THEMATIC REVIEW

RET-mediated modulation of tumor microenvironment and immune response in multiple endocrine neoplasia type 2 (MEN2)

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Abstract

Medullary thyroid carcinomas (MTC) arise from thyroid parafollicular, calcitonin-producing C-cells and can occur either as sporadic or as hereditary diseases in the context of familial syndromes, including multiple endocrine neoplasia 2A (MEN2A), multiple endocrine neoplasia 2B (MEN2B) and familial MTC (FMTC). In a large fraction of sporadic cases, and virtually in all inherited cases of MTC, activating point mutations of the RET proto-oncogene are found. RET encodes for a receptor tyrosine kinase protein endowed with transforming potential on thyroid parafollicular cells. As in other cancer types, microenvironmental factors play a critical role in MTC. Tumor-associated extracellular matrix, stromal cells and immune cells interact and influence the behavior of cancer cells both in a tumor-promoting and in a tumor-suppressing manner. Several studies have shown that, besides the neoplastic transformation of thyroid C-cells, a profound modification of tumor microenvironment has been associated to the RET FMTC/MEN2-associated oncoproteins. They influence the surrounding stroma, activating cancer-associated fibroblasts (CAFs), promoting cancer-associated inflammation and suppressing anti-cancer immune response. These mechanisms might be exploited to develop innovative anti-cancer therapies and novel prognostic tools in the context of familial, RET-associated MTC.

Key Words
- RET
- MEN2
- tumor stroma
- immunity

Introduction

Medullary thyroid carcinoma (MTC) is a malignant neuroendocrine neoplasia originating from the C-cell component of the thyroid gland. MTC accounts for about 5% of all thyroid malignant tumors and can occur either as sporadic (about 75% of cases) or familial (about 25% of cases) form. Hereditary MTC occurs in the context of different autosomal dominant syndromes, including multiple endocrine neoplasia type 2 (MEN2). MTC can be the only manifestation in familial MTC (FMTC), can be associated with pheochromocytoma (PC) and parathyroid adenoma/carcinoma in MEN2A or with PC, mucosal neuromas and marphanoid habitus in MEN2B. Activating mutations in the REarranged during Transfection (RET) proto-oncogene are responsible for the occurrence of all these syndromes (Marquard & Eng 1993).
RET encodes for a receptor tyrosine kinase (RTK) that functions as a receptor for neurotrophic factors of the glial cell line-derived neurotrophic factor (GDNF) family: GDNF, neurturin, artemin and persephin. RET activation requires the presence of four co-receptors belonging to the GDNF receptor α1-4 family of glycosylphosphatidylinositol (GPI)-linked proteins. Each coreceptor dictates the specificity for one of the four ligands (Aitakinen & Saarma 2002). Three RET isoforms (RET9, RET43 and RET51) encoding for protein variants differing in the intracellular tyrosines involved in RET activation (Tahira et al. 1990, Lorenzo et al. 1995, Matera et al. 2000) have been described.

RET is physiologically required for the development, maturation and maintenance of central and peripheral nervous systems and of the excretory system. RET function is essential to spermatogenesis, the correct retina development and the formation of gut-specific secondary immune organ (Peyer's Patches). RET loss-of-function (LOF) and gain-of-function (GOF) mutations are responsible for various human diseases (Table 1).

RET proto-oncogene was isolated for the first time in 1985 from a human T-cell lymphoma for its ability to transform NIH 3T3 mouse fibroblasts (Takahashi et al. 1985). In 1987, Fusco and coworkers identified, as transforming oncogene from human papillary thyroid carcinoma (PTC), a novel rearranged form of the RET proto-oncogene (Fusco et al. 1987, Grieco et al. 1990). It was only in 1993 that germline RET autosomal dominant missense mutations were identified in various MEN2A families (Mulligan et al. 1993), mapping in cysteines located at the boundary between RET extracellular and transmembrane region. One year later, various research groups detected, in MEN2B patients, a single missense mutation in the intracellular tyrosine kinase domain of the RET receptor (Fig. 1) (Hofstra et al. 1994). RET protein activation in MTC is due to point mutations that map either in the extracellular portion or in the intracellular tyrosine kinase (TK) domain of the receptor and a robust genotype-phenotype correlation has been observed for most MTC-associated RET mutations (Krampitz & Norton 2014). The constitutive/aberrant RET activation sustains several biological processes, including cell proliferation and survival, motility and invasive ability, tissue remodeling and immune modulation. Finally, the central role of RET in MTC has prompted the development of RET kinase inhibitors to be used in the therapy of MTC (Mologni et al. 2017).

Here, we will discuss how oncogenic RET signaling, in particular that associated with MEN2 syndromes, modulates gene expression and shapes the stromal and the immunologic components of tumor microenvironment, thus influencing tumor growth. Finally, we will discuss how these features might be exploited for therapeutic benefit of MTC patients.

### RET activation and signal transduction

Physiological RET activation after ligand binding results in the activation of its kinase activity leading to the autophosphorylation of various intracellular domains of the RET MEN2 oncoproteins on tumor microenvironment (Fig. 1) (Hofstra et al. 1994). RET protein activation in MTC is due to point mutations that map either in the extracellular portion or in the intracellular tyrosine kinase (TK) domain of the receptor and a robust genotype-phenotype correlation has been observed for most MTC-associated RET mutations (Krampitz & Norton 2014). The constitutive/aberrant RET activation sustains several biological processes, including cell proliferation and survival, motility and invasive ability, tissue remodeling and immune modulation. Finally, the central role of RET in MTC has prompted the development of RET kinase inhibitors to be used in the therapy of MTC (Mologni et al. 2017).

## Table 1
The principal RET genetic alterations associated with human diseases are shown.

<table>
<thead>
<tr>
<th>Type of RET mutation</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss-of-function mutations</td>
<td>Hirschsprung disease (HSCR, aganglionic megacolon)</td>
<td>Edery et al. (1994), Romeo et al. (1994)</td>
</tr>
<tr>
<td>RET point mutations</td>
<td>Congenital anomalies of the kidney or lower urinary tract (CAKUT)</td>
<td>Davis et al. (2014)</td>
</tr>
<tr>
<td>Gain-of-function mutations</td>
<td>Familial and sporadic medullary thyroid carcinoma (MTC)</td>
<td>Eng et al. (1994), Hofstra et al. (1994), Mulligan et al. (1993)</td>
</tr>
<tr>
<td>RET point mutations</td>
<td>Anaplastic thyroid carcinoma (ATC)</td>
<td>Kunstman et al. (2015)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Sporadic parangangioma</td>
<td>Krawczyk et al. (2010)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Urothelial carcinoma</td>
<td>Kato et al. (2017)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Papillary thyroid carcinoma (PTC)</td>
<td>Grieco et al. (1990)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Lung adenocarcinoma</td>
<td>Kohno et al. (2012), Lipson et al. (2012)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Colon carcinoma</td>
<td>Lipson et al. (2012)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Myeloproliferative disorders</td>
<td>Ballerini et al. (2012)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Fallopian tube adenocarcinoma</td>
<td>Kato et al. (2017)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Uterine carcinosarcoma</td>
<td>Kato et al. (2017)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Duodenal adenocarcinoma</td>
<td>Kato et al. (2017)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Breast cancer</td>
<td>Amit et al. (2017)</td>
</tr>
<tr>
<td>RET fusions</td>
<td>Pancreatic adenocarcinoma</td>
<td>Esseghir et al. (2007)</td>
</tr>
<tr>
<td>RET amplifications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RET aberrant expression</td>
<td></td>
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</tr>
</tbody>
</table>

tyrosine residues. This event has two consequences: the maintenance of the active kinase conformation that is mainly mediated by Tyr900 and Tyr905 (Fig. 1) and the generation of docking sites for signal transduction proteins. Upon ligand binding, RET activates numerous signaling pathways including the RAS/extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), the phosphatidylinositol 3-kinase (PI3K)/AKT, c-Jun amino-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK), signal transducer and activator of transcription 1/3 (STAT1/3) and phospholipase C-γ (PLCγ) (Plaza-Menacho et al. 2014). The most important RET tyrosines that are phosphorylated in vivo and involved RET signaling include Y1015 (binding site for PLCγ) and Y1062 (docking site for multiple adaptor proteins) (Santoro et al. 2004, Arighi et al. 2005, Plaza-Menacho et al. 2006; Kurokawa et al. 2003) (Fig. 1).

Germline RET mutations in MEN2 can constitutively activate RET and fall in two groups (Table 2):

1. those involving the cysteine residues in the extracellular cysteine-rich domain, that associate with the MEN2A phenotype, resulting in ligand-independent, constitutive dimerization and activation of the kinase due to the formation of disulfide bridges between RET monomers,

2. those involving the intracytosolic tyrosine kinase domain that associate with MEN2B phenotype. These mutations are believed to affect the stability of RET-inactive dimer conformation, increasing ATP-binding affinity and altering the affinity of RET to downstream substrates.

FMTC-associated RET mutations belong to both groups (Krampitz & Norton 2014). Due to the strong genotype-phenotype association between RET MEN2 mutations and severity of the disease, it is possible to classify MEN2-associated RET mutations in four disease risk levels (A–D, from the lowest to the highest). This classification is important for planning prophylactic thyroidecmy to prevent MTC and to assess the risk of developing MTC-associated neoplastic lesions (American Thyroid Association Guidelines Taskforce 2009). RET mutations, mainly the M918T in the TK domain, can also be found in about 50% of sporadic MTC, and their presence correlates with an aggressive disease phenotype (Romei et al. 1996, Schilling et al. 2001) (Table 2).

With respect to RET MEN2A, RET MEN2B mutants display stronger TK activity, increased Y1062 phosphorylation and more efficient recruitment of adapters and activation of downstream signaling pathways, including ERK, PI3K/AKT and JNK pathways (Salvatore et al. 2000, 2001, Kurokawa et al. 2003).
RET oncogenic proteins can activate, albeit constitutively and more efficiently, the same signaling pathways induced by physiologically ligand-stimulated RET. However, some differences in downstream signaling between wild-type RET and different MEN2 mutants may account for differential gene expression, that can be translated in differences in tumor microenvironment, and ultimately in disease severity in MTC (Engelmann et al. 2009).

### The role of microenvironment in thyroid cancer: FTC vs MTC

Human tumors must not be considered as simple masses of proliferating cells, but complex tissues, in which various cellular and non-cellular components, associated to cancer cells, constitute the so-called tumor microenvironment (TME). TME consists of extracellular matrix (ECM), mesenchymal cells (i.e., fibroblasts, pericytes, adipocytes and other stromal cells), immune-inflammatory cells, blood and lymphatic vessels. TME and cancer cells are in close contact and can profoundly influence each other thus promoting tumor initiation, progression and metastatic conversion (Egeblad et al. 2010a,b, Hanahan & Coussens 2012, McAllister & Weinberg 2014). Malignant cells actively contribute to remodel the pre-existing stroma creating a new microenvironment that can have inflammatory or desmoplastic characteristics.

In thyroid cancer, stroma composition changes among the different histotypes. An immune/inflammatory infiltrate is associated to papillary thyroid carcinoma (PTC), while anaplastic thyroid carcinoma (ATC), metastatic PTC and MTC display a pronounced desmoplastic stromal reaction correlating to aggressiveness and lymph node metastasis (Koperek et al. 2007, 2013; Mai & Hogan 2016, Sun et al. 2016).

### Desmoplastic stroma

**Desmoplastic stroma: cellular component**

Desmoplastic stroma is characterized by newly formed fibrotic tissue composed of fibroblasts and myofibroblasts surrounded by a thick collagenous matrix similar to wound-healing stroma. Cancer-associated fibroblasts (CAFs) therefore represent the main cellular entity involved in desmplasia and their activation to myofibroblasts can be interpreted as an attempt of the tumor tissue to heal the injury produced by the infiltrative and destructive growth of cancer cells, thereby flagging the invasive and malignant character of the tumor. CAFs play a crucial role in carcinogenesis because they are responsible for synthesis, deposition and remodeling of extracellular matrix (ECM). They provide paracrine growth factors that influence cancer cell behavior and support development, growth and progression of different tumors, therefore being considered indicators of invasive cancer behavior.

### Table 2  The principal RET mutations in MTC are reported.

<table>
<thead>
<tr>
<th>RET domain</th>
<th>Codons</th>
<th>Phenotype</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular domain</td>
<td>G321</td>
<td>MEN2A/FMTC</td>
<td>Altered folding and protein maturation; ligand-independent constitutive dimerization; formation of disulfide bridges between monomers; activation of the kinase</td>
</tr>
<tr>
<td></td>
<td>G533</td>
<td>MEN2A/FMTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K603</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C609</td>
<td></td>
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</tr>
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<td></td>
<td>C611</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>C618</td>
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<td></td>
<td>C620</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>C630</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C634</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular domain</td>
<td>L790</td>
<td>MEN2A</td>
<td>Ligand-independent dimerization; enhanced phosphorylation of intracellular substrates; increased STAT3 phosphorylation</td>
</tr>
<tr>
<td></td>
<td>Y791</td>
<td>FMTC</td>
<td>Facilitated transition to active conformation</td>
</tr>
<tr>
<td></td>
<td>E768</td>
<td>FMTC</td>
<td>Improved access to ATP binding site</td>
</tr>
<tr>
<td></td>
<td>V804</td>
<td>MEN2A/FMTC</td>
<td>Altered conformation of activation loop; activation of monomeric RET; STAT3 phosphorylation</td>
</tr>
<tr>
<td></td>
<td>S891</td>
<td>MEN2A/FMTC</td>
<td>Promotion of protein active form</td>
</tr>
<tr>
<td></td>
<td>A883</td>
<td>MEN2B</td>
<td>Affected stability of RET inactive dimer conformation, increasing ATP binding affinity; altered affinity of RET for downstream substrates; increased Y1062 phosphorylation; more efficient recruitment of adapters and activation of downstream signaling</td>
</tr>
<tr>
<td></td>
<td>M918</td>
<td>MEN2B/sporadic MTC</td>
<td></td>
</tr>
</tbody>
</table>

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MTCs are aggressive tumors that tend to metastasize early to lymph nodes (10–30% of the cases) and are characterized by a considerable variation in overall survival. Recent reports have suggested that only MTC with a desmoplastic stromal reaction (desmoplasia) develop regional lymph node metastasis, suggesting this as a prognostic marker.

Immunohistochemical studies have shown that, in the MTC stromal compartment, three fibroblast activation markers (fibroblast activation protein α, (FAPα), Tenascin C (Tn-C) and α-smooth muscle actin (α-SMA)) are highly expressed compared to normal thyroid gland and correlated to the degree of desmoplasia (Koperek et al. 2007). Moreover, recent evidences have suggested that stromal activation is an early event in MTC development as FAPα and Tn-C can be found already in the stroma of microcarcinomas and in area of C-cell hyperplasia (CCH) (Koperek et al. 2007). Interestingly, these markers have also been described in areas of autoimmune thyroiditis associated to some cases of MTCs, suggesting a function in inflammatory tissue remodeling. Finally, Tn-C expression was seen in the stroma of both hereditary and sporadic cases of MTCs (Koperek et al. 2009, Steiner et al. 2016). Although the mesenchymal tissue of the thyroid is prone to develop fibrosis in both inflammatory (i.e., Riedel’s, Hashimoto, De Quervain’s, fibrotic nodules) as well as in neoplastic conditions (Mai & Hogan 2016), not many studies have been conducted to identify the characteristics of MTC desmoplastic stroma.

It has been recently proposed that focal tumor hypoxia could trigger the remodeling of the ECM and increase the ability of cancer cells to invade lymphatic vessels (Koperek et al. 2011); moreover, the switch of superoxide dismutase 3 expression from cancer cells to cancer mesenchymal stromal cells has been suggested to modulate cancer growth and migration (Laatikainen et al. 2010, Parascandolo et al. 2017). Interestingly, PTC with mutations in the RET/PTC-RAS-BRAF pathway has been described to show high expression of CAF-related proteins (Sun et al. 2016). Since MTC also displays mutations in the same oncogenes, the development of desmoplastic stroma in MTC could be related to the activation of this signaling cascade. Finally, a recent report has indicated that mutations activating the β-catenin pathway are associated to PTC with a prominent mesenchymal cellular component (Rebecchini et al. 2017), suggesting that the activation of β-catenin pathway downstream of RET could be responsible for CAF activation.

In general, the finding that markers of CAF activation are associated to loco-regional metastasis in MTC (as well as in different histotypes of thyroid malignancies) suggests their utility in the molecular characterization of MTC with the aim to define the surgical strategy to remove cervical lymph nodes. Desmoplastic poorly vascularized stroma, besides supporting tumorigenesis, is also a barrier for chemotherapeutic drugs to enter the tumor; therefore, therapeutic strategies combining stroma inhibition with cancer-directed drugs have shown promising results in preclinical and clinical set-up.

Desmoplastic stroma-derived mediators and the induction of EMT

Tumor-promoting activity of CAF could be linked to their ability to produce soluble factors promoting tumorigenesis and metastasis, as well as tumor angiogenesis and recruitment of immune-inflammatory cells, thus shaping TME into a cancer-promoting context (Kalluri 2016). In breast cancer, CAFs produce the chemokine CXCL12/SDF-1, that can recruit endothelial progenitor cells into the tumor mass thus promoting angiogenesis; SDF-1 can directly stimulate the CXCR4 cognate receptor expressed on breast carcinoma cells promoting lymph nodal metastasis (Orimo & Weinberg 2006). Interestingly, CXCR4 has been identified as a transcriptional target of wild-type RET (Lu et al. 2011). Furthermore, CXCR4 mRNA could be induced by both PTC-associated RET mutants (RET/PTC1 and RET/PTC3) in follicular thyroid epithelial cells (Castellone et al. 2004, Borrello et al. 2005) and by MTC-associated RET mutants (RET MEN2B). Accordingly, the treatment of RET-positive neuroblastoma (NB) cells with Vandetanib, a RET tyrosine kinase inhibitor (TKI), correlates with the downregulation of CXCR4 expression and decreases cell migration and invasion (Ding et al. 2014).

Using gene expression profiles, it has been possible to identify differences in RET MEN2A- and RET MEN2B-induced transcriptional programs that could explain their different biological activity. Jain and coworkers identified CXCR4 as a gene expressed at higher levels in RET MEN2B compared to MEN2A. The induction of CXCR4 by RET/ MEN2B has been related to the specific activity of RET MEN2B oncoprotein: the mutation M918T is particularly efficient in activating the STAT3 pathway, which is the main regulator of CXCR4. Accordingly, CXCR4 expression has been detected by immunohistochchemistry (IHC) in many human TC samples, including MTC, but not in benign thyroid tumors or normal thyroid tissues.

CXCR4, similar to other chemokine receptors, can mediate cell motility, invasiveness and matrix remodeling by stimulating the production of matrix metalloproteases. Moreover, CXC chemokines, including SDF-1α020, are proangiogenic, and CXCR4 can be expressed on vascular endothelium, thus promoting vessel sprouting (Chatterjee et al. 2014a, b; Guo et al. 2016). Thus, activating mutations in MEN2B and, to a lesser extent in MEN2A, can activate a transcriptional program that regulates tissue remodeling, cell motile and invasive ability and metastatic capacity of MTC cells. Also, other genes involved in the process of epithelial-to-mesenchymal transition (EMT) (i.e., transforming growth factorβ (TGF-β) pathway’s components) were preferentially increased in RET MEN2B MTC in comparison to MEN2A (Jain et al. 2004a).

The activation of the EMT program triggers a complex cellular response, with downregulation of epithelial properties and acquisition of mesenchymal features. As a consequence, cancer cells activating EMT acquire a motile and invasive phenotype (Shibue & Weinberg 2017). Oncogenic RET has been associated with EMT features. Both PTC isoforms and MTC-associated RET mutants can induce the expression of EMT-related genes and EMT-associated biological activities (Watanabe et al. 2002, Kurokawa et al. 2003, Jain et al. 2004b, Ameur et al. 2009). RET MEN2 mutants have been shown to induce EMT-related genes both in cell cultures and in MTC samples, as shown by differential display and microarray analysis. In a study comparing inherited and sporadic MTCs, the authors observed that MTCs carrying germline RET MEN2A mutations were similar to sporadic non-metastatic MTCs, whereas cases with germline RET MEN2B mutations displayed molecular signatures similar to those expressed by sporadic metastatic MTCs. These signatures included EMT-related genes, belonging to matrix remodeling and cell-adhesion genes. Accordingly, treatment of an MTC cell line expressing the RET MEN2A mutant allele (TT) with sunitinib, a RET kinase inhibitor, inhibited EMT-related gene expression (Jain et al. 2004b, Ameur et al. 2009). RET51- and RET9-specific RET depletion in TT cells, obtained by using RNAi or a RET kinase-dead (RetKD) mutant, caused a drop in the expression of EMT transcription factors ZEB1 and TWIST1 (Lian et al. 2017) and inhibited EMT-related activities (anoikis resistance, anchorage-independent growth and invasion). Moreover, this approach demonstrated that RET51 was more efficient than RET9 in sustaining EMT-related activities (Lian et al. 2017).

**Immune-inflammatory component in tumor microenvironment**

Another important component of the tumor microenvironment is represented by immune-inflammatory cells. Several epidemiological observations support the view that cancer development and progression are profoundly affected by the immune system. Chronic inflammation, either caused by infections or by autoimmunity, increases the risk of developing certain cancer types (Balkwill & Mantovani 2001), suggesting that cancer cells can co-opt immune cells and immune-mediated mechanisms at their own advantage to favor tumor development and progression (Colotta et al. 2009). In TME, chemoattractant factors produced by cancer cells, as well as other microenvironmental factors (e.g. tumor hypoxia), favor the flux of immune cells around tumors (Crusz & Balkwill 2015). Both innate and adaptive immune cell infiltrates have been described in tumors, and how the different populations contribute to the development and progression of cancer has been the object of intense investigation (Palucka & Coussens 2016).

Accordingly, human PTC samples, carrying RET/PTC rearrangements, display a significant immune infiltrate. Both innate and adaptive immune cell population have been described in TC. These cells have been extensively characterized in TC derived from the follicular cell, including PDTC and ATC. The density and the composition of the immune infiltrate in follicular TC have been correlated with different clinic-pathological features (Ward 2014). Among the innate immune cells, both regulatory and cytolytic NK, dendritic cells, macrophages and mast cells have been described. Moreover, different classes of lymphocytes, including CD4+ and CD8+ T lymphocytes, FoxP3+ T regulatory (Treg) cells and CD19+B lymphocytes have been observed in these tumors.

Importantly, the activation of the immune-inflammatory transcriptional program elicited by oncogenic RET proteins depends on the signaling pathways mediated by the 1062 tyrosine residue. The immunogenic capacity of RET/PTC has been extensively studied in in vivo models of thyroid carcinogenesis. RET/PTC3 isoform expression was associated with tumor infiltration by cytotoxic CD8+ T cells and myeloid CD11b+ Gr1+...
cells. Interestingly, CD11b+ Gr1+ myelocyte-, but not CD8+ T lymphocyte-infiltration, was dependent on the integrity of RET/PTC3 Y588, that corresponds to the RET multidocking site Y1062. These data suggest that while CD11b+ Gr1+ recruitment might be due to RET Y1062-dependent proinflammatory signaling, the recruitment of CD8 T cells could be the result of the expression of tumor-specific antigens, possibly endowed in the RET/PTC3 oncoprotein (Russell et al. 2004, Shinohara & Rothstein 2004, Pufnock & Rothstein 2009, Neely et al. 2011, Wixted et al. 2012).

**RET-derived inflammatory mediators shaping tumor microenvironment**

In TC, the activation of the RET/RAS/BRAF signaling cascade has been demonstrated to profoundly affect cancer-related immunity. Many reports have in fact shown that the RET/PTC proteins, in the context of differentiated thyroid carcinoma (DTC), can activate a proinflammatory transcriptional program that sustains tumor growth, invasive/metastatic ability and immune escape. The activation of this transcriptional program is possibly dependent on the ability of RET to activate both the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and the STAT1/3 transcriptional factors, both typically involved in the regulation of the immune response (Menicali et al. 2012, Ward 2014).

The ectopic expression of RET/PTC1 or RET/PTC3 in PC Cl3 rat thyroid epithelial cells induces an increase in cyclooxygenase 2 and microsomal prostaglandin E synthase 1 mRNA levels, with a consequent increase in prostaglandin E2 secretion (Puxeddu et al. 2003). Other reports show that RET activation caused the expression of proinflammatory molecules, including chemokines (CCL20, CCL2, CXCL8/interleukin 8 (IL8), CXCL12/SDF1), cytokines (IL1β, CSF-1, SPP1/OPN, GM-CSF and G-CSF) or matrix-degrading enzymes and adhesion molecules (Powell et al. 2003, Borrello et al. 2005, Puxeddu et al. 2005). Importantly, many of these factors mediate pro-tumorigenic biological activities in experimental models and correlate with more pronounced aggressive features of human DTC. The pro-tumorigenic activity of these proinflammatory factors in DTC is related to their ability to bind specific receptors expressed on cancer cell surface. The secretion of immune/inflammatory proteins in tumor microenvironment causes extracellular remodeling, increases vessel density, permeability and adhesive properties. Moreover, as many of these factors possess chemoattractant activity toward immune cells, they can shape and influence the function of tumor immune infiltrate (Russell et al. 2003, Menicali et al. 2012).

Not only RET/PTC, but also ligand-stimulated wt RET and RET MEN2 mutants, as mentioned earlier, could induce the expression of proinflammatory factors. For instance, IL8 has been identified as a RET-regulated gene in human thyroid follicular cells (Borrello et al. 2005) and in neuroectodermal cancer-derived cells (SKNMC) treated with GDNF. Accordingly, IL8 is also produced by RET MEN2A-transfected SKNMC cells, by the MTC cell line TT, carrying an endogenous RET MEN2A mutant, and in the TPC1 cell line, derived from a RET/PTC1-positive PTC. Interestingly, the expression of IL8 depended on multiple RET-mediated downstream signaling pathways (Iwahashi et al. 2002). In the context of cancer, IL8 is involved in the regulation of angiogenesis, cell motility, cell growth/survival, immune cell infiltration/anti-tumor immune responses. In DTC, PDTC and ATC, IL8 functions both as an autocrine and as a paracrine factor. As an autocrine factor, it favors TC cell proliferation, survival and motility. Moreover, by inducing the expression of the SLUG transcription factor, it sustains EMT and stemness features of TC cells (Liotti et al. 2017). As a paracrine factor, it is produced by TC-infiltrating mast cells (MC) (Visciano et al. 2015). In accord to the ability of RET to induce IL8 production, Brouitin and coworkers, identified IL8 as a potential soluble biomarker of therapeutic response to sunitinib in MTC patients (Brouitin et al. 2011).

**Mechanisms of immune surveillance in MTC**

Studies in mice demonstrated that experimental tumors, initiated by treating mice with carcinogenic agents, could be not only recognized but also rejected by the immune system, mainly by virtue of the cytotoxic activity of T lymphocytes (Cali et al. 2017). In human beings, the relationship between cancer and the immune system is complex and only partially understood. Human cancer can be recognized and in principle be eliminated by the immune system through a mechanism defined immune surveillance, but tumors often escape immune-mediated elimination using different mechanisms. The following three phases have been envisaged during cancer development (Dunn et al. 2004):

- **elimination:** both innate and adaptive immune cells cooperate and succeed in eradicating microfoci of transformed cells
- **equilibrium:** immune-mediated killing of cancer cells leads to the selection of resistant clones, due to their intrinsic genetic and epigenetic instability
• escape: these resistant clones eventually overcome immune pressure and expand, thus leading to the development of an established tumor.

The remarkable advances in exome sequencing has allowed the identification of mutant antigens in virtually all types of human cancer, and the availability of broad databases has allowed to define neoepitopes potentially recognized by the immune system. Some cancers, like melanoma, display high numbers of mutations in coding exons, and this feature makes them more ‘immunogenic’, thus potentially rejectable by the immune system. Other tumor types, like TC, display the lowest mutational burden among all solid cancer types. Thus, MTC should in principle show a very low number of neoepitopes (Garraway 2013, Garraway & Lander 2013, Garraway et al. 2013). RET itself, activated either by rearrangements, as in PTC, or by point mutations, as in MTC, may represent a novel antigen (Powell et al. 2003).

MTC being a rare disease, genomic profiling and histological analysis of human samples for the characterization of the immune cell infiltrate has been limited. Many investigators have used mice models to study RET-mediated immunomodulatory activity. Interestingly, in a study comparing expression profiles associated with RET MEN2A, RET MEN2B and RET FMTC mutations, Engelman and coworkers observed that a signature of genes related to the host immune-inflammatory response, related principally to NK cells and cytotoxic T lymphocytes (CTL), were enriched in RET MEN2A and FMTC tumors with respect to RET MEN2B (Engelman et al. 2009). The authors also hypothesized that RET MEN2A/FMTC-induced tumors were more susceptible to cytotoxic cells than RET MEN2B tumors. In fact, NK and CTL cells apoptosis-inducing genes (i.e., FasL, perforin and granzymes) were upregulated in RET MEN2A tumors. Moreover, genes encoding for chemoattractants, adhesion molecules and growth factors involved in the recruitment and activation of NK and CTL cells displayed higher expression in RET MEN2A tumors than in RET MEN2B. Importantly, an interferon-associated signature could also be observed, consistent with a cytotoxic Th1-type of immunity. These data indicated that RET MEN2A, but not RET MEN2B oncoproteins could elicit an effective cytolytic immunity. An IHC analysis of these tumors confirmed that granzyme and perforin1 antibodies could stain the immune infiltrate in RET MEN2A-,- but not RET MEN2B-induced tumors. Consistently, the authors observed that RET MEN2A/FMTC, but not RET MEN2B tumors expressed CX3CL1/fractalkine, a chemokine involved in the recruitment of cytotoxic immune cells, into both experimental tumor models and in human cancer tissues (Engelman et al. 2009). These data suggest two possible interpretations: 1. RET MEN2A tumors are more immunogenic than the RET MEN2B; 2. RET MEN2B tumors, displaying higher expression of EMT factors, can induce immunosuppression more efficiently than RET MEN2A/FMTC. In fact, EMT has been described as a pathological process that confers cancer cells, besides other features, remarkable immunosuppressive properties (Thiery et al. 2009).

**RET and cancer immunotherapy in MEN2**

Cancer immunotherapy is based on two main mechanisms: passive and active. Passive immunotherapy can be achieved by adoptive transfer of molecules (mainly antibodies) or immune cells (cytotoxic CD8+ lymphocytes or NK cells) that target tumor cells. Active immunotherapy includes: (1) vaccination with tumor antigens or with dendritic cells pulsed with tumor cells to expand tumor-specific cytolytic T cells; (2) reactivation of anti-tumor immunity by blocking the activity of immune checkpoint molecules (Emens et al. 2017). In recent years, the possibility to enhance anti-cancer immune response by targeting these immune checkpoints has shown efficacy in many cancer types (Gajewski & Schumacher 2013).

In the normal physiology, once activated, naïve T cells become effector T cells acquiring cytotoxic activity. Effector T cells, during immune response, also upregulate their expression of ‘immune checkpoint’ receptors, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1). These receptors, when engaged with their ligands, inhibit T-cell activation, proinflammatory T helper 1 (TH1) cytokine production and cell-mediated cytotoxicity. This immunosuppressive activity is necessary for preventing prolonged inflammation and autoimmunity. CTLA-4 is a negative regulator of the CD80/CD86 (B7-1/B7-2)-CD28 co-stimulatory molecules required for T-cell priming. CTLA-4, like CD28, binds CD80 and CD86, but with a higher affinity, thus inhibiting costimulation. PD-1 is an inhibitory receptor expressed on activated T cells, but, at variance from CTLA-4, also on a broad range of immune cells. The ligands for PD-1 are the programmed death-ligand 1 and 2 (PD-L1/2), that can be expressed on both hematopoietic and non-hematopoietic cells, often in response to the presence of proinflammatory cytokines. Importantly, cancer cells often express PD-L1 and/or PD-L2, thus avoiding T-cell-mediated destruction.
Immune checkpoint blockade therapies that inhibit CTLA-4-CD80/CD86 and/or the PD-1/PD-L1 axis have proven successful in treating several cancers (Bardhan et al. 2016). However, the expression levels of immune checkpoints in tumors are not always predictive of therapeutical response. Tumor-infiltrating immune cells in the tumor microenvironment, mutational landscape and mismatch-repair deficiency may be important in predicting clinical benefits of immune checkpoint inhibitors (Meng et al. 2015). Recently, the immunological characterization of many tumors has lead to the idea that two classes of tumors can be envisaged based upon immune cellular and molecular features: 1. those displaying the ‘T-cell-inflamed phenotype’, characterized by T-cell infiltration, remarkable chemokine expression and Th1-cytokine profile, where interferon gamma (IFNγ) dictates immune activation. These tumors evade immune attack by actively suppressing immunity using various mechanisms; (2) those displaying the ‘non T-cell inflamed’ phenotype. These tumors are probably not detected by the immune system because of their intrinsic low immunogenicity. The causes of these differences might be related to the oncogenic pathways and the mutation frequency found in cancer cells, or to the immunological response of the host that, in turn, is determined by intrinsic (polymorphisms in immunoregulatory genes) and extrinsic (environmental influences, including microbiota composition) factors. Whatsoever, T-cell-inflamed tumors are more likely to benefit from immune checkpoint therapy than non-T-cell-inflamed neoplastic lesions (Gajewski & Schumacher 2013).

TC derived from the follicular cells, despite showing a low mutational rate (Agrawal et al. 2013), often display a high density of infiltrating T cells or a high T CD8+/Treg ratio similar to tumors with high expression of neoepitopes. These features generally correlate with a better survival. PD-L1 has been found expressed both in immune and in cancer cells in a large set of TC, and higher PD-L1 expression was found in the more aggressive forms (Ahn et al. 2017). For these reasons, clinical trials of the monoclonal antibody pembrolizumab, that targets PD-1 have been started in radioiodine refractory DTC (NCI NCT02973997).

MTC also displays a low mutational rate (Agrawal et al. 2013). Consistently, they often present with few tumor-infiltrating immune cells and low expression of PD-L1 (Bongiovanni et al. 2017). This would suggest that MTC can be considered non-T-cell-inflamed tumors. However, there are also indications that MTC may exhibit some immune reactivity.

IL17 family of cytokines has been described to exert a complex role in cancer because it can induce angiogenesis and immunosuppressive functions, thus exerting a protumorigenic activity. On the other hand, this cytokine has been associated with anti-tumor immune responses by recruiting, into the tumor site, effector CD8+ T cells or polarizing CD4+ T cells to a Th1 phenotype producing IFN-γ (Yang et al. 2014, Alinejad et al. 2017, Qian et al. 2017). In MTC, IL-17 expression was associated with poor outcome (Carvalho et al. 2017). In another report, a significant increase in Treg FoxP3+ lymphocytes in the peripheral blood, in lymph nodes and in thyroid tissues of MTC patients has been observed. Moreover, similar to what was observed in other cancer types, an increase of Tregs in the blood is correlated with the severity of the disease (Muller et al. 2010). These data, taken together, indicate that a proinflammatory activity is present in MTC, possibly sustained, among the others, by Th17 cells. However, the presence of Tregs suggests that immune escape mechanisms are also active, possibly mediated by the expansion of these cells. No data are so far available regarding the expression of immune checkpoints in MEN2.

Other RET MEN2-mediated signaling pathways could induce escape from anti-tumor immune response. Activated RET, including RET MEN2, can activate the Wnt pathway, that inhibits the recruitment of antigen-presenting cells (i.e., dendritic cells) (Gujral et al. 2008, Castellone et al. 2009, Prazeres et al. 2011, Tartari et al. 2011). Interestingly, it has been shown that an immune response could be elicited in MTC patients by vaccination with tumor cell-pulsed autologous dendritic cells, indicating that MTC cells display antigens that could activate antigen-presenting cells. In a transgenic mouse model of MTC, the Ret/Cal mice, in which the RET MEN 2A(C634R) transgene is specifically expressed in C-cells under the transcriptional control of the calcitonin promoter, the vaccination with autologous dendritic cells pulsed with a xenogenic calcitonin increased the number of cytotoxic calcitonin-specific T cells, thus decreasing tumor growth (Papewalis et al. 2008). RET itself might represent a good target for vaccination. In the MT/ret 304/B6 mouse model, spontaneous tumors develop due to overexpression of the RET gene. A RET peptide derived from the extracellular portion of the receptor, administered together with CpG oligonucleotides, was not effective in inducing anti-tumor immunity. However, when an inhibitor of the the insoleamidine 2,3-dioxxygenase (IDO) enzyme (1-methyltriptophan) was given together with the RET peptide (Zeng et al. 2009), effective anti-tumor
immunity was elicited. Interestingly, IDO expression can be increased by activated RET in a STAT1-dependent manner (Moretti et al. 2014). These data confirm that RET induces the expression of immunosuppressive molecules and that anti-cancer immunity can be elicited only when these molecules are blocked.

Conclusions

RET MEN2 mutant proteins are capable of eliciting a complex biological response, as a result of diverse signal transduction pathways downstream the activated receptor. It has been shown that, in MEN2, a strong genotype-phenotype association exists that correlates the intensity and the quality of RET activation with the severity of the disease. Data obtained by gene expression profiles identified signatures that could differentiate RET MEN2B mutants from those associated with MEN2A and FMTC syndromes. Among these genes, those associated with stroma remodeling and EMT, are preferentially observed in RET MEN2B mutants, whereas those associated with a Th1, cytotoxic immune response are enriched in RET MEN2A mutants (Fig. 2).

Patients with advanced MTC that cannot be cured by surgery had no therapeutic options until the emergence of targeted therapies. In the past 10 years, many TKI have been evaluated, including RET-blocking compounds (Plaza-Menacho et al. 2014, Viola et al. 2016). Based upon the preclinical data on MTC cell cultures, various clinical trials have been conducted that confirmed the efficacy of RET-targeting drugs. In 2011, vandetanib, the first RET TKI, was approved by the FDA for the therapy of advanced MTC. Later on, another TKI, cabozantinib, was approved, and many other inhibitors are being tested. Despite its efficacy, TKI treatment displays many limitations, including primary and secondary resistance, toxicities and side effects and the ability to induce a cytostatic rather than a cytotoxic effect. For these reasons, some patients must stop treatment (Valietto et al. 2017). Moreover, the cytostatic effect of the drug imposes that such therapy should be administered lifelong. In many tumor types, the combination of targeted therapy with other agents, including immunotherapy, has shown promising results in enhancing drug efficacy, overcoming resistance and reducing side effects (Keller et al. 2017). Thus, it is possible that RET-targeting TKI, together with immune checkpoint or IDO inhibitors, may represent a novel therapeutic option for patients with advanced and progressive MTC.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of this review.
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