiogenesis (Fig. 1) (<http://cancer.sanger.ac.uk/cosmic>). This knowledge drove numerous clinical investigations with multi-kinase inhibitors (MKIs) and led to the approval of two systemic treatments (cabozantinib, vandetanib) for patients with advanced and progressive MTC not amenable to surgical or other locally targeted modalities. The ZETA phase III

study evaluated the efficacy of vandetanib in patients with advanced MTC, measurable disease by response evaluation criteria in solid tumors (RECIST), and a calcitonin (Ctn) of at least 500 pg/mL (Wells *et al.* 2012). Vandetanib led to a significantly prolonged progression-free survival of 30.5 months (predicted) compared with placebo (19.3 months) (HR 0.46; 95% CI, 0.31–0.69, $P < 0.001$). The overall response rate observed in the trial was 45% in the vandetanib group compared with 13% in the placebo group. Although it was not designed to evaluate overall survival, there was no difference in overall survival between the treatment groups. The phase III EXAM trial with cabozantinib randomized MTC patients to study drug or placebo, who had progressive disease prior to study enrollment (Elisei *et al.* 2013). The median progression-free survival was significantly longer in the cabozantinib-treated patients (11.2 months) compared with the placebo group (4.0 months) (HR 0.28; 95% CI 0.19–0.40, $P < 0.001$), with a partial response of 28% for cabozantinib and 0% in placebo-treated patients. Responses were observed regardless of *RET* mutation status. Overall survival, a secondary endpoint in this study, was not different between the treatment groups (HR 0.85; 95% CI, 0.64–1.12; $P = 0.24$) (Schlumberger *et al.* 2017). MKIs are associated with numerous side effects including palmar-plantar erythrodysesthesia, photosensitivity, stomatitis, taste changes, hypertension, diarrhea, nausea, anorexia, fatigue and uncontrolled hypothyroidism. QTc prolongation was observed in 14% patients treated with vandetanib. Cabozantinib is a more potent inhibitor of

Figure 1

Pathogenic pathways in medullary thyroid carcinoma. Pathogenic drivers of disease include: mutations leading to activated RET or RAS receptors; overexpression of EGFR, MET and VEGFR; genetic loss of CDKNs; and loss of ATF4 protein. ATF4, activating transcription factor 4; BRAF, serine/threonine-protein kinase B-Raf; CDK, cyclin-dependent kinase; CDKN, cyclin-dependent kinase inhibitor; E2F, family of DNA-binding transcription factors; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase kinase; MET, hepatocyte growth factor; RAS, rat sarcoma viral oncogene homolog; Rb, retinoblastoma; RET, rearranged during transfection; VEGFR, vascular endothelial growth factor receptor.

VEGFR-2, which leads to a higher risk for hemorrhage, venous thrombosis, intestinal perforation, and fistula formation. Thus, it is advised to implement a patient-focused evaluation when recommending a therapeutic agent that conveys oncologic benefit balanced by a lower burden of side effects (Cabanillas *et al.* 2014).

The development of novel, potent RET-specific inhibitors has led to a significant amount of excitement due to the responses observed in patients with thyroid cancer with *RET* alterations treated in phase I clinical trials and the overall well-tolerated side effect profiles. Since the MEN 2019 meeting, the updated results for BLU-667 and LOXO-292 were presented at the Annual Meetings of the American Society of Clinical Oncology (ASCO) Meeting in June 2019 and the 44th European Society for Medical Oncology (ESMO) Congress in September 2019, respectively (Hu *et al.* 2018, Wirth *et al.* 2018, 2019, Taylor *et al.* 2019). As of December 19, 2018, 60 patients with *RET*-mutated MTC received BLU-667 (pralsetinib). Among the 49 response-evaluable MTC patients, the objective response rate (ORR) was 47% (95% CI: 33, 62) with 2 complete responses (CR), 21 partial responses (PR), 25 stable disease (SD), and 1 progressive disease (PD) with a disease control rate (DCR) of 98% (Taylor *et al.* 2019). Ninety-six percent of the responding patients continue on treatment. Responses occurred regardless of prior MKI use or *RET* mutation (including patients with a *RET* V804M gatekeeper mutation), and the duration of response was ≥ 6 months in 15 patients. Treatment-related adverse events (AEs) were generally low-grade and reversible with no grade 4/5 AEs and 28% with grade 3 AEs including: leukopenia, increased ALT and AST/serum creatinine/phosphate, hypertension and neutropenia. As of June 17, 2019, 226 *RET*-mutated MTC received LOXO-292 (selpercatinib). For the first 55 consecutively enrolled MTC patients previously treated with cabozantinib and/or vandetanib, the ORR was 56% (95% CI: 42, 70) with 3 CRs, 28 PRs, 2 unconfirmed PRs, 19 SD, and 3 PD. Of the three patients with *RET* V804M/L gatekeeper mutations, one had a CR and two had PRs. Median duration of response had not been reached. Treatment-related AEs of $\geq 15\%$ were dry mouth, diarrhea, hypertension, and increased AST and ALT with most AEs being grade 1–2. 1.7% of all 531 patients in the safety data set discontinued therapy due to treatment-related AE (Wirth *et al.* 2019). Based on the favorable overall response rate and duration of response, selpercatinib received accelerated approval by the US Food and Drug Administration on May 8, 2020 for patients over 12 years of age with advanced or metastatic *RET*-mutated MTC who require systemic therapy.

Two additional RET inhibitors (TPX-0046 and BOS172738) are also being evaluated in ongoing phase I trials (Drilon *et al.* 2019, Schoffski *et al.* 2019). Notably, TPX-0046, a dual inhibitor of RET and SRC, also has activity against the solvent front mutation *RET* G810R, which is associated with acquired resistance to MKIs and other selective RET inhibitors under investigation (Drilon *et al.* 2019, Solomon *et al.* 2020).

Other than *RET* mutations seen with hereditary and sporadic MTC, approximately 14% of sporadic MTC will harbor a *RAS* mutation, most commonly *HRAS* or *KRAS*, which, at this time, is not a targetable mutation (Fig. 1) (<http://cancer.sanger.ac.uk/cosmic>). There have been investigations into other drivers of MTC described in this review.

Targeting CDKs and RB in medullary thyroid cancer

Over the past several decades, it has been well-defined that neuroendocrine cells, including C-cells of the thyroid, are highly sensitive to perturbations in cell cycle regulators in murine models (Williams *et al.* 1994, Harvey *et al.* 1995, Yamasaki *et al.* 1998). Cyclin-dependent kinases (CDKs) are a family of proteins that are required for cell cycle progression and/or gene transcription. CDKs that facilitate cell cycle progression are negatively regulated by a group of CDK inhibitors such as p18 and p27. Loss of expression of CDK inhibitors enhances CDK activity, cell proliferation, and eventually can lead to cancer development, thereby classifying them as tumor suppressors (Fig. 1). The first described tumor suppressor gene, *RB1*, encodes the retinoblastoma protein that is a downstream phosphorylation target of CDKs; an activity that results in RB inactivation allowing cell cycle progression. In addition, loss of RB expression through a variety of mechanisms also can lead to cancer formation. In parafollicular cells, mice with heterozygous loss of Rb or p18 develop medullary thyroid cancer (MTC) and other neuroendocrine tumors associated with loss of expression of the second allele (Williams *et al.* 1994, Harvey *et al.* 1995, Yamasaki *et al.* 1998, van Veelen *et al.* 2008). This striking incidence of these rare tumors in these generalized models suggest a tissue-specific sensitivity to carcinogenesis in this genetic context. In addition, CDK inhibitor loss (e.g. p18 and Rb) cooperates with mutant RET to enhance cancer aggressiveness (Park *et al.* 1999, van Veelen *et al.* 2008). Finally, activation of CDK5 independently is sufficient to cause MTC in mice and its inhibition reverses MTC development and progression (Pozo *et al.* 2013). While there are differences in the

biology of C-cells in mice and humans (Madsen *et al.* 2012), these data have generated interest in analyzing human MTC tumors and cell lines for activation of CDKs to determine if CDK activity is a biomarker for aggressive disease and represents a therapeutic target.

In cell lines, it has been shown that the mitogenic effects of mutated RET require downregulation of p27 and p18 (Joshi *et al.* 2007) and that inhibition of RET results in increased expression of p27 (Vitagliano *et al.* 2004). These data suggest that, while CDK inhibitors might not be synergistic with RET inhibitors, CDKs themselves may be biomarkers and/or therapeutic targets in MTC independent of RET. To determine the frequency of CDK inhibitor loss in human MTC, several groups screened for and identified mutations or loss of expression of p16 and p18 in MTC (van Veelen *et al.* 2009, Grubbs *et al.* 2016, El Naoufal *et al.* 2017). Similarly, a different set of investigators, using high resolution array genomic hybridization (Array CGH) from a second group of patients, determined that p18 loss is associated with more aggressive MTC tumor behavior (Flicker *et al.* 2012). In addition to loss of CDK inhibitors, overexpression of CDKs has been described in MTC, which results in phosphorylation and loss of nuclear RB and is associated with MTC progression, and its inhibition reduces MTC cell growth and has been shown to increase the growth of human MTC cells (Pozo *et al.* 2013). Because loss of CDK inhibitors and overexpression of CDKs result in loss of nuclear RB function (either by phosphorylation or loss of expression), RB phosphorylation or loss of nuclear RB has been evaluated as biomarkers for MTC (Anwar *et al.* 2000, Valenciaga *et al.* 2017). In the larger of these studies for MTC, loss of nuclear RB was associated with death from disease independent of activating RET mutations (Valenciaga *et al.* 2017). Together these data demonstrate that human MTCs are characterized by genetic alterations resulting in Rb inactivation and suggest that CDKs may represent therapeutic targets for this disease.

The work described has been extended into the preclinical space by examining the potential therapeutic use of CDK inhibitors in MTC cell lines. Several CDK inhibitors are FDA approved for other cancers including flavopiradol and dinaciclib (CDK 1, 2, 5, 9 inhibitors) and palbociclib, ribociclib, and abemaciclib (CDK4/6 inhibitors), while other CDKs have been targeted preclinically (CDK5, 7, and 9). Studies using flavopiradol and dinaciclib report low nM IC₅₀ values for RET-mutated human MTC cells (Valenciaga *et al.* 2018). The sensitivity to dinaciclib was present even in cell lines selected for resistance to the FDA-approved multi-kinase inhibitor,

vandetanib. In addition, dinaciclib was synergistic with vandetanib *in vitro*. Interestingly, inhibition of CDK4/6 is cytostatic rather than cytotoxic to MTC cells. Several groups have focused on more specific CDK targeting. As noted previously, in a genetic mouse model, MTC development and progression were dependent on CDK5 activation and CDK5 inhibitors demonstrate activity against human MTC cell lines (Pozo *et al.* 2013, Pozo & Bibb 2016). In addition to work on CDK5, it was observed that, at the IC₅₀ for Dinaciclib, only CDK9 was inhibited. CDKs 7 and 9 regulate gene transcription by RNA polymerase 2 rather than cell cycle. Treatment of RET-mutated MTC cells with the CDK7 inhibitor THZ1 demonstrated nM-range IC₅₀ in association with loss of RET gene expression. Subsequent studies confirmed the presence of a 'super enhancer' in intron 1 of RET that may be responsible for the high degree of sensitivity to RNA polymerase II inhibition (Valenciaga *et al.* 2018). Taken together, the data suggest that activation of CDKs and/or loss of their negative regulators are biomarkers for aggressive MTC and may be new non-RET therapeutic targets that are either downstream and/or independent of RET. Further studies and use of better tolerated and orally available agents may be important avenues for clinical trials in MTC.

Role of ATF4 in the pathogenesis of MTC

Cancer cells are often exposed to nutrient starvation, hypoxia, oxidative stress and other metabolic dysregulation. These various stress factors can interfere with correct protein folding leading to the accumulation of misfolded or unfolded protein, causing a condition called endoplasmic reticulum (ER) stress. In response to ER stress, cells activate a signal transduction pathway called unfolded protein response (UPR) to restore ER homeostasis. UPR restores homeostasis by reducing protein synthesis, which enhances degradation of misfolded protein and increases translation of activating transcription factor 4 (ATF4). Under overwhelming ER stress, the UPR promotes apoptosis, and ATF4 is a key deciding factor in cellular fate (Ameri & Harris 2008) (Maurel *et al.* 2015) (Wang *et al.* 2018). Therefore, induction of ER stress to a critical threshold could be a promising strategy to induce cancer cell death (Tabas & Ron 2011). Neuroendocrine cancers with secretory function like medullary thyroid cancer display enhanced ER stress, due to inappropriately high levels of protein synthesis, mutation load that leads to the accumulation of misfolded proteins and make them reliant on their UPR and ER associated degradation (ERAD) pathway (Clarke *et al.* 2014).

RET is recognized for its essential role in cell survival. There is a mechanistic link between RET activation and integrated stress response through regulation of ATF4 transcriptional activity that may explain, in part, the incomplete responses observed in patients treated with FDA-approved tyrosine kinase inhibitors (vandetanib and cabozantinib). In fact, RET activation promotes cancer cell survival by direct phosphorylation-dependent repression of ATF4 at the promoter of pro-apoptotic targets *NOXA* and *PUMA* thereby circumventing stress-induced apoptosis (Fig. 1) (Bagheri-Yarmand *et al.* 2015). Inducing ATF4 expression in MTC promotes RET degradation and inhibits RET downstream signaling pathways including ERK, AKT and mTOR pathway (Bagheri-Yarmand *et al.* 2017). It has been reported that ATF4 mediates ER-stress-induced apoptosis in response to drugs triggering ER stress, such as the proteasome inhibitor bortezomib, fenretinib (Armstrong *et al.* 2010), inhibitor of ATPase p97 (Anderson *et al.* 2015); HA15 (a thiazole benzenesulfonamide), which targets GRP78/bip (Cerezo *et al.* 2016), ERAD inhibitor (eeyarestatin) (Wang *et al.* 2010). The combination of an ERAD inhibitor, eeyarestatin, with tyrosine kinase inhibitors (sunitinib and vandetanib) synergistically triggers irresolvable oxidative stress by inducing ATF4 mRNA leading to apoptosis in response to ER stress (Bagheri-Yarmand *et al.* 2019). The oxidative stress induced by this combination therapy is, in part, due to increase in KLF9 expression which is an ATF4 target gene and reported to promote oxidative-stress-induced cell death (Zucker *et al.* 2014). Furthermore, the analysis of MTC tumor tissues revealed that low protein levels of ATF4 was associated with increased RET protein levels and poor overall survival compared to patients whose tumors had high levels of ATF4 (Bagheri-Yarmand *et al.* 2017).

Recently, a small molecule ONC201 is reported to trigger cytotoxicity by inducing a stress response that is mediated by the induction of stress-related genes (Ishizawa *et al.* 2016, Kline *et al.* 2016). The mechanism of stress response induced is different between hematological malignancies and solid tumors, but ATF4 induction appeared to be a common mediator of ONC201-induced apoptosis in hematologic malignancies and solid tumors. Therefore, ATF4 could be used as a pharmacodynamic marker of ONC201 efficacy. The mechanism by which ONC201 induces stress-related genes and inhibits AKT, ERK and mTOR signaling pathway need to be elucidated. In summary, cancer-specific induction of ER stress and oxidative stress to activate programmed cell death could be a promising new approach to cancer treatment.

Immune environment in medullary thyroid cancer

In a study including 16 MTC tumors, very low expression of programmed cell death protein 1 ligand (PD-L1) in malignant and immune cells was reported (Bongiovanni *et al.* 2017). PD-L1 expression on malignant cells was $\leq 1\%$ in 15/16 cases, with one case showing 5% positive cells. No correlation between PD-L1 expression and clinicopathological stage or survival was observed in this small study. A larger study including 87 MTC samples evaluated percentages of programmed cell death protein 1 (PD-1) and PD-L1 positive cells within the tumor. In this study, using a threshold of $>1\%$ positive cells, PD-1 expression was reported in 26% and PD-L1 expression in 22% of MTC tumors. Notably, most PD-L1 positive MTCs showed weak to moderate staining intensity (Bi *et al.* 2019).

Immunotherapy has been found to be an effective treatment modality in various solid tumors and hematologic malignancies. In MTC, various trials with vaccines and an anti-CTLA (cytotoxic T-lymphocyte-associated protein 4) inhibitor with radiation have been conducted.

Vaccines

From the late 1990s to the early 2000s, a targeted immunotherapy approach using tumor vaccines was attempted in patients with MTC. However, a major problem is the lack of defined tumor antigens. In MTC, CEA and calcitonin may represent feasible targets for the induction of cytotoxic immunity. Therefore, dendritic cell (DC) vaccination loaded with calcitonin and CEA has been tried in a prospective clinical trial (Schott *et al.* 2001). One trial included seven MTC patients (four patients with sporadic MTC and three patients with MEN2). In that trial, DCs were administered subcutaneously every week for 4 weeks and then at 4–8-week intervals. This treatment regimen was well-tolerated with no adverse effects, except fever in one patient. One of seven patients enrolled had regression of liver and lung metastases and two patients had mixed responses. A more refined DC therapy approach was subsequently attempted. Because no specific tumor antigens are yet discovered in MTC, tumor lysate was tried in another prospective study including ten sporadic MTC patients (Stift *et al.* 2003, 2004). This vaccine was well-tolerated with two patients developing fever up to 24–48 h after administration. Clinical benefit was demonstrated in six patients, which included one PR, one minor response, and two SD.

After autologous DC vaccination showed minimal efficacy as described previously, allogeneic tumor

lysate-based DC therapy was tried. A pilot trial of autologous DCs pulsed with tumor cell lysate derived from allogeneic MTC cell lines in ten patients with metastatic MTC was performed (Bachleitner-Hofmann *et al.* 2009). DC vaccinations were well-tolerated and safe. After a median follow-up of 11 months, three patients had SD, while seven patients had PD.

A yeast-based therapeutic cancer vaccine targeting the CEA protein is under investigation in an MTC cohort, as part of a phase 2 clinical trial (NCT01856920). GI-6207 (Recombinant *Saccharomyces cerevisiae*-CEA (610D)) is an 'off the shelf' experimental cancer vaccine made with baker's yeast. Upon administration, this vaccine may stimulate a host cytotoxic T-lymphocyte response against CEA-expressing tumor cells, which may result in tumor cell lysis. Preclinical studies have shown that GI-6207 can induce an immune response to CEA as well as therapeutic anti-tumor responses (Wansley *et al.* 2008). A previous phase I GI-6207 study has demonstrated safety and enhanced immune response in some patients (Bilusic *et al.* 2014). One patient with MTC was included in the phase 1 clinical trial, but was taken off study at 3.5 months for potential toxicity that was attributed to a possible manifestation of a strong immune response at the site of a metastatic focus. Final results from this study have not been released. Due to a limited number of patients in the MTC vaccine trials, conclusions regarding treatment efficacy of such approaches should be drawn with caution. Better strategies are needed.

In search for other tumor-associated antigens, a study noted that 65.2% of MTC tumors expressed NY-ESO-1 gene (Maio *et al.* 2003). This was higher than that seen in other malignancies (e.g. melanoma, hepatoma, breast, prostate, or ovarian cancer). Furthermore, antibodies to NY-ESO-1 were present in these patients, consistent with development of a spontaneous humoral immune response to this antigen. A more effective approach in MTC may be immunization with NY-ESO-1 peptides, which appear to be quite immunogenic.

Immunotherapies targeting immune checkpoint molecules such as CTLA-4, PD-1 and PD-L1 have been rapidly explored as promising treatment strategies in various types of cancers, including thyroid cancers. Very sparse data using these agents are available in MTC. In a phase 1 trial, one patient with MTC treated with an anti-PD-1 agent, nivolumab, has achieved a PR (Yamamoto *et al.* 2017). In a thyroid cancer expansion cohort, part of a phase I/II trial of ipilimumab (anti-CTLA-4 antibody) and hypofractionated stereotactic radiation therapy in patients with advanced solid malignancies (NCT02239900),

three MTC patients were included. Two heavily pretreated patients (one *KRAS* and one *RET* M918T mutated) had rapid progressive disease and one patient (*RET* M918T mutated) had SD for 9 months (Dadu *et al.* 2018). A clinical trial using pembrolizumab (anti PD-1 antibody) as a single agent is currently being investigated (NCT03072160). The patients are divided in two groups based on their treatment history with an immune stimulating cancer vaccine (recombinant *saccharomyces cerevisiae*-CEA).

Challenges and unmet needs in medullary thyroid carcinoma

Lack of normal human C-cell lines

Further progress in understanding MTC and its pathogenesis is hindered by several factors: (1) a relatively small number of *in vitro* reagents for preclinical studies, (2) the absence of non-*RET*-mutated MTC cell lines, (3) technical challenges with the existing reagents, (4) difficulty expanding MTC cells *ex vivo*, and (5) the absence of data on normal C-cell biology. The state-of-the-science and barriers toward progress in the field of MTC were discussed in detail at the 16th International Workshop on Multiple Endocrine Neoplasia (MEN2019).

The majority of preclinical data on MTC derives from two human MTC cell lines: TT (*RET* C634W mutated) and MZ-CRC1 (*RET* M918T) (Berger *et al.* 1984, Cooley *et al.* 1995). Data generated using these cell lines have been useful to identify anti-RET functions of currently approved or developing compounds, and these cells can be grown *in vitro* in a variety of conditions, albeit slowly, and *in vivo* as s.c. and orthotopic xenografts (Rossfeld *et al.* 2017). However, it is notable that a cell line with the second most common driver mutation in *HRAS* does not exist and there are no normal human C-cell lines for fundamental studies in the field (the interspersed nature of C-cells within the thyroid makes their isolation impractical). Finally, MTC researchers recognize that these cells, while useful, are technically challenging to grow and expand and are relatively resistant to transfection and infection making them difficult to utilize for more global molecular and chemical screens. Genetically engineered mouse models (GEMM) have been developed for MTC. For example, mice develop MTC when mutated *RET* is overexpressed in the C-cells and when cell cycle regulators are perturbed (Williams *et al.* 1994, Harvey *et al.* 1995, Pozo *et al.* 2013). However, mice with a single copy of mutated *RET* only develop C-cell hyperplasia

(Smith-Hicks *et al.* 2000), and GEMMs, while valuable, are costly to create and maintain and are not amenable for drug testing and/or development on a larger scale. Alternative models, such as ectopic expression of mutated *ret* in drosophila eye lens, have been utilized to identify RET inhibitors that have been verified in thyroid cells and eventually in clinical trials (Vidal *et al.* 2005, Wells *et al.* 2012, Levinson & Cagan 2016). Thus, while *in vivo* models of MTC can be highly successful and informative, they are time consuming, highly specialized, and as of now cannot be compared to normal C-cell biology. Thus, the development of new cellular models and data sets are required to enable expansion of knowledge regarding both a basic understanding of normal C-cell physiology and MTC biology and to allow for high-throughput preclinical testing in models that reflect MTC more broadly.

Over the past decade, the prior understanding, that C-cells develop from ectoderm precursors (Pearse & Carvalho 1967), has been challenged and likely disproven with the advent of modern stem cell and lineage-tracing technologies (Johansson *et al.* 2015, Nilsson & Williams 2016, Kameda 2019). This may not be a complete surprise to clinicians as ‘collision tumors’ in which both MTC and follicular cell derived thyroid cancer are found in the same tumor (Sadat Alavi & Azarpira 2011). Moreover, immunohistochemical studies demonstrated that MTCs express the transcription factor NKX2.1 (also known as thyroid transcription factor 1, TTF1), similar to endoderm-derived thyroid follicular cells and lung cells (Johansson *et al.* 2015, Nilsson & Williams 2016, Kameda 2019). Another study had identified thyrotropin receptor (TSH-R) mRNA expression in some human MTC specimens (Elisei *et al.* 1994). Elegant studies have generated functional thyroid organoids *in vitro* through an endoderm developmental program (Martin *et al.* 2000, Antonica *et al.* 2012, Saito *et al.* 2018). Subsequently, lineage tracer studies have more fully characterized thyroid development and the roles and timing of the three transcription factors that create thyroid follicular epithelium (NKX2.1, FOXE1, PAX8). The thyroid derives from primordial endoderm cells located in the pharyngeal pouch that are separated by ectodermal cells into the thymus, parathyroid, and ultimobranchial body, the latter of which is destined to become the thyroid gland through the aforementioned three transcription factors (Mansouri *et al.* 1998, Kusakabe *et al.* 2006, Ozaki *et al.* 2011). It has previously been thought that the C-cells derived from the ectoderm that separates this body (Pearse & Carvalho 1967). With a better understanding of stem-cell biology, this population has been shown to be derived from an

endoderm-derived SOX-17 expressing precursor that expresses NKX2.1 and that NKX2.1 drives calcitonin expression (Johansson *et al.* 2015, Nilsson & Williams 2016, Kameda 2019). In fact, mice with loss of PAX8 do not develop a thyroid follicular epithelium but have two ultimobranchial bodies that are comprised of Nkx2.1 positive cells that express calcitonin (Mansouri *et al.* 1998). These data confirm that C-cells are an endoderm-derived cell type. Although not yet performed for human C-cells, stem-cell derived endoderm has served as the basis to develop neuroendocrine cell lineages and neuroendocrine tumors of the pancreas and lung. The importance of this information is that endoderm stem-lineages are malleable for development into tissues and tumors from derived stem cells, particularly when performed using 3D cellular matrices and/or organoid or ‘organ on chip’ technologies (Huh *et al.* 2010, Xu *et al.* 2016, Hassell *et al.* 2017).

In addition to using stem-cell technologies and biomimetic tissue matrices to develop normal C-cells, similar approaches have potential to more successfully propagate human MTCs *ex vivo* and/or develop them experimentally from the C-cell models. Finally, even without cell propagation, methods to more robustly analyze normal C-cell RNA signatures for comparison studies should be developed using single cell technologies to create baseline expression profiles. It was agreed at the MEN 2019 meeting that the development and testing of these systems should be a priority of basic researchers to enable an increased pace of translation toward clinical practice.

Lack of knowledge of drivers of MTC beyond *RET* and *RAS*

While *RET* and *RAS* alterations have been identified as oncogenic pathways for MTC, mutations of these genes account for only two-thirds of the disease. Attempts to identify additional oncogenic drivers have been limited and uninformative. To date, only two studies report relatively large volume exomic sequencing with 25 and 18 MTC tumor samples evaluated, respectively, with no identifiable novel recurrent driver mutation (Agrawal *et al.* 2013, Qu *et al.* 2020). Another possible reason is that MTC oncogenesis is also caused by gene copy number change, a poorly studied pathway compared with gene mutations. Copy number change has been reported in several high-density comparative genomic hybridization (CGH) array studies of MTC, with deletion of chromosome 1p, 19p and 22 being commonly reported (Marsh *et al.* 2003, Ye *et al.* 2008, Flicker *et al.* 2012).

Loss of *CDKN2C* (cyclin-dependent kinase inhibitor 2C), located on chromosome 1p, has been associated with poor prognosis in MTC patients and its role is further substantiated by a mouse knockout model with concordant findings (van Veelen *et al.* 2008, Grubbs *et al.* 2016, Maxwell *et al.* 2020). More broadly, the *CDKN*/retinoblastoma (RB) tumor suppressor pathway has been proposed as an alternative pathway in MTC (Valenciaga *et al.* 2017). Homozygous deletion of *Rb1* leads to the development of aggressive MTC in mice, and decreased tumor RB expression is linked with worse prognosis in patients with MTC (Song *et al.* 2017, Valenciaga *et al.* 2017). Further investigation is needed to elucidate the role of the RB pathway in MTC oncogenesis. This is a worthwhile endeavor given that targeted therapies that inhibit CDK are being investigated across many cancer types.

A recent study, utilizing whole transcriptome sequencing in 29 MTC specimens, found that the tumors had a mesenchymal-like or a proliferative-like molecular subtype (Qu *et al.* 2020). A more aggressive phenotype was associated with the proliferative-like cluster, though the authors noted that further study with larger specimen numbers was warranted.

The taskforce discussed the importance of identifying additional pathogenic drivers in MTC and proposed an approach similar to the TCGA effort in order to achieve this goal. Given the rarity of this malignancy, collaboration with multiple centers worldwide will be essential. Identification of focused efforts to perform RNAseq of fresh frozen tumor specimens from primary, metastatic, and recurrent-progressive lesions from *RET*+, *RAS*+ and *RET/RAS* WT tumors was highlighted as a reasonable next step. Additionally, given the genotype-phenotype variability of somatic MTC, including among individuals who share common mutations, further correlation of tumor behavior and pathogenic drivers is necessary. Likewise, this effort is best approached in a collaborative manner with multiple centers contributing to allow adequate patient numbers.

The unknown role of immuno-oncology in MTC

Although tumor immunology has changed the landscape of treatment for cancer, not all patients (including those with MTC) benefit from immunotherapy approaches. Many lessons have been learned from other tumor types and it is now recognized that it is necessary to evaluate multiple components within the tumor microenvironment. Type, density and

location of immune cells within the tumor site are all important and are linked to response to therapy and survival in other cancers. This analysis will allow for a better characterization of a 'hot' or immunogenic vs a 'cold' or nonimmunogenic tumor microenvironment. Preliminary data on a large subset of MTC tumors showed that both intra- and peritumoral expression of markers for cytotoxic, helper, memory T cells, regulatory T cells are low (Dadu *et al.* 2018). These findings suggest that MTC tumors have a 'cold' immune microenvironment. 'Cold' tumors tend to have lower expression of PD-L1, mutational burden, and antigen presentation markers (e.g. major histocompatibility complex class I) (Galon & Bruni 2019). These types of 'cold' tumors are the most challenging to treat. It was discussed at the MEN 2019 meeting that a potential approach will be to find a strategy to overcome the lack on immune response and turn the tumors into 'hot' tumors. Combining a priming therapy that enhances T-cell response, such as vaccines or adoptive T-cell transfer with immune checkpoint inhibitors could be a rational approach. Other promising priming strategies include radiation, chemotherapy and targeted therapies.

Mechanisms of resistance to treatments for MTC

Although patients with MTC can demonstrate PRs and SD with cabozantinib and vandetanib, many will eventually develop progression after a period of time despite therapeutic doses. The presence of *RET* V804M/L mutations has demonstrated resistance to vandetanib and cabozantinib *in vitro* with much higher IC₅₀ values compared with other *RET* mutations (Carlomagno *et al.* 2004, Bentzien *et al.* 2013, Mologni *et al.* 2013). The mechanisms of acquired drug resistance *in vivo* may be related to acquisition of the *RET* V804M/L gatekeeper or *RAS* bypass mutations as demonstrated in a cohort of patients with somatic *RET*-mutated MTC treated with cabozantinib and/or vandetanib (Hu *et al.* 2019). Emergent *RET* G810 solvent front mutations have been reported in patients with *RET*-altered non-small-cell lung cancer and medullary thyroid cancer treated with the selective RET inhibitor, selpercatinib (Solomon *et al.* 2020). Glycine substitution in this region of the RET kinase prevents adequate binding of selpercatinib to the ATP-binding site. Solomon *et al.* reported that the inhibitory activity against *RET* G810S/R/C was significantly lower *in vitro* when exposed to selpercatinib, pralsetinib, cabozantinib and vandetanib. Thus, the consensus of the MEN 2019 meeting was that further investigation into

treatment-emergent mutations is needed as possible resistance mechanisms to targeted systemic therapies.

Future directions

The era of targeted therapy for thyroid cancer began shortly after the turn of the century with the recognition that neoplastic growth depended on the upregulation of angiogenesis and pathogenic ‘driver’ mutations encoding kinases that activate critical intracellular pathways. Since then, there was an exponential development of targeted systemic therapies studied in successfully completed clinical trials led by an international community of investigators. Since 2011, two drugs were approved for use in advanced, progressive MTC (cabozantinib and vandetanib). It is very likely that highly potent RET-specific inhibitors will be approved in the near future. However, there are no effective treatments with a low side-effect profile for non-RET mutated MTC and a curative treatment has yet to be identified.

Future therapeutic trials may need to combine agents targeting different pathways to achieve greater ORRs and hopefully lead to cures without significant toxicity. A MKI may be able to generate sufficient immunogenic cells in the tumoral microenvironment to enhance the efficacy when combined with a checkpoint inhibitor. Use of a CDK inhibitor or ATF4 modulator in combination with a MKI or RET inhibitor may be considered.

For decades, surgery has been considered the most effective treatment with curative intent in early stages of MTC, but with palliative intent in patients with clinically appreciable lymph node or distant metastases. Approximately 38% of node-negative and 90% of node-positive MTC patients have residual disease after surgery, underscoring the need for more effective treatment options in this patient population. A neoadjuvant approach provides theoretical advantages of downstaging tumor (higher R0/R1 resection rate) and thereby increases surgical resectability and reduces surgical morbidity/complexity. It also has the potential of eradicating micrometastatic tumor cells to improve locoregional control and/or survival after surgical resection. To date, there have been no trials evaluating a neoadjuvant treatment for MTC, particularly because of the difficulties in patient accrual due to concerns regarding safety in surgery following neoadjuvant therapy. Additionally, it is critical to use endpoint measurements with meaningful clinical efficacy. Currently, no response evaluation criteria have been developed and gained acceptance for use in

the neoadjuvant therapy followed by surgery paradigm. To overcome these barriers, planning neoadjuvant trials with multidisciplinary input are paramount. The currently approved agents for MTC, vandetanib and cabozantinib, are not ideal for neoadjuvant use due to their antiangiogenic properties, which have the potential to result in bleeding and poor wound healing. With the advent of RET inhibitors with relative low side-effect profiles with minimal antiangiogenic activity, implementing a RET inhibitor neoadjuvantly in patients with more locally advanced disease may lead to better surgical outcomes and reduce long-term recurrences.

The remarkable achievements attained over a relatively short time promise a hopeful future for patients with MTC. From the MTC Workshops at the MEN 2019, there was consensus that further research is much needed in the following areas: understanding the fundamental biology of C-cells by developing a normal C-cell model, establishing other mechanisms of oncogenesis beyond RET and RAS, understanding the mechanisms of heterogeneity of clinical behavior, clarifying mechanisms of drug resistance, and most importantly finding a treatment regimen that leads to sustainable control or cure for what has long been considered a chronic cancer.

Declaration of interest

R D received research support from Eisai, Merck, and Astra Zeneca and was a member of the advisory board of Bayer. R B Y, M R, and E G G have nothing to declare. M Z received research support from Eli Lilly and Co. G J C has nothing to declare. R F G was member of the Data Safety Monitoring Committee of Novo Nordisk. B R was member of the advisory board of Eisai, Loxo Oncology and speaker at Eisai and Loxo Oncology. K R S has nothing to declare. M H received research support from Eli Lilly and Co and was member of the advisory board of Blueprint Medicines, Eli Lilly and Co, and Loxo Oncology.

Funding

This work did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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Received in final form 14 May 2020

Accepted 29 May 2020

Accepted Manuscript published online 29 May 2020