Molecular Diagnostics in Treatment of Medullary Thyroid Carcinoma

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1. Introduction

Medullary thyroid carcinoma (MTC) is accounting for 5 - 8% of all thyroid cancers and arises from calcitonin producing parafollicular C cells of the thyroid gland. Mainly MTC is sporadic in nature, but in 20 - 30% of cases it is present in an autosomal dominant inherited pattern with defined phenotype referred as multiple endocrine neoplasia type 2 (MEN 2) and familial medullary thyroid carcinoma (FMTC). The identification of missense germline mutations in the \(RET\) proto-oncogene between 1993 and 1998 as the cause of MEN 2 and FMTC ushered in the molecular age. Specific mutations in the \(RET\) gene encoding a transmembrane tyrosine kinase result in "gain-of-function" of the receptor with definite changes in downstream signal transduction pathways. Intriguingly, examination of the mutated codons led to the growing recognition of a striking genotype-phenotype correlation between the transforming activity inherent in these mutations and disease onset and aggressiveness, implicating that manifestation and clinical progression is conditioned by the type of mutation. Detection of the mutant alleles in kindred members predicts disease inheritance and provides the basis for prophylactic thyroidectomy in children. This seminal discovery, enabling predictive testing, paved the way for an evidence-based practice of clinical cancer genetics. In case of novel \(RET\) mutations it is exceedingly important to clarify whether it represents a harmless polymorphism or a causative pathogenic mutation. For this purpose, we established a molecular diagnosis program that, in conjunction with clinical data, allows individualized risk stratification for patients.

2. RET proto-oncogene and MTC

2.1 RET proto-oncogene – Genotype to phenotype

Transfection studies using DNA from human T cell lymphoma led to the isolation of a transforming gene called \(RET\) (REarranged during Transfection) that consists of two linked sequences caused by cointegration during transfection (Takahashi et al., 1985). The resulting chimeric gene encodes a fusion protein comprising an N-terminal region with a dimerization motif fused to a tyrosine kinase (TK) domain. Subsequently, the name RET has been retained to designate the gene coding for the tyrosine kinase domain of this fused oncogene. The human \(RET\) gene is localized on chromosome 10q11.2 (Donghi et al., 1989) and spans 21 exons. Homologues of \(RET\) have been identified in higher and lower vertebrates as well as in \textit{Drosophila melanogaster} (Hahn and Bishop, 2001). The \(RET\) proto-
oncogene encodes a transmembrane receptor of the tyrosine kinase family with three major isoforms that arise through alternative splicing of the 3’-terminus, leading to expression of proteins that differ by their last 51 (RET51), 43 (RET43) or nine (RET9) amino acids, respectively (Manie et al., 2001). It is expressed primarily in neural crest and urogenital precursor cells, and is implicated in developmental processes, such as maturation of peripheral nervous system lineages, kidney morphogenesis or spermatogonia differentiation (Durbec et al., 1996; Meng et al., 2000; Schuchardt et al., 1994). Among them, RET9 and RET51 are the major isoforms consisting of 1072 and 1114 amino acids, respectively. The signaling complex associated with RET9 markedly differs from RET51-associated factors, which might have an influence on the higher transforming potential of the RET51 isoform (Le Hir et al., 2000; Tsui-Pierchala et al., 2002). The RET protein is composed of three functional domains: The intracellular tyrosine kinase domain, a transmembrane region and a stretch of four extracellular cadherin-like domains that are implicated in ligand binding. The extracellular domain consists of four cadherin-like regions and a cysteine-rich tract, which facilitates receptor dimerization upon ligand stimulation (Iwamoto et al., 1993; Takahashi et al., 1998). This region also contains several glycosylation sites (Takahashi et al., 1991). The fully glycosylated form (170-175 kDa) of RET is found in the plasma membrane, while the 150-155 kDa species is believed to be an immature, partially processed form found only in the endoplasmic reticulum (ER). RET serves as a functional receptor for neurotrophic factors of the glial cell-line derived neurotrophic factor (GDNF) family: GDNF, neurturin, artemin and persephin (Ichihara et al., 2004; Takahashi, 2001). Binding to and activation of RET occurs via glycosylphosphatidylinositol-anchored as well as soluble co-receptors that are designated as GDNF-family receptors (GFRs) α1-4 (Airaksinen and Saarma, 2002; Manie et al., 2001) (Fig. 1 A). Ligand stimulation leads to activation of the RET receptor by dimerization and subsequent autophosphorylation of intracellular tyrosine residues. These, in turn, serve as docking sites for a number of interacting molecules activating downstream signal transduction pathways (Chiariello et al., 1998; Hayashi et al., 2001; Murakami et al., 1999). Although tyrosine residues 905, 981, 1015, 1062 and 1096 are all phosphorylated upon ligand binding, it is the phosphorylation of tyr1062 that plays a crucial role in RET signaling as it acts as a multifunctional docking site for many adaptor or effector proteins (Jijiwa et al., 2004).

Autosomal dominant gain of function mutations in the RET proto-oncogene have been identified as the key cause for the development of the multiple endocrine neoplasia type 2 (MEN 2) syndrome, which can be further divided into three distinct clinical manifestations MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC) (Bocciardi et al., 1997; Carломagno et al., 1997; Hofstra et al., 1994; Mulligan et al., 1993; Santoro et al., 1995). In addition, 30-70% of sporadic medullary thyroid carcinomas harbor a mutation in the RET gene. Mutations render the RET receptor constitutively active and display striking genotype-phenotype correlations. Patients with MEN 2A always develop medullary thyroid carcinoma (MTC), but also pheochromocytoma (50%) and parathyroid hyperplasia or adenoma (20-30%). MEN 2B, in contrast, is the most aggressive subtype and is characterized by the same features as MEN 2A, but with earlier onset and developmental abnormalities such as mucosal neuromas, intestinal ganglioneuromas, ocular and skeletal abnormalities (marfanoid habitus). The most indolent subtype FMTC is characterized by the incidence of MTC-only (Brandi et al., 2001). Mutations identified in more than 98% of MEN 2A patients affect one of six cysteine residues in the cysteine-rich
region at codons 609, 611, 618, 620 (exon 10), 630 or 634 (exon 11) and cause ligand-independent homodimerization through covalent intermolecular disulphide bonds, resulting in subsequent constitutive activation of the RET kinase which, in turn, leads to permanent downstream signaling (Santoro et al., 1995) (Fig. 1 C). Approximately 87% of MEN 2A mutations affect codon 634 (Eng et al., 1996; Hansford and Mulligan, 2000). In contrast, mutations found in MEN 2B patients affect residues in the tyrosine kinase domain and activate the RET receptor in its monomeric state, thereby changing the substrate specificity towards other cellular substrates and downstream signaling pathways (Borrello et al., 1995; Murakami et al., 1999; Santoro et al., 1999) (Fig. 1 B).

Fig. 1. Schematic mechanisms of RET tyrosine kinase activation in healthy status versus MEN 2B and MEN 2A. A) Normal RET activation by glial cell line-derived neurotrophic factor (GDNF). GDNF binds to GFRα and leads to RET dimerization and autophosphorylation. B) Constitutive RET activation by MEN 2B mutations. C) Ligand independent RET activation by MEN 2A mutations. CAD Cadherin like domain; Cys Cysteine-rich domain; TMD Transmembrane domain; TK Tyrosine kinase domain

Moreover, increased autophosphorylation of tyrosine 1062 has been described (Bocciardi et al., 1997; Salvatore et al., 2001; Santoro et al., 1995). MEN 2B is primarily associated with a single missense mutation of codon 918 (M918T), which is detectable in more than 90% of MEN 2B patients (Carlson et al., 1994; Eng et al., 1996; Hofstra et al., 1994; Mulligan et al., 1993). A smaller number of MEN 2B cases contain mutations at codon 883 (A883F) (Gimm et
al., 1997; Smith et al., 1997). Mutations identified in FMTC patients (for example at codons 790, 791 or 844) are found in the cysteine-rich region as well as in the tyrosine kinase domain, and lead to low level activation of the RET kinase corresponding to the indolent penetrance phenotype of FMTC (Arighi et al., 2005; Manie et al., 2001). An overview about mutated codons at specific sites of the RET oncogene and the correlating clinical phenotype is mapped in Fig. 2.

**Fig. 2.** Domain structure of the RET proto-oncogene: CAD Cadherin like domain; Cys Cysteine-rich domain; TMD Transmembrane domain; TK Tyrosine kinase domain. Arrows point to the affected protein domains and specify the mutated codons and the correlating clinical phenotype.

### 2.2 Medullary thyroid carcinoma – Standard diagnosis and treatment
In most cases, the prognosis for MTC patients is good after early diagnosis and intervention. Since 1994, genetic screening using DNA from peripheral blood has been available for MEN 2. This method allows diagnosis prior to the onset of symptoms. Moreover, the MEN 2-associated mutations, involving RET exons 8, 10, 11, 13, 14, 15 and 16 are tested routinely. Currently, early genetic screening for RET mutations is considered the standard of care for
MEN 2 (Eng, 1999). Preoperative measurement of serum calcitonin is another highly sensitive method to establish the diagnosis of MTC. However, since germline \textit{RET} mutations have been identified as a cause of MEN 2, the use of additional measurement of calcitonin is questionable. Though calcitonin levels can be helpful in determining the extent of disease and the extent of surgery required (Gimm, 2001).

At present treatment of MTC is restricted to surgical removal of neoplastic tissue, and cure is only achieved when the disease is restricted to the thyroid gland. The general recommendation to perform total thyroidectomy seems to be justified since MTC is often multifocal and not susceptible to radiiodine ablation. Overall, \textit{RET} mutations are classified into three groups based on the level of risk (or aggressiveness) for MTC (Sippel et al., 2008). Level 3 mutations (codon 883, 918, and 922) have the most aggressive course, with metastatic disease presenting in the first years of life. Because of the high risk for malignancy at an early age, thyroidectomy is recommended within the first 6 months of life and preferably within the first month of life (Brandi et al., 2001). Level 2 \textit{RET} mutations (codon 611, 618, 620, and 634 mutations) are considered high risk for MTC and the current recommendation is that these patients undergo thyroidectomy before the age of 5 years. Mutations at codon 609, 768, 790, 791, 804 and 891 are classified as level 1. Patients carrying these mutations are still considered high risk for MTC but with the lowest risk of the \textit{RET} mutations. MTC in these patients tends to develop later in life and takes on a more indolent course. Because clinically apparent disease is rarely reported prior to 10 years of age, many recommend waiting until then to perform a thyroidectomy. Based on more detailed research and knowledge the stratification system for \textit{RET} germline mutations was recently reclassified by the American Thyroid Association (ATA) into categories of increasing risk: class A (codons 768, 790, 791, 804, 891), class B (codons 609, 611, 618, 620, 630), C (codon 634), and D (codon 883, 918), with level A representing lowest risk (FMTC) and level D representing the highest risk (MEN 2B) class (Kloos et al., 2009).

Patients with unresectable or metastatic disease display a poor prognosis because radiation and chemotherapy have only a limited role (Cohen and Moley, 2003). Although MTC is less radiosensitive, radiotherapy is used as a palliative for symptomatic bone, central nervous system and mediastinal metastases. Some studies suggested a specific radiotherapy for MTC based on the selective uptake of $\text{[\text{I}^3\text{H}] MIBG}$ and $\text{[\text{I}^{111}\text{In}] pentetreotide}$ (Forssell-Aronsson et al., 1995; Troncone et al., 1991) also concerning their role in diagnosis of neuroendocrine tumors and to some degree localisation of metastases (Kaltsas et al., 2001). Other nuclear approaches using radionuclide-labeled antibodies combined with pretargeting strategies to improve uptake of these antibodies have raised interest in targeted radiotherapy for MTC (Juweid et al., 1996; Juweid et al., 2000; Kraeber-Bodere et al., 1999; Mirallie et al., 2005). Since activated \textit{RET} is proven to be causative for the development of MTC, molecular strategies to inhibit its activity or expression in cancer cells are highly promising. Targeting the enzymatic activity of tyrosine kinases by small molecule inhibitors like STI571 (Gleevec® or Imatinib), BAY 43-9006 (Sorafenib), allyl-geldanamycin, or arylidine 2-indolinone (RPI-1) selectively inhibit \textit{RET} kinase activity and cell growth (Cohen et al., 2002; Lanzi et al., 2000). For example, oral daily RPI-1 treatment reduces the growth of human medullary thyroid carcinoma xenografts in mice by 81\% (Cuccuru et al., 2004). Also, two indolocarbazole derivatives, CEP-701 and CEP-751, have been shown to effectively block \textit{RET} phosphorylation at nanomolar levels and MTC cell growth (Strock et al., 2003), whereas the pyrazolo-pyrimidine PP1 inhibits tumorigenesis induced by \textit{RET}/\textit{PTC} oncogenes and...
causes degradation of activated membrane-bound RET receptors through proteosomal targeting (Carlomagno et al., 2002b; Carniti et al., 2003; Strock et al., 2003). In addition, the pyrazolo-pyrimidine PP2, and the 4-anilinoquinazoline ZD6474 (Vandetanib) displayed a strong inhibitory activity towards constitutively active oncogenic RET kinases (Carlomagno et al., 2003; Carlomagno et al., 2002a). However most of the tyrosine kinase inhibitors are multikinase inhibitors and are also active against multiple signaling molecules (Santarpia et al., 2009). Moreover, some RET mutations (e.g. valine 804) cause resistance to these drugs (Carlomagno et al., 2004). Finally the efficacy in human patients has to be proven by treatment. Until now first clinical trials with several kinase inhibitors showed no beneficial or only moderate effects of the drugs (Table 1). Thus, development and evaluation of novel treatment strategies, including gene therapeutic approaches are further needed.

<table>
<thead>
<tr>
<th>Kinase inhibitor</th>
<th>Clinical trial</th>
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<tr>
<td>Imatinib</td>
<td>Phase II</td>
<td>de Groot et al., 2007</td>
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<td>Sorafenib</td>
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<td>Vandetanib</td>
<td>Phase III</td>
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<td>Sunitinib</td>
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<td>De Souza et al., 2010</td>
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<td>Motesanib</td>
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Table 1. Summary of some tyrosine kinase inhibitors used in clinical trials

Gene therapy is attractive for thyroid cancer treatment because of the possibility to selectively target therapeutic genes by application of tissue-specific promoters, such as the thyroglobulin or the calcitonin promoter. A range of therapeutic strategies were under investigation utilizing various genes: pro-drug activating genes (herpes simplex virus thymidine kinase / ganciclovir and purine nucleoside phosphorylase/ fludarabine), the nitric oxide synthase II gene in a direct toxin therapy, gene for IL-12 in an immune stimulation strategy, and the expression of the sodium iodide symporter gene in radiotherapy application (Spitzweg and Morris, 2004). Moreover, molecular mimics (competition) directed toward specific mutations of RET by using the mutant EC-RET C634Y that is able to inhibit the membrane bound receptor RETC634Y through interfering with its dimerization (Cerchia et al., 2003).

Another attractive approach is to block oncogenic signal transduction either by reducing RET expression or by interfering with receptor autophosphorylation using dominant-negative RET protein. In this regard, adenovirus (Ad)-mediated expression of RET containing mutations in the N-terminal region of the extracellular domain such as HSCR32 associated with Hirschsprung’s disease or FLAG has been shown to substantially inhibit receptor maturation, thereby preventing its transport to the membrane (Drosten et al., 2002; Drosten et al., 2003). These molecules proved to be highly active against MTC in cultures of human TT cells, which harbour the RETC634W mutation, and after inoculation of ex vivo infected tumor cells into nude mice. A second dominant-negative RET protein (RETATK), lacking the intracellular tyrosine kinase domain, showed the ability to block oncogenic activated RET autophosphorylation by forming an inactive dimer with the mutated RET receptor. Transduction of TT cells with RETATK resulted in decreased cell-cycle progression, but also, more importantly induction of cell death by apoptosis. Activity of RETATK against MTC was also demonstrated in RET transgenic mice, which develop orthotopic tumors in the thyroid. Injection of an Ad vector expressing dominant-negative RET protein into MTC of the thyroid glands significantly decreased the tumor size after two weeks (Drosten et al.,
2004). To enhance tumor specificity of anti-RET Ad vectors in order to target systemically spreaded medullary thyroid carcinoma cells, we have recently identified a number of MTC-specific peptides that can be used to efficiently redirect the therapeutic gene to primary MTC, their migrating populations, and potentially tumor metastases under \textit{in vivo} conditions (Böckmann et al., 2005a; Böckmann et al., 2005b; Schmidt et al., 2011).

3. Combining tools – RET molecular diagnostics

\textit{RET} gene analysis widely used to identify carriers at risk of developing medullary thyroid cancer, occasionally uncovers novel sequence variants of unknown clinical significance. For these newly identified or rare mutations in the \textit{RET} gene, the causative role of the mutation and the genotype-phenotype relationship must be evaluated to define the mutation’s codon-specific risk level. For this purpose, we have implemented a molecular diagnostic approach that allows us to classify \textit{RET} mutations into one of the four clinical risk groups. Based on such molecular diagnosis recommendations for treatments of patients with hitherto undefined \textit{RET} mutations are made. The program established allows direct translation of a genetic event into individualized clinical settings and is critical for treating physicians to decide whether a prophylactic thyroidectomy is necessary or not. Considering the potential risks after thyroidectomy e.g. reduced flexibility of the vocal cord and subsequent restrictions for the patient like hormone replacement therapy, molecular diagnostics in MTC-treatment have a direct impact on affected patients.

3.1 From clinical RET mutation analysis to functional in vitro and in vivo characterization

\textit{Clinical classification and DNA based screening}

Diagnosis of putative carriers of \textit{RET} genes with novel mutations starts with clinical work up and subsequent \textit{RET} mutation analysis. Therefore preoperatively basal and pentagastrin-stimulated calcitonin levels, as a marker for MTC tumors, are determined by appropriate chemiluminescence assay. By utilizing histology analysis after total thyroidectomy tumor staging is performed according to the International Union Against Cancer tumor-node-metastasis (TNM) classification from 1997. To detect mutations genomic DNA is isolated and five fragments covering the exons 8, 10, 11 and 13 to 16 of the \textit{RET} proto-oncogene are amplified with exon specific PCR primers using high-fidelity PCR systems. The resulting fragments are sequenced to specify the point mutation.

\textit{Construction and characterization of mutant RET expressing cell lines in vitro}

On the basis of the clinical findings the first goal is to determine whether a particular mutation is capable of converting RET into a dominantly transforming oncogene. At this point wet lab work starts to generate a RET51 mutant with defined specific mutation. pLPCX vectors are used to express the mutated \textit{RET} gene. The pLPCX vector contains elements derived from Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV), and is mostly designed for retroviral gene delivery and expression. The cDNA fragment encoding the human tyrosine kinase RET51wt is ligated downstream from the CMV promoter of pLPCX retroviral vector (\textbf{Fig. 3 A}). pLPCX expression vectors containing the selected \textit{RET} point mutation are generated by site directed mutagenesis using primers harboring the desired codon and pLPCX RET51wt plasmid as a template. All constructed plasmids are
routinely sequenced to confirm the presence of the desired mutations. NIH3T3 fibroblasts were chosen as in vitro model because they do not express endogenous RET protein and are generally accepted as a reliable transformation system to study oncogene function. In order to investigate the effects of mutated RET proteins on cellular transformation, NIH3T3 mouse fibroblasts are stably transfected with the pLPCX-RET expression vector. Single puromycine-resistant clones are separated by limited dilution, cultivated and finally checked for RET protein expression (Fig. 3 B). After construction of the desired cell line a plethora of assays is applied to determine specific cellular parameters that help to evaluate aggressiveness of the investigated receptor mutant (Fig. 3 C).

Fig. 3. Schematic workflow for generation and characterization of mutant RET-expressing NIH3T3 stable cell lines and in vitro assays. A) Site directed mutagenesis of RET51 wildtype containing retroviral plasmid to gain oncogenic RET point mutations. B) Transfection of NIH3T3 with mutant RET gene followed by clonal selection and subsequent control of RET protein levels of stably expressing NIH3T3-RET cell lines. C) Established in vitro assays from selected RET clones for evaluation of the transforming potential of single RET mutants.
1. **Tyrosine kinase activity** induced by mutated RET proteins: Protein extracts from NIH3T3 transfectants stably expressing mutated forms of RET are immunoprecipitated with an anti-RET antibody and subjected to a kinase assay.

2. Analysis of growth properties by determining the **proliferation rate** through cell counting or cell viability assays.

3. **Apoptosis resistance**: DNA-Damage induced cell death is activated by chemotherapeutics like doxorubicine or cisplatin. Subsequent killing of cells expressing oncogenic RET mutants is measured by flow cytometry. Here, propidium iodide staining is used to analyze cellular DNA content and look for sub-diploid or apoptotic cell population, respectively.

4. **Anchorage-independent growth** as one of the most important oncogenic properties of cancer cells is determined by soft agar assays. Therefore, stable NIH3T3 transfectants are seeded in semisolid medium and cultured over a period of 30 days. Formed cell colonies are stained and counted under the microscope.

5. **Migration and invasiveness** are major characteristics describing the aggressiveness and metastatic behavior of cancer cells. NIH3T3 fibroblasts expressing distinct mutant RET proteins are tested for their motility by matrigel invasion assays in Boyden chambers. Boyden chamber inserts contain membranes that are permeabilized by small pores coated with Matrigel™-Basement-Matrix. Hereon cells are seeded in serum-free medium. Medium containing a high concentration of fetal calf serum is added as chemoattractant in the lower chamber. Staining of cells enables examination of migrated cells under a fluorescence microscope.

In all experiments established, already characterized NIH3T3 transfectants harboring well known point mutations in the RET gene like Y791F (FMTC), C634R (MEN 2A) and M918T (MEN 2B) with a defined genotype to phenotype correlation (Mise et al., 2006) are carried along as standard to classify new point mutations of as yet unknown oncogenic potential. In addition, short-term cultured parental NIH3T3 cells are employed as untransformed negative control.

**Establishing tumor allografts in nude mice through injection of NIH3T3-RET cell lines**

To investigate the transforming potential and aggressiveness of a particular RET mutant in vivo, experimental research in animal models is needed (Fig. 4). As in vivo transformation model, athymic nude mice are used. NIH3T3 stable transfectants are subcutaneously injected into the hind flank of 6 to 8 weeks old mice. Parental NIH3T3 cells are used as negative control. After injection tumor formation is monitored over time. To estimate the growth rate, tumor dimensions are measured with calipers every 2 days. Tumor volumes are calculated by the rotational ellipsoid formula: $V = \frac{A \times B^2}{2}$ (A-axial diameter; B-rotational diameter). Finally, after sacrificing the animals, tumors are removed, weighted, embedded in paraffin and submitted to immunohistochemistry. In detail, tumor sections carrying RET mutations are cut, dewaxed, rehydrated and probed with antibody against Ki-67 nuclear antigen, which recognizes actively proliferating cells. The percentage of proliferating cells can be determined by counting Ki-67 positive cells under a bright-field microscope. At the same time tumors can also be subjected to gene expression analysis. Therefore, tumor tissues are snap frozen in liquid nitrogen and stored at -80°C. RNA and protein extracts from samples are isolated by grinding 1 g of frozen tissue into a fine powder using a cold mortar and pestle by standard procedures for subsequent western blot and/or qPCR analysis.
Fig. 4. Induction of RET tumor formation by subcutaneous injection of stable NIH3T3 transfectants. Measurement of tumor volume and immunohistochemical examination of cancer cell proliferation allows estimation of tumor aggressiveness.
3.2 Fingerprinting of RET-derived tumors by microarray analysis

Our main concern is to find genotype-associated molecular signatures that could predict the onset and aggressiveness of MEN 2-RET-related MTCs with a defined point mutation. Therefore, we generated transcriptomic profiles of RET-derived tumors differing in their clinical appearance (FMTC, level 1; MEN 2A, level 2; MEN 2B, level 3). This genetic fingerprint library is compared with the expression profile of a tumor with an unclassified RET-mutation. Consequently a hitherto clinically undefined mutation is classified into the three risk levels.

**Identification of the differential gene expression pattern for a specific RET point mutation**

First, a GeneChip expression analysis is performed for the investigated RET-derived tumor (Fig. 5). Total RNA from frozen tumor tissue is used to prepare biotinylated cRNA targets, which are hybridized to Affymetrix Mouse 430 2.0 GeneChips. Hybridization and washing of gene chips is routinely performed on an Affymetrix GeneChip Hybridization Oven and Fluidics Station. Afterwards microarrays are analyzed by laser scanning (Affymetrix GeneChip Scanner). Background-corrected signal intensities are determined and processed using MAS5 function of the R/Bioconductor affy package (www.r-project.org/www.bioconductor.org). All calculations including normalization of microarray data, statistical tests, clustering, and further filtering methods are accomplished by up to date gene expression analysis software (e.g. GeneSpring GX 9.0 Agilent Technologies). Genes whose transcripts are not detected in any of the investigated mutations are excluded from statistical analysis to reduce the number of false positive genes. To determine differentially expressed genes, expression data are statistically analyzed using t-test and multiple testing correction (Benjamini and Hochberg False Discovery Rate). Cut-offs are set empirically to three fold and \( P \leq 0.01 \).

**Analyzing and classifying the array data**

The methods described above generate a gene list and expression profile that is unique for a certain point mutation. The obtained expression profile is normalized and clustered together with our pre-existing RET tumor transcriptomic database. This allows us to estimate the potential outcome of a RET point mutation on the genetic level. Information about biological processes and signaling pathways that participate in RET-induced cellular transformation are from outstanding importance, because this could reveal attractive therapeutic targets such as small molecules inhibitors. To extract therapy relevant information, functional annotation clustering is applied by using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/). This online database provides a comprehensive set of functional annotation tools to understand biological meaning behind large list of genes. In detail, functional annotation clustering condenses an input gene list into smaller, much more organized biological annotation modules in a term-centric manner. It allows investigators to focus on the annotation group level by quickly organizing many redundant/similar/hierarchical terms within the group. Annotation clusters, such as immune response, transcriptional regulation, chemokine activity, cytokine activity, kinase activity, signaling transduction, cell death and so on, could be found on the top of the output as expected for this study. With these results one can quickly focus on the major biology at an annotation cluster level. The enrichment score is to rank the overall importance (enrichment) of annotation term groups. It is the geometric mean of all the enrichment P-values of each annotation term in the group.
Fig. 5. Microarray data mining and expression analysis strategies to classify RET tumors with uncharacterized point mutations. Functional annotation clustering can be used to identify targets with therapeutic potential.

Expression profiling identified a statistically significant modification of 1494 genes, 628 down- and 866 upregulated in MEN 2B compared with MEN 2A/FMTC tumors. By contrast, no obvious alterations were observed among individual MEN 2B and MEN 2A type mutations, or between MEN 2A and FMTC. Functional clustering of differential genes revealed RET-MEN 2B specific upregulation of genes associated with novel growth and survival pathways. A central finding of this study was the extent of changes in genes whose products affect the immune response. In particular, we observed a remarkable accumulation of genes encoding NK cell receptors, T-lymphocyte antigens, regulators of NK- and T-cell proliferation/attraction, and apoptosis molecules important for the ability of NK cells and cytotoxic T cells to kill their targets in the tumors initiated by RET-MEN 2A/FMTC mutations,
while expression of these genes was nearly completely suppressed in RET-MEN 2B related cancers. Quantitative real-time PCR on tumors versus cultured NIH-RET cell lines demonstrated that they are largely attributed to the host innate immune system, whereas expression of CX3CL1 involved in leukocyte recruitment is exclusively RET-MEN 2A/FMTC tumor cell dependent. In correlation, massive inflammatory infiltrates were apparent only in tumors carrying MEN 2A/FMTC mutations, suggesting that RET-MEN 2B receptors specifically counteract immune infiltration by preventing chemokine expression, which may contribute to the different clinical outcome of both subtypes (Engelmann et al., 2009). In summary, our data support a model of RET oncogene-specific interference with the host immune system, in which chemokine production by RET-MEN 2A/FMTC cancer cells initiates an antitumor immune attack, while RET-MEN 2B receptors avoid tumor infiltration as a mechanism of evasion that may be critical for the different clinical outcome of both subtypes.

4. Conclusion and future perspectives

In 1993, activating mutations in RET were identified as a cause for the development of MTC. In the following years, extensive research has been dedicated to exploring the mechanisms involved in RET-mediated tumorigenesis. All acquired data emphasize the essential role of mutated RET in the process of MTC development and already indicate a role for RET as an anticancer target. In recent years, many different studies have experimentally verified that RET inhibition might have an adverse effect on MTC progression, and that oncogenic activated RET is indeed a highly promising target for the development of a targeted strategy. Our results obtained from functional investigations of RET oncogene mutations impressingly demonstrate how clinical practice is empowered by molecular information that dictates medical management, lending future credence to the concept of gene-informed personalized healthcare. In the molecular age, however, it would be farfetched to believe that focussing on the DNA levels is the entire truth to solve an individual’s prognosis. Downstream of the transcriptional level regulation of mRNA awaits to be the next step in oncogenesis research. At this point small non-coding microRNAs (miRNAs) appear on stage. The discovery of miRNAs and their impact on functions in many biological and physiological processes has opened a new broad area of possible interactions in the regulatory network of cells. Furthermore, in the past few years it became evident that these regulatory RNAs also have an emerging role in development and progression of tumors. Extensive profiling of miRNAs in many cancer types revealed significant differences in their expression patterns, making them an interesting tool for cancer treatment. Until now the expression and functions of miRNAs in thyroid cancers has been described for follicular, anaplastic and papillary thyroid carcinomas. The studies demonstrated that in these cancer types distinct miRNAs are up and down regulated. In turn, these miRNAs regulate several transcription factors and effector molecules that are implicated in proliferation, cell adhesion, apoptosis and finally lead to oncogenesis and de-differentiation. Thus, miRNAs can act as oncogenes or tumor suppressors in thyroid cancers. To date complete miRNA profiles of MTCs harboring distinct RET mutations are missing. An opportunity that must be exploited for moving towards individualized medicine in cancer treatment beyond ATA risk stratification or, even more important, for prevention of metastasis. The technical procedure developed in our laboratory to identify MTC-associated miRNAs contributing to the oncogenic potential of the mutated RET receptor are illustrated in Fig. 6.
Fig. 6. Real-time PCR based screening for microRNA profiles in various human MTCs harboring RET point mutations. Clustering is used to reveal differential expression patterns of distinct microRNAs in tumors compared to healthy donor tissue.

5. Acknowledgment

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Molecular Diagnostics in Treatment of Medullary Thyroid Carcinoma


This book aims to provide readers with a general as well as an advanced overview of the key trends in endocrine disorders. While covering a variety of topics ranging from thyroid carcinogenesis and pituitary adenomas to adrenal tumors and metabolic bone disease, this book also focuses on more specific issues not yet fully elucidated (e.g. the molecular pathways involved in thyrotropin beta gene regulation or monogenic phosphate balance disorders). Readers of different fields and background will have the opportunity to update their knowledge and more importantly to clarify areas of uncertainty and controversies in several topics of endocrine disorders.

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