Medullary thyroid carcinoma and biomarkers: past, present and future

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The clinical management of patients with persistent or recurrent medullary thyroid carcinoma (MTC) is still under debate, because these patients either have a long-term survival, due to an indolent course of the disease, or develop rapidly progressing disease leading to death from distant metastases. At this moment, it cannot be predicted what will happen within most individual cases. Biomarkers, indicators which can be measured objectively, can be helpful in MTC diagnosis, molecular imaging and treatment, and/or identification of MTC progression. Several MTC biomarkers are already implemented in the daily management of MTC patients. More research is being aimed at the improvement of molecular imaging techniques and the development of molecular systemic therapies. Recent discoveries, like the prognostic value of plasma calcitonin and carcino-embryonic antigen doubling-time and the presence of somatic RET mutations in MTC tissue, may be useful tools in clinical decision making in the future. In this review, we provide an overview of different MTC biomarkers and their applications in the clinical management of MTC patients.

Keywords: biomarker, doubling-time, imaging, prognosis, RET, targeted therapy.

Introduction

Medullary thyroid carcinoma (MTC), which is derived from the parafollicular calcitonin producing C-cells of the thyroid gland, predominantly develops as a sporadic tumour in about 60% of the cases and is hereditary in 40% of the cases. MTC is the principal disease feature of multiple endocrine neoplasia type 2 (MEN 2), an autosomal dominantly inherited cancer syndrome caused by activating germ-line mutations in the RET proto-oncogene [1–3].

Medullary thyroid carcinoma is suspected via physical examination in combination with elevated plasma calcitonin levels [4]. Cytological and histological confirmation is necessary to diagnose MTC. Ultrasoundography (US), computed tomography (CT) and magnetic resonance imaging (MRI) are used to localize the tumour extent. Classification of MTC is based on the pathological tumour node metastasis (TNM) system [5]. The survival of MTC patients strongly correlates...
with stage at diagnosis: the 10-year overall survival rate of MTC patients initially diagnosed in stage I and II is 90–100%, whilst it is 55–85% for patients initially diagnosed in stage III and 20–55% for stage IV patients [6–11].

The appropriate initial treatment of MTC patients is total thyroidectomy and lymph node dissection of at least the central compartment of the neck [12]. Postoperative plasma calcitonin levels can be measured to determine whether biochemical cure has been achieved, i.e. whether plasma calcitonin levels are normalized after surgery [13]. Postoperative hypercalcitoninaemia indicates persistent MTC, which occurs in 30–55% of patients after primary surgery [6, 7, 14, 15]. Additionally, in 5–12% of patients who were initially biochemically cured, it appears that several years after total thyroidectomy the basal plasma calcitonin levels increase, indicating recurrent disease [6, 7, 16]. Epidemiological studies have shown that during the past 30 years neither a change in stage at diagnosis nor improvement in survival has occurred for MTC patients [9, 10]. Together, this indicates that incorrect classification, inadequate surgical treatment and lack of systemic adjuvant therapy are still the largest problems in the management of these patients.

A biomarker is defined as a factor that can be measured objectively and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a specified therapeutic intervention. Biomarkers can be used to identify individuals at risk for cancer, to detect (early) disease, monitor response to treatment, determine prognosis, detect recurrence, and predict response to particular therapeutic agents [17, 18].

Biomarkers can be determined in easily obtainable body fluids, like blood or urine, using various biological assays. But also more invasive techniques requiring tumour tissue for biomarker determination using immuno-histochemistry and DNA and RNA analyses, are widely used. In addition, several imaging methods are based on the detection of specific biomarkers. In an attempt to optimize biomarker studies, a tumour marker utility grading system (TMUGS) is developed. For each biomarker a grade of utility is assigned, accompanied by a level of evidence (LOE) that scores the quality of the research. The LOE categories range from I to V. Level V evidence is obtained from case reports and clinical experience and is considered weak, whilst level I evidence is derived either from at least one prospective randomized controlled trial specifically designed to test the marker or from a meta-analysis and/or overview of level II or III studies and is considered definitive [19]. In textbox 1 the characteristics and clinical applications of a ‘biomarker’ are defined.

Several biomarkers are currently used for the diagnosis and follow-up of MTC patients. In this review, we provide an overview of different MTC biomarkers and their applications in MTC diagnosis, visualization and identification of disease progression (Table 1). Furthermore, we describe different biomarkers as targets for molecular systemic therapies. Finally, we discuss the clinical utility of these biomarkers at present and how these are likely to affect clinical decision making in the future.

**Biomarkers**
- Chromosomal alterations (loss, gain, translocations)\(^a\)
- Gene alterations (mutations, polymorphisms, hypermethylation)\(^a\)
- Specific or altered gene expression (RNA or protein level)\(^b\)
- Increased protein levels\(^c\)

**Biomarker applications**
- Identify individuals at risk for cancer
- Detect (early) disease
- Determine prognosis
- Detect recurrence
- Predict response to particular agents
- Monitor response to treatment

\(^a\)Detectable in DNA isolated from tissue and/or blood.
\(^b\)Detectable in tissues or in RNA/proteins isolated from tissues.
\(^c\)Detectable in body fluids.

**Diagnostic and prognostic biomarkers in MTC**

Cure rates and survival rates in patients with MTC strongly correlate with the stage at diagnosis [8, 9, 11, 15]. This emphasizes the importance of early detection of both sporadic and hereditary MTC. In addition, the clinical management of MTC patients, especially regarding patients with occult MTC after...
initial treatment, is still of considerable debate. Factors that can determine disease progression may serve as prognostic biomarkers and could therefore be of value for the clinical decision making in the follow-up of MTC patients. Currently, a lot of research is being aimed at the identification of prognostic biomarkers for MTC. Some putative biomarkers, detectable in blood or resected tumour material, are described below. Clinical evaluation should reveal whether and how these prognostic biomarkers could be used in the clinical management of MTC patients in the future.

**Plasma calcitonin**

Calcitonin is a hormone which is specifically produced and secreted by thyroid C-cells. Therefore, plasma calcitonin levels can be determined as a highly specific biomarker for the early diagnosis of MTC and C-cell hyperplasia (CCH). However, occasionally MTCs do not secrete calcitonin [20, 21]. Normal levels of circulating mature calcitonin are, depending on the assay used, below 10 pg mL\(^{-1}\), although some physicians use 20 pg mL\(^{-1}\) as cut-off value. Elevated basal plasma calcitonin levels indicate CCH/MTC, but elevated calcitonin levels can also be detected in patients with non-C-cell (thyroid) diseases and in some healthy adult individuals [22–26]. Furthermore, calcitonin levels are correlated with age and BMI (especially in men) and cigarette smoking can increase the plasma concentration of calcitonin. The prevalence of hypercalcitoninaemia not because of MTC is, however, low and varies

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LOE, level of evidence; CEA, carcino-embryonic antigen.

*Sensitivity and specificity of plasma CEA is lower than that of plasma calcitonin.*
from 0.3% to 4.5% [27]. Yet, only calcitonin secretion from C-cells can be stimulated by pentagastrin or calcium and hence, a calcitonin-stimulation test, using calcium or pentagastrin, has to be performed to identify true positive cases [22, 26, 28, 29]. It is important to note that all calcitonin immunoassays are not equivalent [27, 30, 31]. Therefore, it is imperative that reference values for each commercially available assay will be provided in future guidelines.

In some studies, a correlation between preoperative basal and stimulated plasma calcitonin level and tumour size, tumour stage and postoperative biochemical cure has been identified [32–35]. However, because both larger tumours without and smaller tumours with lymph node metastases can secrete the same quantity of calcitonin, basal calcitonin levels alone can not reliably distinguish one from the other. In 1984, Miyauchi et al., found a strong relationship between doubling-time of plasma calcitonin levels in MTC patients and MTC recurrence and survival [36], which is also revealed by more recent studies [37, 38]. This suggests that at present calcitonin doubling-time is the most sensitive biomarker for MTC progression. However, this measure may not always be useful for preoperative risk stratification because of the need for prolonged observation. Recently, Machens et al. found a strong dose-dependent inverse correlation between the responsiveness to stimulation with pentagastrin and the number of lymph node metastases, which is a strong prognostic factor, in a single-centre retrospective study [35]. However, to really appreciate the predictive value of pentagastrin responsiveness, these results need to be prospectively confirmed.

**Plasma CEA**

Carcino-embryonic antigen (CEA), generally expressed by neuro-endocrine tissues [39], is not a specific biomarker for MTC, and therefore of little use in establishing the diagnosis of MTC [40]. However, pre-operatively elevated plasma CEA levels (above 5–10 ng mL$^{-1}$) are associated with tumour size, number of lymph node metastases, MTC recurrence and prognosis [41–43]. In addition, CEA doubling-time seems to be strongly related to disease progression [38]. However, the necessity for a lengthy phase of observation makes CEA (and calcitonin) doubling-time not useful for preoperative risk stratification.

Postoperative measurements of CEA have revealed normal levels in patients which do have (occult) metastatic disease in some cases [44–47]. Therefore, CEA is of higher value measured preoperatively, reflecting the extent of disease before the initial operation, than measured during follow-up. Nevertheless, plasma CEA may be used as a marker in MTCs that do not secrete calcitonin.

**Germ-line RET mutations**

The detection of another well-known MTC biomarker, a germ-line RET mutation, is used to diagnose MEN 2. In 98% of MEN 2 families, a germ-line activating RET mutation can be detected [1]. Germ-line RET mutations indicate hereditary MTC and determine the lifetime risk for developing MTC, which is nearly 100% for RET mutation carriers. Therefore, genetic screening is highly recommended in all MEN 2 families [1, 48, 49]. A high prevalence of de novo RET mutations (over 50%) has been identified in MEN 2B patients [50, 51], and to a lesser extent in MEN 2A⁄FMTC patients [52–54]. Also, germ-line RET mutations are frequently detected in apparently sporadic MTC patients [55–57], indicating the importance of genetic testing in all MTC patients, even without a clear indication for hereditary disease [1, 48, 49].

Identification of germ-line RET mutation carriers allows prophylactic surgery as well as biochemical follow-up for metastatic and recurrent MTC and for development of MEN 2-associated phaeochromocytoma and hyperparathyroidism [1]. Genotype–phenotype correlation studies have shown that specific RET mutations are associated with age at first diagnosis and tumour aggressiveness, according to which MEN 2 patients can be stratified into three risk groups [1, 58, 59]. Timing of genetic screening and treatment, especially regarding the age for thyroidectomy, for
MEN 2-associated MTC varies between these three risk groups [1, 2, 60–62].

**Somatic RET mutations**

Somatic RET mutations can be detected in tumour tissue of 23–69% of sporadic MTC patients [63]. It has been demonstrated that the presence of a somatic RET mutation (M918T) in MTCs of sporadic patients correlates with stage of the disease, a high probability to have persistence of the disease after total thyroidectomy, increased chance of recurrence and metastatic potential, and a reduced survival [54, 64–67]. This indicates that the presence of somatic RET mutations may function as a prognostic biomarker. To decide which specific surgical strategy is beneficial for an individual MTC patient, including somatic RET mutation analysis in the preoperative work-up could prove superior in the long run. Such an approach would require a clinically available RET mutation test customized for preoperative fine needle biopsies. Prospective multi-centre studies are needed in which patients with sporadic MTC are assessed for somatic RET mutations [68].

**Desmoplasia**

Retrospective and subsequent prospective studies have demonstrated that only tumours with a desmoplastic stromal reaction show lymph node metastases [69, 70]. The extent of desmoplasia, i.e. the density of collagen deposition, and the expression of fibroblast activation protein-alpha and Tenascin-C, correlated with the presence of lymph node metastases [71]. However, in these studies data on extent of lymph node dissection were scarcely available and the absence of desmoplasia may have coincided with tumour size [71]. Therefore, before desmoplasia can be indicated as a morphological parameter with clinical relevance, prospective data corrected for tumour size and extent of lymph node dissection are needed.

**MMP-2**

The matrix metalloproteinase (MMP) family has the capacity to degrade all components of the extra-cellular matrix and participates in carcinogenesis, including angiogenesis [72, 73]. MMPs are produced in a latent form and are inhibited by tissue inhibitors of metalloproteinases. MMP-2 is the main, and possibly most important, MMP which is active in neoplasms [72]. Recently, a small study in 37 MTC patients, demonstrated a correlation between the immuno-histochemical staining for MMP-2 and the state of apparent cure compared with persistence of MTC [74]. Nevertheless, data on initial surgery are lacking and multivariate analysis correcting for possible confounders was not performed. Therefore, these results need to be validated in larger studies before MMP-2 expression can be considered a useful prognostic biomarker for MTC.

**Biomarkers and molecular imaging of MTC**

Patients with persistently elevated or rising plasma calcitonin levels after initial surgery should be thoroughly evaluated with imaging techniques to define the extent of any local or distant disease [75]. However, morphological imaging methods cannot always localize the sites of MTC metastases, i.e. occult disease. Several molecular imaging techniques like positron emission tomography (PET) and immunoscintigraphy (IS) have been shown to be superior to morphological imaging methods for the detection of small MTC metastases. These molecular imaging techniques are based on the detection of biomarkers which are taken up by MTC cells, or which bind to MTC-specific receptors.

**PET radiopharmaceuticals**

Fluorodeoxyglucose (FDG) or dihydroxyphenylalanine (DOPA) (Fig. 1a) is taken up by cells with a high glucose metabolism or during the synthesis of catecholamines respectively. Labelled with $^{18}$F, both tracers are used in PET (Fig. 1b). This type of imaging depicts (patho-) physiological processes and is described as functional imaging. $^{18}$F-FDG and $^{18}$F-DOPA are used in the imaging of (metastatic) neuro-endocrine tumours like carcinoids and MTCs [76, 77]. For MTC detection, $^{18}$F-FDG-PET provides a higher sensitivity (76–96%) and specificity (79–83%) than morphological imaging methods [78–82] and can be used postoperatively to
detect apparently occult MTC, especially in the cervical and mediastinal regions [78, 81, 83]. However, 18F-FDG-PET is in general not very useful in patients with mildly elevated calcitonin levels (89). A disadvantage of 18F-FDG-PET is that specificity is low because FDG-uptake is also a feature of infection, inflammation and other neoplasms. The special property of neuroendocrine cells such as C-cells includes the uptake and decarboxylation of monoamine precursors and the secretion of a large variety of products. 18F-DOPA enters cells via transmembrane amino acid transport systems [84]. The clinical experience with 18F-DOPA-PET is limited. It has been shown to be superior to 18F-FDG-PET and morphological imaging, and useful in detection of lymph node metastases [85, 86], especially in patients with slowly progressing MTCs, representing well-differentiated disease [87]. The transmembrane amino acid transport systems can, therefore, be considered a biomarker for MTC.

Positive 18F-FDG-PET imaging has been associated with poor prognosis in phaeochromocytoma-paraganglioma and follicular and papillary thyroid carcinoma patients [88, 89]. In a single centre, prospective study of patients with recurrent MTC with rapidly increasing plasma CEA levels, 18F-FDG uptake has been associated with high uptake of glucose suggestive for a high proliferation of cells [90]. 18F-FDG-PET sensitivity was increased in patients with plasma calcitonin levels >1000 pg mL−1 and in
patients with a short plasma calcitonin doubling-time [82, 87, 91]. This indicates that $^{18}$F-FDG uptake can be a sensitive biomarker for the prognosis of patients with advanced MTC.

**Regulatory peptides**

Medullary thyroid carcinoma’s express several receptors for regulatory peptides like the somatostatin receptor 2 (SSTR2) [92, 93] and the gastrin/cholecystokinin B receptor (CCKBR) [94–96]. For instance, the CCKBR is believed to represent the molecular basis for the pentagastrin-induced release of intracellular calcitonin in patients with C-cell disease [94, 97]. Both receptors are G-coupled transmembrane receptors and are activated by somatostatin (SST) and gastrin or cholecystokinin (CCK) respectively (Fig. 2a). The first clinically used SST peptide is the octapeptide octreotide which binds to SSTR2, and in 1994, the OctreoScan [using $^{111}$In-diethylenetriamine-pentaacetic acid (DTPA)-octreotide] was approved to be used as an imaging method for SSTR-positive neuro-endocrine tumours [77]. For the detection of MTC metastases, the OctreoScan provides a moderate sensitivity (about 50%), which can be improved when used in combination with other imaging methods [46, 98–100]. Recently, a new SST tracer, $^{99m}$Tc-EDDA/HYNIC-TOC, was used for the detection of MTCs and provided a sensitivity of 80% [101].

Behr et al. demonstrated that radio-labelled derivatives of gastrin showed an excellent targeting of CCKBR expressing tissues [102, 103]. Recently, they showed that gastrin receptor scintigraphy was superior to SSTR scintigraphy and PET [104]. Several additional studies have revealed that radio-labelled CCKBR binding peptides may be useful for MTC imaging, as well as for the treatment of patients with CCKBR expressing MTCs [105–108]. A drawback of using radio-labelled CCKBR-binding peptides is the high stomach and gall bladder uptake because of CCKBR expression in these organs.

The RET receptor, a receptor tyrosine kinase, is activated by ligand-co-receptor complexes. The co-receptor family of RET consists of four glycosylphosphatidylinositol-anchored receptors, i.e. the glial cell line-derived neurotrophic factor (GDNF) family of receptors alpha (GFRα). These co-receptors bind to the GDNF family of ligands (GFLs) [109, 110] (Fig. 2b). Previously, GFRα4, one of the four co-receptors of RET, was found to be specifically expressed in normal and malignant thyroid C-cells and may be necessary for MTC development [111]. Persephin (PSPN), and not the other GFL members, was identified as the specific ligand for GFRα4, inducing RET-GFRα4-mediated differentiation and maintenance of thyroid C-cells [111, 112]. Therefore, we propose that radio-labelled PSPN-derived peptides may provide unique tools for diagnostic and therapeutic applications for metastatic MTC.

**Anti-CEA antibodies**

Carcino-embryonic antigen, a neuro-endocrine tumour marker, is integrated at the plasma membrane of C-cells [113], making it a suitable target for the detection of MTC with anti-CEA antibodies. Radio-labelled monoclonal anti-CEA antibodies have been generated and are used for IS. Anti-CEA IS provides a high sensitivity (75–100%) to detect apparently occult MTC postoperatively [44–47, 114, 115]. False positive results are low using anti-CEA IS [44, 45, 47]. The imaging results with labelled anti-CEA antibodies seem to be related to the behaviour of the neoplasm; i.e. the positive result of scintigraphy would be associated with higher MTC aggressiveness [116]. Some radiologists prefer to use a bispecific anti-CEA/anti-In-DTPA antibody in combination with an $^{111}$In-DTPA-hapten [45, 47, 115]. In that way, it seems that a 80% diagnostic sensitivity in patients with known lesions and a 70% diagnostic sensitivity in patients with minimal disease can be obtained.

**Biomarkers and targeted therapy**

In contrast to follicular and papillary types of thyroid cancer, MTC is not sensitive to adjuvant treatment with radioactive iodine [117, 118]. Several approaches to develop systemic molecular therapies, requiring targets specifically expressed by MTC cells, are currently in progress [118–120].
Therapies are being developed aimed at the inhibition of the receptor tyrosine kinase RET. The potency of several small-molecule tyrosine kinase inhibitors (TKIs) to inhibit RET has been tested preclinically, revealing several efficient RET inhibitors, like axitinib, CEP-751, imatinib, lestaurtinib, motesanib, PP1, PP2, RPI-1, sorafenib, sunitinib and vandetanib [2, 121–123]. Some of these inhibitors have been or are being tested for the efficacy as an MTC drug in clinical trials [120]. It seems that in patients with all subtypes of advanced thyroid cancer, sorafenib and axitinib have anti-tumour effects and have an overall acceptable safety profile [124, 125]. In an ongoing phase II trial with hereditary MTC patients, encouraging results are obtained for vandetanib [126, 127].

It is important to note that different RET mutants might have different affinities to certain TKIs. This has already been shown for the V804M mutant of RET which, in contrast to the C634R mutant, is resistant to PP1, PP2 and vandetanib [128], whilst sorafenib is a potent inhibitor of both RET mutants [129, 130]. Further clinical investigation is required to test the efficacy of TKIs for patients with different RET mutations. Also, for sporadic MTC patients without somatic RET mutations, the efficacy of these RET-targeted therapies should be further investigated.

**Radio-immunotherapy using anti-CEA antibodies**

Targeting MTCs with radio-labelled anti-CEA antibodies has been used to develop radio-immunotherapy in addition to surgical treatment. Two pilot studies revealed limited anti-tumour effects of $^{131}$I-anti-CEA, lasting up to 26 months in about 50% of MTC patients with metastasized MTC [131, 132]. In 1999, the first phase I/II trial was performed with 15 patients with metastatic MTC using a humanized $^{131}$I-MN-14 F(ab)$_2$ anti-CEA monoclonal antibody (labetuzumab) [133]. The therapy was well tolerated, and yielded a dramatic improvement in one patient, whereas in seven other patients a median reduction of 55% for plasma calcitonin and CEA levels was observed. In 11 out of 12 patients, MTC continued to be radiologically stable for periods ranging from 3 to 26 months [133]. Recently, it has been found that upon selection of patients with high risk for MTC progression (short calcitonin doubling-time), treatment with an anti-CEA antibody successively increased the
overall survival rate compared with untreated high-risk patients [134]. Therefore, it appears that anti-CEA antibody therapy is a successful example of targeted therapy using biomarkers in MTC.

Radio-labelled octreotide

The SSTR is used as a target for the detection of MTC using radio-labelled octreotide [77, 100]. Therefore, this receptor may be used as target for MTC treatment as well. So far, treatment of Octreoscan-positive MTC patients with $^{111}\text{In-DTPA-octreotide}$ or with another SST tracer, $^{90}\text{Y-DOTATOC}$, revealed moderate responses [135, 136]. If there is a place for radio-labelled octreotide in the treatment of these patients needs to be elucidated.

Conclusions

Biomarkers are important tools in the clinical management of MTC patients (Fig. 3). Plasma calcitonin and CEA are easily obtainable and widely used biomarkers in MTC management, but their prognostic and diagnostic value needs to be further elucidated. Response to pentagastrin stimulation may reflect favourable prognosis and plasma calcitonin doubling-time is the
most useful marker to determine aggressiveness of residual MTC during follow-up. Plasma calcitonin screening in patients with nodular thyroid disease may detect MTC in an early stage, but it is unclear whether such an approach is cost-effective. Plasma CEA is mostly used as a prognostic marker pre-operatively and can be used for MTCs that do not secrete calcitonin.

Germ-line RET mutations are used in the diagnosis and timing of treatment of hereditary MTC. Moreover, somatic RET mutations may serve as a prognostic biomarker in sporadic MTC. Furthermore, RET seems to be an attractive target for systemic treatment with small molecule TKIs.

Molecular imaging techniques, like Octreoscan, anti-CEA IS, 18F-FDG-PET and 18F-DOPA-PET, can be used to visualize MTC metastases. 18F-FDG-PET and 18F-DOPA-PET seem to be superior to morphological imaging in identifying small MTC metastases, and a high 18F-FDG-uptake seems to be associated with an unfavourable prognosis. Therefore, molecular imaging could be useful in the decision making of further treatment of MTC patients as localization of unexpected distant metastases would indicate that these patients are not likely to benefit from re-operation in the neck.

Furthermore, several immuno-histochemical features such as the presence of desmoplasia or MMP-2 may be of prognostic significance. Therefore, patients with a short plasma calcitonin doubling-time, mitigated pentagastrin response, presence of desmoplasia or MMP-2 in tumour tissue, somatic RET mutations and/or high 18F-FDG uptake may require a more intensive follow-up strategy, whilst patients with indications for a good prognosis might benefit from a more expectative approach.

Plasma calcitonin and CEA doubling-time can effortlessly be measured as the levels of these biomarkers are already measured during follow-up. For the detection of desmoplasia and somatic RET mutations, fine needle biopsy material or resected MTC tissue has to be used. Further clinical research should reveal whether these prognostic biomarkers could be implemented independently or in combination with other parameters in future clinical guidelines for management of MTC patients.

Conflict of interest statement

No conflict of interest was declared.

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