Transcriptome analysis in mouse tumors induced by *Ret*-MEN2/FMTC mutations reveals subtype-specific role in survival and interference with immune surveillance

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Abstract

Activating mutations in the *Ret* proto-oncogene are responsible for occurrence of multiple endocrine neoplasia (MEN) type 2A and 2B, and familial medullary thyroid carcinoma (FMTC). A striking genotype–phenotype correlation between the mutated RET codon and clinical manifestation implies that tumorigenesis is conditioned by the type of mutation. We investigated gene expression profiles between and within distinct MEN2 subtypes through whole-genome microarray analysis in tumors induced by NIH-3T3 cells transformed with defined RET-MEN2A (C609Y, C634R), MEN2B, (A883F, M918T), and FMTC (Y791F) mutations. Expression profiling identified a statistically significant modification of 1494 genes, 628 down- and 866 upregulated in MEN2B compared with MEN2A/FMTC tumors. By contrast, no obvious alterations were observed among individual MEN2B and MEN2A type mutations, or between MEN2A and FMTC. Functional clustering of differential genes revealed RET-MEN2B specific upregulation of genes associated with novel growth and survival pathways. Intriguingly, RET-MEN2A/FMTC-specific tumors were characterized by a considerable number of genes involved in the host antitumor immune response via stimulation of natural killer/T-cell proliferation, migration, and cytotoxicity, which were completely absent in RET-MEN2B related cancers. QPCR 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-MEN2A/FMTC tumor cell dependent. In correlation, massive inflammatory infiltrates were apparent only in tumors carrying MEN type 2A/FMTC mutations, suggesting that RET-MEN2B receptors specifically counteract immune infiltration by preventing chemokine expression, which may contribute to the different clinical outcome of both subtypes.

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Introduction

The *Ret* gene on chromosome 10q11.2 was originally identified as an oncogene activated by DNA rearrangement that encodes a transmembrane receptor of the tyrosine kinase family (Takahashi *et al.* 1985). RET serves as a functional receptor for neurotrophic factors of the glial cell-line-derived neurotrophic factor (GDNF) family: GDNF, neurturin, artemin, and persephin. Binding to and activation of RET occurs via co-receptors that are designated as GDNF-family

receptors $\alpha 1$ –4. Ligand stimulation leads to the 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. Gene knockout studies revealed that GDNF/RET signaling plays a crucial role in the development of the enteric nervous system and the kidney (Drosten & Pützer 2006, de Groot *et al.* 2006).

Autosomal dominant gain of function mutations in the *Ret* proto-oncogene have been identified as the key cause for the development of multiple endocrine neoplasia type 2 (MEN2), which can be further divided into two distinct clinical manifestations MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC; Lakhani *et al.* 2007). They render the RET receptor constitutively active and considerably influence the disease phenotype, severity, and the age of onset. Patients with MEN2A not only develop medullary thyroid carcinoma (MTC), but also pheochromocytoma (50%) and parathyroid hyperplasia or adenoma (20–30%). MEN2B has the same features as MEN2A, but with earlier onset and developmental abnormalities such as mucosal neuromas, intestinal ganglioneuromas, ocular and skeletal abnormalities (marfanoid habitus). FMTC is characterized by the incidence of MTC only. From all three syndromes, MEN2B cancer tends to be more aggressive and leads to death from metastatic disease in young children. By contrast, familial MTC behaves in the least aggressive fashion, and, although lymph node metastases are frequent, the disease usually follows an indolent course, and virtually never results in death. Mutations identified in >98% of MEN2A 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 disulfide bonds, resulting in subsequent constitutive activation of the RET kinase, which, in turn, leads to permanent downstream signaling. Approximately 87% of MEN2A mutations affect codon 634. By contrast, mutations found in MEN2B patients affect residues in the tyrosine kinase domain and activate the RET receptor in its monomeric state, thereby changing the substrate specificity toward other cellular substrates and downstream signaling pathways. In addition, increased auto-phosphorylation of tyrosine 1062 has been described. MEN2B is primarily associated with a single missense mutation of codon 918 (M918T), which is detectable in more than 90% of MEN2B patients. A smaller number of MEN2B cases contains mutations at codon 883 (A883F). 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; reviewed in de Groot *et al.* (2006), Plaza-Menacho *et al.* (2006).

The molecular link of different *Ret* mutations to distinct clinical MEN2 syndromes is still largely unknown. To further elucidate the mechanism by

which alterations in a major oncogene result in a disparate biology of closely related cancer syndromes, in particular a higher aggressiveness of MEN type-2B mutations, we performed a whole-genome microarray expression analysis using a defined *in vivo* model of NIH-3T3 mouse fibroblasts expressing various RET-MEN2A (C609Y and C634R), RET-MEN2B (A883F and M918T) and FMTC (Y791F) mutations.

Materials and methods

Cell lines and tumor samples

NIH-3T3 fibroblast cell lines stably expressing the long RET⁵¹ isoform carrying the MEN2A mutations C609Y and C634R, Y791F (FMTC), and the MEN2B-derived mutations A883F and M918T have been described previously (Miše *et al.* 2006). Cultures were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (PAA Laboratories, Coelbe, Germany) and 1% penicillin G/streptomycin sulfate (Life Technologies). Tumors were established by s.c. injection of 10⁶ NIH-RET(MEN2A), and NIH-RET(MEN2B) cells into the hind flank of 6- to 8-week-old NMRI/nude mice. Tissue samples were collected from all animals 3 weeks after injection, weighed, snap-frozen in liquid nitrogen immediately after resection, and stored at –80 °C. All mice experiments were approved by and conducted in accordance with institutional animal care guidelines.

RNA isolation and microarray hybridization

Total RNA from frozen tumor tissue was prepared by standard methods. Five microgram total RNA was used to prepare biotinylated cRNA targets, which were hybridized to Mouse 430 2.0 GeneChips according to the supplier's instructions (Affymetrix, Santa Clara, CA, USA). Hybridization and washing of gene chips was performed on an Affymetrix GeneChip Hybridization Oven 640 and Fluidics Station 450 in compliance with standard procedures. Microarrays were analyzed by laser scanning (Affymetrix GeneChip Scanner 3000).

Microarray data processing and analysis

Three independent GeneChip expression analyses were performed for each mutation. Background-corrected signal intensities were 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 were accomplished by the gene expression

analysis software GeneSpring GX 9.0 (Agilent Technologies, Wilmington, DE, USA). Genes whose transcripts were not detected in any of the investigated mutations were excluded from statistical analysis to reduce the number of false positive genes. To determine differentially expressed genes, expression data were grouped according to MEN2B and MEN2A/FMTC mutations and statistically analyzed using *t*-test and multiple testing correction (Benjamini and Hochberg False Discovery Rate). Cut-offs were set twofold and $P \leq 0.01$. Functional clustering of more than twofold differentially expressed genes revealed their regulation. Sets of co-regulated genes identified from microarray analysis were analyzed using WEB-based Gene Set Analysis Toolkit at bioinfo.vanderbilt.edu/webgestalt to predict molecular interaction networks.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with the RNeasy Mini Kit (Qiagen). After DNase I treatment, 1 µg RNA was reverse transcribed using Omniscript RT (Qiagen) and Oligo-dT primer. The cDNA sample was mixed with iQ SYBR Green Supermix (Bio-Rad). QRT-PCR was performed on iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using 1/20 volume of the RT reaction. Relative gene expression was calculated using iQ5 Optical System Software. All specific primer pairs used are listed in Supplementary material.

Tumor histology and immunohistochemistry

Tumor specimens (~ Ø 8 mm) were removed and fixed in 4% formaldehyde for at least 48 h. The tissue was dehydrated, defatted, and paraffinized using a histokinette (Leica, Germany). Poured paraffin blocks were cut in 4 µm thick sections. Sections were backed for 1 h on glass slides and processed for either histology by conventionally staining with hematoxylin/eosin or immunohistochemistry using a Bond-Maxx Immunostainer (Menarini, Berlin, Germany) with antibodies against pan Granzyme (clone N-19, sc-1969, Santa Cruz, CA, USA) and Perforin 1 (clone H-315, sc-9105, Santa Cruz). Slides were dewaxed, pretreated with EDTA for 20s, and incubated with the primary antibodies (dilution 1:400). The pan Granzyme antibody was additionally incubated with an anti-goat IgG H&L antibody (dilution 1:1000, Rockland Inc., Gilbertsville, PA, USA). Immunostaining was visualized using a Mixed-DAB-refine Kit (Menarini) and counterstained with hematoxylin. Slides were scanned and analyzed with a MIXAX slide scanner (Zeiss, Germany).

Results

Differential gene expression in tumors induced by individual subtype-specific *Ret* mutations

To investigate the mechanisms that represent the phenotypic (neoplastic) differences among the MEN2 syndromes, we studied gene expression profiles in growing tumors established by NIH-3T3 cell lines stably expressing specific RET-MEN2A (C609Y, C634R), MEN2B (A883F, M918T) and -FMTC (Y791F) mutations. All NIH-RET transfectants have been extensively characterized for their oncogenic properties *in vivo* and in cell culture compared with parental NIH-3T3 fibroblasts prior to injection (Miše *et al.* 2006; data not shown). Our main concern was to find genotype-associated molecular signatures that could explain the earlier onset and aggressiveness of MEN2B-RET-related MTCs. Three independent, whole-genome Affymetrix GeneChip array analyses were performed on each mutation. On the basis of the selection criteria for genes differentially regulated in MEN2B versus MEN2A/FMTC tumors, 1494 genes were obtained, 866 that were upregulated, and 628 that were downregulated in MEN2B (twofold, $P \leq 0.01$). By contrast, no significant alterations in gene expression profiles were identified between two varying *Ret* mutations within individual MEN2A (C609Y versus C634R) or MEN2B (A883F versus M918T) subtypes, suggesting a mechanistic similarity of MEN2 subtype-associated RET mutations. Apart from a low genotypic intra-subtype variability, major differences in gene expression were also not found in MEN2A (C609Y and C634R) versus FMTC tumor samples. Both MEN2A mutations are located in the extracellular cysteine-rich domain of the *Ret* receptor, whereas the FMTC mutation at codon 791 is found in the cysteine-rich region as well as in the intracellular tyrosine kinase domain. Based on clinical observations, a codon 791 mutation can lead to both FMTC and MEN2A phenotypes, suggesting common mechanisms that are, corresponding to our gene expression data, responsible for the development of both syndromes.

We also performed hierarchical clustering of the microarray data to exhibit the reproducible differential gene expression patterns among MEN2B (A883F + M918T) versus MEN2A (C609Y + C634R) and FMTC (Y791F) subtype mutations. As shown in Fig. 1, genes with similar expression levels are clustered as homogeneous subgroups. To retrieve functional information on differentially expressed genes in our samples, sets of co-regulated genes (up- or down-regulated in MEN2B versus MEN2A/FMTC)

Figure 1 Hierarchical clustering of differentially expressed genes identified by whole-genome gene chip analysis on NIH-RET(MEN2A) (C609Y, C634R), NIH-RET(MEN2B) (A883F, M918T) and NIH-RET(FMTC) (Y791F)-specific tumors. Genes are colored according to the normalized expression values. The colors in the heat map represent expression levels (*red*, overexpression; *yellow*, average expression; *blue*, underexpression/no expression). The tumor samples are ordered in columns and the corresponding gene clusters in rows.

identified from GeneChip arrays were further classified according to their biological process profiles using their Gene Ontology (GO) annotation. Biological processes differentially regulated between MEN2B and MEN2A/FMTC tumors are shown as Supplementary data.

Among the MEN2B tumor-specific gene clusters (shown in Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>) are subclusters enriched of genes involved in cell growth via activation of the phosphatidylinositol 3-kinase (PIK3)/AKT signaling pathway such as related RAS viral (r-ras) oncogene homolog 2 (*Rras2*) or IGF 2 (*Igf2*), the CCRK/MAPK3 mitogen-activated protein kinase (MAPK) pathway (*Fgf7*, *Fgfr2*, *Kras*, *Map3k8*, *Mapkapk3*, *Ngfb*, *Prkca*, *Rps6ka2*, *Rps6ka6*, *Rras2*, and *Grb10*), and the WNT signaling pathway with aberrantly activated frizzled homolog 2 (*Fzd2*), disheveled associated activator of morphogenesis 2 (*Daam2*), calcium/calmodulin-dependent protein kinase II β

(*Camk2b*), and dickkopf (DKK) homolog *Dkk2* and *Dkk3*. Moreover, a large number of genes related to cell cycle progression and oncogenesis (e.g. cyclin E, *Ccn1*; forkhead box M1, *Foxm1*) including several growth factors (*Ecgf1*, *Fgf7*, *Gdf5*, *Gfer*, *Hgf*, *Igf2*, *Ngfb*, and *Met*) were found significantly upregulated. Another subcluster contains genes engaged in cell adhesion and metastasis (*Col4a6*, *Lama4*, *Itga3*, *Itga6*, *Itgb4*, *Cdh2*, *Foxc2*, *Hgf*, *Met*, *Pcdhb3*, and *Twist2*). In addition, we observed increased expression of various genes in RET-MEN2B specific neoplastic tissues that contribute to cell survival by inducing apoptosis resistance (*Ndn*, *Aatf*, *Naip2*, *Traf4*, *Traf6*).

In our attempt to identify biological processes that could discriminate MEN type 2B from 2A/FMTC malignancies, clusters are of particular interest that contain a considerable number of genes involved in the immune system. A total of 56 genes that are expressed at high levels in MEN2A/FMTC tumors and moderately or not in the MEN2B phenotype belonged to the

host immune and inflammation response category (Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Ten of these genes are expressed by natural killer (NK) cells (*Cd244*, *Klra3*, *Klra12*, *Klrb1d*, *Klrc2*, *Klrcl1*, *Klre1*, *Ncam1*, *Ncr1*, and *Nkg7*), others are present on the surface of cytotoxic T lymphocytes (CTLs; for example *Cd28*, *Cd69*, *Ctla4*). Activated CTLs and NK cells are known to induce lethal damage on their target cells by granule exocytosis or the Fas ligand/Fas system (Henkart 1994, Kojima *et al.* 1994). In support of a potential NK- and/or T-cell-mediated antitumor immune response to RET/FMTC expressing cells, a number of genes that are highly upregulated in RET/FMTC tumors code for apoptosis molecules such as Fas ligand (*FasL*), perforin 1 (*Prf1*), and the majority of murine granzymes (*Gzma*, *Gzmb*, *Gzmc*, *Gzmd*, *Gzme*, *Gzmf*, *Gzmg*, *Gzmk*, *Gzmn*). Seven genes encode cytokines, cytokine receptors, or chemokines (*IL15*, *Il2rb*, *Il2rg*, *Il12rb1*, *Il18rap*, *Icos*, *Vav3*, and *Cx3cl1*) critically involved in the stimulation of T- and NK-cell proliferation and their recruitment in cancerous tissues (Allavena *et al.* 1997, Kuniyasu *et al.* 2001, Dimberg *et al.* 2007, Zeng *et al.* 2007). Ten upregulated genes in this category code for signaling molecules (e.g. *Gbp2*, *Gbp4*, interferon-activated genes *Ifi202b*, *Ifi2003*, *Ifi204*, *Ifi205*, and interferon-produced proteins *Ifi44*, *Ifi3*, *Ifitm1*, and *Irf8*). Consistent with the chemotactic effect of IL15 on T and NK cells by stimulating their adhesion to vascular endothelium (Allavena *et al.* 1997), cell adhesion molecules such as *Vcam1* showed enhanced expression in RET-MEN2A/FMTC tumors.

A pattern of genes related to cell adhesion and/or metastasis was also found upregulated in MEN type 2A/FMTC tumors (*Col4a5*, *Dsc2*, *Fblim1*, *Itga1*, *Itga10*, *Itgb3*, *Itgb5*, *Mmp9*, *Mmp16*, *Dmp1*, *Tgfb2*, *Postn*, *Thbs3*). Finally, we identified a subcluster enriched of genes that are engaged in skeletal development such as bone morphogenic proteins (BMPs)/receptors (*Bmp1*, *Bmp5*, *Bmpr1b*, and *Bmpr2*), extracellular matrix components (*Col10a1*, *Col5a2*, and *Mgp*), members of the transforming growth factor β -receptor signaling (TGF β) pathway (*Bmpr2*, *Bmp5*, *Tgfb2*), or TGF β family binding proteins (*Htra1*, *Ltbp4*) with higher expression in RET-MEN2A/FMTC than in RET-MEN2B tumors, while six genes that negatively regulate this pathway were upregulated in MEN2B compared with MEN2A/FMTC tissues (*Bambi*, *Eid2*, *Foxg1*, *Fst*, *Fstl3*, and *Nb1l*). Members of the TGF β /BMP pathways regulate many processes including cellular proliferation, adhesion and differentiation,

hematopoiesis, inflammation, wound repair, and skeletal development. The BMP and activin membrane-bound inhibitor, for instance, was identified as a target of the cancer-promoting WNT pathway also upregulated in the RET-MEN2B tumors (Sekiya *et al.* 2004). However, since MEN2B patients in addition to the early development of invasive tumors frequently develop skeletal abnormalities, the relative lack of TGF β /BMP genes in MEN type 2B versus RET-MEN2A/FMTC tumors may account for the bone defects as well. By contrast, one example of a factor implicated in bone development that is aberrantly expressed in RET-MEN2B induced tumors is GDF5. In fact, inactivating mutations in this gene are associated with numerous alterations in the length and number of bones in the limbs (Thomas *et al.* 1996). According to the potential role of WNT/ β -catenin signaling in new bone formation by functioning as a positive regulator of osteoblasts, and negative regulator of osteoblast-dependent osteoclastogenesis (Day *et al.* 2005, Hu *et al.* 2005), it appears interesting that secreted WNT inhibitors from the DKK family enhance the osteoclastogenic potency of BMP-driven osteoprogenitors (Fujita & Janz 2007), whereas the DAAM2 transduces WNT/PCP signals to the RhoA signaling cascade, resulting in the induction of actin skeletal re-organization and cell movement (Mlodzik 2002). These observations suggest that aberrant *Dkk2*, *Dkk3*, and *Daam2* expression in RET-MEN2B cells could cause abnormalities in developing bones that lead to skeletal deformities in patients.

Evaluation of microarray profiles in RET-MEN2-specific tumors and NIH-RET cell cultures *in vitro* by qRT-PCR

To further validate the microarray profiles, qPCR confirmation was performed for a representative choice of genes from various functional clusters (Fig. 2) including those coding for proteins involved in the host immune response (Fig. 3) for each *Ret* mutation using gene-specific primers. QPCR data are illustrated as fold change of expression referred to the lowest gene expression of an individual target. About 32 out of 39 (~80%) showed a consistent change between the microarray and the qPCR results. In 24 of these, the change detected by qPCR was significantly greater, indicating that the statistical criteria applied to the microarray data succeeded in minimizing false positive results.

Quantitative PCR analysis revealed that the mRNAs for proteins involved in the host immune response by the stimulation of NK- and T-cell proliferation, migration,

Figure 2 (continued)

Figure 2 Expression of selected differentially regulated genes in RET-mutation-specific tumors by quantitative RT-PCR analysis. Gene expression levels are shown for individual RET mutations: (A) upregulation in RET-MEN2B 918, 883; and (B) upregulation in RET-MEN2A/FMTC 634, 609/791-related tumors. Fold expression was calculated after normalization with GAPDH and ACTB using iQ5 Multicolor Real-Time PCR Detection System. QPCR data are illustrated as fold change of expression referred to the lowest gene expression of each target (set as 1).

and apoptosis induction of their target cells were highly upregulated in MEN2A/FMTC tumor tissues. By contrast, low or negligible mRNA expression was detected in mouse tumors induced by RET-MEN2B mutations (Fig. 3, grey bars). To clarify which cells are responsible for the expression of the immunomodulatory and death-inducing factors, qRT-PCR analysis was carried out in cultured NIH-RET cell lines harboring these mutations in the absence of the immune system. In fact, aberrant transcript levels of virtually all molecules tested (*Gzmb*, *Gzmf*, *Prf1*, *FasL*, *Cd244*, *Nkg7*, *Il15*, *Il2rb*, and *Ncr1*) were detected only in *in vivo* growing MEN2A/FMTC tumors, but were either absent or expressed at very low levels (*FasL*, *IL15*, *Cd244*, and *Nkg7*) in cell cultures, suggesting that they are attributed to activated infiltrating immune effector cells such as NK cells and T lymphocytes. Conversely, fractalkine

(*Cx3cl1*) considered as a chemokine involved in the recruitment and accumulation of leukocytes into cancerous tissues is also expressed at comparable high levels in MEN2A/FMTC-transformed cell lines. Importantly, this chemokine is not expressed in both RET-MEN2B tumor specimens.

To substantiate the assumption of enhanced infiltration of immune cells into MEN type 2A/FMTC compared with RET-MEN2B tumors, we performed immunohistochemical examination of *Ret* genotype-specific tumor sections using antibodies specific against pan granzyme and perforin 1, exclusively expressed by NK cells and CTLs as the major mechanism by which these cells exert an immune response on tumor cells (Kawasaki *et al.* 1992, Grossman *et al.* 2003). As shown in Fig. 4, inflammatory infiltrates were detected only in

Figure 3 Differential expression of genes involved in host immune response and recruitment of immune effector cells in RET-MEN2A/FMTC versus RET-MEN2B. Selected immune response genes (*Gzmb*, *Gzmf*, *Prf1*, *Fasl*, *Il15*, and *Il2rb*), NK cell markers (*Cd244*, *Nkg7*, and *Ncr1*) and chemokines (*Cx3cl1*) were verified in established RET-specific tumors (grey bars) and cultured NIH-RET cell lines (black bars) by QPCR. Data are illustrated as fold change of expression after normalization with GAPDH and ACTB using iQ5 Multicolor Real-Time PCR Detection System. The lowest gene expression of each target was set as 1.

RET-MEN2A/FMTC-induced tumors as indicated by a strong granzyme/perforin staining (brown cells). By contrast, there was no detectable immunoreactivity in RET-MEN2B tissues indicative for the complete lack of inflammatory infiltrates in these tumors. NIH-RET tumor cells by itself stained negative. We also attempted to detect fractalkine using anti-CX3CL1 antibody (NEUROMICS) in tumor sections. Immunohistochemical staining showed that fractalkine immunoreactivity was mainly observed in single extracellular conglomerates, suggesting that the chemokine is present in its soluble (secreted) form (data not shown). This is in agreement with a higher expression of the tumor necrosis factor- α -converting enzyme (ADAM17) in MEN2A/FMTC tumors,

inducing the release of CX3CL1 through its cleavage at a membrane-proximal site.

Discussion

We compared the whole-genome expression profiles triggered by specific RET-MEN2A, -B and FMTC mutations using a defined *in vivo* model of growing NIH-RET-related tumors in mice. Based on the selection criteria for up- and downregulated expressions in MEN2B versus MEN2A/ FMTC, 1494 differential genes were observed. Apart from a low genotypic intra-subtype variability, surprisingly no significant alterations in gene expression were found in MEN2A versus FMTC species. A possible

Figure 4 Verification of inflammatory infiltrates in RET-MEN2A/FMTC versus RET-MEN2B-induced tumors by immunohistochemistry. Paraffin sections of subcutaneously established NIH-RET tumor carrying MEN2A-C634R (A–C), MEN2A-C609Y (D–F), FMTC-Y791F (G–I), MEN2B-M918T (J–L), and MEN2B-A883F (M–O) mutations. Immune cells were detected with antibodies specific for pan-granzyme (middle column; B–N) and perforin 1 (right column; C–O). A strong positive reaction (brown cells) was observed in section of RET-MEN2A/FMTC specimens. Hematoxylin and eosin (HE) staining (left column: A–M). Original magnification $\times 5$, scale bar = 500 μm (HE); $\times 20$, scale bar = 100 μm (pan-granzyme/perforin 1).

explanation is that a codon 791 mutation can lead to FMTC and MEN2A phenotypes, suggesting a common mechanism responsible for the development of both syndromes. Whether patients with a mutation at codon 791 have a high or least high risk for aggressive MTC presumably depends on additional perhaps environmental conditions. Our findings, however, indicate that both genotypes *per se* exhibit a similar oncogenic potential. Instead, GO classification and TreeView analysis of discriminating gene clusters in MEN type 2B and RET-MEN2A/FMTC-induced tumors showed obvious differences in characteristic survival pathways and, in particular, the host antitumor immune response between both cancer types.

MEN2B-specific tumors are characterized by individual survival pathways

Transformation by MEN2B-RET is potentially a consequence of aberrant activation of signaling

pathways that are normally regulated by cytoplasmic tyrosine kinases. We found that several genes that are important for RAS/MAPK or PIK3/AKT signaling are expressed at higher levels in MEN2B than in the tumors expressing MEN2A /FMTC-RET. Members of these pathways are essential mediators of cellular responses to extracellular signals that include growth factors, hormones, cytokines, and environmental stress (Chang & Karin 2001), which regulate essentially all aspects of malignant cell behavior, proliferation, survival, migration, and invasion. Signaling cascades such as the RAS/MAPK or PIK3/AKT pathway are activated mainly via phosphorylated tyrosine 1062 in RET (Salvatore *et al.* 2001). In line with this, phosphorylation of RET at Y1062 has been shown stronger in RET-MEN2B than in RET-MEN2A or FMTC expressing cells (Miše *et al.* 2006). However, while cancer is most often thought of as a disease that is caused by inappropriate levels of proliferation, apoptosis shares an equally if not more important

role in cancer progression and metastasis. As an anti-apoptotic pathway, the mitogenic MAPK cascade is characterized by the sequential activation of the RAF, MAP3K1, MAPK1, and RPS6KA2/RPS6KA kinases (Bonni et al. 1999). MAPK-mediated survival also involves proteins of the growth factor receptor-bound (Grb) adapter family Grb7/10/14 that bind to the RET tyrosine kinase receptor. In particular, Raf1 and Grb10 have been shown to be required for the ability of the MAP kinase pathway to modulate the phosphorylation and inactivation of Bad, thereby creating a specific antiapoptotic activity (Kebache et al. 2007). Interestingly, our analysis revealed an upregulation of both Grb10/Grb14 and Rps6ka kinases in MEN2B species, which together with their previously noticed increased apoptosis resistance (Miše et al. 2006), may reflect the MAPK prosurvival function in these tumors. Consistent with the assumption that apoptosis suppressive activities in MEN2B versus MEN2A/FMTC cases likely contribute to disease aggressiveness, additional cell survival-related transcripts were found considerably expressed in RET-918 and RET-883 tumors. In particular, growing evidence has demonstrated that the hepatocyte growth factor (HGF) and Met protein overexpressed in many human cancers including oncogene-transformed human thyroid cells (Eccles et al. 1996) play a major role in tumor progression, invasion, and metastasis (Jeffers et al. 1996). Papillary thyroid carcinoma in which both components are activated in an autocrine fashion (Trovato et al. 1998) are characterized by highly invasive behavior and early metastatic spread (Ruco et al. 2001). Since *Ret* can directly induce overexpression of Met in human thyroid epithelial cells (Ivan et al. 1997), an intriguing possibility to explain the higher aggressiveness of the MEN2B phenotype is that activated RET-MEN2B mutations and the HGF-Met signal act in a synergistic manner at the early stage of tumor development, likely by converging on a common target such as the RAS/MAPK or PIK3/AKT cascades, thereby providing an extra growth advantage over cells expressing the MEN2A/FMTC mitogenic RET signal. Because suspension-induced apoptosis (anoikis) resistance plays an important role in tumor progression and metastasis, it is of interest that HGF provides protection against anoikis through activation of MAPK and AKT signaling pathways in head and neck squamous cell carcinoma (Zeng et al. 2002).

Additional significance that apoptosis resistance plays an essential role in RET-MEN2B tumor progression is provided by the upregulation of TWIST2. As one of the E-cadherin repressors, TWIST regulates the epithelial-to-mesenchymal

transition and promotes tumor metastasis (Yang et al. 2004). TWIST2 antagonizes TRP53-induced apoptosis (Maestro et al. 1999). Another protein whose expression is particularly high in the RET-MEN2B tumors that potentially confers resistance to apoptosis is neurally differentiated embryonal carcinoma cell-derived factor (necdin). Previous results have shown that this protein blocks E2F1-induced apoptosis in neuroblastoma cells (Kobayashi et al. 2002). Moreover, necdin interacts with the transactivation domain of p53, thereby inhibiting p53-mediated apoptosis of osteosarcoma cells (Taniura et al. 1999). Thus, identifying the endogenous binding proteins of necdin in cells expressing RET mutations is likely to shed light on the molecular basis for a putative apoptosis inhibitory function in these cells and how this may contribute to enhanced tumor aggressiveness.

Dysregulation of host antitumor response mechanisms during RET-MEN type 2B tumorigenesis

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 type 2A/FMTC mutations, while expression of these genes was nearly completely suppressed in both RET-MEN2B species. A comparative analysis of the transcript levels in transplanted tumor tissues and in cultured NIH-3T3 cell lines harboring single *Ret* mutations revealed that the majority of immunomodulatory genes are exclusively upregulated in mice growing tumors, indicating that these molecules are attributed to the host innate immune system. In support of this notion, we found massive inflammatory infiltrates in sections from RET-MEN2A/FMTC tumors as evidenced by a positive perforin and pan granzyme staining that were barely detectable in neoplastic tissues of RET-MEN2B origin. The strong representation of immune- and inflammation response-related genes in RET-MEN2A/FMTC-induced tumors and their apparent lack in more aggressive RET malignancies points to a critical perhaps differential role of RET oncoproteins in the early modulation of immune response mechanisms. Tumor-infiltrating leukocytes can negatively regulate tumor progression by producing cytostatic or cytotoxic molecules. At the molecular level, the granule exocytosis pathway and

Figure 5 Model of RET receptor subtype-specific interference with the innate immune system of nude mice. RET-MEN2A/FMTC-transformed cells produce CX3CL1 (fractalkine), whereas cells induced by RET-MEN2B mutations anticipate chemokine expression (1). The secreted membrane-bound and soluble forms of CX3CL1 cause recruitment (chemotaxis and adhesion) of NK cells, cytotoxic lymphocytes, and monocyte–macrophages expressing its receptor CX3CR1 (2). IL15 produced by activated monocyte–macrophages, for example, attracts T and NK cells to tumor endothelium and stimulates their proliferation via interaction with the IL2 receptor β (3). Tumor-infiltrating activated NK and T cells secrete cytotoxic granules (4) that potentially leads to enhanced killing of MEN type 2A/FMTC-specific tumors, while RET-MEN2B receptors avoid immune infiltration as a mechanism of evasion (5).

Fas–FasL system account for virtually all of the measurable contact-mediated cytotoxicity delivered by NK cells and CD8+ T cells (Russell & Ley 2002, Lieberman 2003). The granzyme pathway utilizes perforin to traffic granzymes appropriately into the cytosol of target cells (Shi *et al.* 1997, Keefe *et al.* 2005), where granzymes A and B (and orphan granzymes) induce cell death by clearing critical substrates (Russell & Ley 2002, Lieberman 2003). Thus far, in mouse experimental tumor systems, perforin activities have been demonstrated to protect the host against tumor initiation (van den Broek *et al.* 1996), primary tumor growth (van den Broek *et al.* 1996, Smyth *et al.* 1998), and tumor metastasis (Smyth *et al.* 1999). Intriguingly, in our transplanted model, nearly all mouse granzyme encoding genes (A, B, C, D, E, F, G, K, and N; Grossman *et al.* 2003) are extensively expressed in the RET-MEN2A/FMTC-related tumors, suggesting that this immune surveillance system may operate at early stages of tumorigenesis, possibly leading to tumor growth control. This assumption is substantiated by our previous findings, showing that neoplastic cell growth of faster proliferating NIH-RET mutants associated with MEN2A and FMTC is slowed down shortly after tumor cell transplantation into nude mice, and can then

be overcome by basically less proliferative RET-MEN2B expressing cells (Miše *et al.* 2006). The observed growth behavior of RET-MEN2A/FMTC specimens may be due to the formation of an effective antitumor immune response that eventually increases susceptibility to NK- and T-cell lysis, thereby contributing to growth control, and perhaps a less aggressive phenotype. In fact, cytotoxic T- and tumor-infiltrating NK cells have been associated with a favorable prognosis in a variety of human malignancies (Coca *et al.* 1997, Ishigami *et al.* 2000, Takanami *et al.* 2001, Hsia *et al.* 2005).

The data herein suggest that, whereas RET-MEN2A and FMTC mutations are related to antitumor immunity, oncogenic receptor tyrosine kinase signaling specifically initiated by MEN type 2B mutations causally determines immune defense. In agreement with a contributory role of MEN2A/FMTC in cancer-associated inflammation, NIH-3T3 transfectants with these mutations show a significantly enhanced expression of *Cx3cl1* both *in vitro* and when transplanted into mice. This chemokine is critically important for infiltration (chemotaxis and adhesion) of various lineages of lymphocytes characterized by a high content of intracellular perforin and granzyme, and monocytes/macrophages expressing its receptor

CX3CR1 in human carcinomas (Guo *et al.* 2003, Ohta *et al.* 2005). Previous reports have demonstrated that the antitumor effects elicited by fractalkine gene transfer into subcutaneously implanted murine cancer cell lines depend on NK and T cells (Guo *et al.* 2003, Lavergne *et al.* 2003, Xin *et al.* 2005). Consistent with our data, high endogenous fractalkine expression in neuroblastoma (Zeng *et al.* 2007), colorectal cancer (Ohta *et al.* 2005), and gastric adenocarcinoma (Hyakudomi *et al.* 2008), where it is mainly observed in the plasma membrane and cytoplasm of tumor cells, correlated with a higher density of tumor-infiltrating immune cells. In case of RET oncogene-induced tumors, this activation is dependent on the kind of RET mutation, since fractalkine is not expressed in MEN2B tumors lacking immune infiltrates. The exact mechanism by which MEN type 2A/FMTC RET maintains chemokine production, or more relevant, RET-MEN2B counteracts immune infiltration by anticipating CX3CL1 expression is presently unknown and subject to further investigations. It was reported that *Cx3cl1* mRNA and protein expressions are induced by various inflammatory stimuli including IL1, TNF- α and IFNG in endothelial cells (Fratelli *et al.* 2001), but these factors were not found differentially regulated in RET-MEN2A/FMTC versus RET-MEN2B tumors, implicating that other oncogene-related mechanisms may account for the effect. Together with the studies demonstrating that a high expression of fractalkine in cancer cells drastically reduce their metastatic potential, thus resulting in a better prognosis for patients (Ohta *et al.* 2005, Vitale *et al.* 2007), this phenomenon raises the possibility of increased tumor cell killing in MEN2A/FMTC specimens, and may explain the faster progression of MEN2B tumors to clinically significant forms by a RET-MEN2B receptor-specific prevention of molecules involved in recruitment of T- and/or NK-cell cytotoxicity, thereby allowing developing cancer cells to grow unchecked in the absence of otherwise intact host immune responses.

So far, a differential display analysis of NIH3T3 fibroblasts transfected with RET-MEN2A mutation 634 and MEN2B mutation 918 has been reported (Watanabe *et al.* 2002). In addition, expression analysis of tumors from MEN2A and MEN2B patients was performed (Jain *et al.* 2004). Although, we identified a much larger spectrum of differentially expressed genes, data from the present approach are generally consistent with these studies, indicating that genes involved in the process of cell growth and epithelial to mesenchymal transition are characteristic for MEN2A and MEN2B tumors respectively. For

single targets such as pleiotropin, which is specifically upregulated in MEN2A compared with MEN2B tumors (Watanabe *et al.* 2002), we obtained similar results using a different technology. The differences in genes identified to their experiments including those genes associated with the host immune response presumably reflect our *in vivo* versus *in vitro* design or the mouse versus human expression profiling of tumors at an early stage of development.

In summary, our data support a model of *Ret* oncogene-specific interference with the host immune system (Fig. 5), in which chemokine production by RET-MEN2A/FMTC cancer cells initiates an anti-tumor immune attack, while RET-MEN2B receptors avoid tumor infiltration as a mechanism of evasion that may be critical for the different clinical outcome of both subtypes.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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