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

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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).

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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 (Airaksinen & 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

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

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

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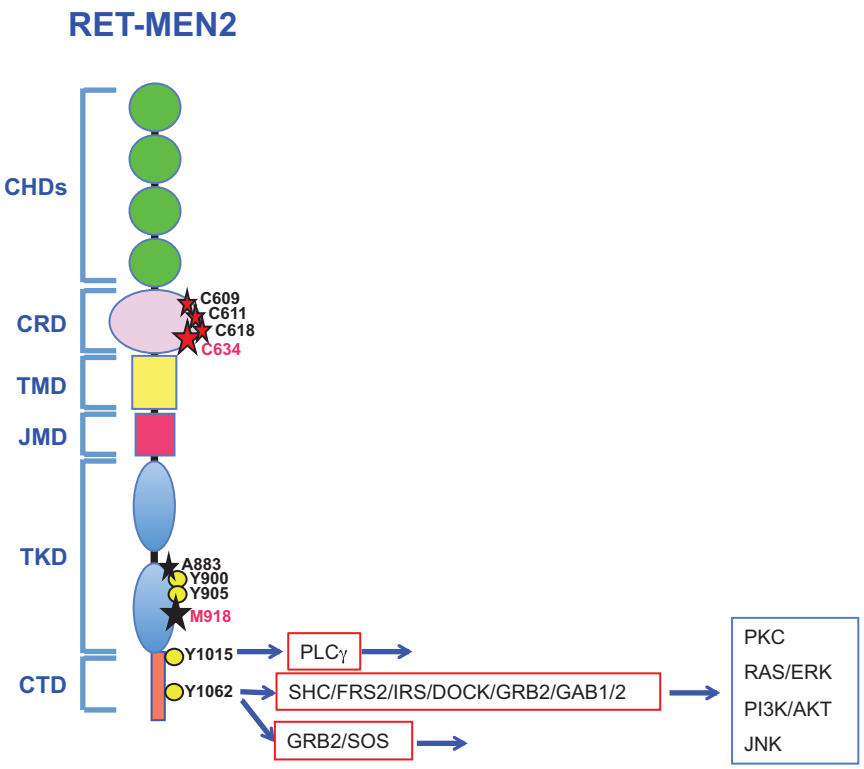

Figure 1
A representative scheme of *RET* receptor tyrosine kinase structure is depicted. The *RET* gene is located on chromosome 10q11.12. RET encodes for a receptor tyrosine kinase (RTK) with quite peculiar features: RET extracellular domain contains 4 cadherin-homology domains and a cysteine-rich region. Its tyrosine kinase domain contains two subdomains divided by a short kinase insert. Specific structural domains are indicated: CHD, cadherin-homology domain; CRD, cysteine-rich domain; CTD, carboxy-terminal domain; JMD, juxta-membrane domain; TKD, tyrosine kinase domain; TMD, trans-membrane domain. Selected heritable mutations identified in patients with MEN2A/FMTC (red stars) or MEN2B (black stars) are shown. Substitutions involving C634 and M918 (highlighted in red) are the most common mutations involved in MEN2A and MEN2B syndromes, respectively. RET tyrosines phosphorylated upon activation are indicated as yellow circles. Y900 and Y905 represent autocatalityc tyrosines, whereas Y1015 and Y1062 represent major docking sites for signaling proteins. Y1015- and Y1062-binding proteins are indicated in red boxes, and the activated downstream signaling pathways are indicated in blue boxes.

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 thyroidectomy 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).

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Table 2 The principal RET mutations in MTC are reported.

RET domain	Codons	Phenotype	Effects
Extracellular domain	G321	MEN2A/FMTC	Altered folding and protein maturation; ligand-independent constitutive dimerization; formation of disulfide bridges between monomers; activation of the kinase
	G533		
	K603		
	C609		
	C611		
	C618		
	C620		
	C630		
	C634		
Intracellular domain	L790	MEN2A	Ligand-independent dimerization; enhanced phosphorylation of intracellular substrates; increased STAT3 phosphorylation
	Y791		
	E768	FMTC	Facilitated transition to active conformation
	V804	FMTC	Improved access to ATP binding site
	S891	MEN2A/FMTC	Altered conformation of activation loop; activation of monomeric RET; STAT3 phosphorylation
	A883	MEN2B	Promotion of protein active form
	M918	MEN2B/sporadic MTC	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

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*. 2010*a*,*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 desmoplasia 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

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(Bhowmick *et al*. 2004, Desmouliere *et al*. 2004, Mueller & Fusenig 2004, Orimo *et al*. 2005).

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 immunohistochemistry (IHC) in many human TC samples, including MTC, but not in benign thyroid tumors or normal thyroid tissues

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(Yuan *et al*. 2004, Gonzalez *et al*. 2009, Torregrossa *et al*. 2012, Shin *et al*. 2013, Wang *et al*. 2013, Rossi *et al*. 2015, Zhu *et al*. 2016, Kim *et al*. 2017), suggesting an association with invasiveness/aggressiveness. Moreover, nuclear enrichment of STAT3 and elevated expression of CXCR4 have been detected in metastatic MEN2B MTC patients (Yuan *et al*. 2004).

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-1α$^{\Phi}$020, are proangiogenic, and CXCR4 can be expressed on vascular endothelium, thus promoting vessel sprouting (Chatterjee *et al*. 2014*a*,*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 factor β (TGF-β) pathway's components) were preferentially increased in RET MEN2B MTC in comparison to MEN 2A (Jain *et al*. 2004*a*).

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*. 2004*b*, 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*. 2004*b*, 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 cytolitic 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+

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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, Broutin and coworkers, identified IL8 as a potential soluble biomarker of therapeutic response to sunitinib in MTC patients (Broutin *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

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- 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 gene 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 (Engelmann *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 cytolitic 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 (Engelmann *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 cytolitic 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.

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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 pro-tumorigenic 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 indoleamine 2,3-dioxygenase (IDO) enzyme (1-methyltriptophan) was given together with the RET peptide (Zeng *et al*. 2009), effective anti-tumor

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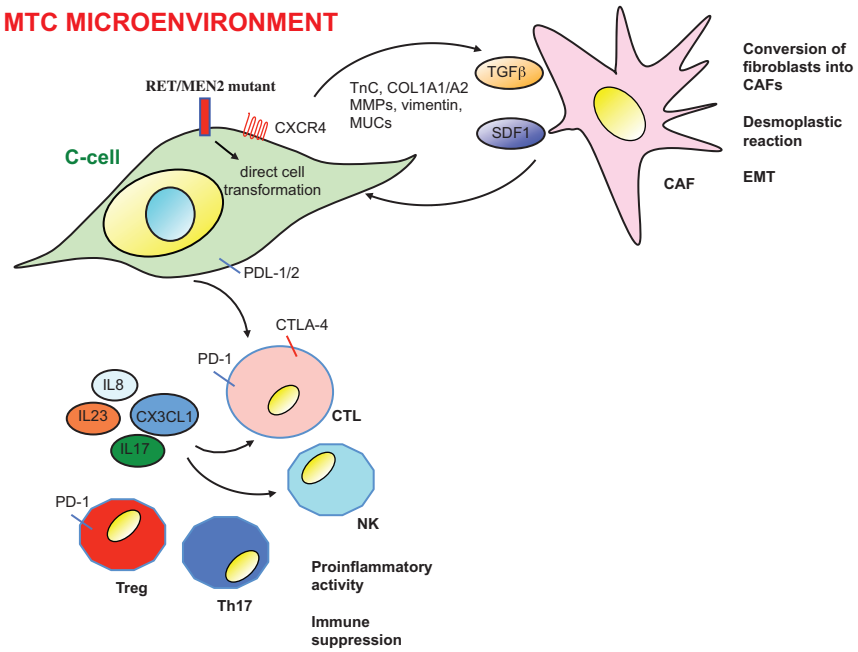

Figure 2
RET MEN2-associated mutants shape tumor microenvironment in MTC. Activation of RET in MEN2 results not only in the oncogenic conversion of the C-cell, but also in the production of factors that influence tumor microenvironment. RET-transformed C-cells can produce Tenascin C (TnC), collagens (COL1A1/2), vimentin, matrix metalloproteases (MMps) and cytokines (SDF-1, TGFβ). These factors induce fibroblast conversion into myofibroblasts, also defined as cancer-activated fibroblasts (CAFs), desmoplastic reaction and the epithelial-to-mesenchymal transition (EMT). Activated RET in the context of MEN2-associarted MTC can induce the production of immune-inflammatory molecules that can recruit and activate immune cells. CX3CL1 is induced by RET MEN2A and is involved in the recruitment of NK and CTLs. Signaling pathways activated by RET MEN2B can induce immunosuppression by recruiting Tregs and by the expression of immunomodulatory molecules, including PD L1/2. IL23 and IL17 are expressed in MTCs and can be responsible for Th17 presence and activity into tumor site. Some factors, including osteopontin (OPN), IL8 and stromal cell-derived growth factor 1 (SDF1), can increase the malignant potential of MTC cells by autocrine activation of cognate receptors.

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 (Valerio *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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