Molecular genetics of medullary thyroid carcinoma: multistep tumorigenesis

Moleculaire genetica van medullaire schildkliercarcinomen: meerstaps tumorontwikkeling
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 1 april 2008 des ochtends te 10.30 uur

door

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geboren op 3 oktober 1980 te Capelle aan den IJssel

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Printed by: Optima Grafische Communicatie, Rotterdam
ISBN: 978-90-8559-369-0

The research presented in this thesis was financially supported by the Dutch Cancer Society (UU2712). The printing of this thesis was financially supported by the Dutch Cancer Society and Siemens Medical Solutions Diagnostics.

“Vreemd dat je kunt kiezen voor alles wat je wilt,
maar kiezen kun je nooit voor die ene man en vrouw,
dat maakt ze zo bijzonder, die kozen voor jou.” (Harry Jekkers)

Voor mijn ouders

ABBREVIATIONS

APC, adenomatous polyposis coli
AR, ankyrin repeat
ARTN, artemin
ATC, anaplastic thyroid carcinoma
CCH, C-cell hyperplasia
CCKBR, gastrin/cholecystokinin B receptor
CDK, cyclin dependent kinase
CEA, carcino-embryonic antigen
CT, calcitonin
DOPA, dihydroxyphenylalanine
FDG, fluorodeoxyglucose
FMTC, familial medullary thyroid carcinoma
FTC, follicular thyroid carcinoma
GDNF, glial cell-line derived neurotrophic factor
GFRα, GDNF family receptor alpha
HPT, hyperparathyroidism
INK4, inhibitors of CDK4
IS, immunoscintigraphy
LOH, loss of heterozygousity
MEN, multiple endocrine neoplasia
MTC, medullary thyroid carcinoma
NRTN, neurturin
NSAIDs, non-steroidal anti-inflammatory drugs
PC, pheochromocytoma
PET, positron emission tomography
PSPN, persephin
PTC, papillary thyroid carcinoma
RET, rearranged during transfection
SNP, single nucleotide polymorphism
SST, somatostatin
SSTR, SST receptor
SVSC, selective venous sampling catheterisation
TK, tyrosine kinase
TKI, TK inhibitor

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CHAPTER 1

Introduction:
Medullary thyroid carcinoma development, a multistep process

To be submitted in altered form

1.1. Multistep MTC development

1.1.1. Thyroid gland and cancer

The thyroid gland consists of two lobes, connected by the isthmus, lying on either side of the ventral aspect of the trachea. The thyroid gland consists of two types of cells (Table 1). First, the epithelial follicular cells, arranged in spherical groups around a protein-rich colloid, forming a functional unit of the thyroid gland, i.e. the follicle (Figure 1). Thyroid follicular cells actively take up iodide, which is oxidized to active iodine and incorporated into tyrosine residues of thyroglobulin. The combination of two iodinated tyrosine residues is required for the synthesis of the thyroid hormones tetra-iodothyronine or thyroxine (T_4) and subsequently tri-iodothyronine (T_3), which are released into the peripheral blood stream. In the liver and kidney, T_4 is further converted to T_3, the more biologically active hormone. The thyroid hormones activate transcription by binding to nuclear steroid hormone receptors, regulating protein synthesis and metabolism in many different organs (1).

Second, about 0.1-0.001% of all thyroid cells are the neuroendocrine C-cells, embryonically derived from the neural crest. Primordial cells from the neural crest migrate ventrally and become incorporated within the last pharyngeal pouch, where they move caudally to the future thyroid gland. Next, the cells are incorporated near the hilus of the thyroid gland and subsequently distributed throughout the gland. In the adult mammalian thyroid, C-cells are situated within the follicular wall immediately beneath the basement membrane or between the follicular cells (Figure 1). When, stimulated by calcium, they produce and secrete the polypeptide hormone calcitonin (CT), a 32 amino acids long protein, which inhibits resorption of intact bone by inhibiting osteoclast secretory activity (1).

Thyroid cancer can originate from both types of thyroid cells. The vast majority of thyroid cancers originate from the follicular cells; papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC). About 5-10% of thyroid cancer is medullary thyroid carcinoma (MTC), which originates from the C-cells. The estimated number of newly diagnosed cases of thyroid cancer in the United States in 2007 is 33,550 (2). In 2004, 17,300 new cases of thyroid cancer have been registered in the European Union (3). The Netherlands registered 373 new cases of thyroid cancer in 2003 (4). Follicular types of thyroid cancer occur three times more frequently in women than in men, while the incidence of MTC is not different for men and women.

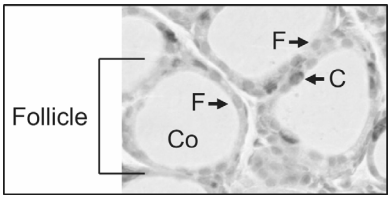

Figure 1. Follicles of a mouse thyroid gland.
F, follicular cells; C, C-cells; Co, colloid.

Table 1. Thyroid cell characteristics.

Characteristics	Follicular cell	C-cell
Embryonic lineage	Endodermal	Ectodermal
Cell type	Epithelial	Neuroendocrine
Hormone production	T_4, T_3	Calcitonin
Tumor development	PTC, FTC, ATC	MTC

1.1.2. Medullary thyroid carcinoma

In 1959, MTC was first described as a clinicopathologic entity (6). Several years later, in 1966, MTC was suggested to be derived from the thyroid C-cells (7). In 1968 and 1969, it was shown that CT was present in MTCs and elevated plasma CT levels were detected in MTC patients (8, 9). MTC is usually a well-defined lesion with a slightly lobulated outer contour and a greyish-white or tan appearance. MTCs are highly vascularized and the presence of amyloid deposits is typical (5). MTCs are known to spread to lymph nodes in the neck and mediastinum at early stages, and eventually also to distant sites, like bone, liver and lung.

Classification of MTC is based on the pathological Tumor, Node, Metastases system (pTNM) and is also referred to as stage I (tumor less than 2 cm in diameter without evidence of disease outside of the thyroid gland), stage II (any tumor between 2 and 4 cm without evidence of extrathyroidal disease), stage III (any tumor greater than 4 cm, or level VI nodal metastases or microscopic extrathyroidal invasion regardless of tumor size) and stage IV (any distant metastases, or lymph node involvement outside of level VI, or gross soft tissue extension) (10) (Table 2).

Sporadic MTC patients, are diagnosed by physical examination, imaging techniques and measurements of elevated plasma CT with or without elevated levels of carcino-embryonic antigen (CEA), a neuroendocrine tumor marker. In these patients, nodal metastases are present at the time of diagnosis in over 50% of cases (11). The survival of MTC patients strongly correlates with stage at diagnosis: the 10-year overall survival rate of MTC patients in stage I and II is 90-100%, while it is 55-85% for patients in stage III and 20-55% for stage IV patients (12-15).

The appropriate initial treatment for patients who are diagnosed with MTC is total thyroidectomy and careful lymph node dissection of the central compartment of the neck (11). Measurement of postoperative plasma CT levels is a sensitive method to determine whether the operation has been curative (16, 17). In contrast to the thyroid follicular cells, C-cells do not take up and store iodine. For this reason, in contrast to papillary and follicular thyroid carcinoma, additional treatment with radioactive iodine is not effective for MTC (18). Furthermore, chemotherapy and radiotherapy are usually ineffective for MTC (13, 19). An effective systemic treatment in addition to surgery is currently not available for MTC patients.

Table 2. Classification of MTC.

Stage	T	N	M
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1a	M0
	T2	N1a	M0
	T3	N1a	M0
Stage IVA	T4a	N0	M0
	T4a	N1a	M0
	T1	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
Stage IVB	T4b	AnyN	M0
Stage IVC	AnyT	AnyN	M1

T0, no evidence of primary tumor; T1, tumor ≤ 2cm limited to the thyroid; T2, tumor > 2cm but ≤ 4cm limited to the thyroid; T3, tumor > 4cm limited to the thyroid or any tumor with minimal extrathyroid extension; T4a, tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve; T4b, tumor invades prevertebral fascia or encases carotid artery or mediastinal vessels; N0, no regional lymph node metastasis; Nla, regional lymph node metastasis to level VI (pretracheal, paratracheal, and prelaryngeal/Delphian lymph nodes); N1b, regional lymph node metastasis to unilateral, bilateral, or contralateral cervical or superior mediastinal lymph nodes; M0, no distant metastasis; Ml, distant metastasis (20).

1.1.3. Multiple endocrine neoplasia type 2

About 40% of MTC cases occur as the most important clinical manifestation of multiple endocrine neoplasia type 2 (MEN 2). MEN 2 is an autosomal dominantly inherited cancer syndrome with a penetrance for MTC of nearly 100% (Figure 2). MEN 2 is divided into three subtypes MEN 2A, MEN 2B and familial MTC (FMTC), all characterized by development of MTC. In addition, MEN 2A patients may develop pheochromocytoma (PC), a neuroendocrine tumor of the adrenal medulla, and hyperparathyroidism (HPT). MEN 2B patients may also develop PCs, and intestinal and mucosal ganglioneuromatosis in addition to MTC. MEN 2B patients have a characteristic marfanoid habitus. In some cases, MEN 2A and FMTC patients suffer from Hirschsprung disease (22).

In 1993, activating missense mutations in the *RET* (REarranged during Transfection) proto-oncogene, on chromosome 10q11.2, were shown to constitute the genetic basis for MEN 2A (23, 24). One year later, a specific germline *RET* mutation, resulting in a methionine to threonine substitution at position 918 in the RET protein, was identified in MEN 2B families, and as a somatic mutation in sporadic MTC (25-27). These mutations lead to constitutive activation of the receptor tyrosine kinase RET (28, 29), which activates several downstream signaling pathways like the RAS/ERK and PI3K/PKB signaling cascades, regulating cell proliferation, differentiation and survival (30, 31).

Identification of germline *RET* mutation carriers by presymptomatic screening allows for prophylactic treatment. Genotype-phenotype correlation studies have shown that specific *RET* mutations are associated with age at first diagnosis and tumor aggressiveness, according to which MEN 2 patients can be stratified into three risk groups (22, 32, 33). Timing of genetic screening

and treatment for MEN 2-associated tumors varies between these three risk groups (34, 35). Risk group 1 (least high risk) is recommended for prophylactic thyroidectomy at the age of 5 to 10 years, risk group 2 (high risk) should be operated on before the age of 5, and risk group 3 (highest risk) is treated in the first year of life, however, the timing of central lymph node dissection is still a matter of considerable debate (22, 36, 37). After prophylactic surgery, *RET* mutation carriers are recommended for life-long biochemical follow-up for metastatic MTC as well as for development of PC and/or HPT (22).

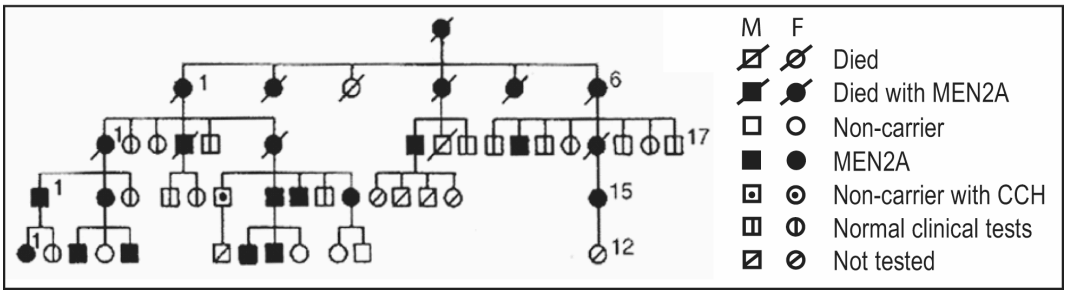

Figure 2. Pedigree of a MEN 2A family showing an autosomal dominant inheritance pattern. (adapted from (21)). Reprinted with permission from the Massachusetts Medical Society.

1.1.4. Molecular genetics of cancer, a multistep process

Tumorigenesis is induced by genetic alterations, affecting genes that control, amongst other processes, cell proliferation, differentiation and survival. These genetic alterations can occur at the level of chromosomes, via deletion or amplification of an entire chromosome (arm) or a specific part of a chromosome, or via chromosomal translocations. Alterations can also occur at the level of individual genes, via sequence variations (mutations and polymorphisms) or promoter hypermethylation, or at the level of gene expression.

Tumorigenesis is a process of accumulating genetic alterations, eventually leading to an imbalance between cell division and cell death signals (38). This multistep process is best described for colon cancer. Normal epithelial cells of colon crypts transform into neoplastic cells following initial alterations on chromosome 5/loss or mutation of *APC*. Subsequent activation of *KRAS*, LOH of chromosome 18q and LOH of chromosome 17p/mutations in *P53* result in further progression to adenoma and finally carcinoma formation (38, 39) (Figure 3).

This complex process takes several years to decades. Therefore, cancer is called a ‘disease of the elderly’. However, some types of cancer, like leukemia, lymphomas or brain tumors, occur relatively frequently in children or adolescents. In hereditary forms of cancer, germline mutation carriers are born with a genetic defect present in every cell of their body. The first oncogenic event

is already present, causing a dramatic acceleration of cancer development. Therefore, familial cancer is generally diagnosed at significantly earlier ages compared to non-familial (sporadic) cancers.

The ability to generate transgenic mice and knockout mice has made it possible to determine which oncogenes and/or tumorsuppressor genes collaborate in tumor formation. An early study showed that mice expressing cMyc or Hras in mammary gland tissue developed breast carcinomas with a relatively long latency period and at low frequency. Double-transgenic mice expressing both *cMyc* and *Hras* transgenes developed breast tumors at a greatly accelerated rate and at much higher frequency compared to both single-transgenic strains (Figure 4) (40).

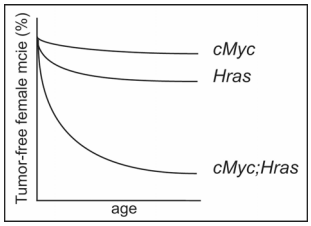

Figure 4. Oncogene collaboration in mammary carcinoma development in double-transgenic mice. Mammary carcinomas are developed in a higher frequency and with an earlier age-of-onset in *cMyc;Hras* mice compared to both single transgenic strains, indicating collaboration of *cMyc* and *Hras* in mammary carcinoma development (adapted from (40)).

1.1.5. Multistep tumorigenesis of MTC

The tumorigenesis of MTC is also considered to be a multistep process. It is thought, at least for the familial forms, that MTC is developed from C-cells via C-cell hyperplasia (CCH) (Figure 5). Two distinct pathological features of CCH are described: physiologic (also reactive) CCH and neoplastic CCH. Perry *et al*., described these two types as morphologically distinct entities. Histologically, C-cells in physiologic CCH can only be detected by anti-CT immuno-histochemistry, while in most of the neoplastic CCH cases, C-cells can be identified in conventional hematoxylin eosine stainings, showing a large and mildly to moderately atypical morphology (41). No clear correlation between C-cell number or hypercalcitonemia with the type of CCH has been observed (41, 42). Physiologic CCH is associated with hypercalcemia, hyperparathyroidism, chronic lymphocytic thyroiditis and follicular thyroid tumors, and might progress into MTC, however its malignant potential is not yet documented (43).

In MEN 2 families, neoplastic CCH can be detected preoperatively in non-symptomatic persons, which are identified by genetic testing. Therefore it is suggested that neoplastic CCH is induced by germline *RET* mutations (43). Although neoplastic CCH is detected in a small subset of sporadic MTC patients, identified by plasma CT measurements, the malignant potential is unclear (44, 45). Normally, CCH is seen more often in men than in women. Not all CCH detected in men is neoplastic (about 20%), whereas it is suggested that the MTC risk in women with CCH is about approaches 100% (42).

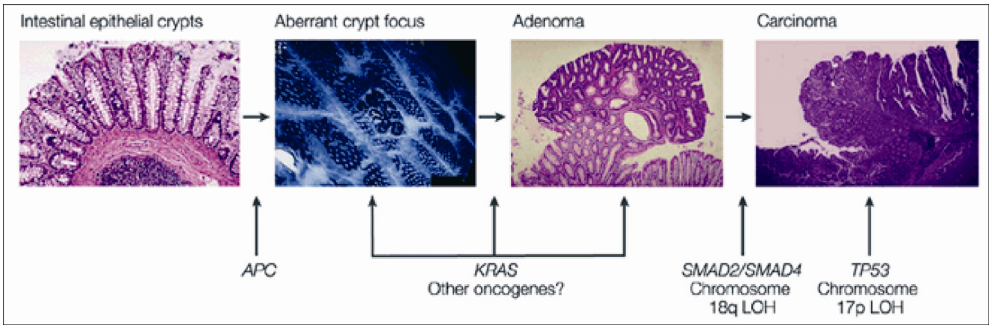

Figure 3. The multistep tumorigenesis of human colorectal cancer. Initial oncogenic events in *APC* induce formation of aberrant crypts. Alteration in *KRAS*, *SMADs*, *P53* and other genes result in progression to colon adenoma and subsequently carcinoma (adapted from (39)). Reprinted with permission from R. Fodde.

The time line of progression from CCH to invasive MTC depends on the *RET* mutation (43). MEN 2 patients, especially MEN 2B patients harboring the M918T mutation, develop MTC as early as the first year of life. However, other MEN 2 patients, harboring *RET* mutations in e.g. codon 618 or 804, generally develop MTC later in life (35). These correlations indicate that the level of RET activation determines the MEN 2 phenotype and that activation of RET might be sufficient for MTC formation in patients expressing a RET mutant with high transforming activity. This is supported by the relatively small amount of chromosomal alterations detected in hereditary forms of MTC (46, 47). Nevertheless, in MEN 2 families in which members carry the same *RET* mutation, the age at diagnosis can vary from younger than 10 years to older than 65 years (48, 49), suggesting that other genetic events are involved in hereditary MTC development.

Although somatic *RET* mutations can be detected in a subset of sporadic MTC cases, the genetic mechanisms underlying sporadic MTC tumorigenesis are largely unknown. Several chromosomal alterations have been detected in sporadic MTCs, more frequently than in familial MTCs, suggesting involvement of several genes (46, 47).

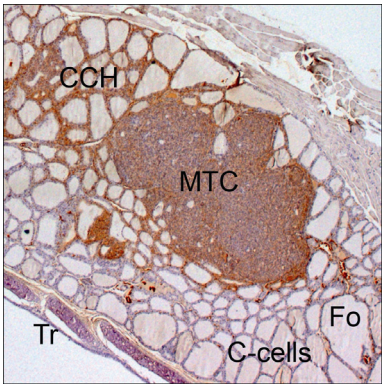

Figure 5. Mouse thyroid gland showing multistep MTC tumorigenesis. Nuclei are stained with haematoxylin (purple), C-cells are stained for CT expression (brown). Fo, follicle; Tr, trachea.

1.2. Molecular alterations during MTC tumorigenesis

1.2.1. Chromosomal alterations in hereditary and sporadic MTC

Before the discovery that mutations in the proto-oncogene *RET* were responsible for the development of MEN 2-associated MTC, the genetic disease cause was linked to chromosome 10 (50). However, loss of chromosome 10 polymorphic DNA markers was not frequently detected in DNA from MTC samples (46, 47, 51, 52), suggesting that a genetic mechanism other than loss of a tumor suppressor gene on chromosome 10, was involved in MEN 2 development. In PTC, a follicular cell-derived thyroid carcinoma, chromosomal rearrangements involving *RET* on chromosome 10q11.2 are involved, resulting in RET/PTC fusion proteins (53). Such translocations are not detected in MTC. In 1993, it was discovered that activation of the proto-oncogene *RET*, by germline mutations, actually caused MEN 2 (23, 24).

Several studies have revealed that MTCs are genetically stable tumors compared to other types of cancer: allelic loss was detected for 4.8% (22/462) of informative chromosome arms in MTC, compared to 14.4% (56/388) in PC (47). Another study revealed similar results, 4.7% (4/86) in MTC compared to 16.1% (42/261) in PC (52), suggesting that relatively few and very specific alterations are involved in MTC tumorigenesis. In 60-65% of MTCs, chromosomal alterations are detected, of which losses on chromosome arms 1p, 3q, 13q and 22q, and gains on chromosome 19 are the most commonly detected alterations (Table 3). Interestingly, loss of 3q, 13q and 22q and gain of 19 is detected more often in sporadic MTC compared to MEN 2-associated MTC (46, 47), suggesting partly different genetic mechanisms in these two types of MTC. However, loss of

Table 3. Most frequently detected chromosomal alterations in hereditary and sporadic MTC

Ref.	**(52)**	**(47)**		**(54)**		**(46)**		**Total**	
Tech.	**LOH**	**LOH**		**CGH**		**CGH**			
	Spor	Spor	MEN 2	Spor	MEN 2	Spor	MEN 2	Spor	MEN 2
	n=10	n=10	n=19	n=21	n=3	n=29	n=9	n=70	n=31
-1p	10%	10%	33%	9%	0%	21%	11%	14%	23%
	(1/10)	(1/10)	(6/18)	(2/21)	(0/3)	(6/29)	(1/9)	(10/70)	(7/30)
-3q	0%	40%	0%	9%	33%	28%	11%	18%	10%
	(0/10)	(2/5)	(0/9)	(2/21)	(1/3)	(8/29)	(1/9)	(12/65)	(2/21)
-13q	0%	11%	8%	19%	33%	24%	0%	17%	8%
	(0/10)	(1/9)	(1/13)	(4/21)	(1/3)	(7/29)	(0/9)	(12/69)	(2/25)
-22q	20%	38%	7%	0%	0%	17%	0%	15%	4%
	(2/10)	(3/8)	(1/14)	(0/21)	(0/3)	(5/29)	(0/9)	(10/68)	(1/26)
+19	n/a	n/a	n/a	33%	33%	14%	0%	22%	8%
				(7/21)	(1/3)	(4/29)	(0/9)	(11/50)	(1/12)

CGH, comparative genomic hybridization; LOH, loss of heterozygosity; n/a, not applicable; Ref, reference; Spor, sporadic; Tech, technique.

chromosome 1p is detected frequently in both sporadic and hereditary MTC, suggesting that tumor suppressor genes located at this chromosomal region, are generally involved in the tumorigenesis of MTC. Together, these data indicate that in addition to RET activation, other genes are involved in MTC tumorigenesis, and probably even more oncogenic events are required for sporadic MTC development.

1.2.2. Germline *RET* mutations in hereditary MTC

As described in chapter 1.1.3. germline *RET* mutations constitute the genetic basis of MEN 2 (Figure 6). In MEN 2A patients, *RET* mutations are predominantly present in exon 10 and 11, affecting cysteine residues in the extracellular domain of the protein. The most commonly affected cysteine is C634, but also C609, C611, C618, C620 and C630, and non-cysteine residues at other codons are affected by mutations in MEN 2A. In MEN 2B, the most common mutation (over 95% of cases) is present in exon 16, resulting in a methionine to threonine substitution at position 918 in the intracellular tyrosine kinase domain of the RET protein. In addition, a germline A883F mutation has been detected in a small subset of MEN 2B cases. Most of the MEN 2A mutations have also been detected in cases of FMTC, however, some germline *RET* mutations have only been associated with FMTC without clinical presentation of PC. The two MEN 2B mutations have not been detected as germline mutations in cases of MEN 2A or FMTC (35, 55, 56). Interestingly, some patients harboring germline *RET* mutations at codons 609, 611, 618 or 620, suffer from MEN 2A/FMTC in combination with Hirschsprung disease, normally associated with inactivation of RET (57, 58).

Normally, cysteine residues in the extra-cellular domain of RET are involved in intramolecular bonding. In MEN 2A-RET, substitution of a cysteine residue results in a free cysteine which can interact with a free cysteine in other MEN 2A-RET molecules, leading to ligand-independent dimerization, resulting in constitutive autophosphorylation and activation of the receptor. On the other hand, MEN 2B-RET functions as a monomer, and is thought to be activated by a conformational change in the intracellular tyrosine kinase domain (28, 29). The RET mutants have different levels of transforming activity (55), and/or differences in substrate specificity resulting in activation of different signaling pathways (31, 59, 60), which might explain the genotype-phenotype correlations detected for familial MTC.

Several studies show that M918T-RET has the highest transforming activity, however, it can be even further increased by introducing a second mutation, C634R, or by adding RET ligand (61). Although it is rare, some MEN 2 patients present with multiple germline *RET* mutations (62-65). Also, cosegregation of certain germline *RET* mutations with particular *RET* single nucleotide polymorphisms (SNPs) has been detected in FMTC families (66, 67). Cosegregation of two exonic *RET* SNPs, G691S and S904S, was strongly associated with early age-of-onset in a study of 4

Spanish MEN 2A families, which was later confirmed in a larger study of 35 Spanish MEN 2A families (68, 69). Yet, in another large study of 177 families (80 from the UK, 44 from Spain and 53 from France) this modifying effect on age-of-onset could not be confirmed (70), revealing that the modifying effect of these cosegregating *RET* SNPs on MEN 2-associatied MTC development remains unclear.

In MTCs of some MEN 2A/FMTC patients, somatic *RET* mutations could be detected in addition to the germline *RET* mutation (64, 71-73). For one such double mutant, it was shown that the level of activity was significantly higher compared to that of RET affected by either of the two mutations alone (74). Moreover, allelic imbalance of the mutant and wildtype *RET* allele has been shown for MEN 2A-associated MTCs (75). This might suggest that one *RET* mutation initiates MTC tumorigenesis and secondary germline or somatic *RET* alterations are required to obtain a dominant dose of RET activation required for MTC development.

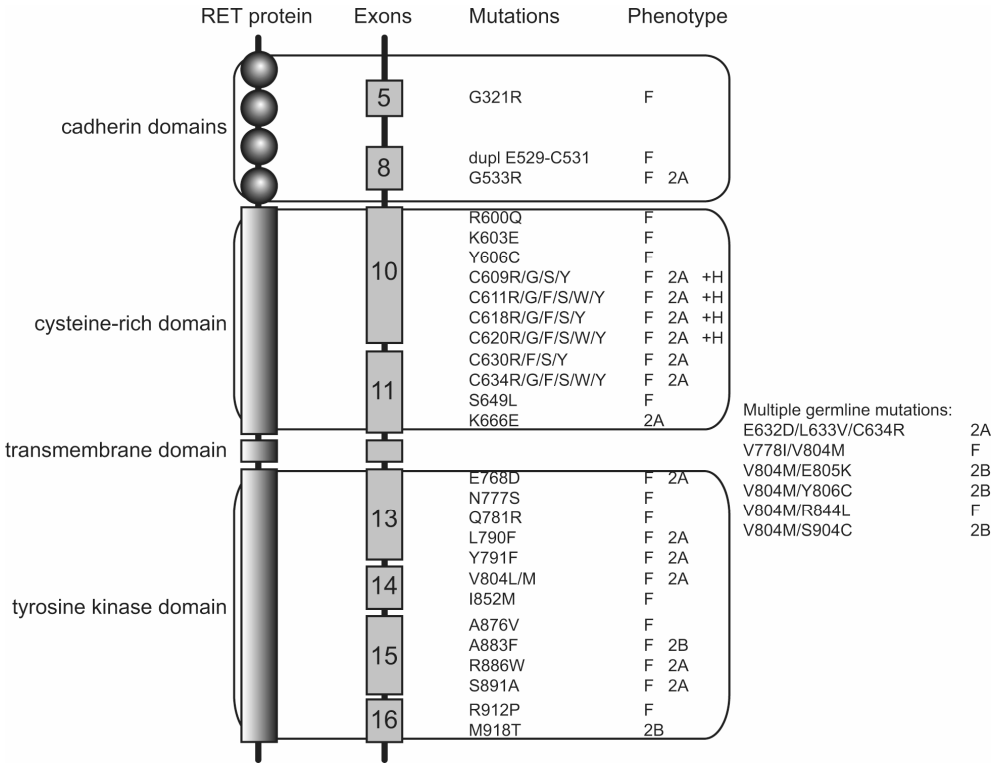

Figure 6. Germline *RET* mutations associated with hereditary MTC. RET consists of four extracellular cadherin domains and an extracellular cysteine-rich domain, a transmembrane domain and an intracellular tyrosine kinase domain. FMTC mutations are predominantly located in the cysteine-rich and tyrosine kinase domain. MEN 2A mutations are predominantly located in the cysteine-rich domain and MEN 2B mutations are located in the tyrosine kinase domain. dupl., duplication; F, FMTC; 2A, MEN 2A; 2B, MEN 2B; +H, MEN 2A/FMTC + Hirschsprung disease. (adapted from (35, 55, 56)).

1.2.3. Somatic *RET* mutations and *RET* polymorphisms in sporadic MTC

In 1994, the M918T mutation, also detected in the germline of MEN 2B patients, was detected as a somatic *RET* mutation in sporadic MTCs (26, 27). Somatic *RET* mutations have been detected in about 23-69% of sporadic MTCs (76). The most common mutation is the M918T mutation, but missense mutations affecting other codons, and double or triple somatic *RET* mutations have occasionally been detected (56, 64, 76-78). As in MEN 2A-associated MTCs, allelic loss of *RET* has been detected in combination with somatic *RET* mutations in some cases of sporadic MTC (64, 79, 80). Furthermore, in addition to single base pair point mutations, individual cases of small deletions in *RET* have been reported in sporadic MTC (56).

Eng *et al*. showed that somatic *RET* mutations are not homogeneously expressed throughout the tumor (71). Furthermore, the absence of a somatic *RET* mutation in the primary tumor of a sporadic patient does not necessarily indicate that MTC metastases of the same patient are mutation-negative as well. Some patients have a mutation in only a subset of their metastases, and different *RET* mutations can be present in tumors from one sporadic MTC patient (64, 71). The heterogeneous expression of somatic *RET* mutations in sporadic MTC, suggests that a mutation of *RET* is not an early event in sporadic MTC tumorigenesis, but rather involved in MTC progression. Although some groups have not been able to detect a relation between the presence of a somatic *RET* mutation and tumor-phenotype (77, 79), others have shown that somatic RET activation in MTC correlates with a poor clinical outcome (increased recurrence and metastatic potential, and reduced survival) (81-85).

Some studies have revealed that some exonic *RET* SNPs, G691S (exon 11), L769L (exon 13), S836S (exon 14) and S904S (exon 15), as well as some intronic *RET* SNPs, IVS1-126G>T and IVS14-24, are overrepresented in sporadic MTC patients compared to controls, suggesting that they can modify the risk for sporadic MTC (66, 86-93). However, these associations could not be confirmed by others (66, 86-88, 90, 94-97). 100% cosegregation of G691S/S904S, as well as several other significant cosegregations, like S836S/IVS1-126G>T and IVS1-126G>T/IVS1-1463T>C, have been detected to be overrepresented in sporadic MTC cases (69, 90, 92). Because a significant association of some of these *RET* SNPs and cosegregations have been identified in only a subset of these studies, their roles in MTCs tumorigenesis remains to be determined.

1.2.4. Alterations in genes that are involved in RET activation

Normally, RET is activated by binding of a ligand-coreceptor complex (Figure 7). The family of RET-coreceptor ligands consists of glial cell-line derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN). The coreceptors, GDNF family receptor alpha (GFRα)-1, GFRα-2, GFRα-3 and GFRα-4, belong to a glycosyl-phosphatidyl inositol (GPI)-linked receptor family (98-100). Because RET activation is involved in MTC development, one could

expect that gain-of-function or overexpression of the genes encoding these proteins might be involved in MTC tumorigenesis. Therefore it is not unexpected that deficiencies of these genes in knockout mice did not result in MTC formation (101-111).

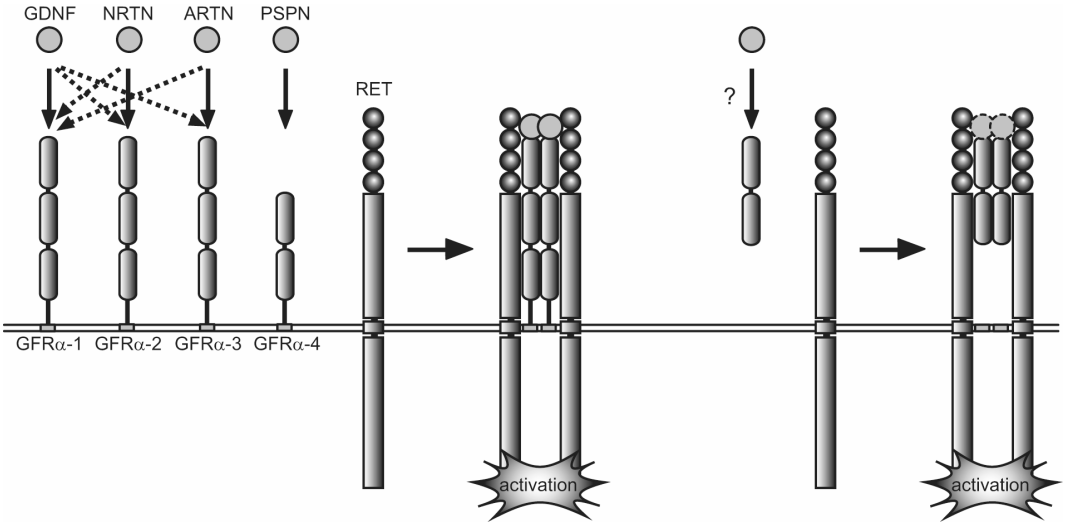

Figure 7. Ligand-coreceptor-mediated RET activation. GFRα-1, -2 and -3 have three cysteine-rich domains whereas GFRα-4 has only two. The GFRα receptors are distributed within lipid rafts (pink). Each ligand preferably binds to one of the GFRα receptors (continuous arrows), although some cross-reactivity has been detected (dotted arrows). So far, PSPN is the only known ligand for GFRα-4. Left panel shows activation via GPI-linked GFRα. Ligand-coreceptor complexes bind to RET, upon which RET dimerizes and is autophosphorylated. Right panel shows activation via soluble GFRα. For GFRα-4 it has been shown, in mice, that the soluble isoform is able to activate RET independent of its ligand PSPN. (adapted from (31, 35, 56, 116, 118, 119)).

Mutation analysis of 12 sporadic and 9 non-*RET* MEN 2-associated MTCs, did not reveal mutations in *GDNF* (112). Gimm *et al*. performed a mutation analysis of the *GFRα-1*, *-2* and *-3* genes in tumor and blood DNA from 31 German sporadic MTC patients. No somatic mutations could be identified, although a germline variant of *GFRα-1*, START-193bpC>G, was overrepresented in the sporadic patients group, compared to 31 healthy controls. Interestingly, immuno-histochemistry on the MTCs from patients harboring this SNP revealed overexpression of GFRα-1, which was localized in the cytoplasm in heterozygous cases but nuclear localization was detected in homozygous MTCs (113). In two other studies, an association of the *GFRα-1* SNP, START-193bpC>G, with sporadic MTC could not be identified, suggesting that the role of this SNP in MTC tumorigenesis remains to be determined (86, 114).

In a large UK study involving 135 sporadic patients and 533 controls, genes encoding all coreceptors and all ligands of RET were sequenced from these sporadic MTCs. A significantly

higher prevalence of the *GFRα-1*, STOP+946bpC>G, and *ARTN*, START-797bpA>T, SNPs was found in sporadic MTC patients compared to the control group (86). The role of these SNPs remains to be determined.

In situ hybridization analysis revealed that GDNF, NRTN, GFRα-1 and -2 were not detectable in normal thyroid tissue and a PTC sample. However, GDNF and GFRα-1 expression could be detected in 13 and 12 out of 15 sporadic MTCs, respectively. In 9 MTCs expression of NRTN and GFRα-2 was observed. Tumors with a high expression of RET also showed high levels of ligand-coreceptor expression, suggesting autocrine stimulation of RET in MTCs (115). Lindahl *et al*. screened various human tissues for GFRα-4 expression and detected expression specifically in thyroid gland tissue. Among different thyroid tumors, MTCs, PTCs and FTCs, GFRα-4 was only expressed by MTCs, indicating that this coreceptor is specifically expressed by thyroid C-cells (116).

Interestingly, a *GFRα-4* sequence variation, START-52pbC>T, which might alter the expression of GFRα-4, has been detected in a MEN 2A patient (117). In addition, a 7bp insertion which may change the balance between GPI-linked and soluble isoforms, has been detected in a MEN 2 family affected by the germline V804L *RET* mutation (117). Recently, it has been shown that the mouse soluble form of GFRα-4 signals independently from PSPN, suggesting that this *GFRα-4* variant could be a modifier of MEN 2 (118).

1.2.5. Alterations in genes that are involved in PC development

MEN 2 is characterized by the development of MTC in combination with PC in about 50% of the cases. Both tumors are derived from cells of neuroendocrine origin. Interestingly, of the most common chromosomal losses detected in MTC, loss of chromosomes 1p, 3q and 22q, is also frequently detected in both sporadic and hereditary PC (120), suggesting an overlap in genes that are involved in the development of these neuroendocrine tumors. Germline mutations in *RET*, *VHL*, *NF1*, *SDHD*, *SDHB* or *SDHC* increase the risk of developing diverse clinical syndromes such as MEN 2, von Hippel-Lindau (VHL), neurofibromatosis type 1 (NF1), or hereditary paraganglioma-pheochromocytoma, respectively, all associated with the development of PCs (121, 122). In addition, these genes have been detected as somatically mutated in a small subset (<10%) of sporadic PC (120). Several studies have been performed to investigate whether these PC-inducing genes are involved in MTC development as well.

VHL is a dominantly inherited familial cancer syndrome caused by mutations in the VHL tumor suppressor gene. VHL is characterised by haemangioblastomas of the retina and central nervous system and clear cell renal cell carcinoma, but also PC. Germline *VHL* mutations may be detected in 5-11% of all PC cases (123). *VHL* is located on chromosome 3p26-p25. Losses of chromosome 3 or 3p are frequently detected in MTC (46, 47, 124). Although one study revealed no LOH at

3p25 in MTC (52), in another study LOH of *VHL* has been detected in 3 out of 6 MEN 2-associated MTCs, which also had allelic imbalance of *RET*. One of the three MTCs with LOH of *VHL*, also showed a somatic frameshift mutation in exon 1 in the remaining allele (125). In addition, MTC has been observed in members of two VHL-families (126), suggesting a role for *VHL* in MTC development.

NF1 is an autosomal dominantly inherited disorder characterized by the development of neurofibromas and gliomas, but also PCs. The *NF1*-gene is located on chromosome 17q11.2 and codes for Neurofibromin (127). In a 14-year old NF1 patient without a germline *RET* mutation, CCH was observed (128), whereas LOH of *NF1* could not be detected in 16 cases of CCH (129), suggesting that *NF1* is not likely to be involved in MTC tumorigensis. NF2, another autosomal dominantly inherited disorder characterized by schwannoma development, is caused by germline mutations in the *NF2*-gene located on 22q12.2 and encoding Merlin. These patients do not develop PCs (127). Loss of chromosome 22q is a frequent event in MTC (46, 47, 52) and LOH of *NF2* has been detected in 6 out of 8 MTCs (130), suggesting a role for *NF2* rather than *NF1* in MTC tumorigenesis.

Germline mutations in genes encoding the succinate dehydrogenase subunit (SDH) proteins have been shown to constitute the genetic basis for the hereditary paraganglioma-pheochromocytoma syndromes (131). Mutations in *SDHB* are associated with maligne pheochromocytomas, which are benign in the majority of cases (132). The *SDHD*, -*B* and -*C* genes, encoding subunits of the succinate-ubiquinone oxidoreductase complex (complex II of the mitochondrial respiratory chain), are located on chromosomes 11q23, 1p36.1-p35 and 1q23.3, respectively. Interestingly, loss of chromosome 1 or 1p is one of the most frequently detected chromosomal alterations in both familial and sporadic MTC (46, 47, 52). LOH spanning *SDHD* was shown in 14% (1/7) of familial MTCs and 0% (0/17) of sporadic MTCs, and of *SDHB* in 40% (4/10) of familial MTCs and 20% (4/20) of sporadic MTCs (133). No somatic mutations could be detected in the *SDHD*, -*B* or -*C* genes in 35 MTC cases (13 familial and 22 sporadic) (133). A germline variation in *SDHD*, H50R, was detected in a family with CCH without a germline *RET* mutation, as well as in 9% (1/11) of MEN 2 patients and 9% (2/22) of sporadic MTC patients (133, 134). Another study described lack of any *SDHD*, -*B* and -*C* alterations in two families with non-*RET* CCH. In three patients with the H50R variation, as well as in ten patients with an *SDHD*, -*B* or -*C* mutation, no CCH could be observed (135). The prevalence of this *SDHD* SNP was not significantly different in sporadic MTC patients (4.3%; 12 out of 277 Swiss, English and Spanish patients) compared to healthy individuals (3.1%; 29 out of 949 Swiss, English and Spanish persons), revealing no association of the H50R-*SDHD* variant with MTC development (133, 135). Other relatively frequently detected SNPs in MTC patients are G12S and S163P in *SDHD* and *SDHB*, respectively (133), however their roles in MTC tumorigenesis remains to be determined.

Together, these results suggest involvement of some of the PC-inducing genes in MTC tumorigenesis. However, the number of studies and the investigated sample sizes are usually low, indicating that the role of these genes in MTC tumorigenesis remains to be further investigated.

1.2.6. Alterations in well-known tumor suppressor genes and oncogenes

The *P53* tumor-suppressor gene is the most striking example of a cancer-related gene because it is somatically mutated in about 50% of almost all types of human cancer (136). Germline *P53* mutations are associated with the Li-Fraumeni syndrome, characterized by the development of sarcomas, adrenal cortical and brain carcinomas, and leukemia (137). *P53*, encoding a transcription factor involved in the regulation of the cell cycle, DNA repair, DNA synthesis, differentiation and apoptosis, is located on chromosome 17p13.1. Loss of the *P53*-locus occurs in 11% (9 out of 79) of human MTCs (47, 52, 130, 138, 139). In contrast to wildtype *P53*, which has characteristics of a recessive tumor suppressor gene, mutant forms can act as dominant oncogenes (136). Detection of P53 with immuno-histochemistry, indicate the presence of stabilized P53 for example by a somatic mutation. Although somatic *P53* mutations are rare in human MTC (4 out of 89) (138-144), nuclear expression is detected in 0-44% of the cases (on average 18%; 28 out of 152) (D.S. Acton, unpublished; (138, 144-147)). Overexpression of P53 seems to occur slightly more frequently in sporadic MTC, compared to hereditary MTC (D.S. Acton, unpublished; (144, 147)).

Several genes controlling the cell cycle G1/S phase transition (Figure 8), are involved in almost all human cancers, especially *RB* and *P16*. Somatic loss of *RB* is predominantly caused by deletions or mutations, and occurs at high frequencies in lung carcinomas (SCLC) and osteosarcomas (148). In addition, germline *RB* mutations predispose to retinoblastomas in children (149). Interestingly, in mice, germline *Rb* deficiency predisposes for MTC (150). *RB* is located on chromosome 13q14, a locus which is lost in about 10-25% of human MTCs (46, 47). In one study, LOH of the *RB* locus was detected in 6 out of 14 CCH cases (129). Despite the relatively frequent loss of the *RB* locus in human MTC, loss of RB expression could not be detected in any of 54 human MTCs (146, 151, 152).

Mutations, deletions or hypermethylation causes loss of *P16* and is found in high frequencies in pancreatic cancer, T cell ALL, glioblastomas, and lung and breast carcinomas (148). In addition, germline *P16* mutations predispose for familial melanoma (153). No homozygous deletions and only 1 case of LOH of the *P16* locus, at 9p21, has been detected in 54 human MTCs (130, 154, 155). In addition, no *P16* hypermethylation was observed in 11 MTCs (156, 157).

Interestingly, a sequence variant of P15, also located at 9p21, and a family member of *P16*, has been detected in 10 out of 31 MTCs (154). Recently, a large SNP analysis of 69 genes in 266 Spanish sporadic MTC patients and 422 controls, revealed association of 7 genes, including *P15*

(and *CDK6*), with sporadic MTC (158). In addition, decreased expression of *P27*, a cell cycle inhibitor of the CIP/KIP family, was observed in 38 out of 64 MTCs and correlated with tumor size and plasma CT level (159). Recently, it has been shown that oncogenic RET downregulates both *P18* and *P27* expression (160).

The proto-oncogene *cMYC*, located on chromosome 8q24.21, encodes a transcription factor that is implicated in cell growth, proliferation, differentiation and apoptosis. Deregulated (constitutive) expression of cMYC occurs in a broad range of human cancers and is often associated with poor prognosis, indicating a key role for this oncogene in tumor progression. The deregulated expression of cMYC is caused by translocation or amplification of the *cMYC*-locus, or by stabilizing mutations (161). NMYC, another member of the MYC family, located at 2p24.1, can also be overexpressed by amplification in human cancer, specifically of neural origin or with neural characteristics (162). No amplification or rearrangements of *cMYC* or *NMYC* could be detected in MTCs (163, 164). However, overexpression of cMYC and NMYC has been shown in 30-100% of human MTCs, while it is not detected in normal C-cells (147, 165-167). It has been shown that NMYC expression in MTC is associated with a reduced survival (163). A difference in expression of cMYC and NMYC between primary MTCs and MTC metastases could not be observed, suggesting that *MYC* is involved in early stages of MTC tumorigenesis (166).

Another family of proto-oncogenes is the RAS GTPase family. HRAS, KRAS and NRAS mediate signal transduction across the plasma membrane (168). *RAS* mutations are frequent oncogenic events in human cancer: the highest incidences are found in adenocarcinomas of the pancreas (90%), colon (50%) and lung (30%), in follicular thyroid carcinomas (50%), and in myeloid leukemia (30%) (169, 170). RAS is one of the main downstream target proteins of RET, making it a possible candidate to be involved in MTC tumorigenesis. Expression of RAS is observed in MTCs (166, 171), however mutations have not been detected (172-174).

Also, no amplifications or rearrangements of the *NRAS* locus could be detected in a small set of MTC samples (164). In contrast to the functions as oncogenes, some tumor suppressor roles have been described for *RAS* genes as well. Loss of *Nras* in *Rb*-deficient mice dramatically increases the metastatic potential of MTCs, indicating that loss of *NRAS* might be involved in MTC tumorigenesis (175). Interestingly, *NRAS* is located on chromosome 1p13.2, a region frequently deleted in human MTC (46, 47). Furthermore, *HRAS* has been identified in a large SNP analysis of 266 sporadic patients as being associated with sporadic MTC (158).

BRAF, encoding a cytoplasmic serine/threonine kinase downstream of RAS, is frequently mutated in human cancers like melanomas, but also in approximately 45% of PTCs (53, 176). Several mutation analyses have failed to detect *BRAF* mutations in human MTCs (177-179).

Like RET, the ERBB family of epidermal growth factor receptors, ERBB1 (or EGFR), ERBB2, ERBB3 and ERBB4, are transmembrane receptor tyrosine kinases. Altered expression or activation of these receptors, particularly EGFR and ERBB2, is a common event in human

epithelial cancers, and correlates with more aggressive disease and poor clinical outcome. Many ERBB-inhibitors have been developed and some are clinically used as anti-cancer drugs (180). The overexpression of ERBB receptors in tumors is usually caused by gene amplifications. The constitutive activation is caused by somatic mutations or by ligands present in the tumor or surrounding stromal cells. In almost all MTCs, although not of epithelial origin, EGFR and ERBB2 expression can be detected, usually at low levels (165, 181-185). Amplifications or rearrangements of *EGFR* have not been detected in 7 MTCs (164), and mutations analyses have not yet been reported. ERBB2 was expressed in all C-cells in CCH, although a more sporadic staining has been observed in MTCs (184). Interestingly, like in other carcinomas, cytoplasmic ERBB2 expression could be detected in a subset of MTCs, however the meaning of this cytoplasmic expression is not yet understood (183, 185).

The PI3K/PKB pathway is one of the main downstream signaling pathways of RET, making these genes possible candidates to be involved in MTC tumorigenesis. No mutations in PI3K or amplification of its locus could be detected in 13 and 14 MTCs, respectively (186). PTEN is a phospholipid phosphatase inhibiting the PI3K/PKB pathway, and is frequently inactivated by deletions, mutations or hypermethylation in human cancer. *PTEN* germline mutations predispose for Cowden's disease, characterized by the development of multiple neoplasms including follicular thyroid adenomas and carcinomas. In mice, *Pten*-deficiency results in, among other tumor types, development of MTC (187). However in men, strong PTEN staining has been observed in 2 out of 2 MTCs, and no hypermethylation of *PTEN* could be detected in 6 MTCs (157, 188).

Together, these results indicate that genes that are frequently altered by deletions, mutations and/or hypermethylation in several types of human cancer, are not commonly altered in human MTCs. However, some studies suggests involvement of some of these well-known cancer-related genes in human MTC tumorigenesis, indicating that this should be further investigated.

1.2.7. Specific gene expression patterns in sporadic and MEN 2-associated MTC

To identify specific gene expression patterns for sporadic and hereditary MTC, a microarray analysis has been performed on 6 sporadic and 6 MEN 2-associated MTCs. A very similar expression pattern was observed with only a few minor differences. Monoamine oxidase B (MAOB) and gamma-aminobutyric acid receptor (GABRR1) were consistently upregulated in sporadic MTCs, while opioid growth factor receptor (OGFR) and synaptotagmin V (SYT5) were up-regulated in MTCs from MEN 2 patients (191).

A second microarray study could also not identify major differences in expression patterns for sporadic MTC (n=11) and MEN 2-associated MTC (n=23), even after correction for the presence of somatic *RET* mutations in the sporadic MTCs (192). Interestingly, the expression profiles could, however, distinguish between MEN 2A- and MEN 2B-associated MTCs. The MEN 2B-specific

gene cluster contains a set of genes involved in matrix remodelling and epithelial to mesenchymal transitions (EMTs), associated with an increased metastatic potential. In addition, a number of the MEN 2B-specific genes modulate tumor growth factor β (TGFβ) signaling, also involved in EMTs. Furthermore, chondromodulin-1 (CHM1), a known regulator of cartilage and bone growth, was expressed at high levels specifically in MEN 2B-associated MTCs, and associated with the skeletal abnormalities characteristic for MEN 2B patients (192).

In another study using differential display, NIH3T3 cells were transfected with either MEN 2A-*RET* (C634R) of MEN 2B-*RET* (M918T). Clusters of genes were detected which were induced or repressed by both MEN 2A- and MEN 2B-RET, however, also a MEN 2A-specific and a MEN 2B-specific gene cluster were observed. Genes included in these specific clusters do not overlap with the microarray results from Jain *et al*. (193), probably due to the difference in sample source.

Several other expression analyses, mostly performed by immuno-histochemistry, have revealed specific protein expression in human MTC. A tissue expression analysis among normal thyroid and thyroid cancer samples revealed expression of prolactin (PRL) in only 10% of MTCs, while the PRL receptor (PRLR) was overexpressed in 70% of MTCs (194). MTC spontaneously arise in 41% of *Prlr*-null mice (195), indicating a suppressive role for prolactin signaling on MTC development in mice, however, this remains to be further elucidated.

In human MTC, expression of several receptors for regulatory peptides, like the somatostatin receptor, cholecystokinin B receptor (CCKBR), Substance P receptor and the glucagon-like peptide 1 receptor, has been observed (196). Some of these receptors are used in the clinical diagnosis and treatment of MTC. Radio-labelled somatostatin or cholecystokinin peptides have been generated, and used to target MTC cells for scintigraphy and/or radio-therapy (196).

Staining of CCH and MTC specimens for the sex steroid receptors, involved in several endocrine-related cancers (197), revealed expression of the estrogen receptor (ER)β in 100% of CCH samples and in 97% of MTCs (198). Contradictory results have been obtained regarding ERα expression (198, 199). The progesterone receptor (PR) and androgen receptor (AR) were rarely expressed in MTC (7% and 14%, respectively). However, in 53% of the CCH samples, the AR was expressed, with a trend to male predominance (61% in men versus 33% in women). The more frequent expression of AR in male CCH might explain the higher CCH incidence in men, compared to women (198).

The TRK family of neurotrophin receptors, TRKA, TRKB, and TRKC, and their Neurotrophin ligands, promote the survival, growth, and differentiation of central nervous system neurons and other neural crest-derived cells. Expression studies in C-cells, CCH and MTCs revealed consistent expression of TRKB with variable levels of TRKA and TRKC in CCH, while in advanced MTCs, TRKB expression was reduced and TRKC expression highly increased. This dynamic expression of the TRK family suggests that these receptors may be involved in MTC progression (200).

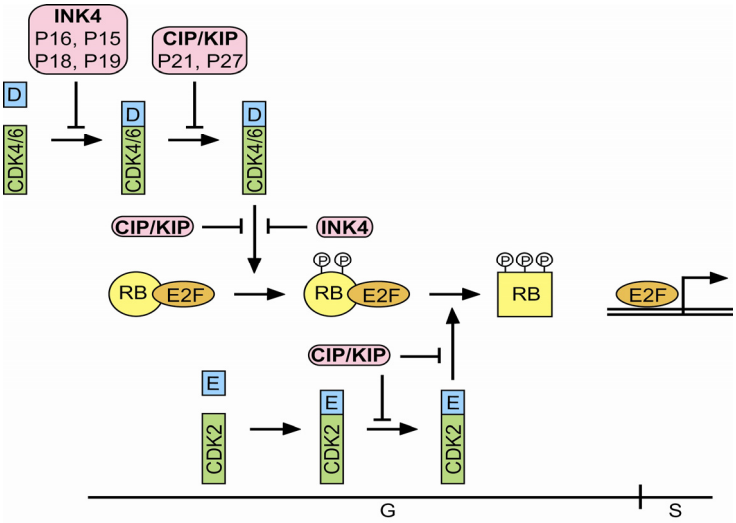

Figure 8. Regulation of the cell cycle G1/S transition: the CDK/RB/E2F pathway. CDK4 and CDK6 are activated by binding of Cyclin D and subsequent phosphorylation. CDK2 is activated by binding of Cyclin E and subsequent phosphorylation. INK4 and CIP/KIP proteins inhibited these processes. Whereas INK4 proteins are specific inhibitors of CDK4 and CDK6, the CIP/KIP proteins also inhibit CDK2. Active CDKs are required for phosphorylation of RB, upon which a conformational change of RB is provoked and the transcription factor E2F released. E2F activates transcription of genes required for G1/S phase transition. D, Cyclin D; E, Cyclin E. (adapted from (189, 190)).

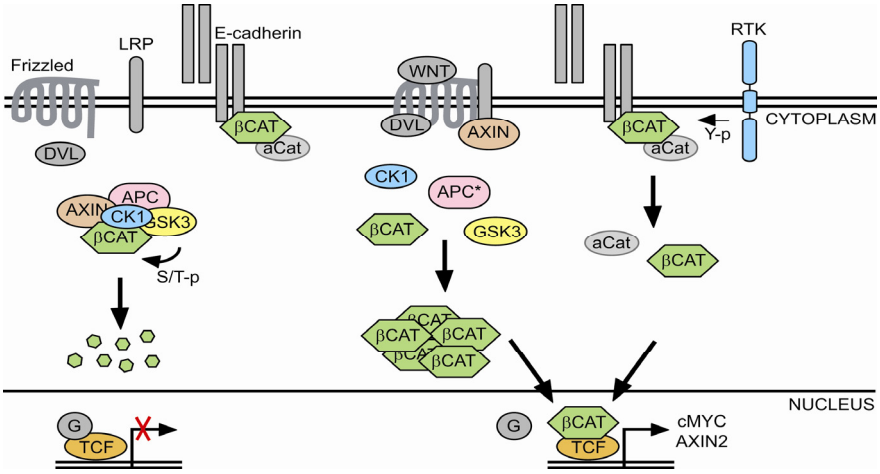

Figure 9. Pathways involved in nuclear localization of β-Catenin. Normally, β-Catenin (βCAT) is bound to E-cadherin and α-Catenin (αCat) forming adherens junctions. Free β-Catenin is trapped by its degradation complex, including Axin, APC, GSK3 and Caseine kinase 1 (CK1). GSK3 and CK1 sequentially phosphorylate serine/threonine residues (S/T-p) of β-Catenin upon which β-Catenin is ubiquitinated and degraded in proteasomes. The transcriptional corepressor Groucho (G) is bound to the transcription factor TCF, preventing it from inducing gene transcription. Activation of the WNT signaling pathway by WNT ligands binding to the Frizzled receptor, or deregulation of the pathway by mutations, for example in *APC* (*), leads to a dissociation of the degradation complex. β-Catenin accumulates and localizes to the nucleus where it competes with the transcriptional corepressor Groucho, resulting in activation of transcription. Upon tyrosine phosphorylation (Y-p) by a receptor tyrosine kinase (RTK) at the plasma membrane, β-Catenin dissociates from the adherens junction and localizes to the nucleus where it activates transcription. DVL, dishevelled; LRP, low-density lipoprotein receptor-related protein. (adapted from (201-205)).

In a large subset of thyroid carcinomas derived from follicular cells, β-Catenin expression is deregulated. Normally, in differentiated cells, β-Catenin is either associated with the adhesion complex at the plasma membrane, or targeted for proteasomal degradation. Two mechanisms have been shown to contribute to the relocalization of β-Catenin to the nucleus (Figure 9). First, upon activation of the canonical WNT signaling pathway by WNT ligands or by mutations in *APC*, *CTNNB1* or *AXIN*, β-Catenin is stabilized and accumulates within the cytoplasm. Subsequently, β-Catenin localizes to the nucleus where it acts as a transcription co-activator, mediating transcription of genes like *cMYC* and *AXIN2*. This mutational activation of the WNT signaling pathway is involved in familial and sporadic colorectal cancer (201, 202). Second, phosphorylation at specific tyrosine residues of β-Catenin by tyrosine kinases, results in a reduced affinity of β-Catenin for E-cadherin. Tyrosine-phosphorylated β-Catenin dissociates from the adherens junctions and localizes to the nucleus (203-205). Nuclear β-Catenin expression is detected in a large subset of human cancers including PTCs (83%) and follicular thyroid cancers (58%) (170, 206). We, and others, have shown nuclear β-Catenin expression in about 50% of human MTCs (207, 208). The role of β-Catenin in MTC development remains to be determined.

1.3. MTC mouse models

1.3.1. Transgenic and knockin *RET* mice

MTC mouse models have been generated by subcutaneously injecting human MTC cells (TT cell line) into nude mice, e.g. (209-211). Within a few days to weeks, these cells grow out into palpable and visible tumors. Recently, tumor tissue from an MTC patient, affected by a germline C634R-*RET* mutation, was successfully transplanted to nude mice (212). These models are generally used to test novel MTC-therapies.

To study multistep tumorigenesis *in vivo*, the best models to use are genetically modified mice. Different transgenic mouse models expressing RET with a MEN 2A or MEN 2B mutation have been generated, developing CCH and/or MTC at different ages and frequencies (Table 4). The first transgenic *RET* mouse model expressed the short RET isoform (RET9) with the most common MEN 2A mutation (C634R) in the thyroid C-cells. One of the generated lines developed CCH from 3 weeks on, and subsequently developed multifocal and bilateral MTC from 8 months on with a complete penetrance of MTC at the age of 14 months (213).

Another group generated a transgenic *RET* mouse model expressing the long isoform (RET51) with the same mutation. Mice, derived from a mixed C57Bl/6J;CBA/ca background, displayed MTC at a frequency of about 35% at 3-4 months of age, and 70% at 12 months or older. Only 6% of mice from another line, derived from a FVB/N background, developed MTC, while PTC was more pronounced in this line (58%) (214). Because of the mixed background in these mice, and

the effect on tumor phenotype, the mice were subsequently crossed back to 4 different genetic backgrounds. A different penetrance of MTC development for the transgene in each strain was observed ranging from 0% (FVB/N) to 98% (CBA/ca) (14% in the BALB/c background and 64% in C57Bl/6J background), suggesting that in these different strains, genetic modifiers are present affecting the penetrance of MTC (215).

Yet a different transgenic mouse model expressing *RET* with the C634R mutation, was generated by using a Moloney murine leukemia virus long terminal repeat, driving the expression to the thyroid gland, heart, liver, colon, parotid gland and brain. In one line, MTC was already detectable from 1 month on, and a complete penetrance was observed in mice between 6-9 months of age. In addition, these mice developed mammary (46%) and parotid gland (23%) carcinomas (216).

Two transgenic *RET* mouse models for MEN 2B have been generated, expressing the human RET9 isoform carrying the M918T mutation. In the first model, the expression was directed to the developing sympathetic and enteric nervous systems and the adrenal medulla by using the human *dopamine β-hydroxylase (DβH)* promoter. These mice developed, in addition to unexpected renal malformations, benign neuroglial tumors which were histologically identical to human ganglioneuromas (217). In the second model, the human *CALC-I* promoter was used to direct the expression of MEN 2B-RET to the thyroid C-cells. MTC was detected in 3 out of 8 founders at the age of 20-22 months. From one of the founders a line predisposed for MTC was generated. In this line, CCH was observed in 77% of animals from 8 months on, which progressed to MTC in 13% of the mice from 11 months on. CCH and MTC development correlated with increased basal plasma CT levels. MTC was never observed in wildtype *RET* transgenic control mice (218).

In addition to these MEN 2B transgenic mouse models, a MEN 2B knockin mouse model was generated by introducing the M919T (equivalent to human M918T) mutation into the mouse *Ret* gene. Both heterozygous and homozygous mice developed a MEN 2B-like phenotype although this was more severe in the homozygous mice. 55% of heterozygotes, 8-12 months old, developed CCH, while at the age of 6-10 months, 86% of homozygotes developed CCH. No MTC was observed in these mice up to 12 months. Nodular chromaffin cell hyperplasia was observed in 16% of heterozygous mice at the age of 8-12 months, and 2% displayed PC. Nodular chromaffin cell hyperplasia and PC, were already detectable at the age of 2-5 months in 33% and 5% of the homozygous mice, respectively. At 6-10 months of age, all homozygous mice displayed PC, as well as ganglioneuroma-like areas (219).

Recently, two other knockin mouse models have been generated by introducing the C620R mutation into the mouse *Ret* gene. In human, this mutation is associated with MEN 2A, FMTC and rarely, Hirschsprung disease. In the first model, homozygous mice displayed severe defects in kidney organogenesis and enteric nervous system development leading to death within 24 hours after birth. The heterozygous mice developed features characteristic of Hirschsprung disease including hypoganglionosis of the gastrointestinal tract. No neoplasia formation (CCH, MTC or

PC) was observed in heterozygotes up to 2 years of age (220). In the second model a similar phenotype was observed. Homozygotes died at the first day postnatally due to kidney agenesis and intestinal aganglionosis. At 20-30 months of age, the heterozygotes derived from the 129/Sv and C57Bl/6J;129/Sv background both showed adrenal gland cortical nodular regeneration or hyperplasia at low frequencies. The heterozygous mice of 20-30 months old, with the 129/Sv background, showed an increased incidence of CCH compared to wildtype littermates, suggesting that the C620R mutation could function as a gain-of-function mutation in mice, although the loss-of-function effect is clearly more pronounced (221).

Table 4. General characteristics of transgenic and knockin *RET* mouse models

Ref.	Genetic background	RET	Promoter	Mutation	Phenotype
Transgenic					
(213)	DBA2	Human *RET9*	Rat CGRP/CT	C634R	MTC
(214)	C57Bl/6J;FVB/N; CBA/ca[1]	Human RET51	Human CT	C634R	PTC, MTC
	C57Bl/6J;FVB/N; CBA/ca[2]				MTC
(215)	CBA/ca	Human RET51	Human CT	C634R	MTC
	C57BL/6J	Human RET51	Human CT	C634R	MTC
	BALB/c	Human RET51	Human CT	C634R	MTC
	FVB/N	Human RET51	Human CT	C634R	No MTC
(216)	C57BL/6J; BALB/c	Human RET51	MLV-LTR[3]	C634R	MTC, mammary and parotid gland carcinomas
(217)	C57Bl/6J	Human RET9	Human DβH	M918T	Ganglioneuroma-like areas, renal malformations
(218)	C57Bl/6J	Human RET9	Human CALC-I	M918T	MTC
	C57Bl/6J	Human RET9	Human CALC-I	None[4]	No MTC
Knockin					
(219)	C57Bl/6J;129/Sv; FVB/N	Mouse RET	n/a	M919T[5]	CCH, PC, ganglioneuroma-like areas
(220)	C57Bl/6J	Mouse RET	n/a	C620R	Hirschsprung's disease[6]
(221)	129/Sv	Mouse RET	n/a	C620R	CCH[6]
	C57Bl/6J;129/Sv	Mouse RET	n/a	C620R	No CCH[6]

[1] from FVB/B background; [2] from C57Bl/6J;CBA/ca background; [3] Expression was directed using the Moloney murine leukemia virus long terminal repeat (MLV-LTR); [4] transgenic mice expressing wildtype human *RET*; [5] equivalent to human M918T; [6] heterozygotes. n/a, not applicable; Ref, reference.

1.3.2. Non-*RET* mouse models that develop MTC

Several other mouse models, not involving *RET*, develop MTC. The first described knockout mouse model which develops MTC is the *Rb*-knockout mouse. RB is involved in regulation of transition from the G1 to S phase of the cell cycle via controlling E2F-mediated transcription (Figure 9). In 1992, the first $Rb^{-/-}$ mice were generated, which turned out to be embryonically lethal (222-224). The heterozygotes developed pituitary tumors with a nearly complete penetrance in the first year of life, as well as CCH and MTC in about 50% of mice (223, 225-227). Backcrossing these mice to different genetic backgrounds either reduced the incidence of MTC (129/Sv) or increased MTC incidence to about 90% (C57Bl/6J) (228).

Because *P53* is the most frequently mutated gene in human cancer, and *RB* and *P53* mutations commonly coincide in many tumors, *Rb;p53* double knockout mice were generated. $P53^{+/-}$ and $p53^{-/-}$ mice develop lymphomas and sarcomas at early ages, but MTC has not been observed (229). In $Rb^{+/-};p53^{+/-}$ mice, the MTC incidence was increased compared to $Rb^{+/-}$ mice. Even though the mean survival of $Rb^{+/-};p53^{-/-}$ mice was reduced to only 4 months, 60% of these mice developed MTC within this period, indicating that *Rb* and *p53* cooperate in the suppression of MTC development (230, 231). In MTCs from $Rb^{+/-}$ mice or $Rb^{+/-};p53^{+/-}$ mice, the remaining *Rb* allele was frequently lost, whereas loss of the wildtype *p53* allele was uncommon (223, 230). Interestingly, Coxon *et al*. detected somatically acquired *RET* mutations, equivalent to human MEN 2A/FMTC mutations, in 4 out of 9 MTCs from $Rb^{+/-};p53^{+/-}$ mice, suggesting that *RET* cooperates with *Rb* and *p53* inactivation in MTC tumorigenesis (232).

Different roles in MTC tumorigenesis have been detected for the E2F-family of transcription factors, which is regulated by RB. C-cell abnormalities have not been observed in *E2f1*, *E2f3* and *E2f4* knockout mice (233-235). Homozygous loss of *E2f1* or *E2f4* strongly reduces the frequency of MTC formation in *Rb*-deficient mice (227, 235). Interestingly, another role in *Rb*-induced MTC tumorigenesis has been detected for *E2f3*. *Rb;E2f3* double knockout mice showed a nearly complete penetrance for MTC, compared to 56% in $Rb^{+/-}$ mice. In addition, $Rb^{+/-};E2f3^{+/-}$ mice and $Rb^{+/-};E2f3^{-/-}$ mice developed one or more distant metastases, especially in lung, liver and kidney, in 23% and 38% of mice, respectively, compared to only 9% in $Rb^{+/-}$ mice (234), revealing a strong cooperative effect of *E2f3* and *Rb* on MTC development and progression.

One of the downstream effectors of RET signaling is RAS. To investigate the role of RAS in MTC tumorigenesis, a transgenic mouse was generated expressing *HRAS* under the control of the rat *CGRP/CT* promoter. About 85% of transgenic mice developed MTC within one year (236). Some of the MTCs coexpressed CT and the follicular cell marker thyroglobulin, suggesting that these mice develop mixed medullary-follicular thyroid carcinomas also seen in human (237).

Loss of *Nras* or *Kras* is shown to significantly reduce the aggressiveness of pituitary tumors arising in $Rb^{+/-}$ mice by enhancing their differentiation, resulting in a prolonged survival (175, 238). In contrast, loss of *Nras* in $Rb^{+/-}$ mice increased MTC incidence to nearly complete

penetrance, MTC size, and promoted progression to metastatic MTC especially to lung, liver and kidney. Only a fraction of primary MTCs has lost the remaining *Nras* allele in $Rb^{+/-};Nras^{+/-}$ mice compared to loss of the wildtype *Nras* allele in all MTC metastases (175). This indicates that loss of *Rb* sets up a cellular context allowing loss of *Nras* to facilitate progression to a more malignant tumor stage. Interestingly, *NRAS* is located on chromosome 1p13.2, a region frequently deleted in human MTC (46, 47). Loss of *Kras* in $Rb^{+/-}$ mice did not reveal a change in MTC incidence at 9 months of age compared to $Rb^{+/-}$ mice, however the age-of-onset was not reported (238), making it impossible to conclude whether *Kras* also collaborates with *Rb* in MTC tumorigenesis.

Another interesting mouse model developing a neuroendocrine tumor spectrum is the *p18* knockout mouse generated by Franklin *et al.* (239). A high frequency of pituitary tumors was observed. In addition, 14% of $p18^{-/-}$ mice older than 12 months, developed CCH and/or MTC (240). Crossing these mice with several other knockout mice has shown synergism in MTC tumorigenesis. In $p18^{-/-};p27^{+/-}$ mice (mean survival 8.5 months), the frequency of CCH/MTC was 54% and even 88% in $p18^{-/-};p27^{-/-}$ mice (mean survival 3.5 months) (240). Not only the increased incidence, but also the earlier age-of-onset indicate that *p18* and *p27* collaborate to suppress MTC development. Only 5% of *p18;p53* double knockout mice developed MTC at the age of 8 months, compared to 2.5% of $p18^{-/-}$ mice at 14 months (241), revealing that the synergism between *p18* and *p53* is not as clear as between *Rb* and *p53*. *p18* knockout mice have also been crossed with *Men1* knockout mice, which develop several endocrine tumors. In humans, germline Men 1 mutations do not predispose for MTC development. 80% of $p18^{-/-};Men1^{+/-}$ mice developed MTC at the age of 12-22 months, compared to 0% in both single knockout strains, indicating that p18 strongly collaborates with *Men1* in MTC suppression (242). $P18^{-/-};Pten^{+/-}$ mice displayed an increased MTC incidence (63% of mice from 6 months onwards compared to 14% and 15% of $p18^{-/-}$ mice and $Pten^{+/-}$ mice, respectively) and earlier age-of-onset (38% of $p18^{-/-};Pten^{+/-}$ mice showed MTC at the age of 3-6 months compared to 0% for both single knockout strains), revealing synergism between *p18* and *Pten* in MTC tumorigenesis (187).

MTC has occasionally been observed in *p27* single knockout mice (240, 243). *P27* knockout mice have been crossed with $Rb^{+/-}$ mice, resulting in an increased MTC incidence (about 80%) and earlier age of onset (5-7 months) in the double knockout mice compared to both single knockout strains (243). This reveals that, like *p27* and *p18*, *p27* and *Rb* collaborate in MTC suppression. *P27* knockout mice have been crossed with *p53* knockout mice, however, MTC has not been observed in *p27;p53* double knockout mice (241). In contrast to *p18*, *p27* did not show a clear synergistic effect with *Men1* in MTC tumorigenesis (242). Recently, *p27* has also been shown to be involved in MTC tumorigenesis in rat. Several rat strains spontaneously develop MTC at late ages: 10-20% of Wistar rats (244, 245), about 20% of Long-Evans rats (246), 50-70% of Wag/Rij rats (247, 248) and 78% of Sprague Dawley rats (249). In the latter strain the MEN x syndrome, involving MTC,

was linked to the distal part of chromosome 4, and recently, a germline *p27* mutation has been detected in these rats (250, 251).

Knockin mice expressing a CDK4 mutant, R24C, involved in human melanoma, and insensitive to INK4 inhibitors (252), develop a wide spectrum of endocrine tumors including MTC in one mouse (253). Crossing these mice with $p27^{-/-}$ mice reduced the life-span of these mice due to early pituitary tumors. A putative synergistic effect on MTC development was not mentioned in this publication (253).

Together, this shows that mice specifically targeted for components of the RB/CDK/E2F pathway develop MTC, revealing that genes involved in regulating the cell cycle G1/S transition are responsible for, and collaborate with each other in, the tumorigenesis of MTC in mice.

Some mouse models not targeted for cell cycle regulatory genes also show MTC development. For example, 41% of *Prlr* knockout mice develop CCH or MTC at one year of age (195). Also, transgenic mice overexpressing cMOS, develop MTC with an incidence of 45% and 66%, depending on the genetic background, B6C3 and FVB/N respectively. MTC was developed in combination with PC in the B6C3 background strain revealing a MEN 2-like phenotype (254).

Summary

Since the discovery of *RET* as the gene responsible for MEN 2, much of effort has been put into the role of *RET* in hereditary and sporadic MTC tumorigenesis. Fundamental research revealing different sequence variations and signaling pathways of RET, has led to our current understanding of the genetic mechanisms underlying MTC development. However, at least for sporadic MTC and probably also for hereditary MTC, other and or additional oncogenic events are required in this process, which is supported by the losses and gains of several specific chromosomal regions. Because of the focus on *RET*, not much attention is put into the additional genes and/or pathways and their possible roles in MTC tumorigenesis. In many studies attempting to discover involvement of non-*RET* oncogenic events in MTC development, very small sample sizes are used. Together, this has led to a lack of information about genes involved in *RET* and non-*RET* induced multistep tumorigenesis of MTC. Lessons can be learned from non-*RET* mouse models developing MTC. Genes affected in these mouse models might be involved in human MTC tumorigenesis as well. This should be further elucidated.

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OUTLINE OF THIS THESIS

Tumorigenesis is a process of accumulating genetic alterations, resulting in neoplastic transformation of cells which eventually leads to tumor growth, the so-called 'multistep process'. The genetic mechanisms underlying the multistep process of MTC development is at present largely unknown. About 60% of all MTCs occur as sporadic cancer and the remaining 40% of MTCs occur as familial cancer, i.e. as part of the multiple endocrine neoplasia type 2 syndrome. This syndrome is caused by activating germline *RET* mutations, which are also detected as somatic mutations in a subset of sporadic MTCs. RET activation initiates hereditary MTC development, however, further oncogenic events are required, but as yet unknown. The underlying genetic mechanisms and the role of RET activation in sporadic MTC formation remain to be elucidated. Previous chromosomal and nucleotide sequence analyses, as well as studies on gene expression profiles in human MTC, have provided some clues about the molecular genetics of MTC. In addition, transgenic, knockout and knockin mouse models add to our understanding of MTC tumorigenesis. An overview of these genetic alterations and mouse models is provided in **chapter 1**.

Because many molecular genetic studies on MTC tumorigenesis have been focussed on *RET*, not much is known about the involvement of non-*RET* oncogenic events in human MTC development. In this thesis we investigated additional genes and/or pathways and their possible roles in MTC tumorigenesis.

A few years ago, our laboratory has generated a transgenic *RET* mouse model, which expresses human RET with the M918T mutation in the thyroid C-cells. This mutation was chosen because, compared to other known *RET* mutations, it has the highest transforming activity *in vitro*, and it is the mutation which is most frequently detected in sporadic human MTC. Because this mouse model develops CCH and MTC, it is an excellent tool to study the molecular genetics of MTC development *in vivo*. Our close collaboration with the clinic and pathology department of our hospital has given us the opportunity to study oncogenic events in DNA isolated from human MTCs as well.

Chromosomal analyses have revealed loss of chromosome 1p as the most frequent chromosomal alteration in both hereditary and sporadic MTC. A common breakpoint on this chromosome is 1p32, at which the gene encoding the cell cycle inhibitor P18, is located. Previous studies have shown that *p18*-deficient mice develop CCH and MTC. Together, these data led us to study *P18* involvement in human MTC development. These studies resulted in the detection of somatic inactivating *P18* mutations in human MTC as well as pheochromocytomas, which is further discussed in **chapter 2**.

RET activation initiates hereditary MTC and is probably involved in MTC progression in sporadic patients, and loss of *p18* initiates MTC development in mice. In addition, we have detected a

cosegregation of inactivating *P18* mutations with activating *RET* mutations in all *P18* mutation-positive human MTCs (**chapter 2**), which led to the hypothesis that RET activation and P18 inactivation collaborate in MTC tumorigenesis. Further, *p18* and *p27* are known to cooperate in MTC suppression in mice. To investigate the role of *RET*, *P18* and *P27* in multistep MTC tumorigenesis, we crossed our *RET* transgenic mice with *p18* and/or *p27* knockout mice, and monitored for MTC development. The results of these studies are discussed in **chapter 3**.

Chapter 4 presents a new RET signaling pathway mediated by β-Catenin. Nuclear β-Catenin is known to be involved in many types of cancer, like colorectal and thyroid follicular carcinomas. In this chapter, we demonstrate nuclear localisation of β-Catenin in human MTC. It has been shown that tyrosine kinases can phosphorylate β-Catenin, upon which β-Catenin loses its affinity for the adhesion complex at the plasma membrane. Free β-Catenin can translocate to the nucleus where it activates transcription of specific target genes. We have found that RET phosphorylates β-Catenin, and studied the consequences of this novel aspect of RET signaling. The results of these studies are discussed in **chapter 4**.

Chapter 5 provides a deeper insight into the clinical management of MTC patients. Among clinicians, there is no consensus about the strategy for postoperative treatment and follow-up of patients with (occult) MTC metastases. This chapter provides an overview of cure and survival rates of patients with persistent or recurrent MTC. Overall, cure rates are low, emphasizing the need for more accurate classification and more effective therapies. In addition, biomarkers are required which can predict prognosis. In this chapter an overview of the currently known MTC biomarkers and their applications in diagnosis, treatment and prediction of prognosis is provided.

The results of the studies described in this thesis are discussed in **chapter 6**, providing an integrated model describing our current knowledge of the molecular mechanisms underlying multistep MTC tumorigenesis.

CHAPTER 2

P18 is a tumor suppressor gene involved in human medullary thyroid carcinoma and pheochromocytoma

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Conditionally Accepted, Int J Cancer

ABSTRACT

Hereditary forms of medullary thyroid carcinoma (MTC) and pheochromocytoma (PC) are associated with activating germ-line mutations in the proto-oncogene *RET*, but additional genetic events underlying both hereditary and sporadic MTC and PC development remain largely unknown. Here, we show for the first time the presence of somatic inactivating mutations in the cell cycle regulator *P18* in human MTC and PC. Each mutation causes an amino acid substitution in the cyclin dependent kinase-interacting region of $P18^{INK4C}$. Since these mutations inhibited $P18^{INK4C}$ function and reduced its stability, our findings implicate *P18* as a tumor suppressor gene involved in human MTC and PC development.

INTRODUCTION

Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominantly inherited cancer syndrome. The clinical expression of MEN 2 varies, although all patients develop medullary thyroid carcinoma (MTC) or its precursor C-cell hyperplasia (CCH). MEN 2 can be subdivided in MEN 2A, MEN 2B and familial MTC. MEN 2A is characterized by MTC, pheochromocytoma (PC), and hyperparathyroidism. MEN 2B is characterised by MTC, PC, and mucosal ganglioneuromas (in the colon, lips and tongue). FMTC patients solely develop MTC. MTC originates from the calcitonin producing neuroendocrine C-cells in the thyroid gland, and PC originates from the neuroendocrine chromaffin cells in the adrenal medulla (1).

MEN 2 patients carry an activating germ-line mutation in the *RET* proto-oncogene, leading to constitutive activation of its encoded transmembrane receptor tyrosine kinase RET. The MEN 2 phenotype correlates strongly with specific *RET* mutations (2, 3). In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein. The most commonly affected cysteine is C634 (4, 5). In MEN 2B, the most common mutation (in 95% of the cases) results in a Methionine to Threonine substitution at position 918 in the intracellular domain of the protein (6-8). Somatic *RET* mutations have been detected in 23-70% of sporadic MTC, the vast majority of which has the M918T mutation, as well as in 10-15% of sporadic PC, with M918T in 50% of the cases (9).

Carriers of the same germ-line *RET* mutation, both related and unrelated, can develop MTC and PC at widely varying ages. This suggests that in addition to the mutated *RET* gene, other tumorigenic events are required for the development of these tumors. Several reports describe that allelic loss of specific chromosomal loci, like 1p, 3q, 13q and 22q, is associated with MTC and PC, strengthening the hypothesis that multiple genes are involved in the development of these neuroendocrine tumors. The most frequently detected chromosomal alteration in MTC and PC is

loss of a specific part or the entire short arm of chromosome 1 (10-12). This suggests that one or more tumor suppressor genes located on chromosome 1p, are involved in the tumorigenesis of MTC and/or PC. The most common break point for the 1p deletions that occur in sporadic and MEN 2-associated MTC and PC is on 1p32, on which the candidate tumor suppressor gene *CDKN2C* (from here on indicated as P18) is located (13-16).

Its gene product, $P18^{INK4C}$, is a member of the family of 'inhibitors of CDK4' (INK4) (17). The INK4 proteins $P16^{INK4A}$, $P15^{INK4B}$, $P18^{INK4C}$ and $P19^{INK4D}$ bind to cyclin dependent kinase (CDK) 4 and/or CDK6, which prevents the formation of active CDK4/6-Cyclin D complexes. Active CDK4 and CDK6 complexes phosphorylate RB, which leads to the release of the transcription factor E2F that in turn activates transcription of genes necessary for G1/S phase transition. Thus, INK4 proteins inhibit cell cycle progression and cell division, explaining the tumor suppressing function of these proteins (17).

Loss of functional INK4 proteins has been detected in different types of human cancer, with loss or mutation of $P16^{INK4A}$ being the most common event in human cancer. Homozygous deletion of chromosome 9p21, the site of both *P16* and *P15*, occurs in bladder cancer, melanomas, acute lymphoblastic leukemia and other types of cancer. Somatic and germ-line *P16* mutations occur most predominantly in pancreatic cancer and melanomas. Additionally, epigenetic *P16* alterations have been detected in gliomas, lung cancer, head and neck carcinomas, bladder cancer, colon cancer and other types of cancer. Epigenetic *P15* alterations have been detected in leukemias and lymphomas. *P19* mutations are rare, but have been detected in a small number of osteosarcomas (18-20). Although loss of chromosome 1p occurs frequently in human cancer, a role of *P18* in tumorigenesis is at present largely unknown. In mice, loss of *p18* induces a MEN-like phenotype. *p18* knockout mice develop intermediate lobe pituitary adenomas with a nearly complete penetrance, and in low frequencies CCH, MTC, PC and parathyroid adenomas (21, 22).

The frequent loss of chromosome 1p32 in MTC and PC, together with the MEN-like phenotype in *p18* knockout mice, prompted us to investigate the potential involvement of *P18* in human MTC and PC development. We have detected several novel somatic *P18* mutations in these human neuroendocrine tumors. Functional analyses revealed that these mutations inhibit $P18^{INK4C}$ function and reduce its stability, indicating that *P18* is a tumor suppressor gene involved in MTC and PC development.

MATERIALS AND METHODS

Patients / tumor material and DNA isolation: Tumors were obtained from the Biobank of the department of Pathology of the University Medical Center Utrecht, Utrecht, The Netherlands. The clinical information associated with these tumors is described in Suppl. Table 1. Formalin fixed paraffin embedded material was used for DNA isolation. Tumor tissue was carefully dissociated from surrounding normal tissue. DNA was isolated specifically from the tumor cells by incubating the sections with a proteinase-K lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 0.25 mM EDTA, 0.5% SDS, 0.2 mg/ml

Proteinase-K) at 55ºC for about 42 hours. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 99.5% ethanol (Merck, Darmstadt, Germany) supplemented with 0.1 mg/ml glycogen (Roche Diagnostics, Basel, Switzerland) and 1.5 M NH_4Ac. DNA isolated from peripheral blood of patients with a P18 mutation in their MTC or PC, was kindly provided by the department of Medical Genetics of the University Medical Center Utrecht, Utrecht, The Netherlands.

Supplementary Table 1. Detailed information about the tumor samples included in this study

Tumor type	Patient			Tissue source
	Age (y)	Gender	Familial / Sporadic	
MTC	15	F	MEN 2A	Primary tumor
	26	M	MEN 2A	Primary tumor
	30	M	Sporadic	Primary tumor
	30	M	Sporadic	Metastasis (lymph node)
	30	M	MEN 2A	Metastasis (lymph node)
	30	M	MEN 2A	Primary tumor
	30	F	Sporadic	Primary tumor
	31	M	Sporadic	Metastasis (lymph node)
	31	M	Sporadic	Primary tumor
	31	M	Sporadic	Metastasis (lymph node)
	32	M	Sporadic	Metastasis (lymph node)
	35	F	MEN 2A	Primary tumor
	35	F	Sporadic	Metastasis (lymph node)
	38	M	MEN 2A	Metastasis (lymph node)
	39	M	Sporadic	Primary tumor
	43	F	Sporadic	Metastasis (lymph node)
	45	F	Sporadic	Primary tumor
	46	M	MEN 2A	Metastasis (lymph node)
	51	F	Sporadic	Metastasis (lymph node)
	51	F	Sporadic	Primary tumor
	51	F	Sporadic	Metastasis (lymph node)
	55	M	MEN 2A	Metastasis (lymph node)
	60	M	Sporadic	Metastasis (lymph node)
	61	M	MEN 2A	Metastasis (liver)
	61	F	Sporadic	Metastasis (lymph node)
	62	F	Sporadic	Primary tumor
	62	F	Sporadic	Metastasis (lymph node)
	76	F	MEN 2A	Metastasis (adrenal gland)
	76	F	MEN 2A	Metastasis (liver)
	76	F	MEN 2A	Metastasis (lymph node)
PC	25	m	MEN 2B	Primary tumor
	25	m	MEN 2B	Primary tumor
	26	m	MEN 2A	Primary tumor
	28	v	MEN 2A	Primary tumor
	28	v	MEN 2A	Primary tumor
	28	m	MEN 2A	Primary tumor

30	v	MEN 2A	Primary tumor
30	m	Sporadic	Primary tumor
32	v	MEN 2A	Primary tumor
33	m	MEN 2A	Primary tumor
33	m	Sporadic	Primary tumor
34	m	MEN 2A	Primary tumor*
34	v	MEN 2A	Primary tumor
34	v	MEN 2A	Primary tumor
36	v	MEN 2A	Primary tumor
36	m	Sporadic	Primary tumor
37	v	Sporadic	Primary tumor
41	m	Sporadic	Primary tumor
45	v	Sporadic	Primary tumor
46	m	MEN 2A	Primary tumor
48	v	Sporadic	Primary tumor
49	v	Sporadic	Primary tumor
55	v	Sporadic	Metastasis (bone)
56	v	Sporadic	Primary tumor
61	m	MEN 2A	Primary tumor
66	m	MEN 2A	Primary tumor
71	m	MEN 2A	Primary tumor
72	m	Sporadic	Primary tumor

*extra-adrenal

Cell culture and transfection: The human osteosarcoma cell line U2OS was maintained in DMEM GlutaMAXTM (4.5 g/L D-Glucose + Pyruvate) (Gibco Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (EU Approved Origin) (Gibco Life Technologies), 100 µg of penicillin/ml and 100 µg of streptomycin/ml (Gibco Life Technologies). For transfection, U2OS cells were grown at 50% confluency. Transfections were performed using FuGENE 6 (Roche Diagnostics Corporation, Basel, Switzerland) or using polyethylenimine (PEI) (Polysciences Inc., Warrington, PA, USA).

Expression vectors: The wildtype *P18* expression vector was generated by cloning the full length coding sequence of human *P18* cDNA into the pCDNA3.1 expression vector. Oligonucleotide-directed mutagenesis was achieved by using Pfu Turbo® (Stratagene, La Jolla, CA, USA). Mutations were confirmed by nucleotide sequence analysis using BigDye® Terminator (Applied Biosystems). The GST expression plasmids, *pGEX-hCDK4* and *pGEX-hCDK6*, were kindly provided by D.S. Franklin (Department of Biochemistry, Tulane University School of Medicine, New Orleans, LA, USA).

Mutation analysis: Primers for *P18* (exon 2 and 3) and *RET* (exon 16) mutation analysis are listed in Suppl. Table 2. PCRs were performed at an annealing temperature of 58ºC, for 36 cycles using AmpliTaq Gold® DNA Polymerase with GeneAmp® 10X PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA). For some tumor samples a semi-nested PCR was performed with 3 µl PCR product. PCR products were purified using a JETquick PCR product Purification Spin Kit (Genomed, Löhne, Germany) or extracted from gel using a JETquick Gel Extraction Spin kit (Genomed). Direct sequence analysis PCRs were performed on purified PCR products using BigDye Terminator (Applied Biosystems). To ensure that the observed sequence variations were not PCR or sequencing artefacts, independent DNA amplifications were performed, and the products were sequenced.

Supplementary Table 2. Primers used for P18 and RET mutation analysis

Gene	Primer	Sequence (5'- 3')	PCR product
P18 exon 2	hP18exon2F	CAT CAT GCA GCC TGG TTA GG	237 bp
	hP18exon2R	TCC CCA CCT CTC TTA ATA CC	
P18 exon 3A	hP18exon3AF	TGG GTC TTC CGC AAG AAC TC	329 bp
	hP18exon3AR	TGG CAG CCA AGT GCA AGG GC	
P18 exon 3B	hP18exon3BF	AGC TGA TGT TAA CAT CGA GG	261 bp
	hP18exon3BR	AGT AGA GGC AAC GTG GGG GA	
P18 exon 2 nested	hP18exon2Fnes	GAC CCT AAA GAA TGG CCG AG	162 bp
	hP18exon2R	TCC CCA CCT CTC TTA ATA CC	
P18 exon 3A nested	hP18exon3AFnes	GTA GCA TAT GCA CTT GAA GG	251 bp
	hP18exon3AR	TGG CAG CCA AGT GCA AGG GC	
P18 exon 3B nested	hP18exon3BF	AGC TGA TGT TAA CAT CGA GG	244 bp
	hP18exon3BRnes	GGA GCC CTC CCC ACG TTT AT	
RET exon 16	RETE16F	AGG GAT AGG GCC TGG GCT CC	195 bp
	RETE16R	TAA CCT CCA CCC CAA GAG AG	

GST-pulldown: Recombinant human *P18* cDNAs (wildtype and mutants) in the pCDNA3.1 expression vector were transcribed and translated in vitro in reticulolysate in the presence of [^{35}S]methionine using the TNT T7 Quick Coupled Transcription/Translation Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Rosetta pLysS competent bacteria (Merck Chemicals Ltd., Nottingham, UK) were transformed with GST expression plasmids. Expression of GST proteins was induced with IPTG (1mM) at 37ºC for 3 hours. Bacteria were pelleted, resuspended in NETN-buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 0.5% NP40) containing protease inhibitors (Complete; Roche Diagnostics) and sonicated two times for 10 sec. GST proteins were purified on Glutathione-Sepharose beads (GE Healthcare, Chalfont St. Giles, UK). [^{35}S]–labeled P18^{INK4C} proteins were incubated with GST proteins in NETN-buffer. Samples were subsequently washed and subjected to SDS-PAGE. Signals were enhanced by incubating the gels in Amplify (Amersham, Arlington Heights, IL, USA) for 20 min. The gels were dried and the [^{35}S]-labeled proteins were visualized by autoradiography. The interactions of the different P18^{INK4C} proteins with CDK4 and CDK6 were quantified as an average of 3 individual experiments.

Immunoblotting and protein stability: For determination of P18^{INK4C} protein stability, cells were transiently transfected with the P18 expression vectors. 24 hours after transfection, cells were incubated with 10 µg/ml cyclohexamide (Sigma, St. Louis, MO, USA) for 0, 1, 2, 4 and 8 hours. For the detection of P18^{INK4C} and Tubulin expression, cells were lysed in RIPA buffer (50mM Tris (pH 7.5), 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% Nonidet P40) containing protease inhibitors (Complete; Roche Diagnostics) and Na_3VO_4 (1mM), scraped and incubated on ice for 30 min. Protein concentrations were measured using a Bradford assay (BioRad, München, Germany) according to the manufacturer's protocol. 20 µg of protein lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Eugene, OR, USA). A polyclonal rabbit anti-p18 (M-168) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 or a polyclonal rabbit anti-tubulin antibody (Abcam, Cambridge, UK) diluted 1:1000 in 4% ELK (Campina Melkunie BV, Eindhoven, Netherlands) in TBST (0.05% Tween-20 in TBS) was used to probe for P18^{INK4C} or Tubulin expression, and ECL (Amersham Biosciences) was used for detection. Levels of expression for the different P18INK4C proteins at the different time points were quantified. The expression level at time point 0 was set at 100%. Half-lifes of wildtype and mutant P18^{INK4C} proteins were calculated as an average of 3 individual experiments.

Cell growth assay: U2OS cells were cultured and seeded at 50% confluency in 6 wells plates and transfected using PEI. For each *P18* construct 20 ng, 100 ng and 500 ng was transfected supplemented with pCDNA3.1 to a total of 500 ng. After 24 hours, cells were selected using neomycin (0.7 mg/ml in culture medium) for 1 week. The selected cells were seeded at four different diltutions in 12-wells plates and cultured for another 10 days. Cells were washed twice with PBS. Subsequently, cells were air-dried and fixed using methanol-acetic acid (3:1) for 10 min. Cells were air-dried and incubated with 3% Giemsa Stain (Fluka Riedel-de Haën, Seelze, Germany) in 10mM phosphate buffer (pH 7) for 30 min. The cells were rinsed with tap water for several times and incubated in water for 30 min. Cells were air-dried and plates were scanned. This experiment was performed 3 times.

RESULTS

Novel somatic *P18* mutations identified in human MTC and PC

To investigate the involvement of *P18* in human MTC and PC development, a mutation analysis on DNA isolated from MTCs and PCs was performed. We analysed 30 MTCs from 15 male and 15 female patients, and 28 PCs from 15 male and 13 female patients. The clinical information associated with these tumors is described in Suppl. Table 1. Sequence variants in the coding exons 2 and 3, and intron-exon junctions of the P18 gene were screened by direct sequencing of PCR products. PCR products of the predicted sizes were amplified from all isolated DNA samples, indicating that homozygous deletions of the coding region of *P18* had not occurred in any of these tumors. However, nucleotide sequence analysis revealed *P18* variants in tumors of six unrelated individuals (Table 1). None of these six *P18* variants were present in blood DNA from the same individuals, indicating that the variants were somatically acquired mutations. These mutations have not previously been reported in human cancer.

In addition, in five out of these 58 tumors, a heterozygous 342C>T substitution was detected in codon 114 of exon 3, which is a known synonymous polymorphism (ENSG00000123080 in Ensembl Genome Browser http://www.ensembl.org).

Each of the mutations detected in human MTC and PC is located in exon 3, and leads to an amino acid substitution in the $P18^{INK4C}$ protein. Four mutations (E51K, A61T, A72T and R79G) were detected in MTCs, and two (P63L and R79K) in PCs (Table 1). In the tumors carrying the P63L and the E51K mutation, the wildtype allele was not detected, whereas the mutations A61T, A72T, R79K and R79G were found in the heterozygous state. Four of the six tumors with a somatic *P18* mutation are from MEN 2 patients carrying a germ-line *RET* mutation as indicated in Table 1. The other two are sporadic MTCs, which are known to have a somatic *RET* mutation in exon 16 in 23-70% of cases (9). *RET* exon 16 mutation analysis indeed revealed the presence of a somatic M918T mutation in both of these MTCs, indicating that in all six tumors the *P18* mutation coincides with an activating *RET* mutation (Table 1).

Table 1. Somatic P18 mutations identified in human MTC and PC

Tumor type	Tissue source	Familial / Sporadic	RET (protein)	P18 (DNA)	P18 (protein)
MTC	Metastasis (lymph node)	Sporadic	M918T som	151G>A	E51K
	Primary	Sporadic	M918T som	181G>A	A61T
	Primary	MEN 2A	C634W germ	214A>T	A72T
	Metastasis (adrenal gland)	MEN 2A	C634W germ	235G>A	R79G
PC	Primary	MEN 2A	C634R germ	188G>A	P63L
	Primary	MEN 2A	C634R germ	236C>T	R79K

som, somatic mutation; germ, germ-line mutation

Mutations affect residues located at sites that are crucial for $P18^{INK4C}$ function

We generated an alignment of the human INK4 protein family using the Ensembl Genome Browser (http://www.ensembl.org) (Figure 1). This shows that some regions of the $P18^{INK4C}$ protein are highly conserved among family members and constitute important structural and functional domains in these proteins. The amino acid residues mutated in MTCs and PCs (indicated in red in Figure 1), are conserved among the entire human INK4 family, with the exception of E51 and A72. The amino acid residues A61, P63 and R79 are also evolutionary conserved, again with the exception of E51 and A72 that are not conserved in zebrafish (Suppl. Figure 1). Interestingly, mutations in *P16* affecting amino acids at positions analogous to all MTC- and PC-associated *P18* mutations (R58X, A68T/L/V, P70L, T79I and R87G/L/P), have been detected in different kinds of human cancer, like melanoma, esophageal squamous cell carcinoma, astrocytoma, pancreatic and bladder cancer (Figure 1) (20, 23, 24).

$P18^{INK4C}$ is a small protein of 168 amino acids, containing five ankyrin repeats (AR), which are known to mediate protein-protein interactions. Each repeat forms a structural unit of 32-33 amino acids, which is comprised of a β-strand helix-turn-helix extended β-strand element (Figure 1) (25). Affected residues A61, P63 and A72 in $P18^{INK4C}$ are located in loop 2, connecting AR II and AR III (Figures 1, 2). E51 is located in α-helix α4 of AR II, while R79 is located in α-helix α5 of AR III (Figure 1). The latter residue directly interacts with G36 of CDK6 and indirectly, via D76 of $P18^{INK4C}$, with R31 of CDK6 (Figure 2B). Loop 2 and AR III are the most important sites for the interaction of $P18^{INK4C}$ with its target proteins CDK4 and CDK6 (25-28).

Taken together, the conservation of some of the mutated amino acids, the presence of mutations at analogous positions in $P16^{INK4A}$, as well as the location of the affected amino acids in an important functional region of the $P18^{INK4C}$ protein, suggest that at least some of the mutations detected in MTC and PC may affect $P18^{INK4C}$ structure and/or function.

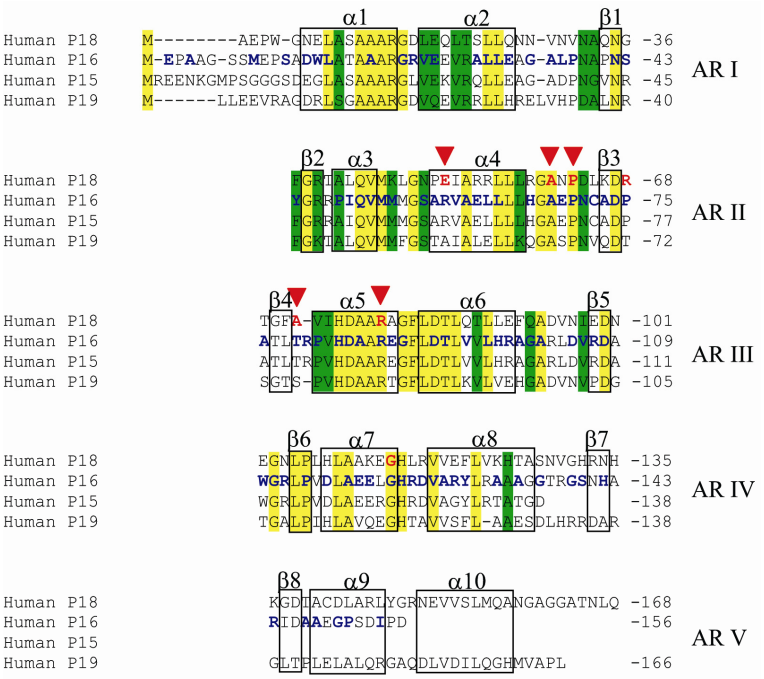


Figure 1. Alignment of human P18^{INK4C} with the other human INK4 proteins. Amino acid sequences were obtained from the Ensembl Genome Browser (http://www.ensembl.org). The amino acid sequences are presented in such a way that the ankyrin repeats are aligned. The α-helices (α1-α10) and β-strands (β1-β8) of the five ARs are indicated in boxes (25). Indicated in yellow: amino acids conserved among all four INK4 family members. Indicated in green: amino acids conserved among three out of four INK4 family members. Indicated in red: amino acids of P18^{INK4C} affected by mutations detected in human tumors or cancer cell lines (37-40). Indicated in blue: amino acids of P16^{INK4C} affected by mutations detected in human tumors or cancer cell lines (20, 41, 44, 49). Red triangles indicate amino acids of P18^{INK4C} affected by mutations detected in MTCs and PCs in this study.

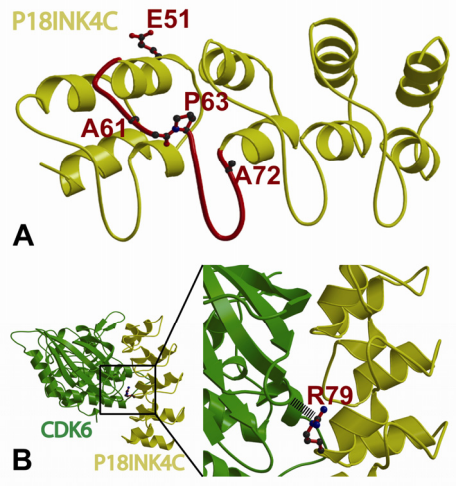


Figure 2. Position of amino acids of P18^{INK4C} affected by mutations detected in MTCs and PCs. A) P18^{INK4C} is indicated in yellow. Loop 2, connecting AR II and AR III, is indicated in red. The amino acids mutated in MTCs or PCs, E51, A61, P63 and A72, are indicated in red in loop 2. B) P18^{INK4C} is indicated in yellow and CDK6 is indicated in green. Amino acid R79 mutated in an MTC and a PC is indicated in red in AR III. Dashed line indicates the direct binding of R79 of P18^{INK4C} to CDK6. Figures were generated using Molscript and rendered using Rasert3D.

Supplementary Figure 1. Alignment of P18^{INK4C} among different species. Amino acid sequences were obtained from the Ensembl Genome Browser (http://www.ensembl.org). The amino acid sequences are presented in such a way that the ankyrin repeats are aligned. Indicated in grey: amino acids conserved among human, mouse, chicken and zebrafish. Indicated in bold: amino acids of P18^{INK4C} affected by mutations detected in human MTCs and PCs.

Mutations affect the interaction of P18^{INK4C} with CDK4 and CDK6

The clustering of MTC- and PC-associated P18^{INK4C} mutations in the CDK-interacting domain suggests that the interactions of the P18^{INK4C} mutants with CDK4 and CDK6 may be affected. To investigate this, GST-pulldown assays were performed with [^{35}S]methionine-labeled wildtype and mutant P18^{INK4C} proteins. These P18^{INK4C} proteins were incubated with purified GST, GST-CDK4 and GST-CDK6, of which the expression was checked with SDS-PAGE and Coommassie staining (not shown). Quantification of the detected interactions revealed that the A61T, P63L and R79K mutants display a strong reduction in binding to CDK4, while binding to CDK6 is reduced even more dramatically (Figure 3). The A72T mutant shows a partially reduced interaction with both CDK4 and CDK6, while the R79G mutant shows a partially reduced binding to CDK6 but hardly interacts with CDK4 (Figure 3). The E51K mutant is not affected in its ability to bind to CDK4 and CDK6 under these experimental conditions (Figure 3). As a control, we introduced an artificial R79P mutation, homologous to the R87P mutation detected in *P16* in different types of human tumors (20). The R79P mutant does not interact with either CDK4 or CDK6, which is consistent with previously published data (28). These results indicate that the *P18* mutations detected in MTCs and PCs, except for the E51K mutation, strongly inhibit P18^{INK4C} binding to CDK4 and/or CDK6.

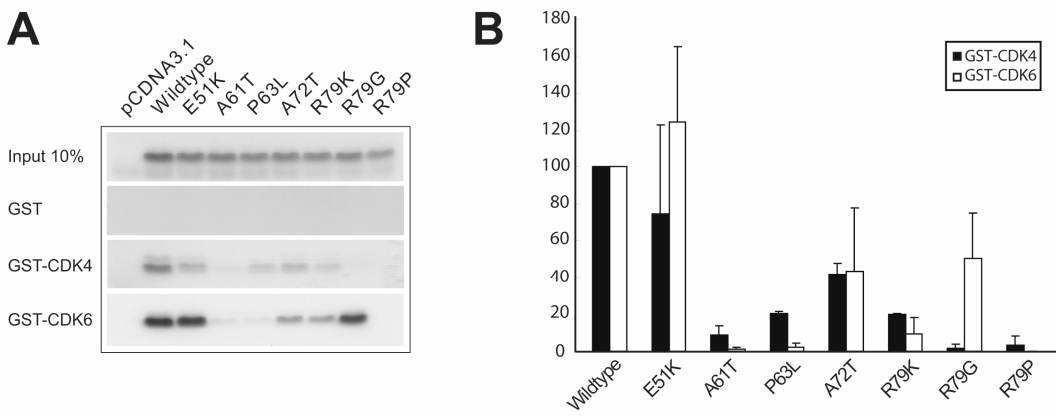
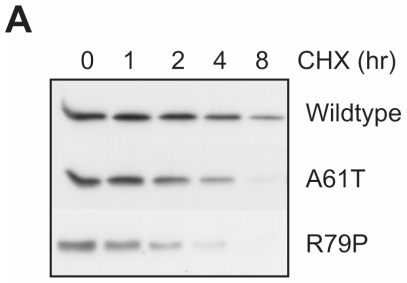


Figure 3. Reduced interaction of $P18^{INK4C}$ mutants with CDK4 and CDK6. GST-pulldowns were performed with 35S-methionine labeled in vitro translated $P18^{INK4C}$. GST, GST-CDK4 and GST-CDK6 were bacterially expressed. A) Interaction between wildtype and mutant $P18^{INK4C}$ proteins, and CDK4 and CDK6 were visualized by autoradiography. B) The interaction levels were quantified in three individual experiments, and averaged. The interaction of the mutant $P18^{INK4C}$ proteins with CDK4 and CDK6 was measured relative to the interaction between wildtype $P18^{INK4C}$ and CDK4 or CDK6, which was set at 100%.



B

P18	Half-life (hr)
Wildtype	5
E51K	2.5
A61T	2.5
P63L	1.75
A72T	3
R79K	2.25
R79G	2.5
R79P	1.5

Figure 4. Reduced stability of $P18^{INK4C}$ mutants. A) U2OS cells were transiently transfected with the different P18-expression vectors and incubated with cyclohexamide for 0, 1, 2, 4 and 8 hours. Three examples of $P18^{INK4C}$ expression (wildtype, A61T and R79P) during cyclohexamide incubation are shown. B) The $P18^{INK4C}$ expression levels during cyclohexamide incubation were quantified in three individual experiments, and averaged. The expression levels without cyclohexamide incubation were set at 100%. Half-lifes of each $P18^{INK4C}$ protein, wildtype and mutants, were measured. CHX, cyclohexamide.

$P18^{INK4C}$ protein stability is decreased by the mutations

Since mutations in $P16^{INK4A}$ and $P18^{INK4C}$ can affect the stability of these proteins (20, 29), we investigated whether the mutations detected in MTCs and PCs affect $P18^{INK4C}$ protein stability. For this purpose, wildtype and mutant P18-constructs were transiently transfected into U2OS cells, a cell line known to express CDK4, CDK6 and RB, and previously used for functional studies on $P18^{INK4C}$ as well as on $P16^{INK4A}$ mutations (28, 30). Subsequently, the transfected cells were

incubated with the protein synthesis inhibitor cyclohexamide (CHX) for 0, 1, 2, 4 and 8 hours. The expression levels of the wildtype and mutant $P18^{INK4C}$ proteins during incubation with CHX were detected by immunoblotting (Figure 4A), quantified and the half-life of each mutant was calculated (Figure 4B). All mutant $P18^{INK4C}$ proteins, detected in human MTC and PC, displayed a shorter half-life (1.75 to 3 hours) compared to that of wildtype $P18^{INK4C}$ (5 hours). The control mutant, R79P, showed even a shorter half-life (1.5 hours). These results indicate that each mutation identified in human MTC and PC decreases the $P18^{INK4C}$ protein stability.

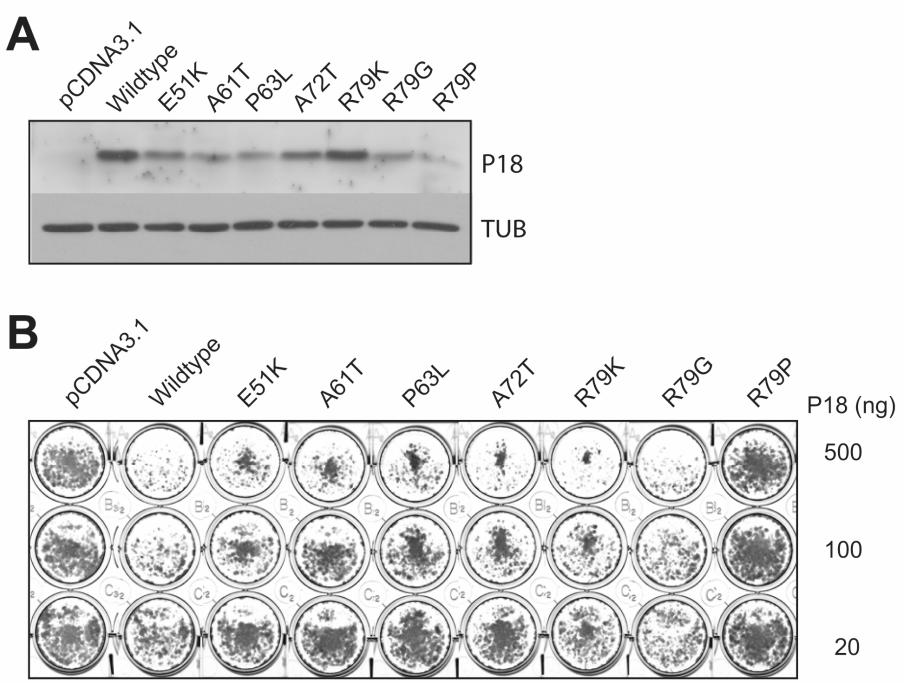


Figure 5. Reduced inhibition of cell growth by $P18^{INK4C}$ mutants. A) $P18^{INK4C}$ expression levels in U2OS cells stably expressing wildtype or mutant $P18^{INK4C}$ were detected by immunoblotting. Tubulin expression was determined to control for equal loading. B) U2OS cells were transfected with different amounts of P18-expression vectors (20 ng, 100 ng and 500 ng). Subsequently, the transfected cells were selected and seeded in a five times dilution series. After 10 days, cells were fixed and stained with Giemsa. This figure shows a representative result of one cellular dilution in one out of three individual experiments. TUB, Tubulin.

$P18^{INK4C}$ mutants are reduced in cell growth inhibition

As shown above, the mutations found in *P18* decrease the interaction with CDK4 and/or CDK6, as well as $P18^{INK4C}$ stability. Next, we investigated whether the $P18^{INK4C}$ mutants could still inhibit cell growth. U2OS cells were transfected with wildtype and mutant *P18*-constructs in different

concentrations. Cells were selected and $P18^{INK4C}$ expression levels were determined (Figure 5A). Cells stably expressing wildtype $P18^{INK4C}$ revealed a decreased growth rate compared to cells transfected with the empty vector. In contrast, cells expressing the MTC and PC $P18^{INK4C}$ mutants all displayed an increased growth rate as compared to cells expressing wildtype $P18^{INK4C}$ (Figure 5B). Cells expressing the control R79P mutant had grown as fast as the empty vector transfected cells (Figure 5B). This indicates that all mutations detected in MTCs and PCs, reduce the ability of $P18^{INK4C}$ to inhibit cell growth, possibly as a result of their reduced interaction with CDK4 and CDK6 (Figure 3) and/or their reduced protein stability (Figure 4).

DISCUSSION

RET activation is known to initiate MEN 2-associated tumor development. However, clinical practice and transgenic mouse studies indicate the requirement for other genetic defects in addition to an activating *RET* mutation. Here we show for the first time the presence of somatic inactivating mutations in cell cycle inhibitor *P18*, located at chromosome 1p32, in human MTC and PC. We have detected six different somatic missense *P18* mutations in MTCs and PCs, five of which cause reduced interaction with CDK4 and/or CDK6. All six $P18^{INK4C}$ mutants are less stable compared to wildtype $P18^{INK4C}$ and are reduced in their ability to inhibit cell growth. This is consistent with the previously published functional analyses on natural and artificial $P18^{INK4C}$ mutants (A61T, A72P and R79P), and *P16* mutations affecting amino acids that are analogous to the $P18^{INK4C}$ amino acids affected in MTCs and PCs (R58X, R87L and R87P) (20, 26, 28-32).

The role of *P18* in human cancer is largely unknown. Although loss of chromosome 1p occurs frequently in human cancer, only a few studies report the involvement of *P18*. Loss of $P18^{INK4C}$ expression is associated with the progression of medulloblastomas, testicular and hepatocellular cancer (33-35) and promotor hypermethylation of *P18* was detected in medulloblastomas and Hodgkin lymphomas (35, 36). The occurrence of *P18* point mutations in human tumors is very uncommon. In one out of 35 oligodendrogliomas a *P18* nonsense mutation, G113X, has been detected, while in another study a deletion of this residue has been detected in one out of 30 oligodendrogliomas (37, 38). In addition, a nonsense *P18* mutation, R68X, has been detected in one out of 67 meningiomas and a missense *P18* mutation, A72P, has been detected in three out of 35 breast carcinomas (39, 40). In many other types of human cancer *P18* mutations could not be detected, e.g. (41-46). Therefore, the occurrence of 6 somatic *P18* mutations in 58 human MTCs and PCs, indicate that *P18* functions as a tumor suppressor gene in the tumorigenesis of these neuroendocrine tumors.

Interestingly, the previously detected P18 mutations affecting R68 and A72 are located in the same region of $P18^{INK4C}$ as some of the mutations detected in MTCs and PCs. This region, i.e. loop 2, is

important for the conformation of $P18^{INK4C}$ and its function (25-28). Furthermore, we have detected two different P18 mutations that both affect the same amino acid residue, R79G in a familial MTC metastasis and R79K in a familial primary PC. Arginine at codon 79 is conserved among different species as well as among all INK4 family members. Moreover, R79 directly interacts with CDK4 and CDK6, explaining the importance of this residue for normal $P18^{INK4C}$ function (25-28). These data indicate that the mutations detected in MTC and PC are positioned in an important functional region of $P18^{INK4C}$.

We have recently found that loss of $p18^{Ink4C}$ expression in RET-induced murine MTC contributes to MTC growth (unpublished data*). In addition, loss of $P18^{INK4C}$ expression is associated with human cancer progression (33-36). Four of the *P18* mutations detected in this study were found in the heterozygous state. Although we can not exclude minimal contamination with normal tissue in our tumor samples used for DNA isolation, this suggests that these tumors have retained one intact *P18* allele. We and others have shown that *P18* acts as a haploinsufficient tumor suppressor gene in mice. Haploinsufficiency of *P18* was seen only in combination with a second carcinogenic stimulus, such as the presence of oncogenic RET or a chemical carcinogen, or in the absence of the Patched gene (unpublished data), (35, 47). Previously, it has been shown that oncogenic RET downregulates $P18^{INK4C}$ expression (48), supporting the hypothesis of *P18* being a haploinsufficient tumor suppressor gene. In all six tumors with inactivating *P18* mutations we detected coincidence with activating *RET* mutations, indicating that the remaining wildtype *P18* alleles in the tumors with a heterozygous *P18* mutation may be downregulated by oncogenic RET. In addition, we show here that the six novel *P18* mutations detected in MTCs and PCs reduce the stability of the $P18^{INK4C}$ protein. We conclude therefore that reduced $P18^{INK4C}$ function, either by inactivating point mutations and/or loss of expression due to protein instability and/or downregulation by oncogenic RET, contributes to MTC and PC development, supporting the model that *P18* functions as a haploinsufficient tumor suppressor gene in human cancer.

ACKNOWLEDGEMENTS

We acknowledge Dr. M.R. Canninga from the department of Pathology of the UMC Utrecht in the Netherlands for providing the human tumor material. We are thankful to Dr. R.B. van der Luijt from the department of Medical Genetics of the UMC Utrecht in the Netherlands for providing peripheral blood DNA of patients with a P18 mutation in their MTC or PC and for the information on germ-line RET mutations. We would like to thank Dr. H. Rehmann from the department of Physiological Chemistry of the UMC Utrecht in the Netherlands for generating the 3D-structure figures. We acknowledge Dr. D.S. Franklin from the department of Biochemistry, Tulane University School of Medicine, New Orleans, LA, USA for providing the GST-CDK4 and GST-CDK6 constructs. This work was supported by project grant UU2712 from the Dutch Cancer Society.

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CHAPTER 3

Synergistic effect of oncogenic *RET* and loss of *p18* on medullary thyroid carcinoma development

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Cancer Research 2008, in press

ABSTRACT

Activating mutations in the *RET* proto-oncogene are associated with both familial and sporadic medullary thyroid carcinoma (MTC) development, however the genetic mechanisms underlying MTC tumorigenesis remain largely unknown. Recently, we have identified somatic inactivating mutations in the cell cycle inhibitor gene *P18* in human MTC, which coincided with activating *RET* mutations, suggesting a role for loss of *P18* in combination with oncogenic *RET* in the multistep process of MTC development. Therefore, we crossed transgenic mice expressing oncogenic *RET* (*RET2B*) with mice lacking *p18* (and *p27*, another cell cycle inhibitor) and monitored for MTC development. *RET2B;p18*$^{+/-}$ mice and *RET2B;p18*$^{-/-}$ mice developed MTC with a highly increased incidence, compared to their corresponding single mutant littermates. In addition, expression of oncogenic *RET* causes an earlier age-of-onset and larger MTCs in *p18*$^{-/-}$*;p27*$^{+/-}$ mice. In a subset of MTCs of *RET2B;p18*$^{+/-}$*(;p27*$^{+/-}$*)* mice, p18^{Ink4c} expression was completely lost. This loss of p18^{Ink4c} expression correlated with higher proliferation rates as well as with larger MTCs, indicating that loss of *p18* in combination with oncogenic *RET* not only increases the risk for MTC development, it also enhances MTC progression. Our data strongly indicate that oncogenic *RET* and loss of *p18* cooperate in the multistep tumorigenesis of MTC.

INTRODUCTION

Multiple endocrine neoplasia (MEN) type 2 is an autosomal dominantly inherited cancer syndrome, which is mainly characterized by a combination of medullary thyroid carcinoma (MTC) and adrenal pheochromocytoma (PC). MEN 2 can be subdivided in MEN 2A, MEN 2B and familial MTC (FMTC). FMTC patients solely develop MTC, while MEN 2A and MEN 2B patients may develop, in addition to MTC and PC, other tumors like parathyroid adenomas (for MEN 2A) and mucosal ganglioneuromas (for MEN 2B). MTC originates from the calcitonin (CT) producing neuroendocrine C-cells in the thyroid gland (1).

MEN 2 is caused by activating germline mutations in the *RET* proto-oncogene, which encodes a transmembrane receptor tyrosine kinase (2). *RET* mutations associated with MEN 2 lead to constitutive activation of RET (3, 4). A strong genotype-phenotype correlation has been described for *RET* and MEN 2 (5, 6). In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein (2). In MEN 2B the most common (95%) mutation results in a Met→Thr substitution at position 918 in the intracellular domain of the protein. The M918T mutation is also found as a somatic mutation in about 30 to 40% of sporadic MTC cases (2).

Previously, we generated transgenic mice expressing the human *RET* oncogene with the M918T mutation in the thyroid C-cells (*RET2B*) (7). MTCs were detected in only 13% (3/23) of these mice at 11 to 24 months of age, while C-cell hyperplasia (CCH), a premalignant stage of MTC, was observed in 77% of these mice from 8 months on. The incomplete penetrance and variable latency period for MTC development in these mice, together with the clinical observation that carriers of the same germ-line *RET* mutation, can develop MTC at widely varying ages, suggest that in addition to the mutated *RET* gene, additional tumorigenic events are required for the development of MTC.

In human MTC, both hereditary and sporadic, the most frequently detected chromosomal alteration is loss of a specific part or the entire short arm of chromosome 1, with the most common break point on 1p32 (8-10), at which the candidate tumor suppressor gene *CDKN2C* (from here on indicated as *P18*) is located. Recently, we have detected several somatic inactivating mutations in *P18* in a subset of human MTCs, indicating that *P18* is a tumor suppressor gene involved in human MTC development (unpublished data*).

p18$^{-/-}$ mice develop CCH and MTC, of which the incidence is enhanced by additional loss of another cell cycle inhibitor, i.e. *p27* (11). Both cell cycle inhibitors p18^{Ink4c}, a member of the INK4 family, and p27^{Kip1}, a member of the CIP/KIP family, inhibit the formation of active cyclin dependent kinase (CDK)-complexes and thus prevent phosphorylation of Rb1, a major player in G1/S phase transition (12, 13). Interestingly, *p27* knockout mice as well as *Rb1* knockout mice also develop CCH and MTC, indicating that the CDK-Rb pathway is involved in the tumorigenesis of MTC (11, 14-16). Moreover, somatic *RET* mutations have been described in MTC of *Rb1;p53* knockout mice (17), suggesting a cooperative effect of oncogenic *RET* and the *Rb*-pathway in MTC tumorigenesis.

Because of the development of MTC in both *RET2B* transgenic mice and *p27*-, *p18*- and *p18;p27* knockout mice, together with the simultaneous occurrence of activating *RET* mutations and inactivating *P18* mutations in human MTC, we hypothesized that *p18* and/or *p27* may collaborate with oncogenic *RET* in the multistep tumorigenesis of MTC. To address this hypothesis, we crossed *RET2B* mice with *p18*$^{+/-}$;*p27*$^{+/-}$ mice and monitored for MTC. Our results indicate a strong synergistic effect of oncogenic *RET* and loss of *p18* on MTC development and progression.

MATERIALS AND METHODS

Mouse strains and genotyping: All experiments with mice were performed with the approval of the Animal Experimental and Ethics Committee of the University of Utrecht, Utrecht, The Netherlands. *RET2B* transgenic mice (C57BL/6 background) and *p18;p27* knockout mice (C57BL/6 background) were previously described (7, 11, 18). For genotype verification, genomic tail-DNA was isolated using a Prot-K lysis buffer (100mM Tris pH8, 5mM EDTA, 0.2% SDS, 0.2M NaCl, 100 ug/ul Proteinase K) at 55^{o}C. Separate PCRs were performed for *RET*, *p18* and *p27* using primers listed in Supplementary Table 1. All PCRs were performed at an annealing temperature of 55^{o}C, for 33 cycles.

Supplementary Table 1: Primers used for genotype verification, LOH analysis and mutation analysis

Primer	Sequence (5'-3')	Product size
RET2B genotyping		
RET-FW	TGG AGA CCC AAG ACA TCA AC	
RET-RV	GGA GAA GAG GAC AGC GGC T	220 bp (human RET transgene)
p18 genotyping and LOH analysis		
P18WT-F	AGC CAT CAA ATT TAT TCA TGT TGC AGG	
P18WT-R	CCT CCA TCA GGC TAA TGA CC	600 bp (wildtype-allele)
P18neo-R	CTC TGA GCC CAG AAA GCG AAG	400 bp (null-allele)
p27 genotyping		
P27KO-1	TCA AAC GTG AGA GTG TCT AAC GG	
P27KO-2	AGG GGC TTA TGA TTC TGA AAG TCG	200 bp (wildtype-allele)
P27KO-3	ATT TTG CTG AAG AGC TTG GCG G	300 bp (null-allele)
p18 mutation analysis		
mP18-E2FW	AGC CTG ATT AGG AGC AAA GG	
mP18-E2RV	GCT GTC ATT TTA GAA ACC CAG GC	275 bp (exon 2)
mP18-E3FW	TTG TTG TGG CTC AAG AGC TGG	
mP18-E3RV	TAG TGA AAC GGA CAG CCA AC	495 bp (exon 3)

Plasma CT measurements: Blood was collected every 3 months via orbital puncture, after isofloran anaesthesia. Plasma was isolated from EDTA-blood by centrifugation. Plasma CT concentrations were measured using a solid-phase, enzyme-labeled, two-site chemiluminescent immunometric assay (DPC, Los Angeles, CA, USA) with an Immulite 1000 analyzer according to the manufacturer's protocol. Interassay variability (measured over 18 assays) shows that this assay is very sensitive and robust: Average concentration for assay-control 1 = 11.2 pg/ml (SD 0.27); variation coefficient = 2.42%. Average concentration for assay-control 2 = 209.5 pg/ml (SD 3.99); variation coefficient = 1.90%. Plasma CT levels were measured in counts per second (cps) and calculated to pg/ml according to the assay-standard curve. Wildtype non-MTC bearing mice have undetectable plasma CT levels (around 35.000 cps). Histological analysis revealed that in our study, plasma CT levels from 1.4 pg/ml onwards (≥ 100.000 cps) indicated microscopically detectable MTCs.

Tissue processing and immunohistochemistry (IHC): Isolated thyroid tissues were fixed in 4% formaldehyde (Klinipath, Deuven, NL) overnight, dehydrated and embedded in paraffin. IHC (for CT, $p18^{Ink4c}$, $p27^{Kip1}$ and PCNA) was performed on paraffin sections of 6µm. For $p27^{Kip1}$ and PCNA staining, antigen unmasking was performed by boiling the slides in sodium citrate buffer (10 mM sodium citrate trisodium salt dihydrate in dH2O; pH=6) for 15 min. Primary antibodies used: rabbit polyclonal anti-CT antibody (Dako, Glostrup, Denmark), diluted 1:5000; rabbit polyclonal anti-p18 antibody (M-168) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:50; Mouse monoclonal anti-KIP1 antibody (BD Transduction Laboratories, San Jose, CA, USA), diluted 1:500; and mouse monoclonal anti-PCNA antibody (PC10) (Cell Signaling Technology, Danvers, MA, USA), diluted 1:4000. Secondary antibodies used: HRP-conjugated swine-anti-rabbit antibody (Dako) and HRP-conjugated rabbit-anti-mouse antibody (Dako), diluted 1:100, at room temperature for 30 min. For p27Kip1 staining, Goat-anti-M/R/Ra IgG DVDPO-500H (Powervision) (ImmunoLogic, Duiven, The Netherlands) was used as secondary antibody. After DAB precipitation, a haematoxylin counterstain was performed. The anti-KIP1 antibody and Powervision were kindly provided by the department of Pathology of the University Medical Center Utrecht, Utrecht, The Netherlands.

Laser capture microdissection (LCM): LCM was used to obtain cell populations of selected areas from paraffin-embedded tissue sections. Sections (10µm thick) on 1 mm PEN Membrane Slides (P.A.L.M. Microlaser Technologies,

Bernried, Germany) were deparaffinized and lightly stained with haematoxylin. Using a P.A.L.M. Microbeam laser capture microdissection system (P.A.L.M. Microlaser Technologies), lesions of MTCs and normal tissues were separately isolated from the sections. DNA was isolated from the microdissected tissue samples using a ProtK lysis (50 mM Tris-HCl pH8, 0.5% Tween-20, 2 mg/ml ProtK) at 55°C for 16 hours.

LOH analysis and somatic mutation analysis: For *p18* LOH analysis and *p18* and *RET* mutation analysis, PCRs were performed directly on DNA-lysates isolated by LCM, using Titanium Taq polymerase (Clontech, Mountain View, CA, USA). *P18* primers are listed in Supplementary Table 1. *RET* primers were described in (17). PCRs were performed at an annealing temperature of 55°C (LOH) or 62°C (mutation analysis), for 33 cycles. For LOH analysis, all three listed primers, discriminating between wildtype and null alleles, were used in one PCR. On gel, wildtype and null alleles are discriminated by different sizes (Supplementary Table 1). As a control, tail-DNA from $p18^{+/+}$, $p18^{+/-}$ and $p18^{-/-}$ mice, as well as LCM-DNA from paraffin sections of normal tissue of $p18^{+/+}$ and $p18^{-/-}$ mice was used. For mutation analysis PCR products were purified using a JETquick PCR purification kit (Genomed, Löhne, Germany). Direct sequence analysis PCRs were performed on purified PCR products using BigDye Terminator (Applied Biosystems, Foster City, CA, USA). As a control, tail-DNA of a wildtype mouse was used.

Statistical analysis: To compare MTC incidences between different genotype groups, the Fisher's Exact test was used. The Mann-Whitney test was used to compare plasma CT levels of mice in different genotype groups. To compare proliferation indices of different MTCs, the Student's T-test was used. P-values < 0.05 were considered as significant.

RESULTS

Generation of compound *RET2B;p18*, *RET2B;p27* and *RET2B;p18;p27* mice

To investigate the role of oncogenic *RET*, *p18* and *p27* in the multistep process of MTC development *in vivo*, we crossed *RET2B* mice (7) with $p18^{+/-};p27^{+/-}$ knockout mice (18). Mating between these mouse strains, both on a C57BL/6 background, yielded all expected genotypes at the anticipated Mendelian ratios. Reproduction was not affected in any of the generated genotypes. For this study, only mice from the third generation were used.

Wildtype mice (*WT*; n=26), *RET2B* mice (n=48), $p18^{+/-}$ mice (n=25), $p18^{-/-}$ mice (n=30) and $p27^{+/-}$ mice (n=22) were generated. To study the effect of oncogenic *RET* with additional loss of either *p18* or *p27* on MTC development, compound *RET2B;*$p18^{+/-}$ mice (n=44), *RET2B;*$p18^{-/-}$ mice (n=31) and *RET2B;*$p27^{+/-}$ mice (n=49) were generated. Franklin *et al.*, showed a synergistic effect of loss of both *p18* and *p27* on MTC development (11). To investigate a putative additional effect of oncogenic *RET* on loss of both *p18* and *p27*, we generated $p18^{+/-};p27^{+/-}$ mice (n=20), $p18^{-/-};p27^{+/-}$ mice (n=24), *RET2B;*$p18^{+/-};p27^{+/-}$ mice (n=44) and *RET2B;*$p18^{-/-};p27^{+/-}$ mice (n=40). $p27^{-/-}$ mice were not generated because in combination with homozygous loss of *p18*, they have a low mean survival of 3.5 months (11, 18).

$p18^{-/-}$ mice with and without additional loss of *p27* display gigantism from early ages on (11, 18). We did not detect any apparent enhancement of the gigantism phenotype in the presence of oncogenic *RET*. All mice were monitored up to 12 months of age. In this period, survival was not

affected in the different genotype groups, except for *p18*$^{-/-}$;*p27*$^{+/-}$ mice, which died, or became moribund, at the age of 8-9 months due to previously described causes (11, 18). The survival of compound *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ mice was comparable to the survival of *p18*$^{-/-}$;*p27*$^{+/-}$ mice. Therefore, MTC development in the latter two genotype groups was monitored up to 9 months of age.

Table 1. MTC incidence in mice of each genotype group up to 12 months of age*

Genotype	3 months	6 months	9 months	12 months
WT	0% (0/26)	0% (0/25)	0% (0/25)	0% (0/25)
RET2B	0% (0/48)	0% (0/46)	0% (0/45)	0% (0/45)
p18$^{+/-}$	0% (0/25)	0% (0/23)	0% (0/23)	0% (0/23)
p18$^{-/-}$	0% (0/30)	0% (0/29)	4% (1/28)	11% (3/27)
p27$^{+/-}$	0% (0/22)	0% (0/22)	0% (0/22)	0% (0/21)
RET2B;p18$^{+/-}$	0% (0/44)	2% (1/43)	21% (9/42)[a]	26% (10/39)[b]
RET2B;p18$^{-/-}$	3% (1/31)	7% (2/30)	33% (10/30)[c]	43% (13/30)[d]
RET2B;p27$^{+/-}$	0% (0/49)	0% (0/49)	4% (2/49)[e]	4% (2/47)[f]
p18$^{+/-}$;*p27*$^{+/-}$	0% (0/20)	0% (0/20)	0% (0/19)	0% (0/18)
p18$^{-/-}$;*p27*$^{+/-}$	0% (0/24)	0% (0/23)	88% (15/17)[g]	ND
RET2B;p18$^{+/-}$;*p27*$^{+/-}$	0% (0/44)	2% (1/44)	7% (3/44)[h,l]	27% (12/44)[i,m]
RET2B;p18$^{-/-}$;*p27*$^{+/-}$	3% (1/40)	31% (11/35)[j,n]	100% (26/26)[k,o]	ND

* MTC was diagnosed by plasma CT levels > 1.4 pg/ml.

[a] P=0.001 for *RET2B;p18*$^{+/-}$ versus *RET2B*; P=0.014 for *RET2B;p18*$^{+/-}$ versus *p18*$^{+/-}$

[b] P<0.0001 for *RET2B;p18*$^{+/-}$ versus *RET2B*; P=0.006 for *RET2B;p18*$^{+/-}$ versus *p18*$^{+/-}$

[c] P<0.0001 for *RET2B;p18*$^{-/-}$ versus *RET2B*; P=0.004 for *RET2B;p18*$^{-/-}$ versus *p18*$^{-/-}$

[d] P<0.0001 for *RET2B;p18*$^{-/-}$ versus *RET2B*; P=0.007 for *RET2B;p18*$^{-/-}$ versus *p18*$^{-/-}$

[e] P=0.269 for *RET2B;p27*$^{+/-}$ versus *RET2B*; P=0.473 for *RET2B;p27*$^{+/-}$ versus *p27*$^{+/-}$

[f] P=0.258 for *RET2B;p27*$^{+/-}$ versus *RET2B*; P=0.475 for *RET2B;p27*$^{+/-}$ versus *p27*$^{+/-}$

[g] P<0.0001 for *P18*$^{-/-}$;*p27*$^{+/-}$ versus *p18*$^{-/-}$; P<0.0001 for *P18*$^{-/-}$;*p27*$^{+/-}$ versus *p27*$^{+/-}$

[h] P=0.117 for *RET2B;p18*$^{+/-}$;*p27*$^{+/-}$ versus *RET2B*; P=0.334 for *RET2B;p18*$^{+/-}$;*p27*$^{+/-}$ versus *p18*$^{+/-}$;*p27*$^{+/-}$

[i] P<0.0001 for *RET2B;p18*$^{+/-}$;*p27*$^{+/-}$ versus *RET2B*; P=0.01 for *RET2B;p18*$^{+/-}$;*p27*$^{+/-}$ versus *p18*$^{+/-}$;*p27*$^{+/-}$

[j] P<0.0001 for *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ versus *RET2B*; P=0.002 for *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ versus *p18*$^{-/-}$;*p27*$^{+/-}$

[k] P<0.0001 for *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ versus *RET2B*; P=0.151 for *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ versus *p18*$^{-/-}$;*p27*$^{+/-}$

[l] P=0.039 for *RET2B;p18*$^{+/-}$;*p27*$^{+/-}$ versus *RET2B;p18*$^{+/-}$

[m] P=0.533 for *RET2B;p18*$^{+/-}$;*p27*$^{+/-}$ versus *RET2B;p18*$^{+/-}$

[n] P=0.013 for *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ versus *RET2B;p18*$^{-/-}$

[o] P<0.0001 for *RET2B;p18*$^{-/-}$;*p27*$^{+/-}$ versus *RET2B;p18*$^{-/-}$

ND, not determined (mice were sacrificed at 9 months).

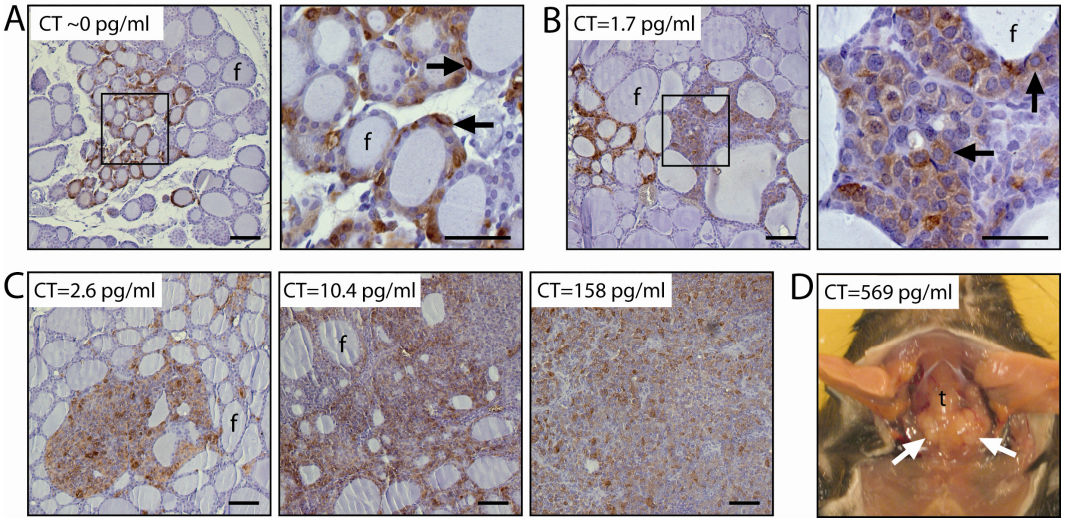


Figure 1. MTC development in mice with elevated plasma CT levels. A) CT staining on the thyroid gland of a wildtype C57BL/6 mouse with undetectable plasma CT level indicating normal C-cell amounts. Right panel shows a higher magnification of the area indicated by the box in left panel. B) CT staining on a thyroid gland of a mouse with a plasma CT level of 1.7 pg/ml indicating a microMTC. Right panel shows a higher magnification of the area indicated by the box in left panel. C) CT staining on thyroid glands of mice with plasma CT levels of 2.6 pg/ml, 10.4 pg/ml, and 158 pg/ml, indicating MTCs of increasing sizes. D) Macroscopic bilateral MTC (indicated by white arrows) in a mouse with a plasma CT level of 569 pg/ml. Black arrows indicate CT positive C-cells. f, thyroid follicle; t, trachea. Scale bars represent 50µm.

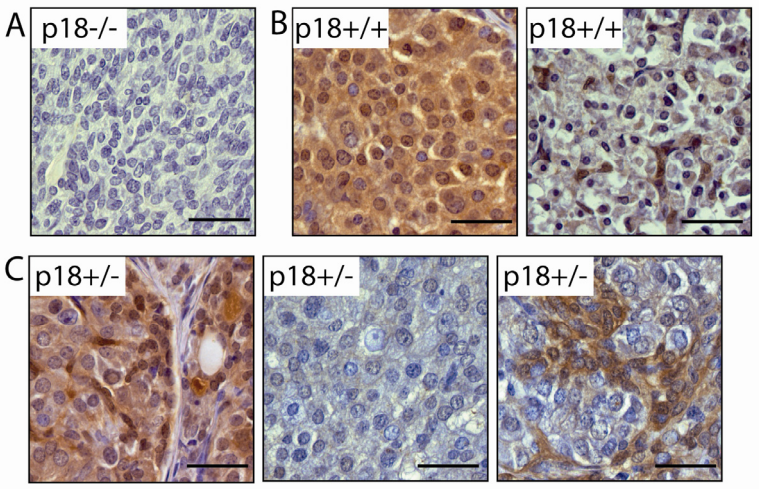


Figure 3. $p18^{Ink4c}$ immunostaining on paraffin sections of murine MTCs. A) negative control staining on *p18*$^{-/-}$ MTC (*RET2B;p18*$^{-/-}$*;p27*$^{+/-}$ mouse). B) $p18^{Ink4c}$ immunostaining on *p18*$^{+/+}$ MTCs (left: positive staining on an MTC of a *RET2B;p27*$^{+/-}$ mouse, right: patchy staining on an MTC of a *RET2B* mouse). C) $p18^{Ink4c}$ immunostaining on *p18*$^{+/-}$ MTCs (left: positive staining on an MTC of a *RET2B;p18*$^{+/-}$*;p27*$^{+/-}$ mouse; middle: negative staining on an MTC of a *RET2B;p18*$^{+/-}$ mouse; right: patchy staining on an MTC of a *RET2B;p18*$^{+/-}$ mouse). Scale bars represent 50µm.

Synergistic effect of oncogenic *RET* and loss of *p18* on MTC development

In *RET2B* mice, the expression of the oncogenic *RET* transgene is directed to the thyroid C-cells due to the used CALC-I promoter. These mice develop CCH and MTC without any other tumors involved in MEN 2 (7). Here, we monitored the development of MTC in the different genotype groups, by measuring plasma CT levels of the mice. Plasma CT levels are used as a specific tumor marker for the clinical diagnosis and follow-up of MTC patients (19). Previously we showed that plasma CT levels can also be used to monitor MTC development in mice (7). Blood was drawn every 3 months and when the mice became moribund. In this way, we were able to monitor MTC development and progression in time. At the time of sacrifice, thyroid glands were isolated for histological analysis. CT immunostainings were performed to identify the presence of an MTC. Bilateral and multifocal MTCs were detected in mice of different genotype groups. The level of plasma CT in wildtype non-MTC bearing mice is undetectable (Figure 1A). Histological analysis revealed that in our study, plasma CT levels of 1.4 pg/ml and higher indicated the presence of an MTC (Figure 1B). Most MTCs were detectable only by microscopy (Figure 1B,C), however, some mice developed macroscopically detectable MTC (Figure 1D).

The numbers and percentages of mice of all different genotype groups which developed MTC within 12 months are summarized in Table 1. Up to 12 months of age, no MTC was detected in the *WT*, *RET2B*, $p18^{+/-}$ and $p27^{+/-}$ groups, whereas 11% (3/27) of $p18^{-/-}$ mice had developed MTC at this age. 43% (13/30) of compound *RET2B;*$p18^{-/-}$ mice displayed MTC at 12 months, which is a significantly increased incidence compared to both *RET2B* mice and $p18^{-/-}$ mice. The MTC incidence in *RET2B;*$p18^{-/-}$ mice was already significantly increased to 33% (10/30) at 9 months of age, indicating a strong synergistic effect of oncogenic *RET* and loss of *p18* on MTC development.

Haploinsufficiency of *p18*, but not *p27*, in *RET*-induced MTC

Previously, it has been shown that treatment of heterozygous *p18* knockout mice with a chemical carcinogen resulted in tumor development at an accelerated rate. The remaining wildtype allele of *p18* was neither mutated nor silenced in tumors derived from heterozygotes, indicating that *p18* is a haploinsufficient gene in tumorigenesis in mice (20). Although MTC developed predominantly in mice of homozygous *p18* knockout groups, a subset of mice in the heterozygous *p18* knockout groups did develop MTC as well. This offered an opportunity to address *p18* haploinsufficiency in MTC development. As is shown in Table 1, loss of a single *p18* allele (with and without additional loss of *p27*) did not result in MTC development in mice up to 12 months, indicating that heterozygous loss of *p18* is not sufficient for MTC development within this period. However, compound *RET2B;*$p18^{+/-}$ mice displayed a significantly increased MTC incidence already at 9 months [21% (9/42)] as compared to both *RET2B* and $p18^{+/-}$ mice, suggesting that *p18* is haploinsufficient in the presence of oncogenic *RET* (Table 1).

RET2B mice which had lost a single *p27* allele did not show a significant increase in MTC incidence compared to *RET2B* mice and $p27^{+/-}$ mice: 4% (2/47) of $RET2B;p27^{+/-}$ mice developed MTC up to 12 months of age (Table 1), indicating that heterozygous loss of *p27* does not affect *RET*-induced MTC development. These data suggest that *RET* and *p18*, but not *RET* and *p27*, cooperate in MTC development.

Table 2: Elevated plasma CT levels indicating CCH in mice that did not develop MTC (plasma CT <100.000cps) at 12 months of age

Genotype	N	Mean CT (cps)	SD	T-test (vs WT)	Mice with MTC (plasma CT >100.000cps)
WT	25	34842	6466		0
RET2B	45	35923	8487	NS	0
$p18^{+/-}$	26	32978	5197	NS	0
$p18^{-/-}$	24	59271	17043	p<0.001	3
$p27^{+/-}$	21	34379	6921	NS	0
$RET2B;p18^{+/-}$	31	42779	10983	p=0.002	10
$RET2B;p18^{-/-}$	17	64816	16925	p<0.001	13
$RET2B;p27^{+/-}$	45	39574	9608	p=0.03	2
$p18^{+/-};p27^{+/-}$	18	42404	6692	p<0.001	0
$RET2B;p18^{+/-};p27^{+/-}$	32	48475	13552	p<0.001	12

cps, counts per second; NS, not significant; SD, standard deviation.

CCH in $RET2B;p27^{+/-}$ mice and $p18^{+/-};p27^{+/-}$ mice at 12 months of age

Up to 12 months of age, *WT* mice did not develop MTC, nor could we histologically detect CCH in these mice. The mean plasma CT level of WT mice at the age of 12 months was indicated as 34842 counts per second (cps) (SD 6466, n=25). To investigate whether mice, of the different genotype groups, that did not develop MTC (plasma CT <100.000 cps) developed CCH, we looked whether they had significantly elevated plasma CT levels at the age of 12 months (Table 2). Plasma CT levels of *RET2B* mice, $p18^{+/-}$ mice and $p27^{+/-}$ mice, did not significantly differ from those of *WT* mice, suggesting that these mice did not develop CCH at the age of 12 months. $P18^{-/-}$ mice, however, did develop a significant increase in plasma CT at the age of 12 months: (59271 cps, SD 17043, n=24), suggesting CCH. In the 3 *RET2B;p18* genotype groups, in which MTC was also detected at 12 months of age, increased plasma CT levels were identified in mice that did not develop MTC.

Interestingly, in the $RET2B;p27^{+/-}$ group and $p18^{+/-};p27^{+/-}$ group, in which MTC development could (almost) not be observed at 12 months, CCH was suggested by elevated plasma CT levels compared to those of *WT* mice. Comparing plasma CT levels of $RET2B;p27^{+/-}$ mice to plasma levels of *RET2B* mice, revealed an increase that was just not significant (p=0.06), while plasma CT

levels of $p18^{+/-};p27^{+/-}$ mice were significantly ($p<0.001$) increased compared to those of $p18^{+/-}$ mice. These data suggests that loss of *p27* does not synergize with oncogenic *RET* in MTC development, whereas is does with loss of *p18*.

Oncogenic *RET* causes earlier MTC onset in compound transgenic mice

The synergism between *p18* and *p27* could also be observed in $p18^{-/-};p27^{+/-}$ mice: 88% (15/17) of $p18^{-/-};p27^{+/-}$ double knockout mice developed MTC at 9 months of age, compared to 4% (1/28) of $p18^{-/-}$ mice (Table 1), indicating that simultaneous loss of *p18* (homozygous) and *p27* (heterozygous) is sufficient for MTC development in mice, as was previously also shown by Franklin *et al.* (11). We wondered whether oncogenic *RET* could increase the MTC incidence even further in the *p18;p27* background. 100% (26/26) of $RET2B;p18^{-/-};p27^{+/-}$ mice developed MTC at 9 months, which was not significantly increased compared to $p18^{-/-};p27^{+/-}$ mice at this age [88% (15/17)] (Table 1). However, 27% (12/44) of $RET2B;p18^{+/-};p27^{+/-}$ mice displayed MTC at 12 months of age, which is a significant increase compared to 0% (0/18) of $p18^{+/-};p27^{+/-}$ mice (Table 1). In addition, a remarkable difference in MTC incidence between $p18^{-/-};p27^{+/-}$ mice and $RET2B;p18^{-/-};p27^{+/-}$ mice was observed at the age of 6 months: 31% (11/35) of $RET2B;p18^{-/-};p27^{+/-}$ mice developed MTC at this age compared to 0% (0/23) of $p18^{-/-};p27^{+/-}$ mice (Table 1), strongly indicating that oncogenic *RET* causes an earlier MTC onset in $p18^{-/-};p27^{+/-}$ mice. All mice which displayed MTC already at the age of 3 or 6 months (n=15), belonged to groups with a combined *RET2B* and heterozygous or homozygous *p18* knockout genotype (Table 1), confirming that the combination of oncogenic *RET* and loss of *p18* strongly promotes (early) MTC development in mice.

Oncogenic *RET* induces larger MTCs in compound transgenic mice

As described above, oncogenic *RET* in combination with loss of *p18* or loss of both *p18* and *p27* resulted in increased MTC incidence as well as decreased age-of-onset. Next, we investigated whether we could detect an effect on MTC growth of oncogenic *RET* in addition to loss of *p18* and *p27*, by comparing plasma CT levels of mice with MTC. We found a correlation between plasma CT levels and MTC size, as was also detected for human MTC patients (Figure 1C) (21). In Figure 2, plasma CT levels of all mice with MTC from different compound genotype groups at 3, 6, 9 and 12 months of age are indicated.

At the age of 9 months, plasma CT levels of compound $RET2B;p18^{-/-};p27^{+/-}$ mice with MTC (n=24) were significantly higher (ranging from 1.4 to 569 pg/ml with a median concentration of 84.25 pg/ml; $p<0.01$) compared to plasma CT levels of $p18^{-/-};p27^{+/-}$ mice with MTC (n=15; ranging from 2.1 to 261 pg/ml with a median concentration of 15.6 pg/ml) (Figure 2).

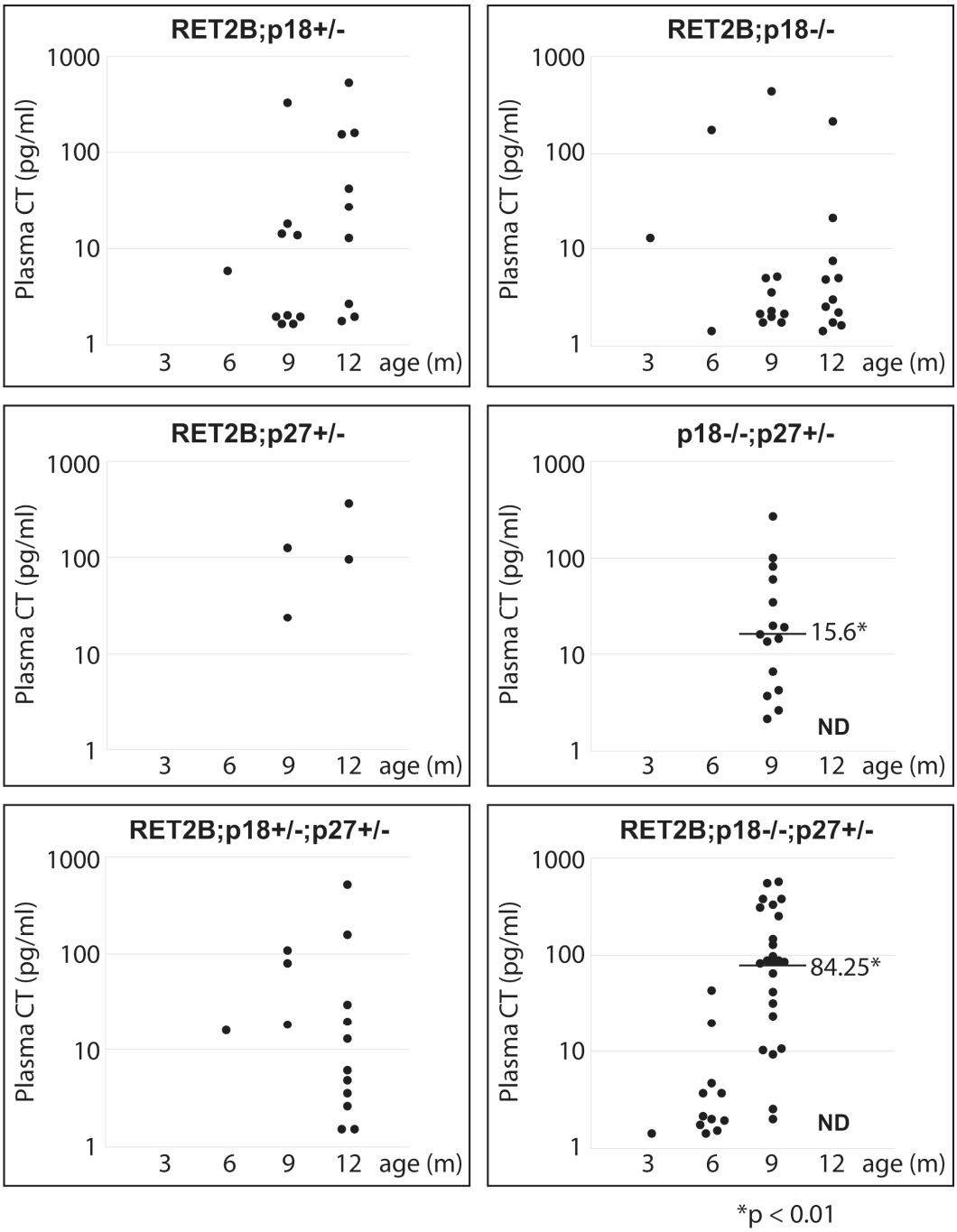


Figure 2. Plasma CT levels of mice with MTC (≥1.4 pg/ml) from compound genotype groups are indicated at 3, 6, 9 and 12 months of age. A logarithmic scale was used to plot the plasma CT levels. ND, not determined (mice were sacrificed at 9 months).

This indicates that already at 9 months of age *RET2B;p18$^{-/-}$;p27$^{+/-}$* mice have larger MTCs compared to *p18$^{-/-}$;p27$^{+/-}$* mice. This was also confirmed by histological analysis.

To investigate whether we could detect a difference in growth rate in these MTCs, we analysed proliferation in 5 MTCs of both the *p18$^{-/-}$;p27$^{+/-}$* mice and *RET2B;p18$^{-/-}$;p27$^{+/-}$* mice. The MTCs were selected from mice of each genotype group which matched according to their plasma CT levels, thus MTCs from comparable sizes. As determined by PCNA immunostainings and subsequent proliferation index calculations, we did not identify a significant difference (p=0.43) in the average proliferation rates between MTCs from the two different genotype groups: 17.7% (SD 2.4) for MTCs from *p18$^{-/-}$;p27$^{+/-}$* mice and 19.6% (SD 4.6) for MTCs from *RET2B;p18$^{-/-}$;p27$^{+/-}$* mice (Supplementary Table 2), suggesting that the larger MTCs detected in *RET2B;p18;p27* mice are due to earlier age-of-onset, rather than to increased growth rate.

Supplementary Table 2. Proliferation rates in MTCs of *p18$^{-/-}$;p27$^{+/-}$* mice and *RET2B;p18$^{-/-}$;p27$^{+/-}$* mice

p18$^{-/-}$;p27$^{+/-}$		*RET2B;p18$^{-/-}$;p27$^{+/-}$*	
Plasma CT (pg/ml)	**PCNA index* (total)†**	**Plasma CT (pg/ml)**	**PCNA index* (total)†**
19.1	16.1% (1420)	22.6	19.3% (1404)
60.1	18.9% (1615)	62.9	16.1% (1419)
80.6	14.9% (1778)	82.9	17.4% (1383)
100	17.7% (932)	95.1	27.5% (2067)
261	20.9% (1658)	252	17.8% (1775)
	mean 17.7% (SD 2.4)‡		mean 19.6% (SD 4.6)‡

***** PCNA index is an average from analysis of 4 microscopic fields per MTC; † total amount of cells analysed;
‡ p = 0.43; SD, standard deviation

Heterozygous loss of *p27* increases MTC incidence in *RET2B;p18$^{-/-}$*, but not *RET2B;p18$^{+/-}$*, compound transgenic mice

As described above, heterozygous loss of *p27* additionally affect MTC development in combination with complete loss of *p18*, but not with oncogenic *RET*. At 9 months of age *RET2B;p18$^{-/-}$;p27$^{+/-}$* mice displayed a significantly higher MTC incidence [100% (26/26)] compared to *RET2B;p18$^{-/-}$* mice [33% (10/30)] (Table 1). In addition, *RET2B;p18$^{-/-}$;p27$^{+/-}$* mice with MTC also displayed significantly higher (p<0.001) plasma CT levels compared to *RET2B;p18$^{-/-}$* mice with MTC (n=10; ranging from 1.7 to 420 pg/ml with a median concentration of 2.2 pg/ml), indicating an additional effect of heterozygous loss of *p27* on *RET2B;p18$^{-/-}$*-induced MTC development (Figure 2).

Comparing plasma CT levels of *RET2B;p18$^{+/-}$;p27$^{+/-}$* mice that did not develop MTC at the age of 12 months to plasma CT levels of *RET2B;p18$^{+/-}$* mice, revealed an increase that was just not

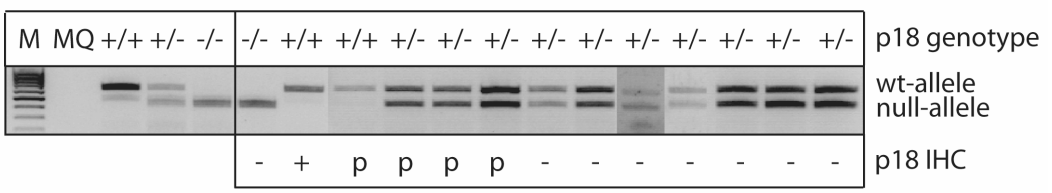
significant (p=0.07), suggesting that additional loss of *p27* does not strongly enhances CCH in *RET2B;p18*$^{+/-}$ mice (Table 2). Surprisingly, *RET2B;p18*$^{+/-}$*;p27*$^{+/-}$ mice [7% (3/44)] displayed a significantly lower MTC incidence at the age of 9 months compared to *RET2B;p18*$^{+/-}$ mice [21% (9/42)] suggesting a protective effect of loss of *p27* on MTC development (Table 1). However, 5 out of these 9 *RET2B;p18*$^{+/-}$ mice displayed plasma CT levels of 1.4 to 2 pg/ml indicating very small MTCs (Figure 2). Moreover, at 12 months of age, these genotype groups displayed equal frequencies of MTC development [26% (10/39) for *RET2B;p18*$^{+/-}$ mice and 27% (12/44) for *RET2B;p18*$^{+/-}$*;p27*$^{+/-}$ mice] (Table 1) as well as no significantly different (p=0.52) plasma CT levels (Figure 2), indicating that it is not likely that loss of *p27* is protective for MTC development.

P27 is known to be a haploinsufficient tumor suppressor gene (22). To investigate haploinsufficiency of *p27* in *(RET2B;)p18*-induced MTC, a P27^{Kip1} immunostaining was performed on 41 MTCs from heterozygous *p27* knockout mice (21 *RET2B;p18*$^{-/-}$*;p27*$^{+/-}$ mice, 7 *RET2B;p18*$^{+/-}$*;p27*$^{+/-}$ mice, 11 *p18*$^{-/-}$*;P27*$^{+/-}$ mice, and 2 *RET2B;p27*$^{+/-}$ mice). As a positive control, we stained MTC sections of 2 *p27*$^{+/+}$ mice. In none of the 41 MTCs complete loss or strongly reduced p27^{Kip1} expression could be observed (not shown), indicating that the remaining *p27* allele is still intact. These results indicate that *p27* functions as a haploinsufficient tumor suppressor gene in *p18*-induced MTC tumorigenesis.

Somatic alterations in *p18* or *RET* in murine MTCs

Next, we investigated whether we could detect loss of *p18* as a somatic event in MTCs from mice without a germline defect in *p18*. We stained MTCs from 7 *RET2B* mice (over 12 months old) and from 2 *RET2B;p27*$^{+/-}$ mice for p18^{Ink4c}. As a negative control, a p18^{Ink4c} immunostaining was performed on MTC sections from a *p18*$^{-/-}$ mouse (Figure 3A). 8 MTCs of *RET2B(;p27*$^{+/-}$*)* mice stained positive for p18^{Ink4c}, while in one MTC of a *RET2B* transgenic mouse a patchy p18^{Ink4c} expression pattern was detected (Figure 3B). We could not detect homozygous loss of *p18* in the DNA isolated from this tumor (Supplementary Figure 1). Subsequently, we performed a mutation analysis on DNA isolated from all 9 *p18*$^{+/+}$ MTCs. In none of these MTCs, a mutation in the coding region of *p18* could be detected.

Coxon *et al*., detected somatic *RET* mutations in 4 out of 9 MTCs from *Rb;p53* double knockout mice (17). These *RET* mutations corresponded with human MEN 2A-associated *RET* mutations. To investigate whether such mutations were acquired in MTCs from *p18;p27* knockout mice as well, we performed nucleotide sequence analysis on DNA isolated from MTCs of 7 *p18*$^{-/-}$*;p27*$^{+/-}$ mice. In none of these MTCs, a somatic *RET* mutation could be identified.



Supplementary Figure 1. *P18* LOH analysis on DNA isolated from MTCs with and without $p18^{Ink4c}$ expression. For controls, tail-DNA from $p18^{+/+}$, $p18^{+/-}$ and $p18^{-/-}$ mice and LCM-isolated DNA from paraffin sections of $p18^{+/+}$ and $p18^{-/-}$ mice was used. LCM-DNA from several murine MTCs was used for LOH analysis. $p18^{Ink4c}$ expression status and genotypes of the corresponding mice are indicated. M, size marker; MQ, water control; p, patchy.

Loss of $p18^{Ink4c}$ expression in MTCs from $p18^{+/-}$ mice correlates with MTC progression

To assess whether $p18^{Ink4c}$ expression was lost in MTCs of heterozygous *p18* knockout mice, $p18^{Ink4c}$ expression was determined in MTCs of *RET2B;*$p18^{+/-}$ (n=9) and *RET2B;*$p18^{+/-}$*;*$p27^{+/-}$ mice (n=8). In 7 out of these 17 MTCs of heterozygous *p18* knockout mice, $p18^{Ink4c}$ expression could be detected, comparable to $p18^{Ink4c}$ expression in MTC of $p18^{+/+}$ mice. However, in 7 out of these 17 MTCs $p18^{Ink4c}$ expression was completely lost and in 3 MTCs a patchy $p18^{Ink4c}$ expression pattern was observed (Figure 3C, Table 3), indicating that somatic loss of $p18^{Ink4c}$ expression occurs frequently in murine MTC.

Next, we investigated whether this somatic loss of $p18^{Ink4c}$ expression was due to LOH or somatic mutations in the *p18* gene. In none of the MTCs which showed (partial) loss of $p18^{Ink4c}$ expression we could detect loss of the wildtype *p18* allele(s) (Supplementary Figure 1), nor could we detect mutations in the coding region of *p18* in MTCs from these 17 *RET2B;*$p18^{+/-}$*(;*$p27^{+/-}$*)* mice, indicating that other mechanisms are involved in the loss of $p18^{Ink4c}$ expression in these MTCs.

Furthermore, we investigated whether loss of $p18^{Ink4c}$ expression affected MTC growth, by comparing the plasma CT levels of *RET2B;*$p18^{+/-}$*(;*$p27^{+/-}$*)* mice with MTCs with (partial) loss of $p18^{Ink4c}$ expression (n=10; ranging from 6.2 to 522 pg/ml with a median concentration of 97 pg/ml) to plasma CT levels of *RET2B;*$p18^{+/-}$*(;*$p27^{+/-}$*)* mice with MTCs with $p18^{Ink4c}$ expression (n=7; ranging from 1.7 to 19.5 pg/ml with a median concentration of 4.8 pg/ml) (Table 3). The plasma CT levels of mice with MTCs without $p18^{Ink4c}$ expression are significantly higher (p=0.001) compared to those of mice with MTCs expressing $p18^{Ink4c}$, indicating that MTCs without $p18^{Ink4c}$ expression were larger compared to those expressing $p18^{Ink4c}$.

Next, we determined the proliferation rates of MTCs of *RET2B;*$p18^{+/-}$*(;*$p27^{+/-}$*)* mice with and without $p18^{Ink4c}$ expression. The average proliferation index of MTCs which had (partially) lost $p18^{Ink4c}$ expression [n=10; 17.9% (SD 4.6)] resembled the average proliferation index of MTCs of *RET2B;*$p18^{-/-}$*(;*$p27^{+/-}$*)* mice [19.6% (SD 4.6)] as shown in Supplementary Table 2. This was significantly higher (p=0.007) compared to the average proliferation index of MTCs of

RET2B;p18$^{+/-}$*(;p27*$^{+/-}$*)* mice which do express p18^{Ink4c} [n=6; 11.5% (SD 2.5)] (Table 3). In conclusion, loss of p18^{Ink4c} expression correlates with larger MTCs and higher proliferation rates, indicating that loss of p18^{Ink4c} enhances MTC progression.

Table 3. Loss of p18^{Ink4c} expression in MTCs of *RET2B;p18*$^{+/-}$*(;p27*$^{+/-}$*)* knockout mice correlate with plasma CT levels and proliferation rates

Genotype	p18^{Ink4c} expression	Plasma CT (pg/ml)	PCNA index* (total)†
RET2B;p18$^{+/-}$	positive	1.7	ND
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	positive	2.5	8.9% (603)
RET2B;p18$^{+/-}$	positive	2.6	10.3% (637)
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	positive	4.8	13.5% (635)
RET2B;p18$^{+/-}$	positive	12.7	9.6% (623)
RET2B;p18$^{+/-}$	positive	13.5	15.4% (633)
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	positive	19.5	11.0% (549)
		median 4.8 pg/ml‡	mean 11.5% (SD 2.5)**
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	negative	6.2	18.8% (645)
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	negative	13.0	14.6% (584)
RET2B;p18$^{+/-}$	negative	27.0	21.4% (496)
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	negative	29.0	10.5% (963)
RET2B;p18$^{+/-}$	negative	42.1	15.3% (625)
RET2B;p18$^{+/-}$	patchy	152.0	22.1% (569)
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	negative	156.0	20.8% (2495)
RET2B;p18$^{+/-}$	patchy	158.0	12.4% (593)
RET2B;p18$^{+/-}$*;p27*$^{+/-}$	patchy	513.0	18.4% (645)
RET2B;p18$^{+/-}$	negative	522.0	24.6% (530)
		median 97 pg/ml‡	mean 17.9% (SD 4.6)**

* PCNA index is an average from analysis of 4 microscopic fields per MTC; † total amount of cells analysed;
‡ p = 0.001; ** p = 0.007; ND, not determined; SD, standard deviation

DISCUSSION

In this paper, we provide experimental evidence that *p18* inactivation functionally collaborates with oncogenic *RET* in murine MTC development. As compared to the single mutant mouse strains, we have found an increased MTC incidence in both *RET2B;p18*$^{+/-}$ and *RET2B;p18*$^{-/-}$ mice from 9 months on. Heterozygous loss of *p27* did not result in increased MTC incidence in *RET2B* mice up to 12 months of age. This indicates that loss of *p18*, but not loss of *p27*, cooperates with oncogenic *RET* in MTC development. The observed earlier age-of-onset of MTC, as well as the larger MTCs in compound *RET2B;p18(;p27)* mice provides further evidence for synergism between oncogenic *RET* and loss of *p18*. Furthermore, somatic loss of p18^{Ink4c} expression in MTCs

of heterozygous *p18* knockout mice resulted in higher proliferation rates and larger MTCs. Loss of *p18* in the presence of oncogenic *RET* greatly enhances MTC incidence as well as MTC progression, which shows that loss of *p18* is a frequent and severe additional oncogenic hit in *RET*-induced MTC tumorigenesis in mouse.

Interestingly, our results are consistent with a previously reported study about the role of *p18* and *p27* in MEN1 (23). *p18*, but not *p27*, collaborates with *Men1* to suppress tumor formation in pituitary, testis, thyroid, parathyroid, and pancreatic islets (23). The synergistic effect of loss of *p18* on tumor formation in both *Men1* knockout mice and in *RET2B* transgenic mice, indicates a general tumor suppressor role for *p18* in endocrine tissues. It has been proposed that INK4 proteins regulate the cell cycle in a cell lineage-specific manner, due to tissue-specific differences in expression patterns (24). In contrast to most other INK4 proteins, $p18^{Ink4c}$ is expressed during mouse embryogenesis, as well as in most adult tissues, like testis, spleen, kidney, skeletal muscle and lung (25). Therefore, the tissue-specific effect of loss of *p18* on endocrine tumorigenesis is not likely due to an endocrine-specific expression pattern of $p18^{Ink4c}$. More likely, a lack of redundancy involving the other INK4 proteins in endocrine tissues might explain the particular importance of *p18* in these tissues.

Previously, biochemical studies have revealed that loss of menin, the *Men1* gene product, downregulates $p18^{Ink4c}$ as well as $p27^{Kip1}$ expression (26, 27). Previously, we have detected that expression of oncogenic *RET* results in downregulation of both $p18^{Ink4c}$ and $p27^{Kip1}$ expression, leading to increased proliferation (28). Therefore, the observed (partial) loss of p18 expression in a subset of MTCs from $p18^{+/-}$ mice without loss of the remaining p18 allele, might be caused by oncogenic RET signaling. However, this oncogenic RET signaling did not result in loss of $p27^{Kip1}$ expression in MTCs from $p27^{+/-}$ mice.

Several reports have shown that $p18^{Ink4c}$ and $p27^{Kip1}$ cooperate in cell cycle arrest of different cell types (29, 30). Loss of inhibition both early and late in G1 (e.g. by loss of $p18^{Ink4c}$ and $p27^{Kip1}$, respectively) affects cell cycle progression more severely compared to loss of inhibition early in G1 alone (31), which could explain the differential effects observed in *RET2B;*$p18^{+/-}$ mice and *RET2B;*$p27^{+/-}$ mice. Different double knockout mouse models, in addition to *p18;p27* knockout mice, like *Rb;p53* knockout mice (16, 32), *p18;p53* knockout mice, *p27;p53* knockout mice (33) and *Rb;p27* knockout mice (14), have revealed a synergism between cell cycle regulatory genes in MTC development. This is the first time that synergism between a receptor tyrosine kinase (RET) and a CDK inhibitor ($p18^{Ink4c}$) is reported to be involved in the multistep process of MTC development. Loss of *p27* in compound *RET2B;*$p18^{-/-}$ mice further increases MTC incidence, which is not associated with loss of $p27^{Kip1}$ expression, indicating that *p27* is a haploinsufficient tumor suppressor gene in *p18*- and *RET2B;p18*-induced MTC development.

Involvement of oncogenic *RET* in human MEN 2 and MTC development is already well established. Our data suggest a cooperative role for *P18* and *RET* in human MTC tumorigenesis.

Germline *P18* mutations have not yet been described in man and somatic alterations in the *P18* gene have rarely been detected in human cancer. However, loss of chromosome 1p32, the location of the *P18* gene, is associated with both sporadic and familial MTC (8-10). Recently, we have detected somatic inactivating *P18* mutations in sporadic as well as hereditary MTCs, with a relatively high frequency compared to other types of human cancer investigated (unpublished data*). Interestingly, all somatic *P18* mutations detected in human MTC coincided with germline or somatic *RET* mutations, suggesting that a cooperation between oncogenic *RET* and inactive *P18* would also contribute to human MTC tumorigenesis.

It has been suggested that *p18* is a haploinsufficient gene in mice. Haploinsufficiency of *p18* has only been detected in the presence of carcinogen treatment (20), or in combination with loss of *p27* or *Patched* (11, 34). In our mouse models, we show that *p18* functions as a haploinsufficient tumor suppressor gene in the presence of oncogenic *RET*. However, the haploinsufficiency of *p18* is not absolute as loss of both *p18* alleles resulted in a higher MTC incidence in all genotype groups and loss of $p18^{Ink4c}$ expression resulted in enhanced MTC growth in $p18^{+/-}$ mice. Taken together, we show that loss of *p18* greatly contributes to *RET*-induced MTC incidence as well as MTC progression. We propose that loss of *p18* (and *p27*) is an additional oncogenic hit in the multistep process of *RET*-induced MTC tumorigenesis.

ACKNOWLEDGEMENTS

We would like to thank H. Boere, S. Versteeg and H. Avezaat of the Animal Facility of the University of Utrecht, Utrecht, The Netherlands for assistance with the mouse experiments. We acknowledge H. Compagner, I. Maitimu and Dr. E. Lentjes of the department of Endocrinology of the University Medical Center Utrecht, Utrecht, The Netherlands for assistance with plasma CT measurements. We are thankful to several colleagues for critically reading this manuscript. This work was supported by project grant UU2712 from the Dutch Cancer Society.

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CHAPTER 4

A novel RET kinase-β-catenin signaling pathway contributes to tumorigenesis in thyroid carcinoma

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Cancer Res 2008, in press

ABSTRACT

The RET receptor tyrosine kinase has essential roles in cell survival, differentiation, and proliferation. Oncogenic activation of RET causes the cancer syndrome multiple endocrine neoplasia type 2 (MEN 2), and is a frequent event in sporadic thyroid carcinomas. However, the molecular mechanisms underlying RET's potent transforming and mitogenic signals are still not clear. Here, we demonstrate that nuclear localization of β-catenin is frequent in both thyroid tumours and their metastases from MEN 2 patients, suggesting a novel mechanism of RET-mediated function, through the β-catenin signalling pathway. We show that RET binds to, and tyrosine phosphorylates, β-catenin and demonstrate that the interaction between RET and β-catenin can be direct and independent of cytoplasmic kinases, such as SRC. As a result of RET-mediated tyrosine phosphorylation, β-catenin escapes cytosolic downregulation by the APC/Axin/GSK3 complex and accumulates in the nucleus, where it can stimulate β-catenin-specific transcriptional programs in a RET-dependent fashion. We show that downregulation of β-catenin activity decreases RET-mediated cell proliferation, colony formation, and tumour growth in nude mice. Together, our data show that a β-catenin-RET kinase pathway is a critical contributor to the development and metastasis of human thyroid carcinoma.

INTRODUCTION

The RET receptor is required for development of urogenital and neural crest derived cell types (1). Under normal cellular conditions, RET is activated by binding of both glial cell line-derived neurotrophic factor (GDNF) ligands and cell surface bound co-receptors of the GDNF family receptor α (GFRα) proteins (2). However, oncogenic activation of RET, by germline point mutations, leads to ligand-independent constitutive kinase activity, giving rise to the inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN 2). MEN 2 is characterized by medullary thyroid carcinoma (MTC), a tumour of thyroid C-cells; and the adrenal tumour pheochromocytoma (PC), as well as other less common tumor and developmental phenotypes (reviewed 3). MTC is the predominant disease feature, with early onset tumours and metastases to lymph nodes and distant organs (4). RET activation contributes to stimulation of RAS-ERK, JNK, PI3K, p38MAPK, SRC, STAT and ERK5 signaling cascades (reviewed 1). However, the identity of the critical secondary oncogenic signals involved in the broad and early metastatic pattern of RET-mediated MTC is still largely unknown.

β-catenin is an ubiquitously expressed multifunctional protein that plays important roles in cell adhesion and signal transduction (5). At the plasma membrane, β-catenin associates with E-

cadherin and α-catenin in linking the cytoskeleton and adherens junctions, while in the nucleus it acts as a mediator of transcription through other DNA binding proteins such as TCF/LEF family members (reviewed 6). Cytosolic free β-catenin interacts with the adenomatous polyposis coli (APC) and axin proteins to form a complex, which in turn recruits glycogen synthase kinase-3 (GSK3) and casein kinase, to form a destruction complex that serine/threonine phosphorylates β-catenin and targets it to the proteosome (reviewed 7).

Abnormal expression, or localization of β-catenin has been reported in many tumour types (5, 8), and β-catenin-mediated loss of cell/cell adhesion has been implicated in anchorage-independent cell growth and cancer metastasis (8). The best characterized mechanism leading to β-catenin-mediated signal transduction is through activation of the WNT pathway by binding of WNT proteins to frizzled or LRP family cell surface receptors (9). However, β-catenin signalling can also be induced in response to overexpression or activation of tyrosine kinases in a WNT-independent fashion, and both these pathways converge to target β-catenin to the nucleus and induce expression of a similar set of β-catenin-specific target genes (10-12). β-catenin tyrosine phosphorylation causes its dissociation from membrane-associated E-cadherin, leading to accumulation of a pool of free cytoplasmic β-catenin (13). This can, in turn, increase the amount of β-catenin reaching the nucleus where it acts as a transcription factor, upregulating expression of genes involved in cell migration, growth, differentiation, and survival (8). Tyrosine phosphorylation of β-catenin, followed by functional downregulation of E-cadherin-mediated cell-cell contact, is potentially critical in initiating cell migration in both normal physiological processes and in tumor metastasis (13).

Loss of membrane-associated β-catenin, often with an accompanying relative increase in cytosolic or nuclear expression, has been noted in anaplastic and poorly differentiated thyroid carcinomas, and in thyroid papillary microcarcinoma (14-16). However, β-catenin had not been previously investigated in RET-mediated tumour development and metastasis. Here, we report that RET interacts with, and activates β-catenin, and that a RET-β-catenin signaling pathway plays roles in RET-mediated tumour growth, invasion and metastasis.

MATERIALS AND METHODS

Expression Constructs: Expression constructs for full-length human RET, GFRα1, and mutant RET constructs have been previously described (17-19). Intracellular (ic)RET expression constructs, generated by fusing cDNA encoding a myristylation signal, two dimerization domains, and the intracellular portion (amino acid 658 to the C-terminus) of RET, have been previously described (18). Axin and wildtype β-catenin expression constructs were a gift from Dr. J Woodgett (Ontario Cancer Research Institute, Toronto, Canada). GST-tagged expression constructs for WT and mutant β-catenin have been reported (20, 21).

Cell Culture and Transfection: The human neuroblastoma cell line SH-SY-5Y was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON). All other cell lines were grown in

Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Burlington, ON), supplemented with 10% FBS. Medium for HEK293-TET-ON cells, used to induce icRET, was further supplemented with 1ug/ml doxycycline. HEK293 cells were transiently transfected with the indicated expression constructs using Lipofectamine 2000 (Invitrogen, Burlington, ON), according to the manufacturer's instructions. RET activation was induced by addition of 100 ng/ml of GDNF (Promega, Madison, WI) for full length RET, or with 1 μM AP20187 dimerizer (ARIAD Pharmaceuticals, Cambridge, MA) for icRET, for the time periods indicated.

Immunoprecipitation, Western, and Far-Western Blotting: Whole cell lysates (WCL) were harvested 48 hours after transfection, and suspended in lysing buffer (20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM sodium orthovanadate, 1% Igepal, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotonin and 10 μg/ml leupeptin) (19). Protein concentration was determined by BCA protein assay kit, according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). For immunoprecipitations, lysates were incubated with a 1:50 dilution of the appropriate primary antibody for 2 hours at 4°C with agitation. Complexes were collected on Protein AG beads (Santa Cruz Biotechnology, Santa Cruz, CA) by centrifugation at 13,000 rpm, washed 3 times with lysing buffer, and resuspended in laemmli buffer. Protein samples were denatured at 99°C for 5 mins, separated on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), as previously described (17, 19). Antibodies used included: anti-RET (c19), anti-ubiquitin (N19), and anti-myc-tag (NE10), antibodies (Santa Cruz Biotechnology, Santa Cruz CA), anti-phospho-RET (pY905) antibody (Cell Signaling, Beverly, MA), anti-β-catenin antibody (BD Biosciences, Mississauga, ON), and anti-V5 tag (axin) (Invitrogen, Burlington ON). An anti-phosphotyrosine antibody (pY99; Santa Cruz) was used to detect tyrosine phosphorylation of β-catenin. For far western analyses, protein lysates were immunoprecipitated for RET and resolved and western blotted, as above. Membranes was incubated for 2 hrs at 4°C in a 0.1% Tween-20/TBS solution containing a probe of 1 μg/ml GST-β-catenin, GST alone or no probe. After three washes, bound proteins were detected with anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz CA) or immunoblotted for RET.

Preparation of Cytosolic Extracts: Cells were harvested by gentle centrifugation, washed twice with PBS, and suspended in ice-cold hypotonic buffer (20 mM Hepes-KOH, pH 7.0, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors) (22). After incubation on ice for 15 min, cells were disrupted by dounce homogenization. Nuclei were pelleted by centrifugation and cytosolic fractions were isolated by collection of the supernatant.

Glutathione S-transferase (GST)-Pull-down Assays: GST-fusion proteins expressed in E. coli, were eluted with 100 mM glutathione elution buffer using a polyprep column (Bio-Rad, Hercules, CA), as described previously (17). For GST pull-down assays, 5 μg of GST-fusion protein and GST-sepharose-beads (GE Healthcare/Amersham Biosciences, Baie d'Urfé, QC) were incubated with whole cell lysates at 4^{o}C for 3 hours, with agitation. Bound proteins were eluted by boiling in laemmli sample buffer containing 2-mercaptoethanol, and resolved by SDS-PAGE and western blotting, as described above.

Reporter Assay: For dual-luciferase reporter assays, TOPFLASH or FOPFLASH vectors (Upstate Biotechnology, Lake Placid, NY) and pRL-TK control were co-transfected into HEK293 cells stably expressing icRET or empty vector. Luciferase activity was measured with a Dual-Luciferase reporter kit (Promega, Madison, WI).

shRNA Production: Four different β-catenin shRNAs, in pLKO.1 lentiviral vectors, were obtained from Open Biosystems (Huntsville, AL). Lentiviral particles containing the different β-catenin shRNA constructs were grown in 293T packaging cells by transfecting a three-plasmid packaging system (23) according to the manufacturer's instructions. Supernatants were collected 48 and 72 hours after transfection, filtered, and pooled. NIH 3T3 stably expressing the oncogenic 2A-RET (C634R) were infected with lentiviral constructs or an heterologous lentiviral control, and polyclonal stable cell lines were generated.

MTT Cell Proliferation Assay: MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assays were performed as described (24). Briefly, HEK293 cells transiently expressing RET or empty vector, seeded in 6-well plates were either infected with lentiviral β-catenin shRNA or untreated. Cells were grown in medium supplemented with 100 ng/ml GDNF. After 3 days, MTT was added to a final concentration of 250 µg/ml for 2 h at 37°C. Culture medium was removed and formazan crystals were dissolved in DMSO. Reduced MTT was measured spectrophotometrically at 570 nm. Statistical significance was calculated by one-way ANOVA.

Apoptosis Assays: NIH 3T3 cells stably expressing 2A-RET were infected with lentiviral constructs for β-catenin shRNA or control vector and cultured for 48 hours. Cells were harvested, fixed in absolute ethanol, and treated with RNaseA at 37ºC overnight. Fixed cells were incubated in 5 mg/ml propidium iodide for 15 minutes at room temperature, and cell cycle analysis performed using an EPICS ALTRA HSS flow cytometer (Beckman Coulter, Mississauga ON). Statistical significance was calculated by one-way ANOVA.

Soft Agar Colony Formation Assays: Soft agar colony formation assays were performed as described previously (17). Briefly, NIH 3T3 expressing 2A-RET or K758M constructs were infected with lentiviral vectors for β-catenin shRNA or control. Approximately 5 x 104 cells were resuspended in 0.2% top agar in culture medium, and plated on 0.4% bottom agar in medium. Culture medium was supplemented every 2 to 3 days. Colonies were counted after 14 days, and statistical significance was confirmed by one-way ANOVA.

Confocal Microscopy: SH-SY-5Y neuroblastoma cells were cultured on glass coverslips coated with 0.2% gelatin. 24 hours prior to fixation, 10 nM retinoic acid was added to the culture medium. Cells were serum starved for 3 hours, then fixed in 3% paraformaldehyde for 40 minutes at room temperature. Cells were then washed in phosphate-buffered saline, permeablised with 0.15% Triton-X-100, and blocked for 30 minutes in 3% BSA. Cells were double-immunostained with primary antibodies specific for β-catenin (BD Biosciences) and RET (c19, Santa Cruz Biotechnology), and species-matched secondary antibodies labeled with Alexa 594 or 488, respectively. Coverslips were mounted on glass slides in Mowiol mounting medium, and observed using a Leica TCS-SP2 confocal microscope. Individual channels were overlayed using Image J software and colocalization was determined using the Image J RG2B colocalization plug-in.

Tumourigenicity in Nude Mice: All in vivo experiments were performed using 6-8 week old athymic nude mice (NIH, Bethesda, U.S.A). Experiments were performed in duplicate using a minimum of 5 animals/treatment group. Mice were maintained in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Care (Queen's University Kingston, Canada). NIH 3T3 parental cells, cells stably expressing 2A-RET constructs, or polyclonal cell lines expressing 2A-RET and β-catenin shRNA (described above) were inoculated subcutaneously into the right flank of the mice. Cells (2 X106 in suspension) were injected on day 0, and tumor growth was followed every 2-3 days by tumour diameter measurements using vernier calipers. Tumour volumes (V) were calculated using the formula: V = AB2/2 (A = axial diameter; B =rotational diameter). Mice were sacrificed at day 14, and tumour tissues were excised for protein extraction and histology. Tumour tissue was homogenized in 10 volumes homogenization buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, 10 µg/ml aprotonin and 10 µg/ml leupeptin). Tissue debris was removed by centrifugation, the supernatant collected, and cells lysed at a final concentration of 1% Triton X-100. Samples were centrifuged to remove insoluble material. Tissue for histology was fixed in neutral buffered formalin and processed by routine methods. Paraffin embedded sections of 5 µm were stained with haematoxylin and eosin for histologic examination.

β-catenin Immunohistochemistry: Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues. Tumour tissue from the CALC-MEN 2BRET mice was excised, fixed, and paraffin embedded, by routine methods. Human MTC samples were obtained from the Department of Pathology of the University Medical Center Utrecht, Utrecht, The

Netherlands. Paraffin sections (6μm) were blocked with 0.5% CASEIN blocking reagent (PerkinElmer Life Sciences, Waltham, MA, USA) in 0.1% Triton-X100 in PBS, and treated with 1.5% hydrogen peroxide to inhibit endogenous peroxidase activity. Sections were incubated with a mouse monoclonal anti-β-catenin antibody (BD Biosciences, San Jose, CA, USA), 1:50 dilution, for one hour at room temperature. Slides were incubated with peroxidase-labeled rabbit anti-mouse secondary antibody (Dako, Glostrup, Denmark), 1:100 dilution, for 30 minutes at room temperature and subsequently with peroxidase-labeled swine anti-rabbit antibody (Dako), 1:100 dilution, for 30 minutes at room temperature. Finally, sections were incubated with DAB and counterstained with Mayer's haematoxylin. Nuclear staining was considered positive when one or more positive nuclei were observed in each microscopic field (40x) (25). Negative control experiments were performed by omitting the primary antibody.

Relative Quantification by Real-Time RT-PCR (qRT-PCR): Relative differences between gene transcript levels were confirmed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Mississauga, ON) and a SmartCycler II (Cepheid, Sunnyvale, CA). Primer and PCR product information are found in Supplementary Table 1. The qRT-PCR assays were repeated at least three times. Crossing threshold (Ct) values were taken at the same threshold for each experiment; folds expression were averaged and mean fold change, relative to the empty vector control, calculated.

Supplementary Table 1. Summary of qRT-PCR primers and products.

Gene symbol	NCBI identifier	Dir	Primer sequence (5'-3')	Product Size (bp)
CTNNB1	NM_001904	F	TGT TCG TGC ACA TCA GGA TAC C	134
		R	ACA TCC CGA GCT AGG ATG TGA AG	
RET	AJ297349	F	AAT TTG GAA AAG TGG TCA AGG C	186
		R	CTG CAG GCC CCA TAC AAT	
CCND1	NM_053056	F	CGA GAA GCT GTG CAT CTA CA	123
		R	AAT GAA ATC GTG CGG GGT CA	
JUNB	NM_002229	F	TCT GCA CAA GAT GAA CCA CG	129
		R	TAG CTG CTG AGG TTG GTG TA	
EGR1	NM_001964	F	AGA TGA TGC TGC TGA GCA ACG	216
		R	ATG TCA GGA AAA GAC TCT GCG GT	
GDF15	NM_004864	F	TGG GAA GAT TCG AAC ACC GA	203
		R	TCG TGT CAC GTC CCA CGA CCT TGA	
VGF	NM_003378	F	ACC TCT CGC GTC GTG ACA CCA	104
		R	AAC CCG TTG ATC AGC AGA AG	
AXIN1	NM_003502	F	AGC ATC GTT GTG GCG TAC T	158
		R	ACA GTC AAA CTC GTC GCT CA	

Dir: direction. bp: base pair F: forward. R: reverse

RESULTS AND DISCUSSION

β-catenin nuclear localization in RET-mediated human thyroid tumours

In preliminary immunohistochemical experiments, using MTCs from CALC-MEN 2BRET transgenic mice, which express a constitutively active, oncogenic RET mutant (2B-RET) (26), we found that 6 out of 7 tumours had nuclear localization of β-catenin (Supplementary Figure S1),

suggesting a role for β-catenin signaling in these tumours and a relationship between RET activation and β-catenin nuclear localization. Further, in human MTC samples from twenty MEN 2- patients with known oncogenic RET mutations (Supplementary Table 2), 8 had nuclear β-catenin expression in a subset of cells, heterogeneously spread throughout the tumours (Figure 1A). In addition, although some MTCs showed β-catenin expression at the cellular membrane, it was less prominent, particularly in tumours with strong nuclear β-catenin expression (Figure 1A). Association of nuclear staining with loss of membranous staining, has also been reported in other cancers, such as colorectal carcinomas (25). Nuclear localization of β-catenin was not detected in normal or hyperplastic C-cells, in mouse or human tissues. Interestingly, nuclear localization was more prevalent in metastases (5/7 cases) than in primary MTCs (3/13 cases) (Figure 1B, Supplementary Table 2), suggesting an association of more aggressive or advanced MTC disease stage and activation of β-catenin. Conversely, in the absence of oncogenic activation of RET, we showed that the endogenous RET and β-catenin proteins colocalized at the plasma membrane, in the neuroblastoma cell line SH-SY-5Y (Figure 1C). Together, these observations indicated that β-catenin signalling may play an important role in progression of RET-mediated tumours and suggested that a novel RET- β-catenin signalling mechanism could be taking place.

Supplementary Table 2. Patient information and β-catenin staining patterns for primary and metastazised human MTCs.

MTC	Age (years)	MEN 2 disease phenotype	RET mutation	β-Catenin staining
Primary	4	MEN 2A	C634W	
	5	MEN 2A	C634R	
	5	MEN 2A	C634G	
	7	MEN 2A	C634R	
	8	MEN 2A	C634R	
	10	MEN 2A	C634G	
	13	MEN 2A	C634R	
	14	MEN 2A	C634Y	nuclear
	26	MEN 2A	C634Y	nuclear
	30	MEN 2A	C634R	
	34	MEN 2A	C634G	nuclear
	36	MEN 2A	C634W	
	37	MEN 2A	C634R	
Metastasis	26	MEN 2B	M918T	nuclear
	30	MEN 2A	C634Y	
	35	MEN 2B	M918T	nuclear
	38	MEN 2A	C618S	nuclear
	45	MEN 2A	C618S	nuclear
	54	MEN 2A	C634R	nuclear
	76	MEN 2A	C634W	

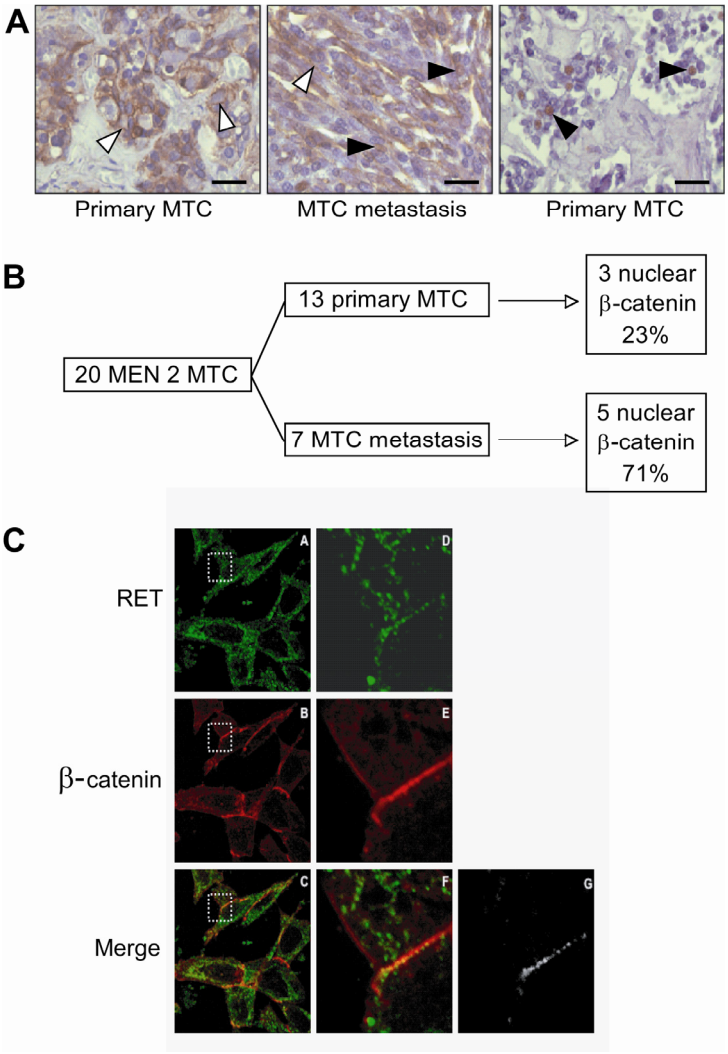
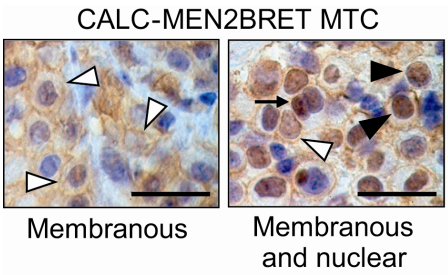


Figure 1. Localization of β-catenin in human MTCs and cell lines. A) Paraffin sections of human primary MTCs (left and right panels) and an MTC metastasis (middle panel) from MEN 2 patients were stained for β-catenin. Membranous and nuclear β-catenin expression is indicated with white and black arrow heads, respectively. The scale bars represent 20 µm. B) Dendrogram showing distribution of nuclear β-catenin expression in our panel of human primary MTCs and metastases from 20 MEN 2 patients with well characterized oncogenic RET mutations. C) Endogenous RET and β-catenin co-localize at the cell membrane. SH-SY-5Y neuroblastoma cells expressing endogenous RET and β-Catenin were serum starved, and fixed in 3% (w/v) paraformaldehyde/PBS. Cells were double immunostained for RET (A, D) and β-Catenin (B, E). Panel C is a merged image of A, and B. Images D, and E and F are enlargements of the dashed boxes shown in A, B and C, respectively. Panel G represents image F after application of a co-localization filter (RG2B colocalization – Image J) which replaces pixels containing signal in both the red and green channel with a grey-scale pixel of similar intensity.

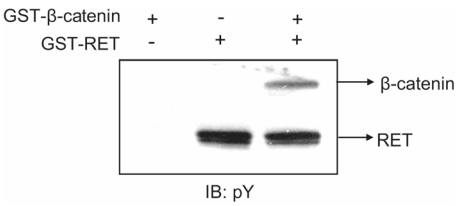


Supplementary Figure S1. Altered localization of β-catenin in response to activated RET in a murine model of RET-induced MTC. Representative micrographs of tumour tissue from CALC-MEN 2BRET transgenic mice that express the activated RET mutant 2B-RET and endogenous β-catenin. Nuclear β-catenin localization was detected in 6 out of 7 MTCs, using a mouse monoclonal anti-β-catenin antibody (BD Biosciences, San Jose, CA, USA). Membranous and nuclear β-catenin expression are indicated by white and black arrow heads, respectively. A black arrow (right panel) indicates a dividing cell. Scale bars represent 20 µm.

RET associates with and tyrosine phosphorylates β-catenin

As tyrosine phosphorylation of β-catenin by several kinases has been correlated with tumorigenesis and metastasis (11-13), we investigated the association of RET with β-catenin. In TT cells, a line derived from a human MTC expressing endogenous RET with an activating mutation (2A-RET), we found that RET induced phosphorylation of endogenous β-catenin. Similarly, in cells co-transfected with RET and β-catenin expression constructs, we showed that treatment with the RET ligand GDNF also induced phosphorylation of β-catenin (Figure 2A). Immunoprecipitation of β-catenin, and immunoblotting with appropriate antibodies, showed that both endogenous and transiently expressed β-catenin and RET associate in complexes (Figure 2A). These complexes could also be detected by immunoprecipitation of RET and immunoblotting for β-catenin (not shown). Our data show that both the ligand-activated wildtype RET (WT-RET) and the oncogenic mutants, 2A-RET and 2B-RET, induce tyrosine-phosphorylation of β-catenin (Figure 2A). In the absence of GDNF, the constitutively active 2B-RET protein, but not WT-RET, was able to induce significant β-catenin tyrosine phosphorylation (Figure 2A). Further, a catalytically-compromised RET mutant, K758M (17), was unable to induce β-catenin tyrosine phosphorylation, even in the presence of GDNF stimulation, suggesting a RET kinase-dependent mechanism of β-catenin phosphorylation (Figure 2A). Interestingly, however, K758M RET was still able to associate with β-catenin, in a phosphorylation-independent fashion, suggesting a constitutive association between RET and β-catenin.

Although β-catenin may associate with receptor kinases and can be tyrosine phosphorylated in cells stimulated by their ligands, EGFR is the only receptor known to directly phosphorylate β-catenin (27). Other receptors may induce activation of SRC, which in turn can phosphorylate β-catenin, as well as other proteins of the adherens junctions (13). To determine whether β-catenin tyrosine phosphorylation could result from a direct interaction with RET, we performed in vitro kinase assays using purified RET kinase (17) and purified recombinant GST-tagged β-catenin (20). We found that the purified RET-kinase can be activated in the presence of ATP and can directly phosphorylate β-catenin in vitro (Supplementary Figure S2).



Supplementary Figure S2. Recombinant purified RET can directly phosphorylate purified β-catenin in vitro. Purified GST-tagged RET protein (5 μg) (1), was incubated with GST-WT β-catenin (5 μg) (2) in the presence of ATP for 30 min at 30°C. Samples were subjected to SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (pY99). Purified RET is phosphorylated, as shown previously (1). In the presence of an active RET kinase, β-catenin is also phosphorylated, suggesting that β-catenin acts a RET kinase substrate.

As endogenous wildtype RET and β-catenin proteins colocalize at the plasma membrane (Figure 1C), we confirmed that they could directly interact by far-western assays. We showed that purified recombinant GST-β-catenin could interact with immunoprecipitated WT-RET, while a purified GST control could not in direct far-western assays (Figure 2B). In vivo, we further showed that the RET- β-catenin interaction can occur independent of SRC, using co-immunoprecipitations in SYF-/- cells, which lack the SRC family kinases SRC, YES, and FYN (Figure 2C). Our data show that RET can associate with, and directly phosphorylate, β-catenin and that the tyrosine phosphorylation of β-catenin is not dependent on SRC activation.

The pattern of tyrosine residue phosphorylation of β-catenin has been shown to be kinase specific. Activation of receptor tyrosine kinases FYN, FER, and MET, leads to phosphorylation at Y142, while Y654 can be phosphorylated directly by EGFR or SRC (11, 12, 20, 27, 28). In pull-down assays using GST-tagged β-catenin tyrosine mutants for either Y142 (Y142F) or Y654 (Y654F) (21), we showed that wild type β-catenin, and to a lesser extent the Y142F mutant, were phosphorylated by WT-RET, but that Y654F β-catenin was not phosphorylated (Figure 2D) suggesting that Y654 was the major site for RET-mediated β-catenin phosphorylation. Tyrosine 654 lies within the putative E-cadherin binding region of β-catenin, and the bulkier phosphorylated residue has been shown to reduce β-catenin binding affinity for E-cadherin leading to dissociation of β-catenin from the membrane (13, 28).

RET activation induces cytosolic translocation of β-catenin and its escape from the axin regulatory complex

Accumulation of cytosolic free β-catenin is tightly regulated by the APC/axin/GSK3 complex, which binds β-catenin and leads to its serine/threonine phosphorylation, targeting it for ubiquitination, and proteosomal degradation (29). We have shown that RET and β-catenin co-localize at the plasma membrane (Figure 1C), but that activation of RET leads to a relative increase in β-catenin in the cytosol (Figure 3A). Notably, the cytosolic level was not further enhanced by the more active 2B-RET kinase, perhaps suggesting that increased RET activity may enhance nuclear β-catenin levels preferentially. Interestingly, in the presence of active RET, we found a relative decrease in β-catenin associated with the APC/axin/GSK3 complex in immunoprecipitations of axin, which interacts directly with β-catenin in this complex (Supplementary Figure S3A). Further, there was an accompanying relative decrease in ubiquitinated β-catenin (Supplementary Figure S3B), suggesting that RET-mediated tyrosine phosphorylation of β-catenin protects it from degradation. This would be consistent with data on RON and MET which also suggest that tyrosine phosphorylation of β-catenin inhibits its interactions with the APC/axin/GSK3 complex (11).

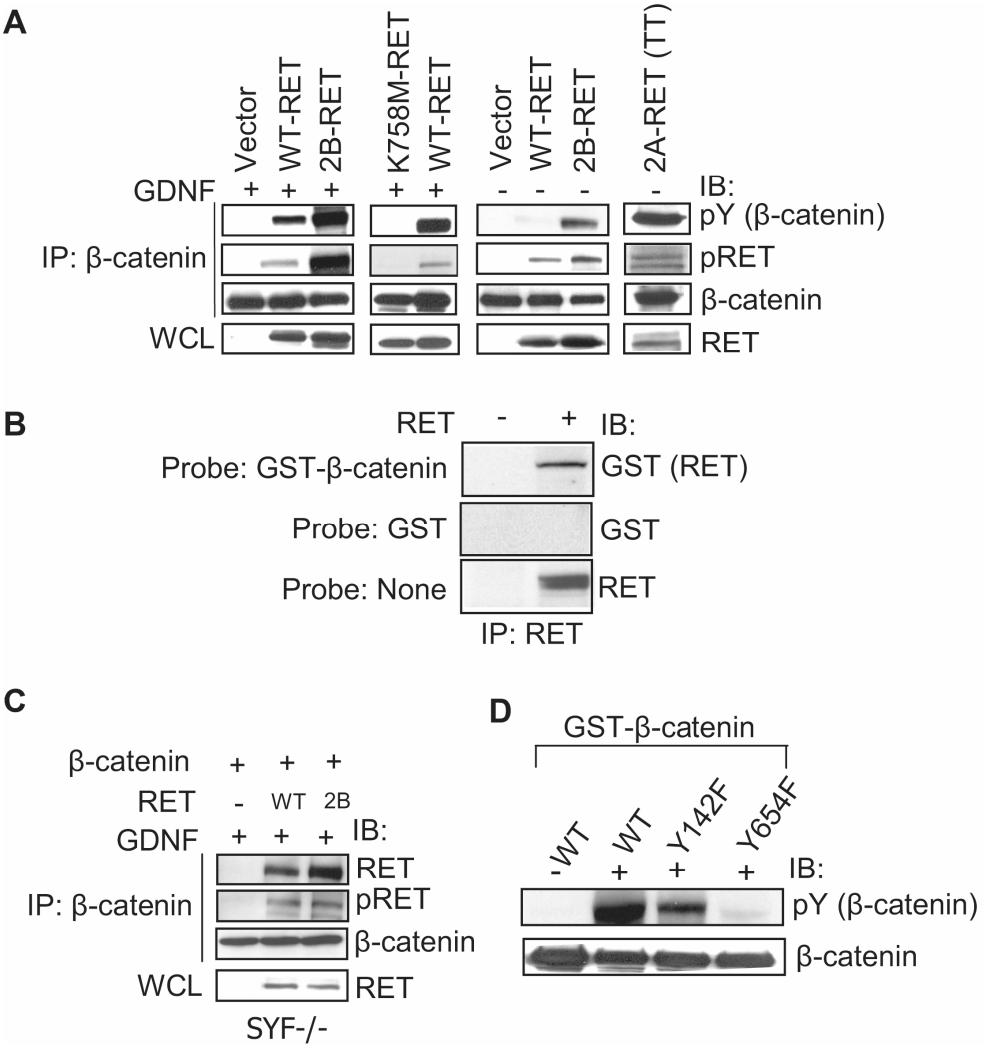


Figure 2. RET associates with, and tyrosine phosphorylates, β-catenin. A) RET induces tyrosine phosphorylation of β-catenin. TT cells expressing endogenous 2A-RET and β-catenin, and HEK293 cells transiently co-transfected with GFRα1, RET (WT-RET, 2B-RET, K758M), and myc- tagged β-catenin constructs, in the presence (GDNF for 15 minutes) or absence of ligand stimulation, were immunoprecipitated for β-catenin and immunoblotted with appropriate antibodies. B). RET and β-catenin interact directly. HEK293 cells transiently expressing WT-RET (+) or vector (-), and myc-tagged β-catenin, were treated with GDNF, as above. Cell lysates were immunoprecipitated with anti-RET antibody (c-19), western blotted and incubated with purified recombinant GST-β-catenin (top), or GST alone (middle), or no probe (bottom) and immunoblotted with either an anti-GST or anti-RET antibody, as indicated. C) SYF-/- cells (lacking SRC family kinases) were transiently co-transfected with myc-tagged β-catenin and the indicated RET construct or vector alone. Cell lysates were immunoprecipitated for β-catenin and immunoblotted with appropriate antibodies. D) β-catenin tyrosine 654 (Y654) is a major RET-phosphorylation site. Cell lysates from HEK293 cells stably expressing WT-RET or empty vector were pulled-down with purified WT or mutant GST-β-catenin. Complexes were immunoblotted with anti-phosphotyrosine or anti- β-catenin antibodies. WCL- whole cell lysates.

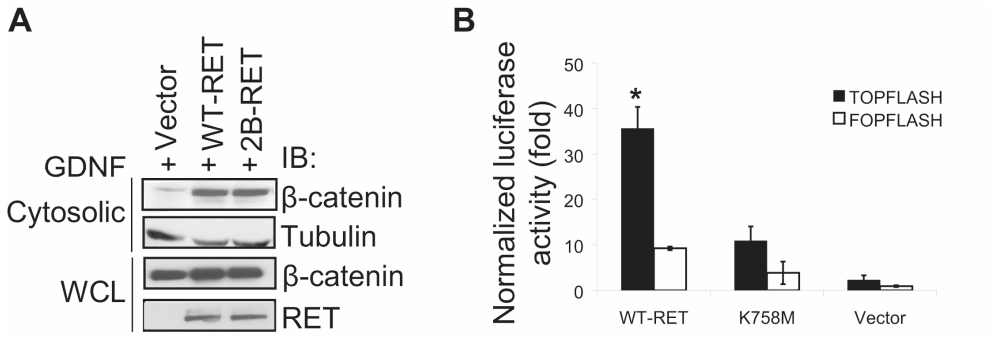
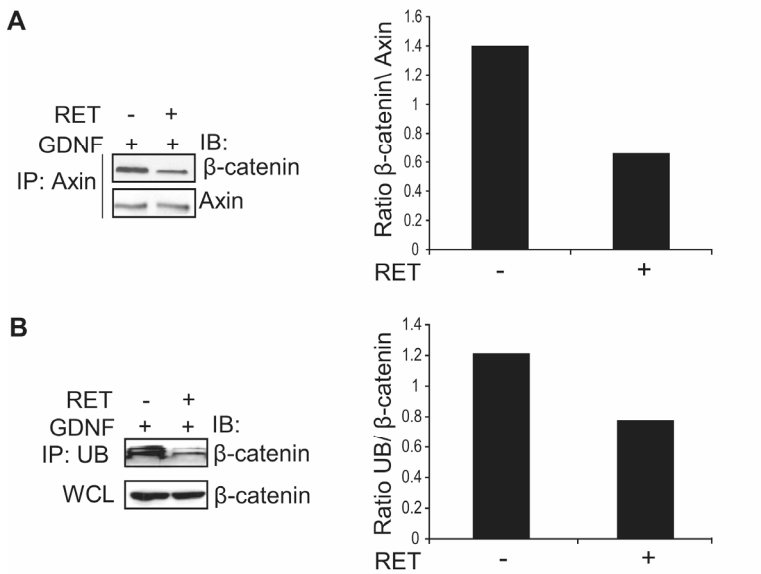


Figure 3. RET alters β-catenin localization. A) Cytosolic β-catenin-levels are relatively increased upon expression and activation of RET. Whole cell lysates (WCL) and cytosolic cell fractions from HEK293 cells expressing GFRα1 and RET (WT-RET or 2B-RET), or empty vector, were immunoblotted for RET, β-catenin, or a tubulin control. B) β-catenin mediates TCF-4 transcriptional activity in RET expressing cells. HEK293 cells transiently transfected with TOPFLASH or FOPFLASH and pRL-TK reporters and inducible icRET constructs were treated with dimerizer for 2h to induce RET activation, and a dual-luciferase assay was performed. Results are the means of three independent experiments ±sd of fold differences.



Supplementary Figure S3. Tyrosine phosphorylated β-catenin escapes the axin regulatory complex in the cytoplasm. A) RET activation reduces the association of β-catenin and axin. HEK293 cells transiently expressing GFRα1 and either WT-RET, or empty vector, were immunoprecipitated with an antibody to axin and immunoblotted for axin or β-catenin. The relative interaction of β-catenin and axin in the presence and absence of RET activation is shown (left) and the relative quantitation is indicated (right). B) β-catenin ubiquitination is reduced in the presence of activated RET. HEK293 whole cell lysates, as described in A) above, were immunoprecipitated with an anti-ubiquitin antibody and immunoblotted for β-catenin. Relative levels of β-catenin ubiquitination, in the presence or absence of ligand stimulated RET, are indicated (left) and the relative quantitation is indicated (right). Each comparison was repeated a minimum of twice and a representative example is shown.

RET-induced β-catenin tyrosine phosphorylation is associated with increased TCF transcriptional activity

β-catenin also has important signalling roles in the nucleus, mediated through its interactions with the TCF family of transcription factors, and other transcriptional regulators, and the subsequent activation of target genes, such as cyclinD1 (8, 11, 12, 27). Initially, to determine whether RET activation could induce a β-catenin transcriptional program, we used the well-characterized TOPFLASH (TCF binding site) and FOPFLASH (mutated TCF binding site) luciferase reporters (11, 30) for detection of β-catenin/TCF-mediated transcription. In cells stimulated with GDNF, TCF-reporter activity was significantly increased (relative to FOPFLASH) in the presence of WT-RET but not in the presence of the kinase-dead mutant (Figure 3B), suggesting that a RET-dependent induction of a β-catenin/TCF transcriptional program may occur.

β-catenin is required for RET-induced cell proliferation and transformation

The role of RET activation in stimulation of β-catenin-mediated transcription, prompted us to investigate whether β-catenin was required for known RET-mediated processes using shRNA-knock-down of β-catenin. We evaluated four lentivirus-produced β-catenin shRNA constructs, pooled those producing the most significant knock-down of β-catenin and its target, cyclinD1 (Supplementary Figure S4), and used these to generate polyclonal β-catenin-deficient cell lines. We used quantitative real time PCR (qRT-PCR) to evaluate the effect of loss of β-catenin on expression of a panel of known RET-target genes, previously identified in gene expression analyses (19, 31-33). RET activation has been shown to increase expression of cyclinD1 and our data demonstrate that both RET and β-catenin are required to induce this in NIH 3T3 cells (Figure 4A; Supplementary Figure S4). Knock-down of β-catenin expression had no significant effect on RET expression or on expression of control transcripts (e.g. axin) not known to be modulated by either RET or β-catenin (Figure 4A). However, it did block, or significantly reduce, RET-induced upregulation of a subset of RET targets, including CyclinD1, JunB, and EGR1, in cells expressing both activated RET and shRNA for β-catenin (Figure 4A). Other RET-targets were unaffected (e.g. GDF15, VGF), consistent with a β-catenin –independent mechanism of RET-mediated stimulation. Interestingly, a RET- β-catenin signaling pathway appeared to be important to induction of cell proliferative targets, including immediate early genes cyclinD1, EGR1 and JunB, but had less impact on targets associated with more differentiated cell functions, such as GDF15 (growth differentiation factor 15) and VGF (VGF nerve growth factor induced). Together, our data show that RET activation may stimulate a β-catenin transcriptional program but that not all RET-targets require β-catenin for expression.

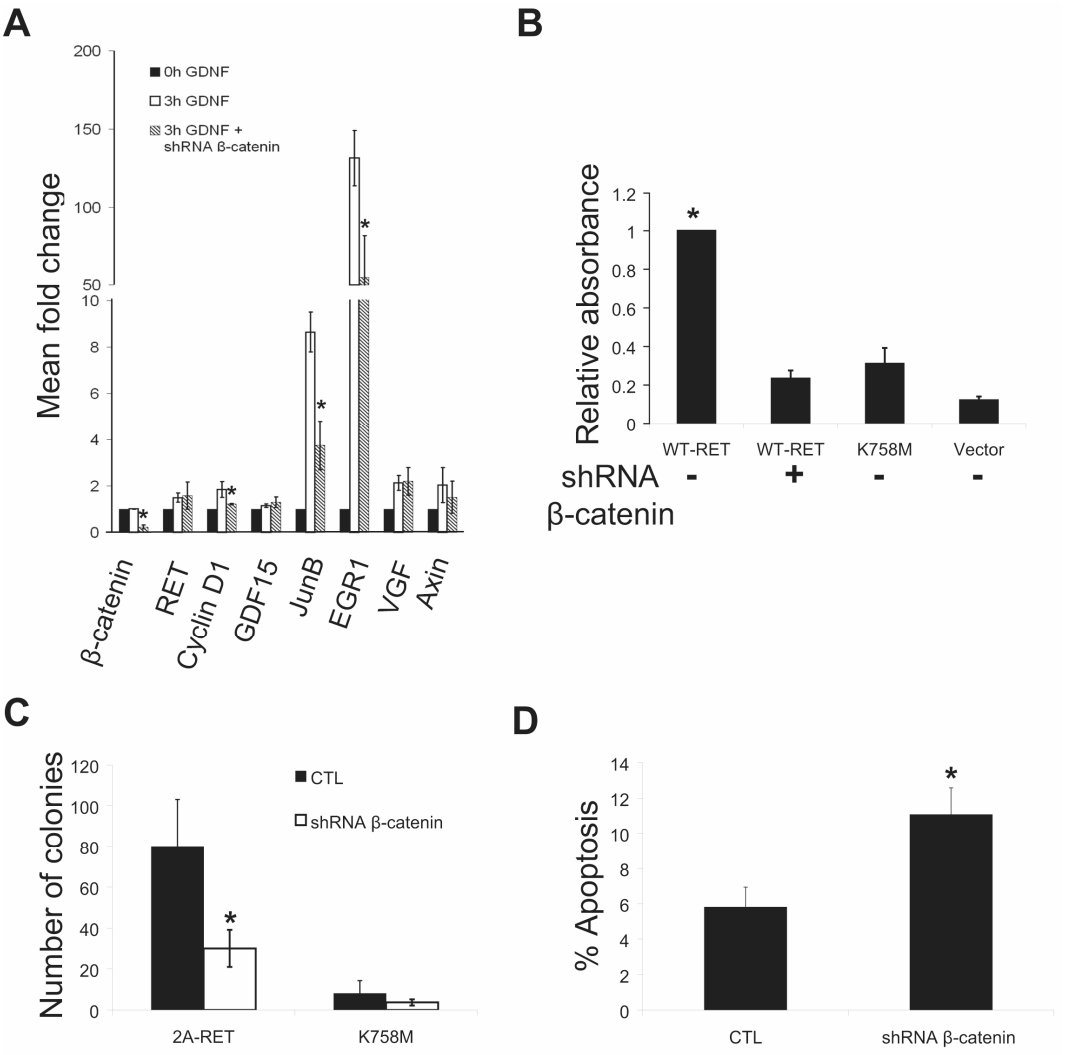
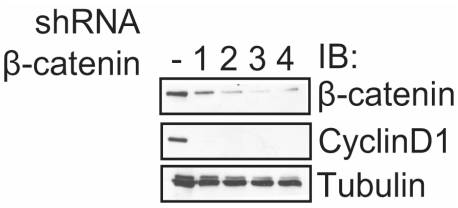


Figure 4. RET activation induces β-catenin-mediated transcription, cell proliferation, and transformation. A) RET and β-catenin have overlapping transcriptional target gene patterns. HEK293 cells stably expressing WT-RET and transiently expressing β-catenin shRNA or a control vector, were treated with GDNF, as above. RNA was isolated after 3 hours and subjected to qRT-PCR, using appropriate primers. Relative changes in expression are shown in comparison to 0h treatment conditions. Fold changes were averaged and expressed as a mean fold change ±sd. B) β-catenin is required for RET induced proliferation. HEK293 cells transiently expressing the indicated RET construct or vector alone were treated with GDNF and with shRNA against β-catenin, or a control, and cell proliferation was measured by MTT assay. Means of at least 3 replicates ±sd are shown. C) Down-regulation of β-catenin reduces RET induced transformation. NIH 3T3 cells expressing constructs for an oncogenic (2A-RET) or a kinase dead (K758M) form of RET were infected with β-catenin shRNA or control lentivirus (CTL) and grown on soft agar. Colony formation was assessed in a minimum of three replicate experiments and is expressed as mean colony number ±sd. D) β-catenin has a modest effect on RET-induced cell survival. NIH 3T3 cells stably expressing 2A-RET and infected with β-catenin shRNA or control lentivirus, as above, were fixed and stained with propidium iodide and percentage apoptotic cells estimated by flow cytometry. Assays were performed in triplicate and means ±sd are shown.



Supplementary Figure S4. Knockdown of β-catenin and cyclinD1 protein levels by shRNA against β-catenin. HEK293 cells expressing 4 different lentiviral- produced shRNAs (1-4) or lentiviral control (-) were monitored for β-catenin and cyclinD1 expression by western blotting, using appropriate antibodies. shRNAs 3 and 4 were pooled for use in the described analyses.

Knockdown of β-catenin expression reduces RET-mediated tumour growth and invasiveness in nude mice

As tyrosine phosphorylation of β-catenin by other kinases has been shown to divert its function from cell adhesion to increased signaling roles (13), and as we found a correlation of β-catenin nuclear expression and metastatasis of MTC, our gene expression data led us to postulate that RET-induced activation of the β-catenin pathway may contribute to cell proliferation and tumourigenesis. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, we showed that GDNF-treatment of HEK293 cells significantly increased cell proliferation in the presence of WT-RET but did not affect cells expressing the kinase dead K758M RET mutant, or an empty vector (Figure 4B). Similarly, GDNF did not stimulate RET-mediated proliferation in the presence of β-catenin shRNA (Figure 4B). A significant decrease in colony formation induced by the oncogenic mutant 2A-RET was also detected in soft agar anchorage-independent growth assays in the presence of β-catenin shRNA (Figure 4C). While β-catenin shRNA expression did increase apoptosis, the level of apoptotic cell death remained quite low in RET-expressing cells in the presence of β-catenin shRNA (Figure 4D), suggesting that a RET-β-catenin signaling pathway has roles in cell proliferation but is not as critical for cell survival.

We next used our β-catenin-shRNA knock-down model to investigate the role of β-catenin in RET-mediated oncogenesis. NIH 3T3 cells stably expressing the 2A-RET oncogenic receptor, with or without β-catenin shRNA, were injected subcutaneously into athymic mice and the ability of cells to form tumour outgrowths was monitored (Figure 5A). NIH 3T3 cells expressing 2A-RET grew rapidly in this model, producing measurable tumours by day 9 (Figure 5A,5B), while parental NIH 3T3 cells produced no detectable lesions (Figure 5B). In the presence of shRNA directed against β-catenin, tumour growth was slower with a notable lag in exponential growth phase. Tumours derived from cells expressing 2A-RET and control vector were nodular and adherent to the abdominal wall with frequent regional invasion and, in some cases, spreading to visceral organs such as kidney (Figure 5C; Supplementary Table 3). In contrast, tumours derived from cells expressing 2A-RET and ß-catenin shRNA were encapsulated, with no adhesion or invasion to surrounding tissues. The average volume of tumours was more than 2-fold less ($P<0.001$) in the animals receiving cells containing both 2A-RET and β-catenin shRNA as compared to 2A-RET and control vector, and this effect was correlated with a reduction in ß-

catenin protein in the corresponding primary tumour tissue (Figure 5D). Together, these data suggest that the highly tumorigenic potential of oncogenic RET mutants is, in part, mediated through a β-catenin signaling cascade.

Supplementary Table 3. Gross pathology and histology of tumour lesions.

Group	Adhesion to abdominal wall	Regional invasion*
NIH 3T3 (parental)	-	-
2A-RET	5/5 (100%)	3/5 (60%)
2A-RET + β-catenin shRNA	2/5 (20%)	0/5 (0%)

* Regional invasion through abdominal wall with occasional spreading to visceral organs.

These observations establish a novel signaling pathway linking the RET receptor tyrosine kinase to β-catenin. We show that RET and β-catenin can interact directly, leading to RET-mediated β-catenin tyrosine phosphorylation, nuclear translocation, and induction of a RET-β-catenin transcriptional program (Figure 6). In cell-based models, our data confirm that β-catenin is an important contributor to RET-induced cell proliferation and colony formation. In vivo, our data showing nuclear β-catenin localization in primary human MTC samples are consistent with activation of a RET-β-catenin pathway in these tumours. Nuclear localization was significantly more common in MTC metastases ($p<.001$) than in primary tumours from MEN 2 patients. Further, β-catenin nuclear localization was observed in 2/2 cases of MTC metastasis bearing the 2B-RET mutation (M918T), the most transforming RET mutant, associated with the most aggressive clinical form of MEN 2, MEN 2B (17), as well as in 6/7 MTCs from the CALC-MEN 2BRET mice which bear the corresponding RET mutation (Supplementary Figure S1). Our mouse transplantation model was consistent with a role for β-catenin in tumour outgrowth and invasiveness, suggesting that activation of the β-catenin signaling pathway by an active oncogenic RET mutant can increase the relative rate of tumour growth and permit infiltration across tissue layers. Together, these data suggest that β-catenin nuclear localization may be an important marker of tumour progression or advanced disease in human MTC. Interestingly, in familial adenomatous polyposis patients, inactivating mutations of APC that lead to increased nuclear β-catenin are also associated with increased risk for papillary thyroid carcinoma, tumours which are also associated with activating RET mutations (3, 34), suggesting that there could be a role for a RET-mediated upregulation of β-catenin in these tumours as well. Targeting β-catenin pathways in thyroid cancer may in future be another avenue for therapeutic intervention in these diseases. Thus, understanding the cellular mechanisms by which RET activates the β-catenin pathway may provide potentially broad insight into the pathogenesis of multiple thyroid tumour types.

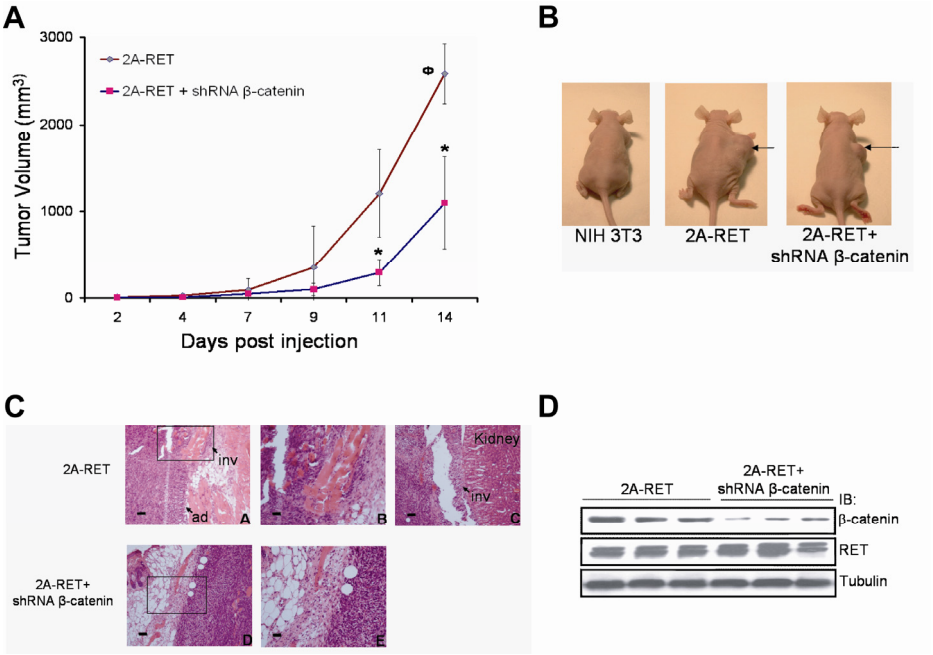


Figure 5.

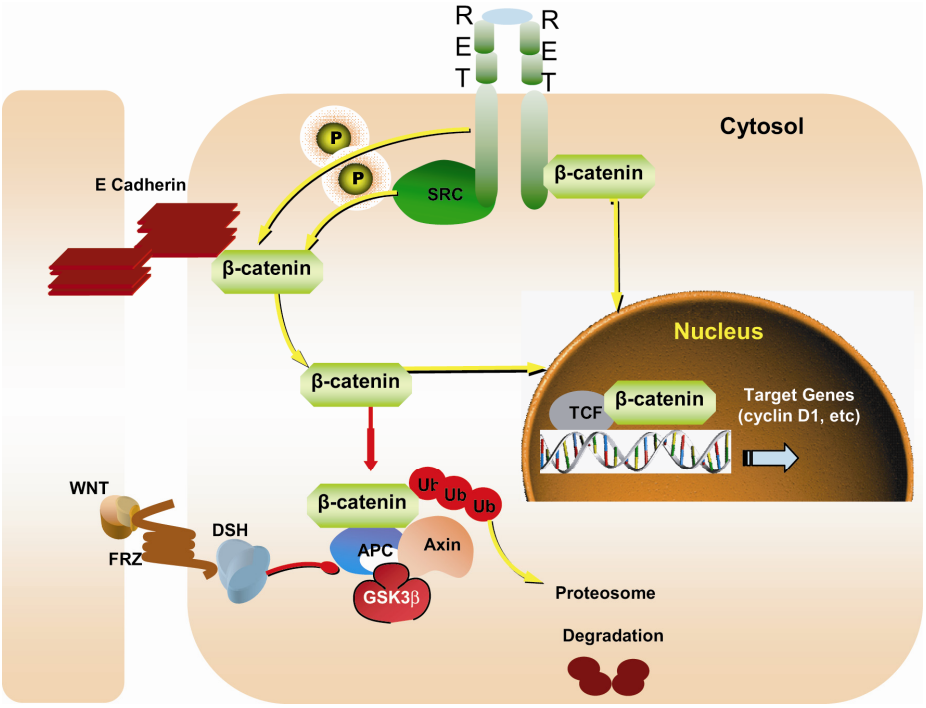


Figure 6.

Figure 5. RET-mediated tumour outgrowth in nude mice is reduced by shRNA to β-catenin. A) NIH 3T3 cells stably expressing 2A-RET together with ß-catenin shRNA or a control vector, were injected into the right flank of nude mice, and tumour volume (mm3) was measured every 2-3 days. B) Photographs of representative animals injected with parental NIH 3T3 cells (left), or cells expressing 2A-RET and endogenous β-catenin (middle), or 2A-RET with ß-catenin shRNA (right) are shown. Arrows indicate tumours. 2A-RET and endogenous ß-catenin coexpression leads to larger, more nodular tumours, compared to 2A-RET in presence of ß-catenin shRNA. C) Coexpression of RET and endogenous β-catenin promotes regional invasiveness and tumour adhesion to the abdominal wall that is absent in the presence of the β-catenin shRNA. Representative hematoxylin and eosin stained micrographs of tumour tissue from mice injected with cells co-expressing 2A-RET and endogenous β-catenin (panels A-C) or cells expressing 2A-RET and β-catenin shRNA (panels D, E) are shown. Panels B and E are higher magnifications of boxed regions shown in A, and D, respectively. Histology of the 2A-RET tumour, infiltrating into the walls of the kidney, is shown in panel C. The scale bars represent 100 μm in panels A, C, and D, and 50 μm in panels B and E. Inv: invasive tumour cells, ad: tumour adhesion to surrounding tissue. D) RET and ß-catenin expression in primary tumours. Proteins extracted from snap-frozen tumour samples were subjected to western blotting with the indicated antibodies.

Figure 6. Proposed model of a RET-β-catenin signalling pathway. β-catenin is constitutively associated with RET at the cell membrane. Upon ligand stimulation of RET, β-catenin is tyrosine phosphorylated and may dissociate from the membrane. Tyrosine phosphorylated β-catenin appears to escape cytosolic regulation by the Axin/GSK3/APC regulatory complex, which degrades free β-catenin, and moves to the nucleus. In the nucleus, β-catenin acts as a transcriptional regulator of genes for growth, and survival that have also been shown to be modulated by RET.

In summary, we have identified a novel RET-β-catenin signalling pathway, which is a critical contributor to enhanced cell proliferation and tumour progression in thyroid cancer. Our studies show that RET induces β-catenin-mediated transcription, cell proliferation, and transformation in vitro and that β-catenin nuclear localization and the resultant RET mediated β-catenin signalling is a key secondary event in tumour growth and spreading in vivo. This novel interaction suggests a mechanism that may underlie the broad and early metastatic potential of MTC. Our data suggest a previously unrecognized role for β-catenin signalling that may have implications for tyrosine kinase mediated tumourigenesis in multiple tumour types.

ACKNOWLEDGMENTS

We would like to thank Dr. M.R. Canninga (Dept. Pathology, UMC Utrecht) for providing the human MTC material. We are grateful to Drs. J. Woodgett, P. Greer, and J. MacLeod for providing us with constructs. We would like to thank Drs. S. Peng, P. Greer, and D. LeBrun, and N. Kaur for helpful discussion.

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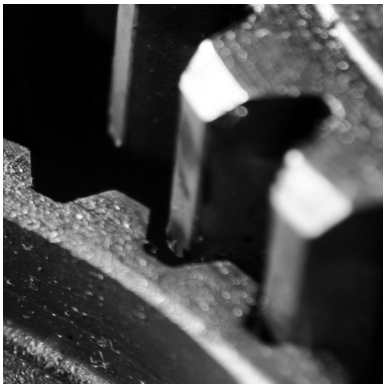
CHAPTER 5



Exploring the use of biomarkers in clinical management of patients with advanced medullary thyroid carcinoma

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Submitted

ABSTRACT

Misclassification of medullary thyroid carcinoma (MTC), due to the limited sensitivity of conventional imaging methods, is still one of the largest problems for accurate treatment of sporadic MTC patients. 30-55% of sporadic MTC patients are not cured after initial surgical treatment. After reviewing the literature, we found that re-operation of patients with postoperative hypercalcitoninemia in whom metastases can be visualized by conventional imaging results in significantly lower biochemical cure rates compared to re-operation of patients with occult metastases. Elective re-operation or re-operation after detection of apparently occult metastases by using alternative localization methods, did not reveal significant differences in biochemical cure. The management of patients with metastasized MTC is still under much debate, because these patients either have a longterm survival, due to an indolent course of the disease, or develop rapidly progressing disease leading to death from MTC metastases. Biomarkers, that can predict MTC prognosis, as well as biomarkers that provide targets for effective adjuvant therapies, are required for guiding decision making for further clinical management of patients with advanced MTC. In this review, we provide an overview of the currently known MTC biomarkers and their applications in diagnosis, treatment and prediction of prognosis.

INTRODUCTION

Medullary thyroid carcinoma (MTC), which is derived from the parafollicular calcitonin (CT) producing C-cells of the thyroid gland, predominantly develops as a sporadic tumor in about 60% of the cases and is hereditary in 40% of the cases. MTC is the predominant disease feature of multiple endocrine neoplasia type 2 (MEN 2), an autosomal dominantly inherited cancer syndrome. MEN 2 can be subdivided in MEN 2A, MEN 2B and familial MTC (FMTC), all characterized by the early onset development of MTC. In addition, MEN 2A patients may develop pheochromocytoma (PC), and hyperparathyroidism (HPT), while MEN 2B patients may develop PC and mucosal ganglioneuromas in the colon, lips and tongue (1, 2).

In 1993, activating missense mutations in the *RET* (REarranged during Transfection) proto-oncogene, at chromosome 10q11.2, were shown to constitute the genetic basis for MEN 2 (3, 4). Specific germline *RET* mutations were detected in families with MEN 2A, and subsequently also for MEN 2B and FMTC (5-7). Also, somatic *RET* mutations can be detected in the tumor tissue of 23-69% of sporadic MTC patients (8) and are associated with poor prognosis (9). These mutations lead to constitutive activation of the receptor tyrosine kinase RET, which activates several

downstream signaling pathways like RAS/ERK and PI3K/PKB signaling cascades, regulating e.g. proliferation, differentiation and cell survival (10, 11).

Classification of MTC is based on the pathological Tumor, Node, Metastases system and is also referred to as stage I, stage II, stage III and stage IV (12). Because of the limited sensitivity of conventional imaging techniques, like ultrasonography, computed tomography and magnetic resonance imaging, staging for MTC is most accurate when performed in combination with postoperatively measured plasma CT levels.

MTC is diagnosed by physical examination and imaging, in combination with measuring elevated plasma CT and carcino-embryonic antigen (CEA) levels (13). In the case of sporadic MTC, nodal metastases are present at the time of diagnosis in over 50% of the cases (14). About 65% of sporadic MTC patients die with advanced MTC metastases (15). The survival of MTC patients strongly correlates with stage at diagnosis: the 10-year overall survival rate of MTC patients in stage I and II is 90-100%, while it is 55-85% for patients in stage III (Table 1) and 20-55% for stage IV patients (16-21).

The appropriate initial treatment for patients who are diagnosed with MTC is total thyroidectomy and careful lymph node dissection of the central compartment of the neck (14). Measurement of postoperative plasma CT levels is a sensitive method to determine whether the operation has been curative (22). Additional treatment with radioactive iodide is not effective for MTC, especially for patients who were initially diagnosed with lymph node metastases (23, 24). Several approaches to develop systemic therapy for MTC treatment are currently in progress (24).

In this review we describe the different approaches in management of MTC patients with postoperatively elevated plasma CT levels. Different studies reveal low cure rates after re-operation, suggesting that the initial treatment is the most important step to obtain cure for these patients. During the last 30 years, no change in stage at diagnosis, nor improvement in survival have been detected for both sporadic and hereditary MTC patients, indicating that misclassification, insufficient familial screening and inadequate treatment are still the largest problems in the management of MTC patients (20, 25). Several biomarkers are currently used for the diagnosis and follow-up of MTC patients. The role of RET, CT, CEA and several other biomarkers, and how these are applied in the clinical management of MTC patients, are described in this review. Novel biomarkers are required for the prediction of disease progression and to offer new targets for therapy for MTC patients.

Postoperatively elevated plasma CT levels: stage IIIA and IIIB

Patients who have postoperatively normalized plasma CT levels are biochemically cured and have the best prognosis, i.e. 10-year survival of 98% (18, 22). Biochemical cure is obtained in 75-90% of the cases which were initially diagnosed without lymph node involvement, but in only 0-30% of

patients with pathological lymph nodes (26). Persistent elevation of plasma CT levels occurs in 30-55% of patients after primary operation for MTC (17, 18, 26-28). Additionally, in 5-12% of patients who were initially biochemically cured, it appears that several years after total thyroidectomy the basal plasma CT levels increase, indicating recurrent disease (17, 18, 29). These high percentages of persistent and recurrent MTC warrent a life-long follow-up, even in the cases of biochemical cure immediately after surgery (29) (Figure 1).

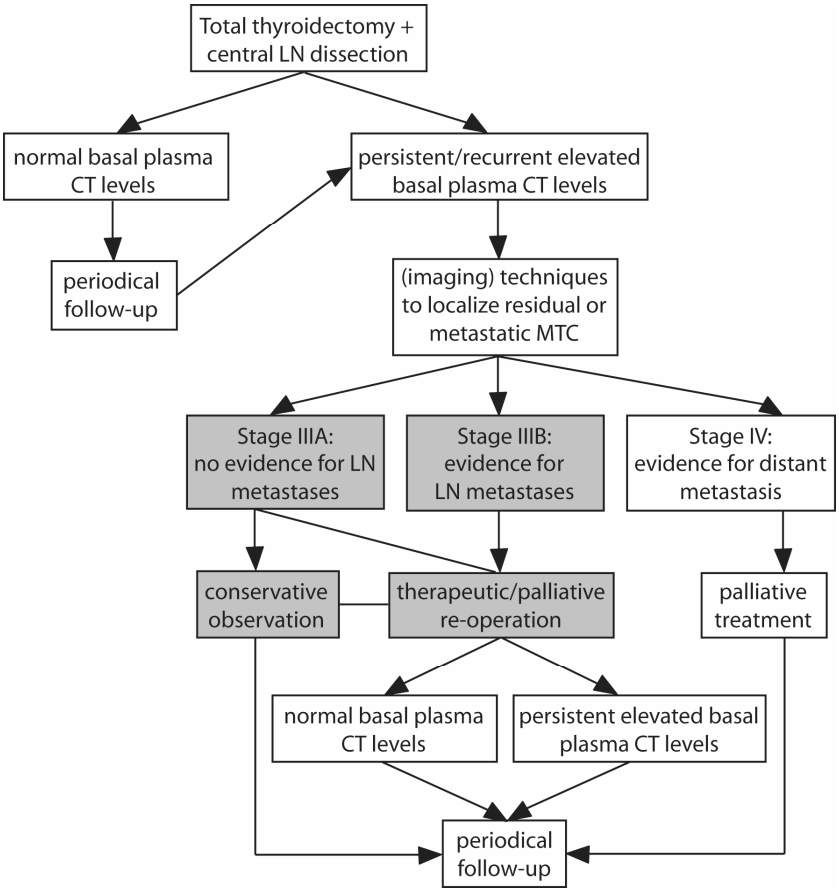

Figure 1. Postoperative management of MTC patients. After initial surgery, normalized plasma CT levels indicate biochemical cure. Postoperative hypercalcitoninemia indicates persistent or recurrent disease. In those cases, conventional or specialized localization techniques could be applied to detect the extend and sites of metastases. Patients without visible distant metastases are classified as stage III patients. In the case of occult MTC, these patients are referred to as stage IIIA patients, in the case of pathological lymph node detection, these patients are referred to as stage IIIB. For stage IIIA patients, a conservative observation strategy can be followed or these patients could be re-operated on, like it is usually performed for stage IIIB patients. After re-operation, measuring plasma CT levels again indicates biochemical cure or persistent MTC. For patients in stage IV, palliative surgical treatment could be performed. In all cases, periodical follow-up is recommended. LN, lymph nodes.

Patients with persistently elevated or rising plasma CT levels after initial surgery should be thoroughly evaluated with imaging techniques to define the extent of any local or distant disease (30) (Figure 1). However, conventional imaging methods can not always localize the sites of MTC metastases, i.e. occult disease. We propose to refer to stage IIIA for patients with postoperative hypercalcitoninemia, but without clinical evidence of local disease in the neck region or mediastinum and without distant metastases, and to stage IIIB for patients with postoperative hypercalcitoninemia with clinical evidence of local metastases but without distant metastases. Usually, re-operation is performed for stage IIIB patients, however, many controversies still exist regarding the follow-up strategy for patients in stage IIIA, probably because these patients either have a longterm survival or develop rapidly progressing disease leading to death from MTC metastases. At this moment, it cannot be predicted what will happen within individual cases.
In general, two approaches can be followed in the management of stage IIIA patients: conservative observation or re-operation (Figure 1). This re-operation could be performed electively, but nowadays re-operation after a metastatic workup using specialized localization techniques, like selective venous sampling catheterisation (SVSC), immunoscintigraphy (IS) with monoclonal antibodies like anti-CEA, or positron emission tomography (PET) with tracers like ^{18}F-fluorodeoxyglucose (^{18}F-FDG), is performed more often, e.g. (31-36). Here, we present an overview of survival rates of stage IIIA and IIIB patients and cure rates for those patients who have been treated by re-operation (Tables 1-4).

Stage IIIA: conservative observation

The first approach is to refrain from surgery, but periodically examine stage IIIA patients by performing plasma CT and CEA measurements and imaging. This expectative approach is maintained until these patients show pathological lymph nodes during follow-up, upon which re-operation is performed to remove the localized metastases. The rationale for the expectative approach is based on the often indolent course of the disease despite, in time, the presence of local, and even distant metastases (16, 37-39).
Another rationale for the conservative surveillance approach is to prevent an increase in morbidity after primary surgery. Due to extensive scarring in the neck, re-operation increases morbidity causing a substantial decrease in quality of life. Several studies describe the complications that are indicated after re-operation of which permanent hypoparathyroidism, thoracic duct injury and recurrent laryngeal nerve injury are the most predominant complications (35, 40-42).
In Table 1, the 5-year relapse-free survival rates, and 5- and 10-year overall survival rates for patients with postoperative hypercalcitoninemia are listed. This indicates that, despite the presence of MTC in these patients, overall survival is still relatively high.

Table 1. Survival rates of stage III patients and patients with postoperative hypercalcitoninemia

Reference	Year of publication	5-year relapse free	5-year overall	10-year overall
Stage III				
(16)	1998			55%
(18)	1998		80%	70%
(17)	2000		85%	85%
(20)	2006		85%	75%
(21)	2007		85%	65%
Patients with postoperative hypercalcitoninemia				
(38)	1990	61%	90%	86%
(39)	2001		90%	72%
(28)	2003	61%	97%	

Stage IIIA: re-operation

The second approach is surgery aimed at the removal of either occult MTC from lymph nodes in the neck and mediastinal compartments or microscopic MTC that can only be localized with specific alternative techniques. After re-operation, metastatic MTC foci can be detected histologically in excised tissue from stage IIIA patients, e.g. (32, 43, 44). Several studies reported the results on biochemical cure after elective re-operation of stage IIIA patients (Table 2).

In case of postoperative hypercalcitoninemia, different alternative techniques can be used to localize metastatic MTC, which is not detectable by conventional imaging. SVSC is a sensitive localization technique, which can be applied to the neck and mediastinum region (34, 45-48). IS, based on immuno-detection of metastases with anti-CEA antibodies, also proved useful for the detection of local lymph nodes in occult disease (31, 33). Recently, it was demonstrated that ^{18}F-FDG-PET has clinical value in postoperative follow-up and may be used additionally to preoperative imaging and for guiding re-operation in patients with biochemical evidence of persistent or recurrent MTC (32). Table 3 summarizes cure rates of stage IIIA patients which have been treated by re-operation after localization of apparently occult MTC with alternative techniques.

After re-operation, both electively and after alternative localization, a 50% reduction of plasma CT levels compared to pre-operative levels is achieved in about half of the patients, e.g. (32, 36, 42, 43). As shown in Tables 2 and 3, biochemical cure is achieved in about 25% of stage IIIA patients (elective re-operation: 27.6% and re-operation after alternative localization: 22.8%; p-value not significant, Fisher's Exact Test). This indicates that the use of alternative localization methods in combination with re-operation does not improve the cure rate over that of patients who are re-operated on electively. Nevertheless, re-operation after using alternative imaging techniques to

localize positive lymph nodes is usually directed specifically towards the site of pathological lymph nodes reducing morbidity, compared to a more aggressive re-exploration surgery when this is performed electively. Moreover, localization with alternative methods can identify the extend of the disease (restaging), which may be used in the decision making of further treatment.

Table 2. Biochemical cure after elective re-operation of stage IIIA MTC patients

Reference	Year of publication	Stage IIIA[1]	Biochemical cure[2]
(147)	1978	8	2
(148)	1986	10	4
(149)	1992	3	1
(40)	1998	29	8
(42)	1999	13	4
(43)	2000	10	2
(150)	2003	12	3
(151)	2007	2	0
totals		**87**	**24 (27.6%)**

[1] numbers indicate number of stage IIIA patients which received re-operational treatment;

[2] numbers indicate number of patients that were biochemically cured after re-operation

Table 3. Biochemical cure after re-operation following alternative localization of stage IIIA MTC patients

Reference	Year of publication	Stage IIIA[1]	Biochemical cure[2]
(48)	1980	7[a]	1
(46)	1989	6[a]	2
(47)	1992	13[a]	3
(27)	1993	32[a]	9
(45)	1994	13[a]	1
(35)	1997	45[a]	17
(152)	1997	18[a]	1
(31)	1998	13[b]	1
(33)	2000	13[b]	2
(34)	2001	5[a]	2
(32)	2004	9[c]	1
(44)	2006	4[c]	0
(36)	2007	6[a]	2
totals		**184**	**42 (22.8%)**

[1] numbers indicate number of stage IIIA patients in which metastases were detected by indicated alternative localization methods upon which they received re-operational treatment;

[2] numbers indicate number of patients that were biochemically cured after re-operation;

[a] SVSC; [b] anti-CEA IS; [c] ^{18}F-FDG-PET

Stage IIIB: re-operation

Next, we compared cure rates after re-operation of stage IIIA patients with cure rates after re-operation of patients without evidence for distant metastases, but in whom postoperatively local metastases or residual MTC can be visualized by conventional imaging, i.e. stage IIIB patients. In Table 4 the results on cure rates for stage IIIB patients after re-operation reported in several studies are presented, yielding an overall cure rate of 13.4%. This suggests that re-operation of stage IIIB patients could be performed with a therapeutic intent, however, in most of the cases, palliation or limiting the progression of MTC to avoid mortality would be the main goal.

Table 4. Biochemical cure after re-operation of stage IIIB MTC patients

Reference	Year of publication	Stage IIIB[1]	Biochemical cure[2]
(148)	1986	1	0
(153)	1989	24	2
(38)	1990	11	0
(154)	1993	36	1
(155)	1993	23	4
(156)	1995	10	0
(157)	1997	26	9
(31)	1998	9	0
(42)	1999	16	3
(43)	2000	18	0
(28)	2003	18	3
(150)	2003	32	10
(151)	2007	33	3
totals		**239**	**32 (13.4%)**

[1] numbers indicate number of stage IIIB patients which received re-operational treatment;

[2] numbers indicate number of patients that were biochemically cured after re-operation

From the studies presented in Tables 2-4 we can conclude that the chance to achieve biochemical cure is significantly higher for stage IIIA patients compared to stage IIIB patients ($p=0.001$; Fisher's Exact Test). This is probably because stage IIIA patients do have metastasized MTC, but these metastases are too small to be detected by the currently available conventional imaging methods. In stage IIIB patients, these MTC metastases are large enough to be detected by these imaging techniques, thereby increasing the chance of development of further metastases (also at distance) and diminishing the chance to cure.

In conclusion, correct preoperative classification and adequate initial treatment is most important in obtaining optimal cure rates. Overall, cure rates for patients with persistent or recurrent MTC are low (Table 2-4). In addition, for patients with distant metastases, palliation is the main goal for treatment (Figure 1), emphasizing the need for systemic therapies for patients with advanced MTC.

Known and novel biomarkers and applications for (metastasized) MTC

Biomarkers can be used to identify individuals at risk for cancer, detect disease earlier, determine prognosis, detect recurrence, predict response to particular therapeutic agents, and monitor response to treatment (49). In textbox 1 the characteristics and clinical applications of a 'biomarker' are defined.

MTCs synthesize and secrete CT, CEA, catecholamine, chromogranin A, serotonin, and histamine metabolites. CT, CEA, and to a lesser extent chromogranin, are currently the most useful plasma biomarkers for diagnosis and follow-up of MTC (50). Another well-known clinically useful biomarker is *RET*, which is used for the identification of persons that have a high risk to develop MTC (germline mutation carriers). Currently, different strategies are being developed in order to generate RET-targeted therapies for MTC. Several other biomarkers are used for the detection of MTC and to develop effective adjuvant therapies. Next, we provide an overview of these and other biomarkers and their applications in MTC diagnosis, treatment and prediction of prognosis.

Biomarkers:

Chromosomal alterations (loss, gain, translocations);
Gene alterations (mutations, polymorphisms, hypermethylation);
Altered gene expression (RNA or protein level).

Biomarker applications:

Identify individuals at risk for cancer;
Detect disease earlier
Determine prognosis;
Detect recurrence;
Predict response to particular agents;
Monitor response to treatment.

Textbox 1

***RET* is a biomarker for the diagnosis of hereditary MTC**

MEN 2 is an autosomal dominantly inherited syndrome with a penetrance for MTC of nearly 100%. When familial MTC is suspected, genetic screening is performed for germline *RET* mutations. The *RET* exons 10, 11, 13, 14, 15 and 16, and when negative the remaining 15 exons, should be sequenced in order to detect germline *RET* mutations (1). In 98% of MEN 2 families, a germline activating *RET* mutation can be detected (1). A high prevalence of de novo *RET* mutations (over 50%) has been identified in MEN 2B patients (51, 52), and to a lesser extend in MEN 2A/FMTC patients (53-55). Also, germline *RET* mutations are frequently detected in apparently sporadic MTC patients (56-58). Because a germline *RET* mutation is the best predictor

for MTC development, genetic screening is highly recommended in all MEN 2 families as well as in patients without a clear indication for hereditary MTC (1, 59, 60).
Once a germline *RET* mutation has been identified in an index-patient, relatives are being screened for the same *RET* mutation. Identification of germline RET mutation carriers allows prophylactic surgery as well as biochemical follow-up for metastatic and recurrent MTC and for development of PC and HPT (1).
Genotype-phenotype correlation studies have shown that specific *RET* mutations are associated with age at first diagnosis and tumor aggressiveness, according to which MEN 2 patients can be stratified into three risk groups (1, 61, 62). Timing of genetic screening and treatment for MEN 2-associated tumors varies between these three risk groups (63, 64). Risk group 1 (least high risk) is recommended for prophylactic thyroidectomy at the age of 5 to 10 years, risk group 2 (high risk) should be operated on before the age of 5, and risk group 3 (highest risk) is treated in the first year of life, however, the timing of central lymph node dissection is still a matter of considerable debate (1, 65, 66).

RET is used as a target for therapy

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 AMG-706, axitinib, CEP-701, CEP-751, imatinib, PP1, PP2, RPI-1, sorafenib, sunitinib and ZD6474 (64, 67-69). Some of these inhibitors are being tested for the efficacy as an MTC drug in clinical trials (70). Imatinib, a potent inhibitor of BCR-ABL, cKIT and PDGFR, has been shown to inhibit RET phosphorylation and MTC cell proliferation in high doses (71-73). The results of some clinical trials with imatinib to treat patients with metastasized MTC are however not very promising (74, 75). More encouraging results are obtained in an ongoing phase II trial with hereditary MTC patients for ZD6474, a VEGFR2 inhibitor, which has been shown to also inhibit RET (76, 77). Clinical trials involving MTC patients for AMG-706, axitinib, sorafenib and sunitinib are ongoing or underway (70).
It is important to note that different RET mutants might have different affinities to certain TKIs. This is already shown for the V804M mutant of RET which, in contrast to the C634R mutant, is resistant to PP1, PP2 and ZD6474 (78), while sorafenib is a potent inhibitor of both RET mutants (79). Further clinical investigation is required to test the efficacy of TKIs for patients with different *RET* mutations. Also, for sporadic MTC patients without a somatic *RET* mutation in their primary MTC, the efficacy of these RET-targeted therapies should be further investigated.
Most of the TKIs used in clinical trials or in preclinical studies with MTC patients are not specific for RET, e.g. ZD6474 is a potent inhibitor of VEGFR2. Inactivation of several tyrosine kinases (TKs) that are expressed in MTCs might be more effective than inhibiting RET alone. More research should be performed to study the expression and involvement of TKs other than RET in

MTCs. In order to be able to stratify MTC patients for effective TKI-therapies, the expression and/or activity of RET and/or other TKs should be evaluated in resected MTC tissue before treatment with TKIs is initiated.
These data indicate that RET-targeting TKIs (in combination with other TKIs or chemotherapy) for treatment of MTC patients are underway, however more and larger clinical studies with a longer follow-up time are awaited.

Plasma CT is a biomarker for the clinical diagnosis and follow-up of MTC

Plasma CT levels can be used as a biomarker for the diagnosis of CCH and MTC. Normal levels of circulating mature CT are below 10 pg/ml, although some physicians use 20 pg/ml as cut off value. Elevated basal plasma CT levels (> 10 pg/ml) indicate CCH/MTC, but elevated CT levels can also be measured in patients with non-C-cell (thyroid) diseases and in some healthy adult individuals (80-82). Procalcitonin, predominantly expressed by lymphocytes, is not a biomarker for MTC, but used for the detection of sepsis (83).
For the diagnosis of sporadic MTC, measurement of basal and/or stimulated plasma CT levels is the most specific and sensitive biomarker. Routine plasma CT measurements could be used for the early diagnosis of sporadic MTC, however false positive cases are observed (82, 84-90). CT-stimulation assays, using calcium or pentagastrin, can be performed in order to identify true positive increases (82, 84, 91).
In some studies, a correlation between preoperative plasma CT level and tumor size, tumor stage and postoperative biochemical cure has been identified (92-94). Although the necessity to use preoperative plasma CT measurements for the diagnosis of MTC is widely accepted and implicated, the predictive value of the absolute preoperative CT level is not yet entirely elucidated.
In 1984, Miyauchi *et al*., found that the doubling time of plasma CT levels in MTC patients has prognostic values for the 3-year survival, recurrence within 5 years, and time interval between surgery and clinical recurrence of MTC (95). Also a more recent study among 65 MTC patients revealed a strong relationship between CT doubling time and survival (96). This suggests that at present CT doubling time is the most sensitive predictive biomarker for MTC progression and can be useful in the decision making for further treatment of patients with occult MTC.
Postoperative plasma CT levels can be measured in order to determine whether biochemical cure has been achieved, in other words whether the operation has been performed adequately. Postoperative hypercalcitoninemia indicates persistent MTC. When, after biochemical cure, during follow-up plasma CT levels increase, recurrent MTC is indicated (29). Because of the relatively high percentage of MTC recurrence, life-long follow-up by measuring plasma CT levels periodically, is recommended for all MTC patients (1).
When persistent or recurrent MTC is undetectable by conventional imaging, CT measurements by SVSC can be performed to localize the occult metastases (45, 47).

CEA is a biomarker for the clinical diagnosis and follow-up of MTC

CEA, a neuroendocrine tumor marker, is usually measured during diagnosis and follow-up of MTC. Preoperatively elevated CEA levels (above 5-10 ng/ml) are associated with tumor size, number of lymph node metastases, MTC recurrence and poor prognosis (97-99). Postoperative measurements of CEA have revealed normal levels in patients which do however have (occult) metastatic disease in some cases (31, 100-102). Therefore, CEA has a higher value measured preoperatively, reflecting the extend of disease before the initial operation.

In addition, CEA can be used for the immuno-detection of MTC. 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 (100, 102, 103). False positive results are low using anti-CEA IS (31, 100, 101). Some radiologists prefer to use a bispecific anti-CEA/anti-In-diethylenetriaminepentaacefic acid (DTPA) antibody in combination with an ^{111}In-DTPA-hapten (31, 33, 101).

Positive anti-CEA scans could be useful in the decision making of further treatment of (occult) MTC patients as it was suggested that MTC detection with anti-CEA antibodies may result from higher CEA expression which could correlate with more aggressively growing forms of MTC (102). Moreover, in patients with occult MTC, unexpected distant lesions in bone and/or liver in addition to positive lymph nodes in the central compartment of the neck and mediastinal region can be detected by anti-CEA scans, indicating that these patients would not benefit from re-operation in the neck (103).

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. Juweid *et al*., published two pilot studies revealing limited anti-tumor effects of ^{131}I-anti-CEA, lasting up to 26 months in about 50% of MTC patients with advanced and metastesized MTC (104, 105). Like for the IS of MTC, the anti-tumor effect using the bispecific antibody against CEA and In-DTPA in combination with the In-DTPA-hapten has been evaluated in nude mice with MTC xenografts (106). In 1999, the first phaseI/II trial was performed with 15 metastatic MTC patients using a humanized ^{131}I-MN-14 $F(ab)_2$ anti-CEA monoclonal antibody (labetuzumab) (107). The therapy was well tolerated, and yielded a dramatic improvement in one patient and in 7 other patients, a median of 55% reduction of plasma CT and CEA levels was observed. In 11 out of 12 patients, MTC has continued to be radiologically stable for periods ranging from 3 to 26 months (107). Recently, it has been found that upon selection of patients with high risk for MTC progression (short CT doubling time), treatment with the bispecific antibody successively increased the overall survival rate compared to untreated high risk patients (108).

Several preclinical studies in mouse models have been performed to evaluate the effectivity of combination anti-CEA/chemotherapy. Some studies have revealed effective MTC growth inhibition by combining anti-CEA with dacarbazine (DTIC), doxorubicin, or both (109-111). Behr *et al*., showed a highly effective therapy in mice, using anti-CEA in combination with doxorubicin and applying bone marrow support (111). However, only a moderate anti-tumor response was observed in a phase I trial using a combination of ^{90}Y-labelled labetuzumab with doxorubicin and peripheral blood stem cell rescue in 15 patients with advanced MTC (112).

Using PET radiopharmaceuticals for imaging

FDG or dihydroxyphenylalanine (DOPA) are taken up by cells with a high glucose metabolism or during the synthesis of dopamine, respectively. Labelled with ^{18}F, both tracers are used in PET. 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) neuroendocrine tumors like carcinoids and MTCs (113, 114). For MTC detection, ^{18}F-FDG-PET provides a higher sensitivity (76-96%) and specificity (79-83%) than conventional imaging methods (32, 115-118) and can be used postoperatively to detect apparently occult MTC, especially in the cervical and mediastinal regions (32, 44, 117). The clinical experience with ^{18}F-DOPA-PET is very limited. It has shown to be superior to ^{18}F-FDG-PET, DMSA-V and conventional (morphological) imaging, and useful in detection of lymph node metastases (119, 120), especially in patients with slowly progressing MTCs (Koopmans *et al*., JNM in press).

Positive ^{18}F-FDG-PET imaging has been associated with poor prognosis in pheochromocytoma-paraganglioma and follicular thyroid carcinoma patients (121, 122). In patients with recurrent MTC with rapidly increasing plasma CEA levels, ^{18}F-FDG uptake has been associated with high uptake of glucose suggestive for a high proliferation of cells (123). In patients with a plasma CT level >1000 pg/ml, the ^{18}F-FDG-PET sensitivity was higher than that in patients with plasma CT <500 pg/ml (124). In addition, Koopmans *et al*. recently have shown that the sensitivity of ^{18}F-FDG-PET was increased in patients with a short plasma CT doubling time, which is a predictor of poor prognosis. Their results suggest that ^{18}F-FDG uptake is a sensitive predictive biomarker for the prognosis of patients with advanced MTC (Koopmans *et al*., JNM in press; (118)).

Regulatory peptides for imaging and treatment of MTC

MTCs express several receptors for regulatory peptides like somatostatin receptors (SSTRs) (125-128) and the gastrin/cholecystokinin B receptor (CCKBR) (129-131). Therefore, these receptors may be used as targets for MTC diagnosis and treatment.

The first clinically used somatostatin (SST) peptide is the octapeptide octreotide which binds to SSTR2, and in 1994, the Octreoscan (using ^{111}In-DTPA-octreotide) was approved to be used as an imaging method for somatostatin receptor-positive neuroendocrine tumors (114). For the detection

of MTC metastases, the Octreoscan provides a moderate sensitivity (about 50%), which can be improved in combination with other imaging methods (102, 132-134). Recently, a new SST tracer, ^{99m}Tc-EDDA/HYNIC-TOC, was used for the detection of MTCs and provided a sensitivity of 80% (135). Treatment of patients with a positive Octreoscan with ^{111}In-DTPA-octreotide or with another SST tracer, ^{90}Y-DOTATOC, revealed moderate responses (136, 137).

Behr *et al*. demonstrated that radio-labelled derivatives of gastrin showed an excellent targeting of CCKBR expressing tissues (138, 139). Recently, they showed that gastrin receptor scintigraphy was superior to SSTR scintigraphy and PET (140). 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 (141-144). A drawback of using radio-labelled CCKBR-binding peptides is the high stomach and gall bladder uptake due to CCKBR expression in these organs.

Previously, GDNF family receptor alpha (GFRα)4, one of the four GPI-linked co-receptors of RET, was found to be specifically expressed in normal and malignant thyroid C-cells. Of 27 different human adult tissues studied, GFRα4 transcripts were detected only in the thyroid gland. Moreover, in 8 out of 8 MTC samples analysed, high expression levels of GFRα4 were detected (145). Co-expression of RET and GFRα4 in humans seems to be restricted to thyroid C-cells and may be necessary for MTC development (145). Persephin (PSPN), and not the other members of the GDNF ligand family, was identified as the specific ligand for GFRα4, inducing RET-GFRα4-mediated differentiation, development and maintenance of thyroid C-cells (145, 146). Therefore, we propose that radio-labelled PSPN-derived peptides may provide unique tools for diagnostic and therapeutic applications for metastatic MTC.

CONCLUSIONS

In this review, we described the problems regarding the clinical management of patients with advanced MTC. Among clinicians, these is no consensus about the strategy for postoperative treatment and follow-up of patients with (occult) persistent or recurrent MTC. Some clinicians prefer to perform aggressive re-operations, or to perform compartment-oriented re-operations after localization of apparently occult disease with alternative imaging techniques. Reviewing the literature revealed that the chance to achieve biochemical cure is significantly higher for stage IIIA patients compared to MTC patients in stage IIIB. In addition, the use of alternative localization methods in combination with compartment-oriented re-operation of stage IIIA patients does not improve cure rates over those of stage IIIA patients who are re-operated electively. Nevertheless, several alternative imaging methods have shown to be superior to conventional imaging, and can

identify the extend of the disease, which may be used in the decision making of further clinical management.

Operations in the neck region, especially when performed sequentially, result in intensive scarring and morbidity reducing quality of life. Therefore, MTC patients should be referred only to select centers that have the surgical skills to deal with extensive disease in the neck and mediastinum. A conservative approach could be a well-considered alternative in cases of occult MTC, because of the often indolent course of the disease and to prevent further morbidity.

Biomarkers can be used in decision making of the clinical management of MTC patients. Germline *RET* mutations are used to determine the risk for hereditary MTC, and for timing of genetic screening and prophylactic treatment in MEN 2 family members. For the diagnosis and follow-up of MTC, plasma CT and CEA levels are measured and several imaging methods involving radio-labelled anti-CEA antibodies, or radiopharmaceuticals and radio-labelled regulatory peptides, which target receptors expressed in MTCs, are used. Radio-labelled anti-CEA antibodies and regulatory peptides may also be used for the treatment of MTC patients. In addition, several therapies are being developed aimed to inhibit RET.

Postoperatively elevated plasma CT level, short plasma CT doubling time, elevated CEA levels, and positive anti-CEA and ^{18}F-FDG-PET scans are associated with a poor prognosis, which may therefore serve as prognostic biomarkers. The biomarkers and their (putative) application in the clinical management of MTC patients discussed in this review, are summarized in Table 5. Further clinical and preclinical investigation should be performed to evaluate the diagnostic, therapeutic and prognostic value of these and novel biomarkers in the clinical management of patients with advanced MTC.

Table 5. Summary of biomarkers which are, or might be, useful for diagnosis, treatment and prediction of prognosis of MTC.

Biomarker	Diagnosis	Therapy	Prognosis
RET	Germline *RET* mutations	Germline *RET* mutations RET-TKIs	Somatic *RET* mutations
CT	Plasma CT levels		Plasma CT doubling time Postoperatively elevated plasma CT levels
CEA	Plasma CEA levels Anti-CEA antibodies	Anti-CEA antibodies	Elevated plasma CEA levels Positive anti-CEA IS
PET radiopharmaceuticals	^{18}F-FDG-PET ^{18}F-DOPA-PET		Positive ^{18}F-FDG-PET scan
SSTR	Somatostatin peptides	Somatostatin peptides	
CCKBR	Gastrin/CCK peptides	Gastrin/CCK peptides	
GFRα4	PSPN peptides	PSPN peptides	

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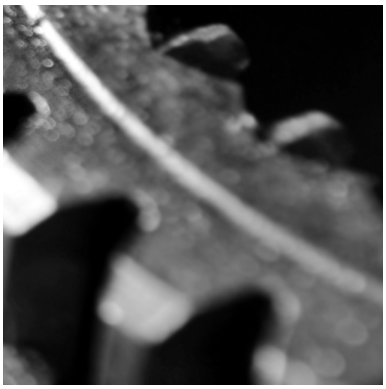
CHAPTER 6



General Discussion

6.1. The role of *P18* in MTC tumorigenesis

6.1.1. *P18* is an endocrine-specific tumor suppressor gene

Loss of *p18* in the germline of mice induces formation of pituitary adenoma, MTC, PC, parathyroid adenomas and testis interstitial cell adenomas (1). Strikingly, these are all tumors of endocrine origin and are, except for the testis tumors, involved in human multiple endocrine neoplasia type 1 (MEN 1) and/or MEN 2 (2). In men, loss of chromosome 1p32, spanning *P18*, occurs frequently in hereditary and sporadic MTCs (3, 4) as well as in other tumors associated with MEN, like PCs, parathyroid adenomas, and pituitary adenomas (5-7). Also, in neuroblastomas, arising from the sympethetic nervous system and of neuroendocrine origin, loss of 1p32 occurs frequently (8). No *P18* sequence variations have been detected in parathyroid adenomas, but a strongly reduced expression has been observed (9, 10). A reduced P18 expression level has also been detected in men and mouse pituitary adenomas (11, 12).

In chapter 2 we have described novel somatic *P18* mutations in hereditary and sporadic MTCs, and in hereditary PCs. These mutations, all located at functionally important sites of the P18 protein, cause reduced P18 stability as well as binding to CDK4 and CDK6. Somatic *P18* mutations are extremely rare in other types of human cancer, e.g. (13-17). So far, only a nonsense and a deletion mutation at codon 113 have been detected in 2 out of 65 oligodendrogliomas, also of neuroendocrine origin, an A72P mutation has been detected in 3 out of 35 breast carcinomas and a nonsense mutation at codon 68 has been shown in 1 out of 67 meningiomas (18-21). Further, promoter hypermethylation has only been detected in a small subset of Hodgkin lymphomas and medulloblastomas (22, 23). Although germline *P18* alterations in men have not been described yet, these somatic *P18* alterations, as well as the endocrine-specific tumor phenotype in *p18* knockout mice, indicate a tumor suppressor role for *P18* in (neuro)endocrine tissues.

Another member of the INK4 family, *P16*, is frequently inactivated in human cancer by homozygous deletions, mutations or hypermethylation (24). However, hypermethylation or loss of *P16* have not been detected in 11 and 49 human MTCs, respectively (25-28). *P16* knockout mice develop lymphomas and sarcomas, but MTCs have not been observed (29, 30). It has been shown that the different INK4 proteins have partially redundant and partially distinct functions (31). Recently, it has been suggested that p16 expression compensates for loss of *p18* in a tissue-specific manner. Compared to wildtype mice, p16 expression was increased 32-fold in adrenal tissues, 18-fold in pituitary, 4-fold in spleen and kidney, but not in lung tissues of $p18^{-/-}$ mice (32). Unfortunately, p16 expression levels have not been assessed in the thyroid C-cells of these mice. Although p16 expression was increased in pituitary glands of $p18^{-/-}$ mice, these mice still developed pituitary adenomas, suggesting that the compensation of loss of *p18* by *p16* in tumor suppression is only partial (32). To elucidate whether *P16* and/or other INK4 members can compensate for the loss of *P18* in C-cells, expression analysis of the four INK4 proteins should be

performed on normal and neoplastic human thyroid tissues and in thyroid tissues from wildtype and *p18* knockout mice.

The tissue-specific tumor suppression function of *P18* might also be explained by differential effects on the target proteins of P18, CDK4 and CDK6. The results on INK4-protein affinity for CDK4 and CDK6 are contradictory, but P18, and perhaps all INK4 proteins, tends to favor CDK6 over CDK4 (31, 33, 34). However, MTC has been detected in an INK4-insensitive *R24C-Cdk4* knockin mouse (35), and even more strikingly, *p18;Cdk4* double knockout mice completely lack the alterations induced by *p18* loss (36), suggesting that *Cdk4* is required for the (tumor suppressor) function of *p18* in mice. Unfortunately, development of C-cell hyperplasia (CCH) and/or MTC has not been assessed in these *p18;Cdk4* knockout mice.

Recently, *CDK6* has been indicated as a low-penetrance gene for sporadic MTC (37). It would be interesting to cross *Cdk6* knockout mice (38) with *p18* knockout mice to reveal the dependency of *p18* on *Cdk6 in vivo*. Expression analysis of CDK4, CDK6, and perhaps also other proteins involved in the RB/CDK/E2F pathway, or P18 regulation, should be assessed in endocrine tissues and tumors to elucidate the tumor suppressing mechanism of *P18* in endocrine tissues.

6.1.2. *P18* is a haploinsufficient tumor suppressor gene

In 1971, A.G. Knudson proposed a 'two-hit' model for retinoblastoma development and tumorigenesis in general (39). Hereditary retinoblastoma is caused by germline mutations in the *RB* tumor suppressor gene. It was thought that tumor suppressor genes were recessive and require mutations in both alleles to lose their function and be involved in tumorigenesis. In hereditary cancers, the first 'hit' is inherited in the germline and therefore present in all cells of the body, and the second 'hit' is acquired somatically in the tumor cells. In case of sporadic cancer, both events are acquired somatically during life to cause tumor initiation. Inactivation of one or both alleles is caused by mutations, LOH, homozygous deletions or hypermethylation. For some tumor suppressor genes the 'two-hit' theory holds true, the so called 'classical' tumor suppressor genes. However, nowadays it is known that mutations in tumor suppressor genes can have gain-of-function or dominant negative effects, or cause haploinsufficiency (40-42).

Haploinsufficiency occurs when one allele is inactivated and the other allele is insufficient to provide the level of functionality which is required for tumor suppression. This effect can be ascibed to a reduction in dosage (43). Haploinsufficiency does not necessarily have to be absolute, but can also be partial, depending on the type of tissue, coinciding genetic alterations, modifiers and/or other factors. Because of the minor effect of some haploinsufficient tumor suppressor genes, it may be difficult to detect their involvement in tumorigenesis. Usually, haploinsufficient events are only detected in combination with other oncogenic or haploinsufficient stimuli (40, 43). Some well-known haploinsufficient tumor suppressor genes, commonly involved in human cancer,

are *P27*, *P53*, *RB*, *APC* and *PTEN* (40, 41, 43-45). Many other (candidate) tumor suppressor genes are listed in (40, 43).

As described in chapter 3, $p18^{-/-};p27^{+/-}$ mice displayed a higher MTC incidence compared to $p18^{-/-}$ mice, and p27 expression was retained in all MTCs of these mice, indicating haploinsufficiency of *p27* in MTCs induced by loss of *p18*. These results are consistent with a previously reported study from Franklin *et al*. (1). We could not identify a haploinsufficient effect of *p27* in *RET*-induced MTC development in mice up to 12 months of age (chapter 3).

Loss of the *P18*-locus on chromosome 1p is a frequent event in human MTCs and other human cancers (3-7), however, inactivating mutations are extremely rare (46). Reduced P18 expression is observed in medulloblastomas, testicular and hepatocellular cancer, and associated with tumor progression (47-49). 4 of the 6 somatic *P18* mutations that we have detected in human MTCs and PCs, were in the heterozygous state (chapter 2). These characteristics are all suggestive for *P18* being a haploinsufficient tumor suppressor gene. In mice, the haploinsufficient effect of *p18* has been shown by treatment of heterozygous *p18* knockout mice with a chemical carcinogen, DMN (Table 1). Tumors that had developed in these $p18^{+/-}$ mice did not display LOH, loss of expression or mutations in *p18*, indicating that *p18* acts as a haploinsufficient tumor suppressor gene (50). In $Ptc^{+/-};p18^{+/-}$ mice, medulloblastoma formation was highly increased compared to $Ptc^{+/-}$ mice. In these tumors, expression of Ptc was lost, whereas p18 expression could still be detected (47). Also in a $Pten^{+/-}$ and $p27^{-/-}$ background, *p18* displayed a tissue-specific haploinsufficient function in tumorigenesis (Table 1) (1, 51).

As described in chapter 3, we have found haploinsufficiency of *p18* in combination with oncogenic *RET* (*RET2B*) (Table 1). No LOH of the remaining *p18* allele nor mutations in the coding sequence could be detected in MTCs from heterozygous *p18* knockout mice. However, (partial) loss of expression could be observed in a subset of these MTCs. The p18 expression status of MTCs from $p18^{+/-}$ mice correlated with the proliferation rate as well as the basal plasma CT levels, indicating that *p18* functions as a haploinsufficient tumor suppressor gene in *RET2B*-induced MTC, but that this haploinsufficiency is not absolute.

Table 1. Tissue-specific haploinsufficiency of *p18* in combination with additional oncogenic triggers on hyperplasia/tumor formation.

Organ	DMN treatment (50)			*p27*$^{+/-}$ (1)			*Pten*$^{+/-}$ (51)			*RET2B* (chapter 3)		
(cell type)	*p18*$^{+/+}$	*p18*$^{+/-}$	*p18*$^{-/-}$	*p18*$^{+/+}$	*p18*$^{+/-}$	*p18*$^{-/-}$	*p18*$^{+/+}$	*p18*$^{+/-}$	*p18*$^{-/-}$	*p18*$^{+/+}$	*p18*$^{+/-}$	*p18*$^{-/-}$
Thyroid gland	0/13[1]	6/20	5/20	1/24	1/4	14/16	3/20	3/17	12/19	0/25	10/39	13/30
(C-cells)	(0)[2]	(30)	(25)	(4)[2]	(25)[2]	(88)	(15)	(18)	(63)	(0)	(26)	(43)
Pituitary gland	0/13	4/20	13/20	13/20	5/5	33/33	0/20	0/17	16/19			
(intermediate lobe)	(0)	(20)	(65)	(65)	(100)[3]	(100)[3]	(0)	(0)	(84)			
Adrenal gland	0/13	2/20	8/20	9/21	5/6	23/23	15/20	13/17	18/19			
(medulla)	(0)	(10)	(40)	(43)	(83)	(100)	(75)	(76)	(95)			
Testis	0/13	4/20	10/20	0/12	1/5	10/10						
(interstitial)	(0)	(20)	(50)	(0)	(20)	(100)						
Lung	3/13	8/20	10/20									
(bronchiolar/alveolar)	(23)	(40)	(50)									
Pancreas				0/14	0/3	1/5						
(islets)				(0)	(0)	(20)						
Stomach				4/23	3/6	9/14						
(squamous)				(17)	(50)	(64)						
Intestine				5/24	0/4	15/17						
(duodenum)				(21)	(0)	(88)						
Prostate							?[5]	?[5]	?[5]			
(epithelial)							(20)	(40)	(100)			

Data are presented as [1] number positive/number examined [2] (%); [3] one case of CCH in *p18*$^{+/+}$ mice and one case of MTC in *p18*$^{+/-}$ mice; [4] earlier onset in *p18*$^{-/-}$ mice compared to *p18*$^{+/-}$ mice; [5] not mentioned

6.1.3. Cross-talk of RET and P18 via the PI3K/AKT pathway

One of the two major downstream signaling pathways of RET is the PI3K/AKT 'survival' pathway (52, 53). The kinase PI3K, which is activated by RET or other receptor tyrosine kinases, activates AKT, which in turn inhibits FOXO transcription factors leading to cell proliferation and survival (Figure 1). This pathway is inhibited by the phopholipid phosphatase PTEN, resulting in cell cycle arrest and apoptosis. *PTEN* is often inactivated by mutations, deletions or hypermethylation in human cancer (54). No loss or hypermethylation of *PTEN*, or mutations in *PI3K* are detected in human MTCs, however, the studies describing investigations of alterations in these genes in MTCs are sparse (26, 55, 56). Because the PI3K/AKT pathway is activated by RET, alterations in *PI3K* or *PTEN* might be unnecessary for development and progression of hereditary MTCs and *RET* mutation-positive sporadic MTCs. Studying large sample sets of sporadic MTCs without a somatic *RET* mutation, could reveal possible activation and involvement of this pathway in human non-*RET* MTC.

In mice, loss of *Pten* results in PC development, but also MTC fhas been detected in 3 out of 20 (15%) $Pten^{+/-}$ mice older than 6 months (51). Combined loss of *Pten* and *p18* resulted in a synergistic effect on MTC incidence (Table 1) (51). Elevated levels of active Akt, pAkt, were detected in prostate epithelial cells of $Pten^{+/-}$ mice, whereas pAkt was only marginally increased in prostate epithelium of $p18^{-/-}$ mice. Interestingly, substantially increased pAkt expression was detected in the prostate epithelial cells of $p18^{-/-};Pten^{+/-}$ mice, indicating that p18 negatively regulates Akt activation in a recessive manner to Pten function (51). Unfortunately, pAkt levels have not been assessed in C-cells/MTCs of these mice. In prostate cell lines, Bai *et al*., showed that AKT could be activated by activation of the RB/CDK/E2F pathway, and that this activation was dependent on PI3K-mediated phosphorylation of AKT (Figure 1) (51).

Because of the synergistic effect on MTC incidence in $p18^{-/-};Pten^{+/-}$ mice, it is likely that this positive regulatory loop is present in MTC cells as well. Both loss of PTEN and activation of RET results in PI3K-mediated AKT activation. Together, this suggests that the synergistic effect of oncogenic RET and loss of P18 in MTC tumorigenesis is mediated via the PI3K/AKT pathway (Figure 1). It would be interesting to cross *RET2B* mice with *Pten* knockout mice to determine whether oncogenic RET and loss of Pten cooperate in MTC development or can compensate for each other. Somatic *RET* mutation analysis in *p18;Pten*-induced MTCs and pAKT expression analysis in *RET2B;p18*-induced MTCs could further elucidate the cross-talk of RET, P18 and the PI3K/AKT pathway in multistep MTC tumorigenesis.

6.1.4. A role for P53 in RET- and/or P18-induced MTC tumorigenesis

The most pronounced tumor suppressor gene involved in human cancer is *P53* (24). Upon activation by oncogenes or stress signals like DNA damage or hypoxia, P53 induces several

cellular responses. P53 mediates cell cycle arrest via upregulation of e.g. P21 (G1-arrest) and 14-3-3sigma (G2-arrest). Several genes that are regulated by P53, control the release of cytochrome c from mitochondria, leading to activation of caspases followed by apoptosis. P53 also mediates senescense, although the mechanism is unclear. In addition, P53 regulates genes involved in extra-cellular matrix remodelling and angiogenesis, both important features in tumor metastasis. Finally, P53 is involved in DNA repair (57). The role of *P53* in human MTC development is not determined yet. Previously, we and others have detected nuclear P53 expression in about 20% of human MTCs (D.S. Acton, unpublished; (58-62)). Loss of *P53* occurs in about 10% of human MTC and mutations are rare (58, 59, 63, 64). This suggests that *P53* might act as a haploinsufficient tumor suppressor gene in human MTC.

P53 knockout mice do not develop MTC, but additional loss of *p53* in *Rb*-deficient mice enhances MTC development (65, 66). We have crossed transgenic mice expressing human oncogenic RET in the thyroid C-cells (*RET2B*) (67), with $p53^{-/-}$ mice to determine their roles in multistep MTC tumorigenesis. $RET2B;p53^{+/-}$ mice (5 out of 25; 20%) developed a significantly increased incidence of MTC at the age of 17 months compared to *RET2B* mice (1 out of 24; 4%), indicating a synergism between oncogenic *RET* and loss of *p53* in MTC development in mice. This synergism was even more pronounced in $RET2B;p53^{-/-}$ mice: at the age of 4 months, 4 out of 6 $RET2B;p53^{-/-}$ mice had developed MTC and the other 2 had CCH (D.S. Acton, unpublished). This clearly shows that loss of *p53* enhances *RET2B*-induced MTC tumorigenesis in mice.

P53 knockout mice have also been crossed with *p18* knockout mice. MTC was detected in a 1 out of 20 $p18^{-/-};p53^{-/-}$ mice and in 1 out of 42 $p18^{-/-}$ mice (68), suggesting that *p18* and *p53* do not collaborate in murine MTC suppression. In another study, MTC development was unfortunately not monitored in *p18;p53* double knockout mice (47). Therefore, the effect of combined loss of *p53* and *p18* in multistep MTC tumorigenesis remains to be further elucidated. It would be interesting to cross *p53* knockout mice with our $RET2B;p18^{-/-}$ mice to detect whether MTC development could be further enhanced by additional loss of *p53*. MTCs from these different mouse models can be used to determine the role of *P53* in MTC tumorigenesis. Currently, we are investigating p53 expression in MTCs from $RET2B;p18^{-/-}$ mice to further elucidate the role of *RET*, *P18* and *P53* in multistep MTC tumorigenesis.

6.1.5. P18 and the RB/CDK/E2F pathway are targets for MTC therapy

In chapter 5, we have discussed the need for systemic therapies in addition to surgery for patients with advanced MTC. Many genes encoding proteins involved in the RB/CDK/E2F pathway have been associated with MTCs, suggesting that these proteins may be important targets for therapy. We have detected inactivating *P18* mutations in human MTCs (chapter 2). Moreover, we have shown that loss of *p18* enhances *RET2B*-induced MTC incidence and growth, and reduces MTC

onset in mice (chapter 3). From *in vitro* studies it is known that P18 has a high affinity for CDK6, and to a lesser extent for CDK4 (33, 34). The phenotype analysis of *p18;Cdk4* double knockout mice, has revealed that the tumor-inducing effect of loss of *p18* requires *Cdk4* (36).
Inhibitors have been generated that selectively inhibit CDK4 and CDK6, like PD0332991 and CINK4, causing a potent Rb-dependent G1 arrest. Inhibitors with selectivity for CDK2 and CDK1, like seliciclib, BMS387032 and AZ703, cause a less potent G1 arrest compared to the CDK4/6 inhibitors. Some inhibitors, like flavopiridol, inhibit all 4 CDKs. The efficacy of some of these compounds as anti-cancer therapy is currently tested in clinical trials (69, 70). CDK inhibitors inhibit cells to progress to the S-phase of the cell cycle, while chemotherapeutic agents inhibit division of cells in the S-phase of the cell cycle. It is thought that using these drugs in combination, will yield an optimal inhibitory mechanism for tumor growth (70). Many studies are being performed to investigate the effect of CDK-inhibitor treatment in combination with chemotherapy.
In addition to the inhibition of CDK4/6/2/1, several of these compounds inhibit CDK9 and CDK7. These two CDKs are involved in the phosphorylation of RNA polymerase II, which facilitates the initiation of transcription of genes involved in the P53 and NFκB pathways, anti-apoptotic genes like *BCL2*, cell cycle regulators like P21 and Cyclin D1, and hypoxia-induced VEGF. This explains that some of these compounds not only cause cell cycle arrest, but also induce apoptosis in tumor cells (69, 70).
Interestingly, sorafenib, a BRAF inhibitor which also potently inhibits RET (71, 72), has been shown to cause a delay in G1/S transition in tumor cells, which was associated with decreased levels of Cyclin D1, P27 and phosphorylated RB (73). This suggests that sorafenib could function as an anti-MTC drug inhibiting 2 important steps in MTC tumorigenesis.
RET2B transgenic mice develop MTC at low frequencies and with a long latency period, making this model unfavorable for testing MTC treatments. The *RET2B;p18*$^{-/-}$ mice and *RET2B;p18*$^{-/-}$*;p27*$^{+/-}$ mice, however, develop MTC in 30-100% of the mice within 6-12 months of age (chapter 3). These represent excellent models for human MTCs, because these are induced by both RET activation and increased RB/CDK/E2F pathway activity. Therefore, these mice may serve as the best in vivo model currently available to test CDK- and/or RET-targeted therapies.

6.2. The role of β-Catenin in MTC tumorigenesis

6.2.1. Nuclear β-Catenin as a prognostic factor in MTC patients

In chapter 4 we have described nuclear localization of β-Catenin in MEN 2-associated MTCs. Nuclear localization of β-Catenin can be induced by activation of the WNT signaling pathway by ligands, by deregulation of β-Catenin degradation by mutations in APC, Axin2, or β-Catenin itself,

or by tyrosine-phosphorylation of β-Catenin by tyrosine kinases like EGFR and Src (74, 75). In the nucleus, β-Catenin acts as a transcriptional activator, upregulating sets of genes involved in cell proliferation, differentiation and survival (75). Cytoplasmic and/or nuclear expression of β-Catenin in several human cancer, has been associated with advanced disease stage, and may serve as a prognostic marker in these cancers (76-79).

In chapter 5, we have discussed the urgent need for predictive biomarkers which could be used in the decision making for postoperative clinical management of MTC patients. To study whether nuclear β-Catenin could function as such a biomarker, we first stained a total of 45 human MTCs of MEN 2 and sporadic patients (Table 2). In both MEN 2-associated and sporadic MTCs, nuclear β-catenin localization was more prevalent in metastases (65%) compared to primary MTCs (28%) ($p=0.014$ Fisher's Exact Test). This suggests that nuclear localization of β-Catenin in human MTCs is associated with more aggressive or advanced MTC disease stage.

To investigate whether nuclear localization of β-Catenin in MTCs correlates with increased proliferation, we stained human MTCs with (n=13) and without (n=17) nuclear β-Catenin for Ki67. A correlation between nuclear β-Catenin expression and proliferation could not be identified (Table 2). To study the prognostic value of nuclear β-Catenin in human MTCs, we are currently comparing the results on β-Catenin localization in primary MTCs of both MEN 2 and sporadic patients with clinical parameters, like stage at diagnosis, plasma CT and CEA levels at diagnosis, recurrence, metastasis, and survival.

6.2.2. Tyrosine phosphorylation of β-Catenin and its role in MTC development

Nuclear localization of β-Catenin could be caused by mutations in genes involved in β-Catenin degradation, or by mutations in β-Catenin itself. We performed a sequence analysis in 5 MEN 2-associated and 11 sporadic MTCs with nuclear β-Catenin, on exon 3 of the β-Catenin encoding gene, *CTNNB1*, which is a hotspot for somatic mutations. In addition, 4 MEN 2-associated and 9 sporadic MTCs with nuclear β-Catenin expression were analysed for mutations in the mutation cluster region of *APC*. No *CTNNB1* or *APC* mutations could be detected (Table 2).

Although we have not sequenced the entire *CTNNB1* and *APC* genes, nor other genes involved in β-Catenin degradation, like *AXIN2* or *GSK3*, the lack of *APC* and *CTNNB1* mutations led us to think of another mechanism for the nuclear translocation of β-Catenin in human MTCs. Tyrosine phosphorylation of β-catenin by tyrosine kinases has been shown to cause a release of β-catenin from the adhesion complex and translocation to the nucleus. As discussed in chapter 4, RET, a receptor tyrosine kinase, is able to phosphorylate β-catenin at or near the plasma membrane in a Src-independent manner, leading to an increase in β-catenin-mediated transcription. The induction of several downstream target genes of RET was shown to be mediated via β-catenin, and β-catenin

was required for RET-induced cell proliferation and transformation as well as for RET-induced tumor growth and invasiveness in mice.

So far, a specific antibody for tyrosine phosphorylated β-catenin has not been generated. It would be interesting to investigate whether the nuclear β-catenin detected in human MTCs is in fact tyrosine phosphorylated and whether this correlates with the presence and type of *RET* mutations. As shown in table 2, we have detected nuclear β-catenin expression in 4 sporadic MTCs without a *RET* mutation in exon 16. Although we cannot exclude somatic mutations in other *RET* exons, this suggests that in addition to tyrosine phosphorylation by oncogenic RET, other mechanisms can induce nuclear translocation of β-catenin in MTCs. It should be further investigated whether this is due to expression and/or activity of other tyrosine kinases, or whether another cause can be detected for the nuclear translocation of β-catenin in these tumors.

It is unclear whether dissociation of β-catenin from the plasma membrane by RET-mediated tyrosine phosphorylation is important in MTC tumorigenesis. In epithelial cells, β-catenin is localized to adherens junctions at the plasma membrane. The membranous localization of β-catenin in a large subset of MTCs indicates that β-catenin is involved in some kind of adhesion complex at the plasma membrane of MTCs as well. However, nothing is known about cell-cell interactions of non-epithelial C-cells and MTC cells. Expression analysis of cadherins and catenins in MTCs might reveal whether adherens junctions are present in these cells. Another possibility is that RET itself is involved in the cellular adhesion of C-cells as it contains 4 cadherin-like domains in its extra-cellular region. However, no data pointing to this hypothesis are available.

It is more likely that tyrosine phosphorylation of β-catenin is important in MTC tumorigenesis due to increased gene transcription caused by the nuclear translocation of β-catenin. This is supported by our observation that in the absence of β-catenin, RET-induced tumor growth and invasiveness are reduced (chapter 4). Expression analysis of known β-catenin target genes, like cMYC and AXIN2, in MTCs could further elucidate the role of nuclear β-catenin in MTC tumorigenesis.

Table 2. Nuclear β-Catenin analysis in primary and metastasized MEN 2-associated and sporadic MTC.

MTC phenotype	Age (years)	*RET* Mutation[1]	β-Catenin staining[2]	Ki67 staining[3]	*CTNNB1* mutation[4]	*APC* mutation[5]
MEN 2 / Primary	4	C634W		<1%		
	5	C634R				
	5	C634G		<1%		
	7	C634R				
	8	C634R		<1%		
	9	C634R				
	10	C634G		<1%		
	13	C634R				
	14	C634R		<1%		

	14	C634R	nuclear	1-5%	none	
	23	M918T		1-5%		
	26	C634Y	nuclear	1-5%	none	none
	30	C634R		1-5%		
	34	C634G	nuclear	1-5%	none	none
	36	C634W				
	37	C634R				
MEN 2 / Metastasis	26	M918T	nuclear			
	30	C634Y		<1%		
	35	M918T	nuclear			
	38	C618S	nuclear			
	45	C618S	nuclear	5-25%	none	none
	54	C634R	nuclear		none	none
	76	C634W				
Sporadic / Primary	29	unknown				
	29	unknown	nuclear	1-5%	none	none
	31	M918T	nuclear	<1%	none	none
	36			1-5%		
	42			5-25%		
	44		nuclear		none	
	50	unknown		<1%		
	53			<1%		
	61	M918T	nuclear		none	none
Sporadic / Metastasis	29	unknown	nuclear	5-25%	none	none
	31	M918T	nuclear		none	
	31	M918T	nuclear	<1%	none	none
	31	M918T	nuclear	1-5%	none	none
	36			1-5%		
	42			5-25%		
	43			5-25%		
	43			5-25%		
	50	unknown	nuclear	<1%	none	none
	53			<1%		
	60	unknown	nuclear	<1%		
	60	M918T	nuclear	<1%	none	none
	61	M918T	nuclear	<1%	none	none

[1] *RET* mutations for MEN 2 and sporadic patients represent germline and somatic mutations, respectively; 'unknown' indicates that a *RET* mutation could not be detected in exon 16; [2] MTCs were indicated as positive for nuclear β-Catenin when at least one positive nucleus was detected in each microscopic field (40x); [3] Ki67 staining is depicted as 3 categories of estimated percentage of positive cells: <1%, 1-5%, or 5-25%; [4] Exon 3 of *CTNNB1* (mutation hotspot) has been sequenced for MTCs with nuclear β-Catenin expression; [5] First 3kb of exon 15 (codons 690 to 1625) of *APC* (mutation cluster region) have been sequenced for MTCs with nuclear β-Catenin expression.

6.2.3. β-Catenin and the WNT pathway are targets for MTC therapy

As discussed in chapter 5, systemic therapies which can be used in addition to surgery, are in great need for MTC patients, especially for patients with persistent or recurrent MTC. Activation of WNT/β-catenin signaling is a frequent event in human cancer, and especially in intestinal cancer. Many drugs targeting the WNT/β-catenin pathway are being developed and tested as anti-cancer therapy.

Non-steroidal anti-inflammatory drugs (NSAIDs), like aspirin, indomethacin, sulindac, celecoxib, and etodolac, are widely used for the treatment of inflammation, fever and pain. They have been shown to inhibit WNT/β-catenin signaling via several different mechanisms. For example, it has been shown that aspirin induces degradation of β-catenin, indomethacin reduces β-catenin expression, while sulindac inhibits the nuclear content of β-catenin. Several NSAIDs are currently being tested in clinical trials for the treatment of several types of cancer as well as for cancer prevention (80-82).

Exisulind, a selective apoptotic anti-neoplastic drug, has also been shown to affect WNT/β-catenin signaling. It induces phosphorylation and therefore degradation of cytoplasmic β-catenin (82). Other compounds that could be used to inhibit the WNT/β-catenin pathway are dietary phytochemicals or flavonoids like curcumin and quercetin, vitamins like vitamin A and D, and/or their derivatives, and GSK3 inhibitors like lithium (80-83).

In chapter 5 several tyrosine kinase inhibitors are described which inhibit RET and/or other tyrosine kinases. The inhibition of those tyrosine kinases may result in inhibition of tyrosine phosphorylation of β-catenin and therefore inhibit WNT/β-catenin signaling. This has been shown for imatinib, a potent inhibitor of BCR-ABL and used for the treatment of chronic myeloid leukemia patients (84). BCR-ABL tyrosine phosphorylates β-catenin resulting in stabilization of β-catenin and increased β-catenin-mediated transcription (85). Reduced expression of β-catenin and relocalization from the nucleus to the plasma membrane were induced by imatinib in thyroid cancer cell lines (86). Because it has been demonstrated that imatinib also inhibits RET, imatinib has been used in clinical trials to treat MTC patients, however, the responses were not very promising (87, 88).

We have detected nuclear β-catenin in 6 out of 7 MTCs from our *RET2B* transgenic mice (chapter 4). This implicates that, despite the low frequency and late onset of MTC development, these mice are useful to test the efficacy of these anti-β-catenin drugs to inhibit MTC growth preclinically. For the treatment of MTC patients with these anti-β-catenin drugs, nuclear β-catenin localization in resected MTC tissue could be assessed to obtain indications for potential sensitivity to these drugs.

6.3. Model for multistep process of MTC tumorigenesis

Tumorigenesis is a complex process of sequentially occurring genetic alterations, eventually leading to an imbalance between cell growth and cell death, the so called 'multistep process' (89). In 2000, D. Hanahan and R. Weinberg, two founders in the cancer biology field, described the 'hallmarks of cancer' (90). According to their model, malignant growth requires six essential physiologic alterations:

1) Self-sufficiency in growth signals
2) Insensitivity to growth-inhibitory signals
3) Evasion of apoptosis
4) Limitless replicative potential
5) Sustained angiogenesis
6) Tissue invasion and metastasis

These alterations are thought to be induced by 3 to 7 genetic events, depending on the type of tumor (91). These genetic events has been more or less unraveled for colorectal carcinomas (89, 92), and for other types of cancer, multistep models are being proposed, like for cutaneous squamous cell carcinoma (93). The current knowledge about the molecular genetics of MTC (chapter 1) and the results discussed in chapters 2-4 of this thesis, enables us to propose a multistep model of MTC tumorigenesis (figure 2).

1) Self-sufficiency in growth signals.

Mitogenic growth signals dedicate cells to move from a quiescent state into an active proliferative state. These signals are transmitted from the extracellular environment into the cell by growth factor receptors that can activate several distinct signaling pathways. The receptor tyrosine kinase RET is such a growth factor receptor, which normally is dependent on GDNF-ligands for its activation (95). In cases of cysteine mutations in MEN 2A and FMTC patients, the RET protein dimerizes independently of ligand-binding, whereas RET autophosphorylates as a monomer, mutated in the tyrosine kinase domain, as in MEN 2B patients and in a subset of sporadic MTC patients, (96, 97).

Several downstream signaling pathways of RET have been detected, like the RAS/ERK and PI3K/AKT signaling cascades, regulating cell proliferation, differentiation and survival (52, 53). As described in chapter 4, we have detected a novel downstream signaling pathway of RET, mediated by β-Catenin, which promotes cell proliferation and tumor growth.

This indicates that the constitutive activation of RET in hereditary MTC and in a subset of sporadic MTC, leads to a self-sufficiency in growth-inducing signals via several different

pathways. However, in a substantial proportion of sporadic MTCs, a *RET* variant cannot be detected at all, suggesting that other mechanisms are involved in providing self-sufficiency in growth signals in those tumors.

2) Insensitivity to growth-inhibitory signals.

To maintain tissue homeostasis, multiple anti-growth signals inhibit excessive proliferation. Cancer cells need to become insensitive to this protective mechanism. The major component of this proliferative switch is the cell cycle G1/S phase transition, which is tightly regulated by the RB/CDK/E2F pathway (98, 99). Many genes encoding proteins involved in this pathway have been associated with human or mouse MTC development.

Loss of chromosome 1p and 13q, spanning *P18* and *RB*, respectively, are frequently detected alterations in human MTC (3, 100, 101). As discussed in chapter 2, we have detected somatic inactivating *P18* mutations in human MTC. In mice, loss of *p18* or *Rb,* results in MTC development, which is enhanced by additional loss of *p27* (1, 65, 66). In addition, loss of *E2f3* in *Rb*-deficient mice increases MTC incidence as well as formation of metastases (102). Further, *P15* and *CDK6* are suggested to be low-penetrance genes for sporadic MTC (28, 37). Together, this emphasizes the importance of this pathway in MTC formation.

Previously, we have detected that oncogenic RET downregulates P18 and P27 expression (103). Collaboration of *RET* and *P18* in MTC tumorigenesis was suggested by the coincidence of somatic inactivating *P18* mutations with germline or somatic activating *RET* mutations in human MTCs. A synergistic effect on MTC tumorigenesis was indeed detected in *RET2B;p18*$^{-/-}$ mice (chapter 3). Additional heterozygous loss of *p27* further increased MTC incidence, indicating a synergism between activation of RET and the RB/CDK/E2F pathway. More detailed investigation is required for the detection of sequence, methylation and expression alterations in genes involved in the RB/CDK/E2F pathway, to further elucidate the role of this pathway in human MTC tumorigenesis. Together, the results from these studies involving human patients and mouse models suggest that activation of the RB/CDK/E2F pathway may function as RET-independent MTC-initiating signal, however, it may also be involved in the progression of RET-induced MTC.

3) Evasion of apoptosis.

As a result of step 1 and 2 of the multistep tumorigenesis process, enhanced proliferation causes tumor cells to expand. However, tumor cells must acquire resistance to apoptosis in order to further increase the tumor cell population. RET-induced activation of the PI3K/PKB pathway may prevent FOXO transcription factors from inducing apoptosis (54).

The most commonly occurring mechanism through which cells evade apoptosis is by loss of wildtype P53. As summarized in chapter 1, mutations in *P53* are rare in human MTC (58, 59, 63, 64), however, loss of the *P53*-locus and nuclear stabilization of P53 is observed in a subset of

human MTCs (D.S. Acton, unpublished; (58-63, 104)), suggesting a haploinsufficient tumor suppressor role for *P53* in human MTC tumorigenesis. The cause of nuclear stabilization of P53 in human MTC, remains to be elucidated.

P53, at least in part, elicits apoptosis as a response to e.g. DNA damage, via the BCL2 family (105). Hinze *et al*., investigated the expression of the pro-apoptotic factor BAX and the anti-apoptotic factors BCL2 and BCLx in 36 hereditary and 20 sporadic MTCs. BCLx and BAX expression was scored as moderate to weak in sporadic MTCs, whereas in MEN 2-associated MTCs it was weak and moderate, respectively (59). However, these investigators, as well as others, have found strong BCL2 expression in almost all MTCs (148 in total), which correlates with the infrequent occurrence of apoptosis in human MTC (59, 61, 62, 106, 107).

We have detected a synergistic effect of oncogenic *RET* and loss of *p53* on MTC incidence as well as MTC onset in mice (D.S. Acton, unpublished). Loss of *p53* has also been shown to enhance MTC formation in *Rb*-deficient mice (65, 66). Moreover, in 4 out of 9 MTCs from $Rb^{+/-};p53^{+/-}$ mice, a somatic activating *RET* mutation could be detected (108), indicating that loss of *P53* may function as a secondary hit to activation of RET or the RB/CDK/E2F pathway in MTC tumorigenesis.

4) Limitless replicative potential.

Mammalian cells have an intrinsic protective mechanism preventing limitless replication. Telomeres cap the ends of eukaryotic chromosomes, preventing these ends from being recognized as DNA breaks, and therefore, from being processed by DNA-repair activities. With each replication, telomeres are shortened until a certain minimal-length is reached, upon which the cell will undergo apoptosis. Telomerase, or TERT, is an enzyme that maintains the critical telomere-length, permitting further replication (109). TERT is upregulated in over 90% of human tumors, and is also detected to be upregulated in 6 out of 9 human MTCs (110-113). Due to the sparse studies investigating TERT in human MTC and the low numbers of MTCs used, the role of TERT in MTC remains to be determined.

Cells in culture will die after a certain number of replications, which can be circumvented by immortalization. Immortalization is achieved for example by downregulating P53 or RB function (114). Previously, we have shown that MTC cells derived from *RET2B* mice did not survive in culture after a few replication rounds, whereas MTC cells from a $RET2B;p53^{+/-}$ mouse have been in culture for over three years (D.S. Acton, unpublished). Wildtype P53 inhibits transcription of TERT (109), however, we have not measured TERT levels in these cells. Furthermore, phosphorylation of TERT by AKT, increases its activity which is stimulated by PI3K and inhibited by PTEN (109).

Thus, RET-induced signaling via PI3K/AKT and/or loss of wildtype P53 could upregulate TERT in human MTCs. This should be further investigated to elucidate the role of TERT in acquiring a limitless replicative potential in human MTC.

5) Sustained angiogenesis.

Oxygen and nutrients must be supplied to all cells in tissues via the vascular system for normal cell function and survival. In an expanding tumor, cells must be in close proximity to capillary blood vessels to maintain viability, and therefore angiogenesis is an important step in tumor growth. Angiogenesis is induced by the vascular endothelial growth factor A (VEGFA) and its receptors VEGFR1 (or Flt1) and VEGFR2 (or Flk1, KDR), which are highly expressed in most types of cancers. Transcription of VEGF and its receptor is induced by the hypoxia-inducible factor 1 alpha (HIF-1α), which is stable under hypoxia. Under normal conditions, HIF-1α is degraded via VHL-mediated ubiquitination (115, 116). LOH of VHL has been detected in 3 out of 6 MEN 2-associated MTCs, which also had allelic imbalance of *RET*. One of the three MTCs with LOH of VHL showed a somatic frameshift mutation in exon 1 of the remaining VHL allele (117).
HIF-1α expression levels have not been studied in human MTCs, but can be induced by the PI3K/AKT pathway, which is activated by RET. VEGFA and VEGFR2 expression have been observed in human MTCs (118, 119), suggesting the presence of VEGF-signaling in human MTC. Some preliminary results from clinical trials reveal partial response of MTC patients to VEGFR-specific tyrosine kinase inhibitors (TKIs) or TKIs inhibiting both RET and VEGFR2 (120). It is not clear whether the clinical response to these TKIs is caused by inhibiting RET, VEGFR2 or both.
Two interesting observations regarding angiogenesis and MTC were reported recently. First, loss of chromosome 1p was associated with high vascularization in neuroblastomas (121). Second, it has been shown that calcitonin (CT) stimulates angiogenesis by directly activating vascular endothelial cells via the calcitonin receptor–like receptor (CRLR), which is expressed by human endothelial cells (122), suggesting a VEGF-independent pathway for angiogenesis in MTC.
Although the mechanism underlying angiogenesis in MTC has not been thoroughly investigated, VEGFA and VEGFR2, induced by RET activity and/or loss of VHL, might be involved. The role of CT in MTC angiogenesis remains to be determined.

6) Tissue invasion and metastasis.

The last step in malignant tumor formation is invasive growth and the ability to metastasize. Cadherin-catenin-complexes are the most common form of adherens junctions, which are crucial for maintaining tissue architecture and cell polarity and can limit cell movement and proliferation. *CDH1* encodes the major epithelial transmembrane cadherin receptor, E-cadherin, which is lost in several types of human cancer. β-Catenin binds to the cytoplasmic domains of E-cadherin and to

α-Catenin. This complex interacts with these complexes from neighbouring cells, forming adherens junctions. For invasive growth, the intercellular connections must be disrupted, which can be achieved by downregulating the expression of cadherin or catenin family members or by activation of signaling pathways that prevent the assembly of adherens junctions (123).
Because C-cells are not epithelial cells, the existence, composition and role of adherens junctions in MTCs remain to be determined. In our xenograft mouse model, discussed in chapter 4, we have detected invasive growth induced by oncogenic RET. In the absence of β-Catenin, this invasiveness is strongly reduced, suggesting that the RET-induced invasive potential requires β-Catenin. This also implicates that the RET-induced invasive potential of MTC is an effect of β-Catenin-mediated transcription rather than of loss of β-Catenin at the plasma membrane leading to deregulated adherens. This is also supported by the results from microarray analyses on RET-induced MTCs, revealing expression of genes involved in matrix remodelling and epithelial to mesenchymal transitions (EMTs), which are associated with an increased metastatic potential (124). omgaande
In mice, loss of *Nras* (or *E2f3*) in addition to loss of *Rb*, strongly enhanced MTC metastasis (102, 125). Interestingly, *Nras* is located on chromosome 1p, which is frequently deleted in both hereditary and sporadic MTC (3, 100). Correlation studies should reveal whether loss of 1p in MTCs associates with more aggressive and metastasizing disease phenotype.
Thus, the invasive and metastatic potential of MTCs, may be acquired by downstream signaling of RET. The role of other downstream pathways of RET, as well as of micro-environmental factors must be further investigated to understand the mechanism of MTC metastasis.

Concluding remarks

In this thesis we have discussed the molecular genetics of multistep MTC tumorigenesis. Via several downstream pathways, RET signaling may be involved in all steps of MTC development. We have identified a novel RET signaling pathway via β-Catenin, which is activated by tyrosine phosphorylation, and involved in tumor growth and invasiveness. The role and prognostic value of nuclear β-Catenin in MTC should be further elucidated. In addition, we have detected somatic inactivating *P18* mutations in human MTC, and loss of *p18* enhances *RET2B*-induced MTC development and progression. Further investigation of sporadic MTCs without a somatic *RET* mutation could reveal whether RB/CDK/E2F activation or other genetic events are involved in MTC tumorigenesis in the absence of oncogenic RET. Several therapies are currently being developed that target WNT/β-Catenin and RB/CDK/E2F signaling. Further investigation should reveal the effectiveness of these therapies for MTC treatment, especially for patients with advanced MTC, and who are insensitive for anti-RET therapies.

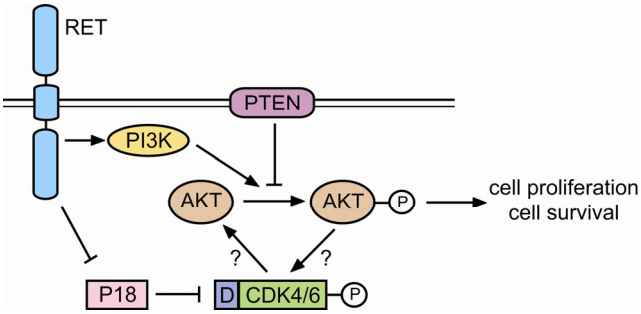


Figure 1. Possible cross-talk of RET and P18 via PI3K/AKT pathway. PI3K, activated by RET, starts a phosphorylation cascade leading to phosphorylation and activation of AKT. Active AKT inhibits transcription factors (e.g. FOXO) involved in cell cycle arrest and apoptosis, resulting in cell proliferation and survival. PTEN negatively regulates this pathway by inhibiting the activation of AKT. PI3K-mediated phosphorylation of AKT activates a positive feedback loop via activation of the RB/CDK/E2F pathway, which is inhibited by P18. Question marks indicate that the particular mechanisms are not fully elucidated yet. (adapted from (51, 54)).

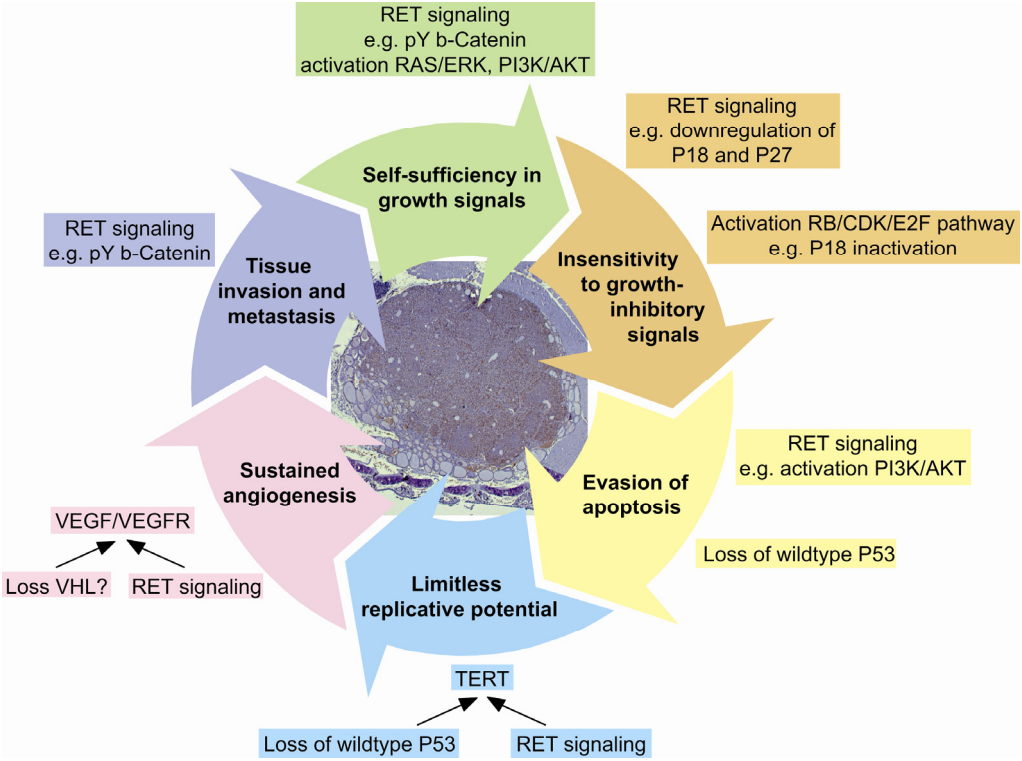


Figure 2. Model of multistep MTC tumorigenesis. Via several downstream pathways, RET signaling may be involved in all six steps of MTC tumorigenesis based on the model for cancer described in (90). In addition, several studies reported the involvement of the RB/CDK/E2F pathway in human and mouse MTC tumorigenesis. Some studies suggest the involvement of P53 in human MTC, but this is more pronounced in mouse MTC. Upregulation of TERT and VEGFR signaling by RET activation and/or other mechanisms might also be involved in human MTC development. pY; tyrosine phosphorylation.

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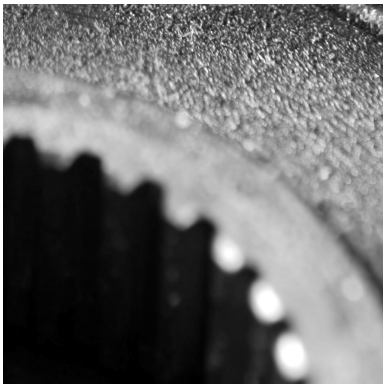
SUMMARY



The genetic mechanisms underlying the multistep process of MTC development is at present largely unknown. About 60% of all MTCs occur as sporadic cancer and the remaining 40% occur as familial cancer, i.e. as part of the MEN 2 syndrome. This syndrome is caused by activating germline *RET* mutations, which are also detected as somatic mutations in a subset of sporadic MTCs. Activation of RET initiates hereditary MTC development and could be involved in sporadic MTC development as well. However, additional oncogenic events are required, but remain to be elucidated. Because many molecular genetic studies on MTC tumorigenesis have been focussed on *RET*, not much is known about the involvement of non-*RET* oncogenic events in human MTC development.

In **chapter 1,** an overview is provided of all *RET* and non-*RET* genetic alterations detected in human MTCs. In addition, an overview of all *RET* and non-*RET* mouse models that develop MTC, is provided. Genes affected in these models might be involved in human MTC tumorigenesis as well, which should be further investigated.

From chromosomal analyses it is know that loss of chromosome 1p, on which the gene encoding the cell cycle inhibitor P18 is located, as the most frequent chromosomal alteration in both hereditary and sporadic MTC. In **chapter 2**, we show the presence of somatic inactivating mutations in *P18* in human sporadic and MEN 2-associated MTCs, and MEN 2-associated pheochromocytomas (PCs). Each mutation causes an amino acid substitution in the cyclin dependent kinase-interacting region of P18. We have shown that these mutations inhibit P18 function and cause reduced stability. Our findings implicate *P18* as a tumor suppressor gene involved in human MTC and PC development.

The somatic inactivating *P18* mutations coincide with activating *RET* mutations in human MTCs, suggesting cooperation between loss of *P18* and oncogenic *RET* in the multistep process of MTC development. To study this further, we have crossed transgenic mice expressing oncogenic *RET* with *p18;p27* double knockout mice, as described in **chapter 3**. We have detected a synergistic effect of oncogenic *RET* and loss of *p18* on MTC development, age-of-onset and MTC size. In addition, somatic loss of p18 expression, correlating with MTC growth, has been detected, indicating that loss of *p18* in combination with oncogenic *RET* not only increases the risk for MTC development, it also enhances MTC progression.

In **chapter 4**, we demonstrate that nuclear localization of β-Catenin is frequent in MEN 2-associated primary MTCs and their metastases, as well as in murine *RET*-induced MTCs. We show that RET is able to interact with, and tyrosine phosphorylate β-Catenin. As a result, β-Catenin escapes cytosolic downregulation by the APC/AXIN/GSK3 complex and accumulates in the nucleus, where it stimulates β-Catenin-specific transcriptional programs. Downregulation of β-Catenin activity decreases RET-mediated cell proliferation, colony formation, and tumor growth in nude mice. These data show that the RET kinase-β-Catenin pathway is a critical contributor to the development and metastasis of human MTC.

Thirty to 55% of sporadic MTC patients are not cured after initial surgical treatment. A literature study, described in **chapter 5**, revealed that re-operation of patients with postoperative hypercalcitoninemia results in low biochemical cure rates. The use of alternative localization methods to visualize apparently occult MTC, did not improve cure rates after re-operation, however, these techniques can identify the extend of the disease. Biomarkers that can predict MTC prognosis, as well as biomarkers that provide targets for effective adjuvant therapies, are required for guiding decision making for further clinical management of patients with advanced MTC. This chapter provides an overview of currently used and putative novel biomarkers, like RET, plasma CT and CEA, radiopharmaceuticals and regulatory peptides.

Chapter 6 provides an integrated model describing our current knowledge of the molecular mechanisms underlying multistep MTC tumorigenesis. Via several downstream pathways, RET signaling may be involved in all the essential steps for tumorigenesis. These pathways may involve activation of β-Catenin-mediated signaling through tyrosine phosphorylation by RET. In addition, loss of G1/S transition control, e.g. via inactivation of P18 or loss of RB, might be an important step in MTC tumorigenesis. Several therapies are currently being developed that target WNT/β-Catenin and RB/CDK/E2F signaling. Further investigation should reveal the effectiveness of these therapies for MTC treatment, especially for patients with advanced MTC, and who are insensitive for anti-RET therapies.

NEDERLANDSE SAMENVATTING

De genetische mechanismen die ten grondslag liggen aan het meerstaps proces van medullair schildkliercarcinoom (MSC) ontwikkeling is tot op heden grotendeels onbekend. Ongeveer 60% van alle MSCen komt voor als sporadische kanker en de overige 40% als familiaire kanker, als onderdeel van het multipele endocriene neoplasie type 2 syndroom (MEN 2). Dit syndroom wordt veroorzaakt door activerende kiembaan *RET* mutaties, die ook als somatische mutaties kunnen worden gedetecteerd in een deel van de sporadische MSCen. RET activering initieert de ontwikkeling van erfelijk MSC en is mogelijk ook betrokken bij de ontwikkeling van sporadische MSCen. Additionele oncogene gebeurtenissen zijn nodig, maar moeten nog opgehelderd worden. Omdat veel moleculair genetische studies betreffende MSCen op *RET* zijn gericht, is er niet veel bekend over de betrokkenheid van niet-*RET* oncogene gebeurtenissen in humane MSC ontwikkeling.

In **hoofdstuk 1** wordt een overzicht gegeven van alle *RET* en niet-*RET* genetische veranderingen die gedetecteerd zijn in humane MSCen. Er wordt ook een overzicht gegeven van alle *RET* en niet-*RET* muismodellen die MSC ontwikkelen. Genen die in deze muizen zijn gemodificeerd, zouden ook een rol kunnen spelen in humane MSC ontwikkeling. Dit zou verder bestudeerd moeten worden.

Uit chromosomale analyses is gebleken dat verlies van chromosoom 1p de meest voorkomende chromosomale verandering is in zowel erfelijke als sporadische MSCen. Op chromosoom 1p ligt een gen dat codeert voor de celcyclus remmer P18. In **hoofdstuk 2** tonen we de aanwezigheid van somatische inactiverende *P18* mutaties in humane MSCen en MEN 2 geassocieerde feochromocytomen (FCen) aan. Elke mutatie veroorzaakt een aminozuur verandering in het cycline afhankelijke kinasenbindend gebied van P18. We hebben aangetoond dat deze mutaties de functie van P18 remmen en een verlaagde stabiliteit veroorzaken. Onze bevindingen impliceren dat *P18* een tumorsuppressorgen is, dat betrokken is bij de ontwikkeling van humane MSC en FC.

De somatische inactiverende *P18* mutaties coïncideren met activerende *RET* mutaties in humane MSCen dat een samenwerking suggereert tussen oncogeen *RET* en verlies van *P18* in de meerstaps ontwikkeling van MSC. Om dit verder te bestuderen, hebben we transgene muizen, die oncogeen RET tot expressie brengen, gekruist met *p18;p27* knockout muizen, zoals beschreven staat in **hoofdstuk 3**. We hebben een synergistisch effect tussen oncogeen *RET* en verlies van *p18* aangetoond op MSC ontwikkeling, leeftijd bij het ontstaan van de tumoren en de tumorgrootte. Daarnaast laten we zien dat somatisch verlies van p18 expressie voorkomt in muizen MSCen en dat dit correleert met MSC groei. Dit indiceert dat verlies van *p18* in combinatie met oncogeen *RET* niet alleen het risico op MSC ontwikkeling verhoogd, maar ook betrokken is bij MSC progressie.

In **hoofdstuk 4** laten we zien dat nucleaire lokalisering van β-catenine frequent voorkomt in zowel MEN 2-geassocieerde primaire MSCen en metastasen, als in muizen RET-geïnduceerde MSCen. RET kan een interactie aangaan met β-catenine, en kan β-catenine tyrosine-fosforyleren. Daardoor

kan β-catenine ontsnappen aan de cytosolische afbraak, gemedieerd door het APC/AXIN/GSK3 complex, en kan het accumuleren in de celkern, waar het β-catenine-specifieke transcriptionele programma's activeert. In de afwezigheid van β-catenine activiteit, wordt RET-gemedieerde celproliferatie, kolonievorming en tumorgroei in naakte muizen geremd. Deze resultaten tonen aan dat het RET kinase-β-catenine signaleringspad een kritische bijdrage levert aan de ontwikkeling en metastasering van humane MSCen.

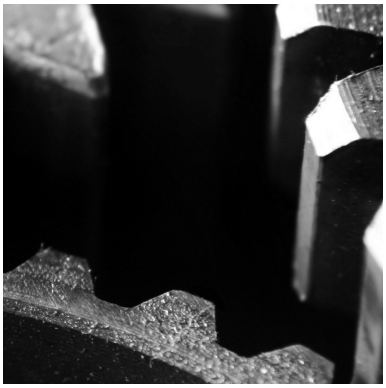
Dertig tot 55% van sporadische MSC patiënten zijn na de initiële chirurgische behandeling niet genezen. Uit literatuurstudie, beschreven in **hoofdstuk 5**, is gebleken dat heropereren van patiënten met postoperatieve hypercalcitoninemie resulteert in lage biochemische genezingskansen. Het gebruik van alternatieve lokaliseringmethoden om schijnbaar onzichtbare MSCen te detecteren, verbeteren de genezingskansen na heroperatie niet, hoewel deze technieken wel de uitgebreidheid van de ziekte kunnen identificeren. Biomarkers die de MSC prognose kunnen voorspellen, en biomarkers die als target kunnen dienen voor effectieve adjuvante therapieën, zijn nodig ter ondersteuning van de besluitvorming in het klinische beleid voor patiënten met gevorderde MSC. Dit hoofdstuk geeft een overzicht van biomarkers die momenteel gebruikt worden in de kliniek, en van mogelijke nieuwe biomarkers, zoals RET, plasma CT en CEA, radiofarmaceutica en reguleringspeptiden.

Hoofdstuk 6 beschrijft een geïntegreerd model die onze huidige kennis over de moleculaire mechanismen, die ten grondslag liggen aan de meerstaps MSC ontwikkeling, weergeeft. Via verschillende signaleringspaden kan RET activering betrokken zijn bij alle essentiële stappen van tumorontwikkeling. Een voorbeeld van deze signaleringspaden is de activering van β-Catenine-gemedieerde signalering door RET-geïnduceerde tyrosine-fosforylering. Daarnaast zou het verlies van de controle op de G1/S transitie, bijvoorbeeld door inactivering van P18 of verlies van RB, een belangrijke stap in MSC ontwikkeling kunnen zijn. Verschillende therapieën, die gericht zijn op het remmen van de WNT/β-Catenine en RB/CDK/E2F signaleringspaden, worden momenteel ontwikkeld. Verder onderzoek moet uitwijzen of deze therapieën effectief zijn voor de behandeling van MSC, vooral voor patiënten met gevorderde MSC en voor patiënten die niet gevoelig zijn voor anti-RET therapieën.

KORTE UITLEG VOOR DE LEEK

In normale weefsels is er een goede balans tussen celgroei en celdood. Als deze balans is verstoord, kan ongeremde celdeling ontstaan. Uiteindelijk kunnen deze ontspoorde cellen een tumor vormen. De balans tussen celgroei en celdood wordt in stand gehouden door allerlei regelmechanismen. Deze regelmechanismen worden aangestuurd door genen in het DNA. Fouten in het DNA kunnen leiden tot ontregelde genen, waardoor de regelmechanismen niet meer goed functioneren. Kanker is dus het gevolg van fouten in het DNA. Meestal ontstaan deze fouten in de loop van het leven, bijvoorbeeld door blootstelling aan sigarettenrook of UV straling. Maar ook tijdens normale celdelingen kunnen fouten in het DNA ontstaan. Soms worden DNA fouten van ouder op kind doorgegeven. Het erven van deze DNA fouten kan leiden tot erfelijke kanker.
Een gezonde cel wordt meestal pas een kankercel, wanneer in deze cel fouten in meerdere essentiële genen hebben plaatsgevonden, dit wordt ook wel het meerstaps model van tumorontwikkeling genoemd. Je kunt het proces van tumorontwikkeling ook wel vergelijken met tandwielen die de wijzers van een klok in gang moeten zetten. Het is moeilijk om de wijzers te laten draaien met één enkel tandwiel. Door interactie met andere tandwielen , gaan de wijzers steeds makkelijker en sneller draaien. Het verschil in grootte van de tandwielen heeft hier invloed op.
Genen werken net als tandwielen. Er zijn genen met een sterk effect op tumorontwikkeling die tumorvorming kunnen initiëren. En er zijn genen met een kleiner effect die essentieel zijn voor de progressie van een tumor tot uitzaaiende kanker. Afzonderlijk hoeven deze genen niet altijd tumorontwikkeling tot gevolg te hebben. Echter, doordat ze net als tandwielen met elkaar kunnen samenwerken, zijn ze in staat om tumoren te laten ontstaan en kanker te laten ontwikkelen.
De afgelopen jaren heb ik onderzoek gedaan naar welke tandwielen (genen) betrokken zijn bij het meerstaps proces van medullair schildklierkanker ontwikkeling. In dit proefschrift beschrijf ik nieuwe tandwielen die nodig zijn om deze tumorontwikkeling in gang te zetten en hoe de verschillende tandwielen in elkaar passen om dit steeds sneller te laten verlopen. Door deze resultaten krijgen we een beter inzicht in hoe medullair schildklierkanker kan ontstaan.

DANKWOORD



Ter afsluiting van dit proefschrift en van een hele leuke en leerzame periode in Utrecht maak ik graag van de gelegenheid gebruik om alle mensen die mij tijdens deze periode hebben geholpen en gesteund, op welke manier dan ook, te bedanken.

Allereerst wil ik mijn co-promoter Dr. J.W.M. Höppener bedanken. Beste Jo, misschien is dit het juiste moment om maar eens toe te geven dat ik waarschijnlijk net zo eigenwijs ben als jij, maar dat is tenslotte een goede eigenschap voor een onderzoeker! Ik wil je bedanken voor de mogelijkheden die jij me hebt geboden mezelf te ontwikkelen als onderzoeker. Ook voor de kans die je me hebt gegeven om een jaar part-time te werken, zodat ik daarnaast voor KWF Kankerbestrijding kon schrijven. Ten tweede, mijn promotoren, Prof. Dr. C.J.M. Lips en Prof. Dr. R. Berger. Beste Kees, uw klinische kijk op mijn onderzoek en input heb ik altijd erg gewaardeerd en hebben mij ook geinspireerd. Ik denk nog regelmatig terug aan ons congresbezoek in New Orleans (van vóór Katrina) en uw verhalen als een ware tour-guide, die deze reis erg leuk en leerzaam maakte. Beste Ruud, ondanks dat je pas later betrokken bent geraakt bij mijn onderzoek wil ik je bedanken voor de ondersteuning die je mij en onze groep hebt gegeven. Jouw daadkracht heb ik erg gewaardeerd.

Dennis, ik wil je bedanken voor alles wat je mij, vooral tijdens mijn stage, hebt geleerd. Je bent een echte mentor voor mij geweest. Ik heb onze wetenschappelijke discussies in de koffiekamer de laatste jaren erg gemist en vind het nog altijd jammer dat we niet langer hebben kunnen samenwerken.

Carola, ook jou kan ik niet genoeg bedanken. Jouw inzet, leergierigheid en enthousiasme zijn erg stimulerend geweest. Ontzettend bedankt voor jouw grote bijdrage aan mijn (ons) onderzoek. Ik heb het heel erg leuk gevonden om met je samen te werken, we hebben erg veel lol gehad (denk aan de uit elkaar spattende tumor…). Ik wens jou en Eelco alle goeds toe in de toekomst. Bedankt dat je mijn paranimf wilt zijn. Lieke, ook jij bedankt dat je mijn paranimf wilt zijn. Ik weet nog goed dat ik dacht, die Lieke kan ik toch niet helemaal alleen op een kamer laten zitten. Vanaf het moment dat wij gingen “samenwonen” was het erg gezellig (soms té) en ik wil je bedanken voor alle steun die je me hebt gegeven. Heel veel succes met het afronden van je Aio-schap en veel geluk, ook voor Bart, in de toekomst.

Ik wil ook alle mensen van onze groep (en oud-groepsgenoten), Mariëtte, Jet, Truus, Gemma, Martine, Gitte, Hugo en Ria, bedanken voor jullie hulp, interesse en gezelligheid. Krista, bedankt voor je inzet voor mijn project. Ook mijn studenten, Akiko, Martijn en Rob, bedankt voor jullie inzet en gezelligheid. Ik heb veel van jullie geleerd. Koen, mijn MEN-maatje, ik wil je bedanken voor je interesse in mijn project en vind het jammer dat we niet intensiever hebben kunnen samenwerken. De congresbezoeken in Washington en Marseille, maar vooral de etentjes na afloop waren erg gezellig. Veel succes en geluk met je carrière en je gezin.

Herma, Sabine en Helma en alle andere mensen van het gemeenschappelijk dierenlaboratorium wil ik bedanken voor de goede zorg voor mijn muisjes.

Verder wil ik iedereen van onze research afdeling bedanken. Mijn kamergenootjes, Willianne en Hannelie wil ik bedanken voor alle momenten dat jullie mijn geklaag hebben moeten aanhoren. Bedankt voor de gezelligheid de afgelopen jaren. Wij hadden toch echt de leukste kamer! Willianne, ik vind het echt heel leuk dat jij flink wat muizenwerk gaat doen. Dat moet toch een beetje in ere gehouden worden onder de Aio's. Heel veel succes en geluk in de toekomst, ook voor Martijn en Jan. Prim, Patricia en Olivier, we zijn (ongeveer) samen begonnen aan dit avontuur en vliegen nu (ongeveer) tegelijk weer uit. Bedankt voor de hulp, het was een leuke tijd. Heel veel succes met jullie postdoc projecten in binnen- en buitenland. All my other fellow-PhD-students, Peter, Janneke, Leyla, Ellen, Gemma, Sabine, Yuan and Raffaella, thanks for the great time we have had. Good luck finishing your PhDs and I wish you all the best for the future. Ook de postdocs, Bart, Saskia, Arjan en Stan, heel erg bedankt. Bart en Saskia, ontzettend bedankt voor al jullie adviezen. Ik vond onze gesprekken over carrière-ontwikkeling erg waardevol. Veel succes en geluk met het ontwikkelen en uitbreiden van jullie eigen groepen (op het werk en privé). Eric en Leo, ik heb jullie interesse in mijn onderzoek en in mijn ontwikkeling als onderzoeker altijd erg gewaardeerd. Bedankt dat ik altijd bij jullie terecht kon. Eric, ik vond het erg leerzaam om met je samen te werken aan het P18-mutatie onderzoek, bedankt. Alle andere collega's en oud-collega's, bedankt voor jullie hulp, interesse en de gezellige tijd.

Ook de mensen van de Speciële Endocrinologie en Metabole Diagnostiek heel erg bedankt. Eef, Inge en Hilda, nogmaals bedankt voor alle hulp bij de calcitonine bepalingen, het was echt een race tegen de klok! Monique, bedankt voor al je hand- en spandiensten. Maria, bedankt voor de ontspannende massages.

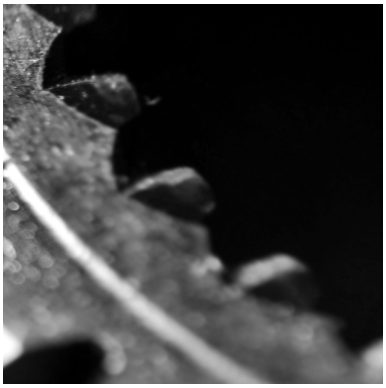
Laura, Anke, Josien en Janneke, ik wil jullie bedanken voor de zeer leerzame tijd bij KWF Kankerbestrijding, met veel frustaties (vooral voor de deadlines), maar uiteindelijk een ontzettend mooi resultaat. Laura, ik zal onze discussie over de definitie 'Biomarker' niet snel vergeten. Ik heb erg veel van je geleerd in de tijd dat ik bij je op je kamer mocht werken, bedankt.

Riccardo en Ron, al snel had ik de weg naar het Erasmus MC gevonden en mocht ik zelfs bij jullie wat proeven komen doen. Ik heb ons contact en jullie ondersteuning altijd heel erg gewaardeerd, bedankt. Ron, ik kijk erg uit naar onze samenwerking in de toekomst. Lois en Taranjit, I would like to thank you for the nice cooperation we have had resulting in a great paper. It was nice meeting you in Los Angeles. Lois, I will keep you to that drink in April! Unfortunately I did not have enough time to come and work with you in Kingston, but I really appreciate the offer. Alain en Marijke, jullie wil ik bedanken voor de hulp bij het selecteren van tumoren voor mijn onderzoek en de kleuringen die bij de Pathologie gedaan konden worden. René en Renske bedankt voor jullie hulp bij het P18-mutatie onderzoek. Paul Krimpenfort, helaas zijn de Calc-Cre muizen er uiteindelijk niet gekomen, toch bedankt voor de samenwerking.

Terry, Martijn, Diana, Eline en Klaas, ik heb de tijd bij GeNeYouS heel erg leerzaam, maar vooral erg leuk en gezellig gevonden. Veel succes met jullie Aio- en postdoc projecten (of met je

opleiding tot militair!) en ‘keep up the good work!’ Ellen, Arnoud, Nga-Chi, Tjakko, Maaike, Eline en Remko, het was een stressvolle klus, maar we hebben een mooi congres georganiseerd!
Renske, onze vriendschap bestaat al sinds de kleuterklas en ik vind het ongelooflijk fijn dat we nog steeds vriendinnen zijn. Ik vind het leuk dat jij en Mathijs altijd geïnteresseerd zijn in mijn werk. Mathijs, succes met jouw promotie-plannen. We zullen nog vaak met zijn vieren een glas (of fles) wijn drinken in onze mooie nieuwe huizen, en zeker volgend jaar op jullie bruiloft. Peter en Janny, familiebanden zijn bij ons aangevuld met een hechte vriendschap. Heel erg bedankt voor jullie interesse en steun en dat we nog maar vaak samen lekker zullen eten en Catannen.
Ook wil ik mijn en Eelco’s familie bedanken. Ook Piet, Ronnie, Judy en Arjan, heel erg bedankt voor jullie interesse. Juud, ik vind het heel jammer dat je er niet bij kunt zijn op mijn promotie, maar geniet van je reis in Zuid-Amerika. Pap, mam, Mark en Dion, heel erg bedankt voor al jullie steun en interesse de afgelopen jaren. Ik ben heel erg trots op jullie en op onze band. En als laatste, Eelco. Zonder jou had ik het echt niet gered. Je bent een enorme steun geweest, zeker in de laatste periode. Bedankt dat je er altijd voor me bent. Verhuizen en promoveren gaan niet echt samen, maar het is ons toch gelukt. We gaan nu (hopelijk) een wat rustiger jaar tegemoet. Ik hou van je.

CURRICULUM VITAE



Wendy van Veelen is geboren op 3 oktober 1980 te Capelle aan den IJssel. In 1998 behaalde zij het Gymnasium diploma aan het Emmaus College te Rotterdam. Aansluitend startte zij met de studie Medische Biologie aan de Universiteit Utrecht. In het kader van het doctoraal examen heeft zij twee onderzoeksstages en een extra-curriculaire onderzoeksstage doorlopen. Tijdens haar eerste stage, op het Hubrecht Laboratorium te Utrecht, heeft zij de rol van Snail en E-cadherine signalering bij epitheliale-mesenchymale transities onderzocht (o.l.v. Dr. L.H.K. Defize en Prof. Dr. A.J. Durston). Tijdens haar tweede stage, op de afdeling Klinische Endocrinologie van het Universitair Medisch Centrum Utrecht te Utrecht, heeft zij de rol van Wnt signalering bij de ontwikkeling van medullaire schildkliercarcinomen onderzocht en heeft zij gewerkt aan het opzetten van een mogelijke radiotherapie (o.l.v. Dr. D.S. Acton, Dr. J.W.M. Höppener en Prof. Dr. C.J.M. Lips). Tijdens haar derde stage, op de afdeling Morphogenèse Cellulaire et Progression Tumorale van het Institut Curie te Parijs in Frankrijk, heeft zij de rol van Wnt en Gli3 signalering bij de vroege ontwikkeling van borstklieren in muizen onderzocht (o.l.v. Dr. J.M. Veltmaat en Prof. Dr. J.P. Thiery). Op 28 april 2003 behaalde zij het doctoraal diploma.

Vanaf 5 mei 2003 was zij werkzaam als assistent in opleiding op de afdeling Metabole en Endocriene Ziekten van het Universitair Medisch Centrum Utrecht te Utrecht. Zij heeft de rol van p18 en β-Catenine bij de meerstapsontwikkeling van medullaire schildkliercarcinomen onderzocht (o.l.v. Dr. J.W.M. Höppener, Prof. Dr. C.J.M. Lips en Prof. Dr. R. Berger). De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

Tijdens haar promotieonderzoek is zij aangesteld als onderzoekster/schrijfster bij de werkgroep Biomarkers en Kanker (o.l.v. Dr. L. van ’t Veer) van de Signaleringscommissie Kanker van KWF Kankerbestrijding. Tijdens deze aanstelling heeft zij gewerkt aan het schrijven van het signaleringsrapport ‘Biomarkers en Kankerbestrijding: gebruik van biomarkers bij erfelijkheidsonderzoek, diagnostiek en behandeling’ en de literatuurstudie ‘Biomarkers geïdentificeerd bij veelvoorkomende soorten kanker’ die gepubliceerd zijn in maart 2007.

Vanaf 14 januari 2008 is zij begonnen als postdoc op de afdeling Gastroenterologie en Hepatologie van het Erasmus Medisch Centrum te Rotterdam en verricht zij onderzoek op het gebied van moleculaire biologie bij darmkanker (o.l.v. Dr. M.J.M. Smits en Prof. Dr. E.J. Kuipers).

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