Novel candidate genes of thyroid tumourigenesis identified in *Trk-T1* transgenic mice

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Abstract

For an identification of novel candidate genes in thyroid tumourigenesis, we have investigated gene copy number changes in a *Trk-T1* transgenic mouse model of thyroid neoplasia. For this aim, 30 thyroid tumours from *Trk-T1* transgenics were investigated by comparative genomic hybridisation. Recurrent gene copy number alterations were identified and genes located in the altered chromosomal regions were analysed by Gene Ontology term enrichment analysis in order to reveal gene functions potentially associated with thyroid tumourigenesis. In thyroid neoplasms from *Trk-T1* mice, a recurrent gain on chromosomal bands 1C4–E2.3 (10.0% of cases), and losses on 3H1–H3 (13.3%), 4D2.3–E2 (43.3%) and 14E4–E5 (6.7%) were identified. The genes *Twist2*, *Ptma*, *Pde6d*, *Bmpr1b*, *Pdlim5*, *Unc5c*, *Srm*, *Trp73*, *Ythdf2*, *Taf12* and *Slitrk5* are located in these chromosomal bands. Copy number changes of these genes were studied by fluorescence *in situ* hybridisation on 30 human papillary thyroid carcinoma (PTC) samples and altered gene expression was studied by qRT-PCR analyses in 67 human PTC. Copy number gains were detected in 83% of cases for *TWIST2* and in 100% of cases for *PTMA* and *PDE6D*. DNA losses of *SLITRK1* and *SLITRK5* were observed in 21% of cases and of *SLITRK6* in 16% of cases. Gene expression was significantly up-regulated for *UNC5C* and *TP73* and significantly down-regulated for *SLITRK5* in tumours compared with normal tissue. In conclusion, a global genomic copy number analysis of thyroid tumours from *Trk-T1* transgenic mice revealed a number of novel gene alterations in thyroid tumourigenesis that are also prevalent in human PTCs.

Endocrine-Related Cancer (2012) **19** 409–421

Introduction

Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid gland (Knauf & Fagin 2009). At the molecular level PTC is characterised by genetic alterations of components of the MAP kinase pathway (Nikiforov 2008). These include structural chromosome rearrangements affecting genes encoding *RET* (*RET/PTC*) and *NTRK1* (*TRK-T*) tyrosine kinase receptors that undergo in-frame recombination with various partner genes (Bongarzone *et al*. 1989, Greco *et al*. 2010). Depending on the fusion partner, various *RET/PTC* and *TRK-T* chimeric oncogenes have been identified.

Specifically, the *Trk-T1* oncogene results from a paracentric inversion of chromosome 1q25 that fuses

1351–0088/12/019–409
DOI: 10.1530/ERC-11-0387
Online version via http://www.endocrinology-journals.org

the 5′ end of the translocated promoter region to the 3′ end of *NTRK1* genes generating the constitutively active and oncogenic kinase *NTRK1* (Greco *et al.* 2010). Transgenic mice featuring the thyroid-specific expression of *Trk-T1* under the transcriptional control of the thyroid-specific bovine thyroglobulin (Tg) promoter were generated previously (Russell *et al.* 2000). Twenty-three percent of *Trk-T1* mice of age $\leq$7 months and 78% of mice $>$7 months developed thyroid nodules characterised by malignant features, such as the proliferation of follicular epithelial cells containing scant cytoplasm, mitotic figures and papillae with fibrovascular stalks (Russell *et al.* 2000, Kim & Zhu 2009). Neoplasms observed in *Trk-T1* thyroids were focal, suggesting the occurrence of clonal genetic events cooperating with the initiating *Trk-T1* transgene. Such genetic lesions that cooperate with *Trk-T1* in this process are still unknown.

Comparative genomic hybridisation (CGH) allows the identification of copy number gains and losses of chromosomal regions in tumours and subsequent discovery of genes that may play a role in the tumourigenic process. CGH can be applied at different resolutions depending on the targets used for the hybridisation such as metaphase chromosomes (metaphase CGH; resolution 5–10 Mb) or DNA microarrays (array CGH; 1 Mb to kb resolution, Chung *et al.* 2004).

In this study, we applied metaphase CGH and array CGH to study gene copy number alterations (CNA) in thyroid tumours developed in *Trk-T1* transgenic mice. We identified four recurrent CNA. For these CNA, we used the synteny approach on www.ensemble.org to define the syntenic human regions. These human regions were reconciled with array CGH data generated in our lab from human PTCs (Unger *et al.* 2008, Hess *et al.* 2011). We further determined candidate genes located in these altered regions from the published literature.

In human PTC samples copy number gains of the genes *TWIST2*, *PTMA* and *PDE6D* were identified by fluorescence *in situ* hybridisation (FISH) in 10/12 samples for *TWIST2* and in 12/12 samples for *PTMA* and *PDE6D*. Moreover, human thyroid tumours consistently featured loss of the genes *SLITRK1*, *SLITRK5* and *SLITRK6* (4/19, 3/19 and 4/19 samples).

Materials and methods

Transgenic mice

Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Facility of the Università 'Federico II', Naples, Italy and all studies were conducted in accordance with Italian regulations for experiments on animals. *Tg-Trk-T1* mice were euthanised and thyroids were immediately fixed in buffered formalin for 4 h at room temperature. Tissues underwent automated processing and paraffin embedding; 5 µm sections were cut and haematoxylin and eosin stained (H&E) for microscopic analysis of the tissue morphology. Thirty *Tg-Trk-T1* thyroid samples were studied and classified according to the WHO criteria for the evaluation of mouse thyroid tumours (Jokinen & Botts 1994). The thyroid tissues were considered normal when composed by variable-sized follicles covered by a monolayer of flattened epithelial cells or as hyperplastic when showing secondary occurrence of small follicles with scant colloid and tall epithelial cells that were merged with normal areas. Finally, neoplastic thyroid lesions were defined as well-demarcated nodules with a distinct papillary and/or follicular architecture; in a few cases, invasion of the surrounding glandular parenchyma or stroma was detected, thereby allowing the classification of carcinomas of the papillary subtype.

Human tissues

For validation experiments human samples from already existing post-Chernobyl PTC cohorts were available at Helmholtz Zentrum München and Imperial College London. Thyroid tumour samples (FFPE tissue sections) from 30 patients presenting with PTC developed after the Chernobyl reactor accident were obtained from the Chernobyl Tissue Bank (CTB; 16 cases), from the Centre of Thyroid Tumours in Minsk, Belarus (seven cases) and from the Institute of Endocrinology and Metabolism, Kiev, Belarus (seven cases). In addition, RNA from frozen tissues of 67 PTC cases was obtained from the CTB for mRNA gene expression analyses. All PTC samples were characterised for the presence of *RET/PTC* rearrangements by RT-PCR and for the V600E *BRAF* mutation, the most frequent *BRAF* mutation in PTC, by direct DNA sequencing (Powell *et al.* 2005).

All tumours were diagnosed as PTC according to the TNM Classification of Malignant tumours and further classified according to their dominant histological architecture (papillary, follicular and solid) (Hedinger *et al.* 1989). Appropriate informed consent was obtained from the patients or their guardians and the study was approved by the ethics committee in accordance with the declaration of Helsinki.

CGH of mouse tissues

CGH was applied to detect genomic copy number changes in murine thyroid nodules. Nodule (thyroid) and reference (liver) DNA were isolated using the QIAmp Mini Kit (Qiagen). DNA concentration and purity were determined photospectrometrically (NanoDrop, Thermo Scientific, Wilmington, DE, USA). Indirect labelling of the reference DNA with digoxigenin and of the tumour DNA with biotin was performed by nick translation using a standard protocol. Hybridisation of the labelled DNA on metaphase spreads with a normal karyotype from the 102/ELxC3H/EL mouse strain was performed according to Richter *et al*. (2003). The chromosomes were counterstained with DAPI and actinomycin D prior to image acquisition and analysis as described by Richter *et al*. (2003).

Array-based CGH

Genomic DNA was isolated from fresh frozen tissues or from cultured tumour cells and fluorescence labelled with Cy3 (Invitrogen). Genomic reference DNA was labelled with Cy5 (Invitrogen) and co-hybridised to 1 Mb BAC mouse arrays (Chung *et al.* 2004). DNA labelling, hybridisation and data processing were performed as described by Unger *et al*. (2008). Further data analysis was performed using the web-based array CGH evaluation platform CAPweb (Liva *et al*. 2006, http://bioinfo.curie.fr/CAPweb). After import of raw data into CAPweb, the normalisation procedure using the R-package MANOR (Neuvial *et al*. 2006) and segmentation of the data set using the R-package GLAD (Hupe *et al*. 2004) were performed using default parameters. The GLAD algorithm considers probes called as gain and a log2 ratio greater than one as high level amplifications. Data were exported to the array data visualisation tool VAMP (La Rosa *et al*. 2006). Common regions of alterations of the analysed cases were determined using the minimal-alteration algorithm (Rouveirol *et al*. 2006), which is implemented in the VAMP software (http://bioinfo-out.curie.fr/projects/vamp/).

Identification of candidate genes

Based on a synteny approach, the murine chromosome alterations were translated into the human syntenic chromosome bands using the (V58) Ensembl database (www.ensembl.org). A comparison of the human chromosome bands with CNA from PTC samples published by Unger *et al*. (2008) and Hess *et al*. (2011) revealed CNA derived from the mouse model that are prevalent either in PTC from young patients (Unger *et al*. 2008, Hess *et al*. 2011) or in PTC from adults (Unger *et al*. 2008).

Further, candidate genes located in these syntenic altered chromosomal regions were analysed by Gene Ontology (GO) term enrichment analysis (Zheng & Wang 2008) in order to reveal gene functions potentially associated with thyroid tumourigenesis.

Fluorescence *in situ* hybridisation

BAC clones were selected from the RPCI23 and -24 mouse libraries (Osoegawa *et al*. 2000) and from the human 32k re-array library (Osoegawa *et al*. 2001). After growth for 15 h at 37 °C plasmid DNA was isolated from the bacterial cultures according to standard protocols (Birnboim & Doly 1979). The following gene spanning (digoxigenin) and reference (biotin) BAC clones were selected and labelled (as described above): mouse: RP23-396K20 (*Ptma*, *Pde6d*, reference clone: RP23-34E15), RP23-441M15 (*Twist2*, reference clone: RP23-34E15), RP23-90A10 (*Slitrk5*, reference clone: RP23-34E15), RP11-127O5 (*Taf12*, *Ythdf2*, reference clone: RP23-59B17), RP23-331P21 (*Srm*, reference clone: RP23-59B17), RP23-254N4 (*Trp73*, reference clone: RP23-59B17); and human: RP11-690I21 (*PTMA*, *PDE6D*, reference clone: RP11-912D12), pooled RP23-717F15 and RP23-275G07 (*TWIST2*, reference clone: RP11-912D12), RP11-613F15 (*SLITRK1*, reference clone: RP11-139J24), RP11-243B06 (*SLITRK5*, reference clone: RP11-139J24), RP11-398A22 (*SLITRK6*, reference clone: RP11-139J24). Murine and human BACs were checked for binding specificity by hybridisation to normal metaphase spreads. Hybridisation of murine BAC clones to interphase cells was performed as described above. The human BAC clones were hybridised onto 8 μm paraffin-embedded tissue sections according to Unger *et al*. (2004). Images from the mouse interphase preparations were taken using the Metafer 4 System (MF-054) (V3.6.7; Metasystems, Altlussheim, Germany), while the human FFPE tissues were analysed by taking 3D images with the AxioImager system equipped with ApoTome (Zeiss, Oberkochen, Germany). The FISH signals of at least 100 cells were analysed and classified as positive for a deletion when cell nuclei showed two green and less then two red signals or as positive for an amplification when cell nuclei harboured more than two red signals in addition to two green signals.

qRT-PCR

Total RNA was isolated from fresh frozen tissue using the RNeasy kit (Qiagen) including on-column

Table 1 Copy number changes in 30 thyroid tumours from *Trk-T1* transgenic mice

Case	Histology	Copy number changes (CGH/aCGH[a])		FISH validation[b] (frequency of cells with aberrant FISH signals in tumour tissues)
		Gain	Loss	
TRK-T1-1	PTC	2(H4), 7(E2, F3→ter), 8(D1→ter), 14(E5)	12(A1.3→A3, C1), 14(A1→A3), X(A2)	ND
TRK-T1-2	PTC	15(E3→F1), 17(E5)	5(A3), 7(A2→B3), 12(A1.3→A3), 14(A1→A3), 16(C1.3→C2), X(A2→A4)	ND
TRK-T1-170	PTC	1(A1→A3), 6(B3), 10(D3), 16(C3.1→C3.2, C4)	2(H1→H3), **4(D1→E2)**, 7(F2→F3), 15(E1→E2)	ND
TRK-T1-236	Hyperplasia	15(E3→F2)	3(G2→G3, H3), 4(C6→D, E2), 19(D3)	ND
TRK-T1-238	Hyperplasia	11(E1→E2)	8(A2→A3), 14(A1→B)	ND
TRK-T1-239	PTC	11(B1.3→B5), 15(E2→F1) 10(A1→D3)[a], 15(A1→D1)[a]	**4(E→ter)**, 13(D2.2), **14**(C3, **E4→E5**), X(F4→F5) **3(A1→H4)**[a], **4(A1→E2)**[a]	del 4 (*Taf12*, *Ythdf2*): 24.0% of cells del 4 (*Trp73*): 24.1% of cells del 14 (*Slitrk5*): 27.3% of cells
TRK-T1-250	PTC	15(A1→D1)[a]	**3(H1→H3)**, 4(C1→C2), 14(A2) 2(A3→B, H1→H4)[a], **4(C7→E2)**[a], 5(B2, F, G1→G3)[a], 8(A1.2→E2)[a], 10(B5.3→C1), 11(B1.2 →E2)[a]	del 4 (*Taf12*, *Ythdf2*): 11.3% of cells del 4 (*Srm*): 25.5% of cells del 4 (*Trp73*): 22.8% of cells
TRK-T1-252	PTC		**4(C7→E2)**[a], 5(A1→C3.2)[a], 12(B3→C1)[a]	del 4 (*Taf12*, *Ythdf2*): 25.4% of cells
TRK-T1-254	PTC	8(D1→E2), 16(C4)	12(A2→B1), 13(B3), 14(A1→A3)	ND
TRK-T1-255	Hyperplasia		**14**(A1→A3, **E2.2→ter**) 2(C1.2→D)[a], 7(A1→A2, A3→C)[a], 8(A4→B1.2)[a]	ND
TRK-T1-256	PTC	3(C→E2), 11(A3.3, B1.3, C→D) 7(A1→F5)[a]	**4(E1→E2)**[a]	ND
TRK-T1-261	PTC	**1(C5→E2)**, 10(B2, C1→D1) 1(A1→D, E2→F, G2→H6)[a], 10(A1→D3)[a], 14(E4→E5)[a], 17(A3.3→B3, C)[a], 19(A)[a/c]	**3**(A1→A3, **H3→ter**), **4(D1→ter)** 3(A1→H4)[a], 4(A1→E2)[a], 5(A3→C2)[a], 9(A1→C)[a]	amp 1 (*Ptma*, *Pde6d*): 38.0% of cells amp 1 (*Twist2*): 43.5% of cells del 4 (*Taf12*, *Ythdf2*): 16.5% of cells del 4 (*Srm*): 23.3% of cells
TRK-T1-262	PTC	**1(C3→E2)**, 9(B→D), 10(C1), 13(A5→C1) 10(A1→D3)[a], 13(A1→D2.3)[a]	**3**(cen→A3, **F2→ter**), **4(C5→ter)**, 6(A1→A2) **3(A1→H4)**[a], **4**(**A1→C1**, **C3→E2**)[a], 5(A3→E1)[a], 7(A1→F5)[a], 11(B1.3→B4, C→E2)[a]	amp 1 (*Ptma*, *Pde6d*): 56.4% of cells amp 1 (*Twist2*): 59.7% of cells del 4 (*Taf12*, *Ythdf2*): 10.5% of cells
TRK-T1-263	PTC		1(H3→ter), **4**(C5, **C7→ter**), 11(E2→ter)	ND
TRK-T1-265	PTC	**1(C5→E1)** 7(A1→F5)[a]	**4(A1→E2)**[a]	amp 1 (*Ptma*, *Pde6d*): 26.2% of cells amp 1 (*Twist2*): 19.2% of cells
TRK-T1-268	PTC	15(A1→D1)[a], 19(A→tD3)[a]	**4(D3→ter)** 2(H1→H4)[a], **4(D2.3→E2)**[a], 6(A1→G3)[a], 7(A1→F5)[a], 12(A1.1→F2)[a]	del 4 (*Taf12*, *Ythdf2*): 76.3% of cells del 4 (*Srm*): 86.6% of cells del 4 (*Trp73*): 76.3% of cells
TRK-T1-270	PTC	3(F1→H2)[a]		ND
TRK-T1-024	Neoplasia		19(A→ter)[a]	ND
TRK-T1-028	Neoplasia		19(A→ter)[a]	ND
TRK-T1-033	Neoplasia	7(A1→F5)[a]	5(F→G1.1)[a], 19(A→ter)[a]	ND
TRK-T1-040	Hyperplasia	11(B5→C, D→E1)[a]		ND
TRK-T1-054	PTC	7(C, D1→ter)[a]		ND
TRK-T1-057	PTC	6(A1→G3)[a], 7(A1→F5)[a]	1(H5→H6)[a], **4(A1→E2)**[a], 5(A1→G3)[a], 9(A1→F4)[a], 11(A1→ E2)[a]	ND

Table 1 *continued*

Case	Histology	Copy number changes (CGH/aCGH[a])		FISH validation[b] (frequency of cells with aberrant FISH signals in tumour tissues)
		Gain	Loss	
TRK-T1-061	PTC			ND
TRK-T1-062	PTC		1(E4→F)[a], 5(F, G1→G3), 7(A2→B1)[a], 17(A3.3→B3)[a]	ND
TRK-T1-063	PTC		1(E4→F)[a]	ND
TRK-T1-066	PTC	16(A2→C4)[a]		ND
TRK-T1-068	PTC			ND
TRK-T1-070	PTC	5(D→G3)[a/c]	**4(D2.3→E2)**[a], 9(A1→F4)[a]	ND
TRK-T1-072	PTC	7(A1→F5)[a], 8(A1.2→B1.2)[a]	1(A1→H6)[a], **4(A1→E2)**[a], 5(A1→F)[a], 11(A1→E2)[a], 12(A1.1→F2)[a]	ND

aCGH, array CGH. Bold: recurrent CNA revealing consensus regions 1C4–E2.3 (10.0% of cases), 3H1–H3 (13.3%), 4D2.3–E2 (43.3%) and 14E4–E5 (6.7%). ND, not determined.
[a]Data from array CGH experiments.
[b]Comparison of frequencies in normal and tumour tissues, FDR-adjusted Fisher's exact test, FDR < 0.1.
[c]High level amplification: the GLAD algorithm (Hupe *et al.* 2004) considers probes called as gain and a log2-ratio greater than one as high-level amplifications.

DNA digestion. RNA concentration and purity were measured using a spectrophotometer (NanoDrop) and integrity of RNA was determined on a microfluidic chip using an Agilent Bioanalyser. mRNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR was carried out in an Applied Biosystems 7500 Fast System machine using exon-spanning gene-specific TaqMan assays with 40 cycles according to the manufacturer's protocols (Applied Biosystems). A TaqMan assay for Actin B was used as endogenous control for each measurement. Relative expression levels were calculated using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001).

Results

Recurrent CNA in *Trk-T1*-transgenic mice

We investigated 30 thyroid lesions from *Trk-T1* transgenics including four hyperplasias, three unspecified neoplasias and 23 thyroid carcinomas for genomic CNA by array CGH. We detected a recurrent gain of chromosome bands 1C4–E2.3 (10.0% of cases) and recurrent losses of chromosome bands 3H1–H3 (13.3%), 4D2.3–E2 (43.3%) and 14E4–E5 (6.7%) (Table 1).

Genes located in the altered chromosome regions were identified using the Ensembl database (www.ensembl.org) and genes that were published in association with cancer were selected as candidate genes (Table 2 and Fig. 1). The list includes the genes *Twist2*, *Ptma*, *Pde6d* (chromosome 1), *Slitrk5* (chromosome 14), *Bmpr1b*, *Pdlim5*, *Unc5c* (chromosome 3), and *Srm*, *Trp73*, *Ythdf2*, *Taf12* (chromosome 4). Analysis of the GO terms of these genes showed gene functions linked to cell differentiation and development, apoptosis and intracellular signalling. Copy number changes for *Twist2*, *Ptma*, *Pde6d*, *Slitrk5*, *Srm*, *Trp73*, *Ythdf2* and *Taf12* were validated in FISH experiments (Fig. 2A and B and Table 1).

Gene CNA in human post-Chernobyl PTC

Recently, gene CNA were identified in human PTC (Unger *et al.* 2008, Hess *et al.* 2011). While Hess *et al.* (2011) reported global genomic copy number changes of PTCs from young patients from Ukraine, the publication of Unger *et al.* (2008) also included data from adult cases and, therefore, conclusions on age-related changes can be drawn. In order to check whether genomic copy number changes that were found in the mouse model are also prevalent in human PTCs, the appropriate mouse regions were translated

Table 2 Comparison of altered regions in PTC from transgenic mice and humans

Murine chromosomal band	Human chromosomal band	Frequency of aberration (%) in PTC from young patients[a]	Frequency of aberration (%) in PTC from adults[b]	Candidate genes
1C4→E2.3	2q14.1–14.3	Gain (0)	Gain (0)	
	2q37.1–37.3	Gain (61.3)	Gain (0)[c]	*TWIST2*, *PTMA*, *PDE6D*
	5q21.1	Gain (7.5)	Gain (40.0)[c]	
	18q21.33–22.1	Gain (0)	Gain (0)	
3H1→H3	1p31.1–22.2	Loss (68.8)	Loss (50.0)	
	4q22.3	Loss (55.9)	Loss (0)[c]	*UNC5C*, *BMPR1B*, *PDLIM5*
4D2.3→E2[d]	1p36.32–35.3	Gain (66.7)	Gain (0)[c]	*YTHDF2*, *SRM*, *TAF12*, *TP73*
14E4→E5	13q31.2–33.1	Loss (67.7)	Loss (45.0)	*SLITRK5*

Between males and females and clinico-pathological parameters (lymph node metastasis (*N*), tumour size (T), histological variant) no differences were found for the human chromosomal bands.
[a]CNA in 93 PTC from young patients (data from Hess *et al.* (2011) and Unger *et al.* (2008)).
[b]CNA in 20 PTC from adults (data from Unger *et al.* (2008)).
[c]Significantly different from childhood cases with $P<0.001$.
[d]Consensus region derived from CNA of 30 murine thyroid tumours.

into homologous human regions using the Ensembl synteny database. Table 2 shows a comparison of syntenic aberrant regions in murine and human tumours. Noteworthy, most of the gene copy aberrations identified in the mouse model were confirmed in human cancer samples. Gains were reported on 2q37.1–37.3 (syntenic to gain 1C4–E2.3) in 61.3% of human PTC from young patients, on 5q21.1 (syntenic to gain 1C4–E2.3) in 7.5% of PTC from young patients and in 40.0% of PTC from adults and on 1p36.32–35.3 (syntenic to 4D2.3–E2) in 66.7% of PTC from young patients. On the other hand, losses on 1p31.1–22.2 (syntenic to loss 3H1–H3) were reported in 68.8% of PTC from young patients and in 50.0% of PTC from adults, on 4q22.3 (syntenic to 3H1–H3) in 55.9% of PTC from young patients and on 13q31.2–33.1 (syntenic to loss 14E4–E5) in 67.7% of PTC from young patients and in 45.0% of PTC from adults.

We sought to confirm the most frequent gain on chromosome 1 and one of the most frequent losses on chromosome 14 identified in the mouse model by FISH analysis of the corresponding syntenic regions on paraffin-embedded sections from human PTC samples. Therefore, we selected cases with DNA copy number gains on 2q37.1–37.3 (investigation of candidate genes *TWIST2*, *PTMA*, *PDE6D* from syntenic murine region on 1C4–E2.3) and cases with DNA copy number losses on 13q31.2–33.1 (investigation of candidate gene *SLITRK5* from syntenic murine band 14E4–E5) (Table 2). In the human genome *SLITRK5* is part of a gene cluster comprising three homologs which are likely of being functionally similar (Aruga & Mikoshiba 2003). Hence, we also investigated the copy number status of the two genes *SLITRK1* and *SLITRK6* which are the other members of the *SLITRK* gene cluster on chromosome 13q31.1–31.2 (Aruga *et al*. 2003). In order to define the copy number status (gain, normal or loss) of a particular case, frequencies of cells with aberrant FISH signals were compared between normal thyroid tissue (controls) and tumour tissue. The differences of frequencies were tested using Fisher's exact test. A P value <0.1 was accepted to reject the null hypothesis (same frequencies of aberrant cells in normal and tumour tissues) and to consider a case as significantly altered. As shown in Table 3 all investigated human PTCs ($n=12$) showed a gain of *PTMA* and *PDE6D* and the majority of cases also showed a gain of *TWIST2* (ten out of 12 cases; 83%). Moreover, in four out of 19 cases (21%) a deletion of *SLITRK5* was observed (Fig. 2C, D and E). Finally, four out of 19 cases (21%) showed a deletion of *SLITRK1* and three out of 19 cases (16%) a deletion of *SLITRK6*. Two cases (Table 3) showed a deletion of all *SLITRK* genes (case 9) or of two *SLITRK* genes (case 18) simultaneously.

Altered expression in human PTC samples

We characterised the mRNA expression of ten selected cancer-related genes. The genes *PDE6D*, *PTMA*, *BMPR1B*, *PDLIM5*, *UNC5C*, *SRM*, *TAF12*, *TP73*, *YTHDF2* and *SLITRK5* were selected from syntenic frequently altered chromosomal regions on 1p, 2q, 4q and 13q (Table 2) in human PTC. Due to the limited amount of RNA available a maximum of ten genes could be investigated by qRT-PCR in 67 human PTC cases and five normal thyroid tissues (Hess *et al*. 2011). We have selected these ten genes because they were equally distributed on the above-mentioned altered four chromosomal bands. Although *TWIST2* appeared

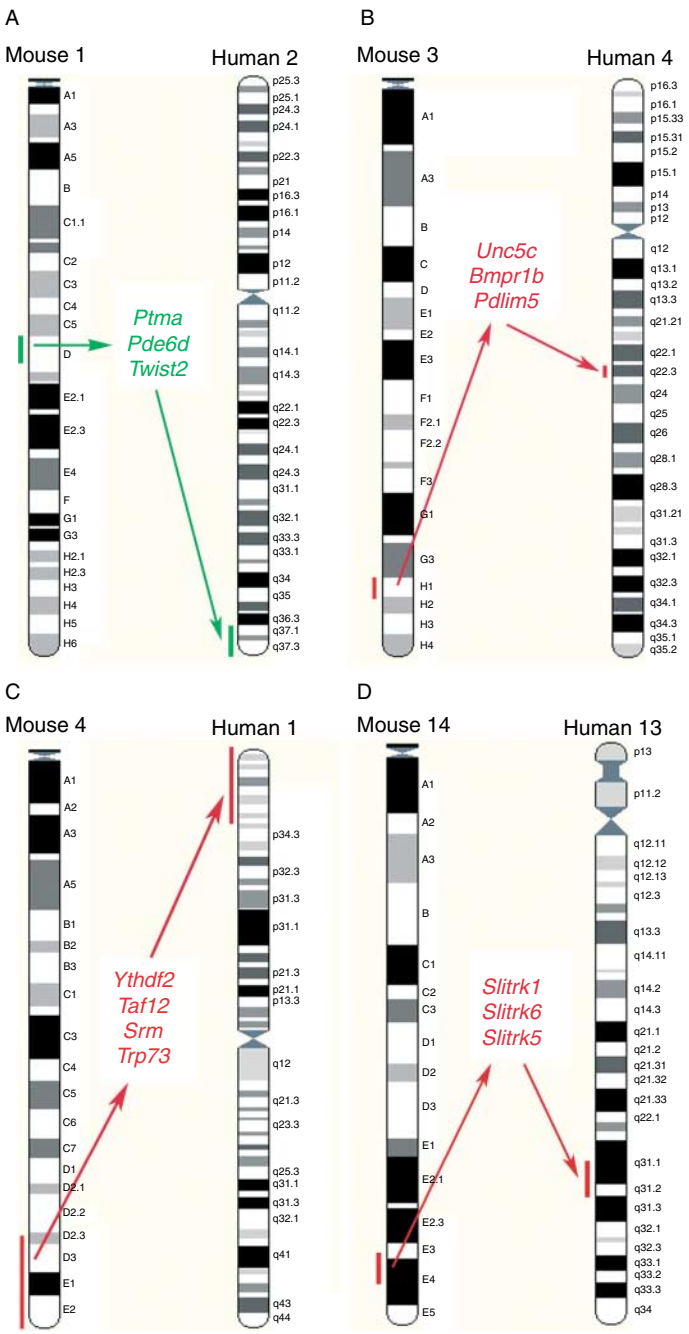

Figure 1 Overview of syntenic chromosomal regions in mouse and human. Chromosomal bands and genes representing gains are shown in green, bands and genes showing a loss are red. (A) Amplified syntenic chromosomal bands on mouse chromosome 1 and human chromosome 2 with candidate genes revealed by database-driven analysis. (B, C and D) Deleted syntenic chromosomal bands and genes that were revealed by database-driven analysis.

to be an interesting candidate gene as well, the above-mentioned material limitations and the fact that it was very closely located to *PTMA* and *PDE6D* within the same chromosomal band led to the exclusion of *TWIST2* from the gene expression analyses. The gene expression data are together with the available copy number data (55/67 cases; Hess *et al*. 2011) summarised in Supplementary Table 1 and Figure 1, see section on supplementary data given at the end of this article. As demonstrated in Fig. 3, the genes *UNC5C* and *TP73* showed a significantly elevated expression in tumours compared to normal tissues, and *SLITRK5*

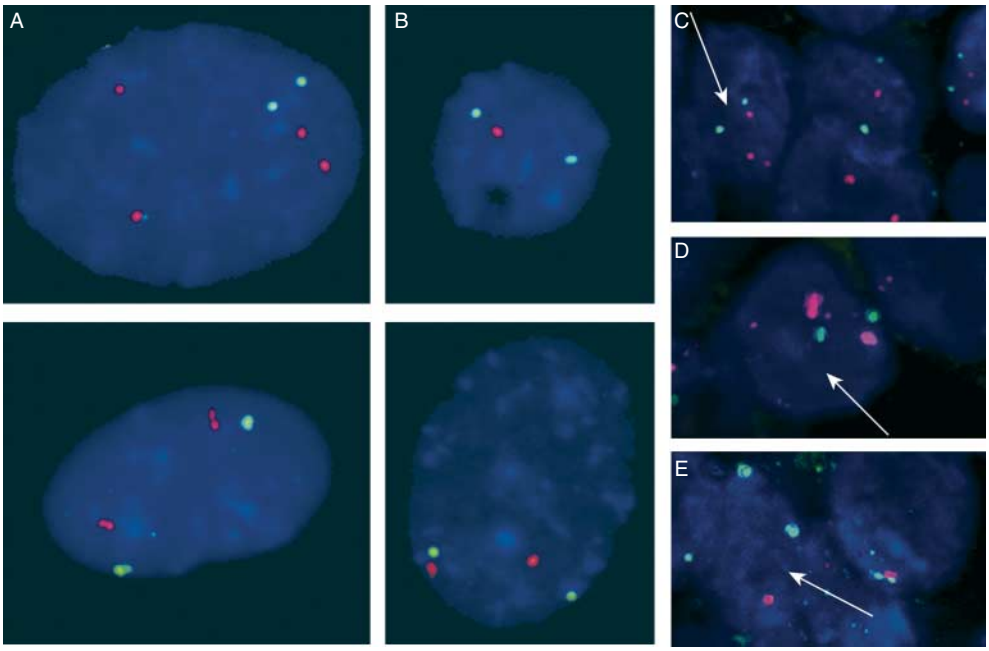

Figure 2 FISH analysis of CNA. (A and B) shows FISH analysis of murine cases (A) TRK-T1-261 and (B) TRK-T1-239. Reference FISH signals are labelled in green while gene-specific FISH signals are labelled in red. (A) A gain of *Ptma* and *Pde6d* and (B) a deletion of *Slitrk5* are shown (upper row of cells) in comparison to cells with unchanged FISH signals (lower row of cells). (C, D and E) shows FISH analysis on human PTC (C) for *TWIST2* showing gained red FISH signals in case 16, (D) for *PTMA* and *PDE6D* showing amplified FISH signals in case 20 and (E) for *SLITRK5* showing a deleted red FISH signal in case 9. Examples of aberrant cells are marked by arrows.

a significantly reduced gene expression in tumours compared to normal tissues ($P < 0.05$; Mann–Whitney *U* test, Fig. 3). Thus, for *SLITRK5* and *TP73* a robust alteration in gene expression was in accordance with the genomic CNA.

Discussion

Rearrangements of the tyrosine kinase-encoding proto-oncogenes *NTRK1* (*TRK-T*) and *RET* (*RET/PTC*) have been found in human PTC (Bongarzone *et al*. 1989, Nikiforov 2008, Knauf & Fagin 2009, Greco *et al*. 2010). Transgenic mouse strains proved that these oncogenes are able to initiate thyroid tumourigenesis (Jhiang *et al*. 1996, Santoro *et al*. 1996, Powell *et al*. 1998, Russell *et al*. 2000). However, genetic lesions that cooperate with these frequent gene alterations of the MAP kinase pathway in thyroid carcinogenesis have not been identified so far. To tackle this issue, we have selected the *Trk-T1* mouse model in our study in order to investigate clonal gene changes that may occur somatically to cooperate with the *Trk-T1* transgene. Thus, initially we have investigated CNA in 30 thyroid tumours from *Trk-T1* transgenic mice and found recurrent genomic CNA on chromosomes 1C4–E2.3 (10%), 3H1–H3 (13.3%), 4D2.3–E2 (43.3%) and 14E4–E5 (6.7%). A validation of potentially affected candidate genes located in these chromosomal regions was exemplarily performed for *Ptma*, *Pde6d*, *Twist2* on murine chromosome 1, *Taf12*, *Ythdf2*, *Srm*, *Tp73* on murine chromosome 4 and *Slitrk* genes on murine chromosome 14 by FISH.

We further asked whether gene copy alterations that have been detected in thyroid tumours from *Trk-T1* transgenic mice were also involved in human thyroid carcinomas. Therefore, gene copy number and mRNA gene expression of these candidate genes were investigated in human PTC samples. From our data, we found that alterations of *TWIST2*, *PTMA*, *PDE6D* (murine chromosome 1D, human chromosome 2q37), *UNC5C* (murine chromosome 3H1, human chromosome 4q22.3), *TP73* (murine chromosome 4D2.3, human chromosome 1p36.32) and *SLITRK5* (murine chromosome 14E4, human chromosome 13q31.2) were located within the most frequently altered chromosomal regions in human thyroid carcinomas (murine CNA on 1C4–E2.3, 3H1–H3, 4D2.3–E2, 14E4–E5 corresponding to human CNA on 2q37.1–37.3, 4q22.3, 1p36.32–35.3 and 13q31.2–33.1 respectively) (Table 2). It has been shown in a recent publication on a homogeneous, age-matched tumour set of exposed and non-exposed PTC (Hess *et al*. 2011)

Table 3 FISH on PTC with CNA on human chromosome 2q37.1–37.3 and 13q31.1–31.2

Case number	Sex	Age at surgery (years)	Histological variant	TNM	RET/PTC rearrangement/BRAF mutation (V600E)	Copy number alterations[a]	FISH aberrant cells (%)		
1	Female	51	Mixed (FP)	T1N0Mx	Negative/negative	Gain 2q37.1–37.3	*TWIST2* (19.8)[b]	*PTMA/PDE6D* (63.0)[b]	
2	Female	34	Classic	T3N1aMx	NA/positive	Gain 2q37.1–37.3	*TWIST2* (6.9)[b]	*PTMA/PDE6D* (59.4)[b]	
3	Female	30	Classic	T1N1aMx	Negative/negative	Gain 2q37.1–37.3	*TWIST2* (20.0)[b]	*PTMA/PDE6D* (54.9)[b]	
4	Male	11	Follicular	T4N1M0	NA/negative	Gain 2q37.1–37.3	*TWIST2* (5.7)	*PTMA/PDE6D* (73.3)[b]	
5	Male	61	Mixed (FS)	T3N0Mx	Negative/positive	Gain 2q37.1–37.3	*TWIST2* (20.6)[b]	*PTMA/PDE6D* (88.1)[b]	
6	Female	32	Classic	T2N1Mx	Negative/positive	Gain 2q37.1–37.3	*TWIST2* (11.9)[b]	*PTMA/PDE6D* (69.3)[b]	
7	Male	41	Mixed (PS)	T3N1Mx	Negative/positive	Gain 2q37.1–37.3	*TWIST2* (15.8)[b]	*PTMA/PDE6D* (79.4)[b]	
8	Female	14	Mixed (FS)	T3N1abM1	Positive[c]/negative	Loss 13q31.1–31.2	*SLITRK1* (17.8)	*SLITRK5* (7.9)	*SLITRK6* (19.5)
9	Female	14	Solid	T3N1abM0	Positive[d/c]/negative	Loss 13q31.1–31.2	*SLITRK1* (26.7)[b]	*SLITRK5* (27.0)[b]	*SLITRK6* (28.7)[b]
10	Female	14	Follicular	T3N1aM0	Positive[d]/negative	Loss 13q31.1–31.2	*SLITRK1* (17.5)	*SLITRK5* (17.8)	*SLITRK6* (19.8)
11	Male	15	Mixed (SF)	T1N1aM0	Negative/negative	Loss 13q31.1–31.2	*SLITRK1* (14.9)	*SLITRK5* (13.9)	*SLITRK6* (15.4)
12	Female	16	Classic	T2N0M0	Negative/positive	Loss 13q31.1–31.2	*SLITRK1* (15.7)	*SLITRK5* (25.0)[b]	*SLITRK6* (14.0)
13	Female	18	Follicular	T3N1aM0	Negative/negative	Loss 13q31.1–31.2	*SLITRK1* (21.0)	*SLITRK5* (13.5)	*SLITRK6* (17.5)
14	Female	17	Classic	T3N1aM0	Negative/negative	Loss 13q31.1–31.2	*SLITRK1* (5.0)	*SLITRK5* (19.0)	*SLITRK6* (17.0)
15	Male	16	Follicular	T3N1aM0	Negative/negative	Loss 13q31.1–31.2	*SLITRK1* (6.5)	*SLITRK5* (20.4)	*SLITRK6* (17.2)
16	Female	13	Follicular	T1N0M0	Positive[d]/negative	Gain 2q37.1–37.3	*TWIST2* (17.6)[b]	*PTMA/PDE6D* (93.1)[b]	
17	Female	18	Solid	T2N1aM0	Positive/negative	Loss 13q31.1–31.2	*SLITRK1* (9.9)	*SLITRK5* (16.0)	*SLITRK6* (12.7)
18	Male	15	Classic	T1N0M0	Negative/NA	Loss 13q31.1–31.2	*SLITRK1* (20.6)[b]	*SLITRK5* (12.0)	*SLITRK6* (28.4)[b]
19	Male	15	Mixed (FS)	T3N1abM0	Negative/NA	Loss 13q31.1–31.2	*SLITRK1* (25.5)[b]	*SLITRK5* (20.7)	*SLITRK6* (15.1)
20	Male	17	Classic	T1N0M0	Negative/negative	Loss 13q31.1–31.2	*SLITRK1* (20.8)	*SLITRK5* (9.3)	*SLITRK6* (15.1)
						Gain 2q37.1–37.3	*TWIST2* (5.8)	*PTMA/PDE6D* (94.0)[b]	
21	Female	17	Mixed (PS)	T1N0M0	Negative/positive	Loss 13q31.1–31.2	*SLITRK1* (16.8)	*SLITRK5* (13.0)[b]	*SLITRK6* (20.7)
22	Male	13	Solid	T3N0M0	Positive[c]/negative	Loss 13q31.1–31.2	*SLITRK1* (16.8)	*SLITRK5* (21.7)	*SLITRK6* (17.8)
23	Female	12	Solid	T2N0M0	NA/negative	Gain 2q37.1–37.3	*TWIST2* (7.7)[b]	*PTMA/PDE6D* (93.1)[b]	
24	Female	35	Classic	T4bN0M0	Negative/negative	Loss 13q31.1–31.2	*SLITRK1* (15.9)	*SLITRK5* (9.9)	*SLITRK6* (25.7)[b]
25	Female	35	Classic	T4aN1aM0	Positive[d]/positive	Loss 13q31.1–31.2	*SLITRK1* (22.5)[b]	*SLITRK5* (19.0)	*SLITRK6* (25.0)
26	Female	32	Follicular	T4bN1aM0	Positive[d]/negative	Loss 13q31.1–31.2	*SLITRK1* (12.6)	*SLITRK5* (23.0)[b]	*SLITRK6* (14.5)
27	Male	32	Classic	T4aN1aM0	Positive[d]/negative	Loss 13q31.1–31.2	*SLITRK1* (18.1)	*SLITRK5* (6.8)	*SLITRK6* (6.7)
28	Female	36	Solid	T4N1bMx	Positive[d]/negative	Loss 13q31.1–31.2	*SLITRK1* (19.4)	*SLITRK5* (13.9)	*SLITRK6* (8.0)
29	Female	32	Follicular	T2aN0Mx	Positive/negative	Gain 2q37.1–37.3	*TWIST2* (9.2)[b]	*PTMA/PDE6D* (10.0)[b]	
30	Female	31	Solid	T4aN1bMx	Negative/positive	Gain 2q37.1–37.3	*TWIST2* (44.1)[b]	*PTMA/PDE6D* (28.6)[b]	

FP, follicular-papillary; FS, follicular–solid; PS, papillary–solid; SF, solid–follicular variant; NA, not available. Sex, age median, histological variants, TNM status are not significantly different for the two CNA gain 2q37.1–37.3 and loss 13q31.1–31.2 (Wilcoxon rank test for age, χ^2 test for the other parameters, $P>0.05$).

[a]Copy number data from Unger *et al.* (2008) and Hess *et al.* (2011).

[b]Comparison of frequencies in normal and tumour tissues, FDR-adjusted Fisher's exact test, FDR <0.1.

[c]RET/PTC3 rearrangement.

[d]RET/PTC1 rearrangement.

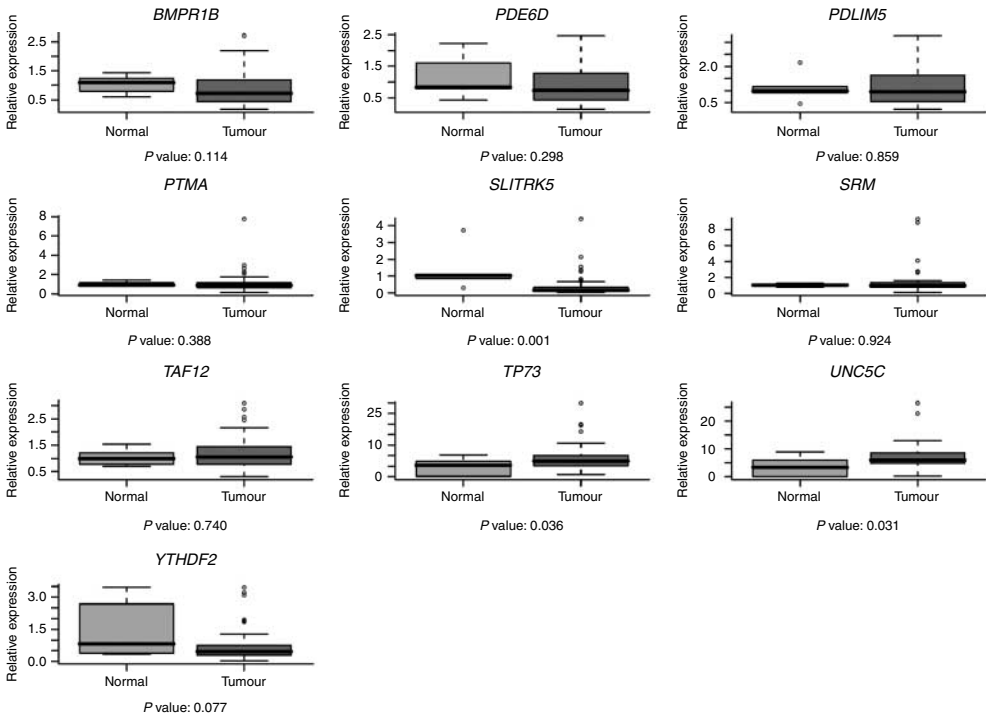

Figure 3 Expression levels of candidate genes in normal thyroid tissue compared to PTC. The box plot for *SLITRK5* shows a significant down-regulation of this gene in tumour tissues ($P<0.05$), while a significant over-expression of the genes *TP73* and *UNC5C* is visible in tumour tissues.

that these syntenic CNA are not associated with exposure to ionising radiation. A de-regulated mRNA expression could be confirmed for the candidate genes *UNC5C*, *TP73* and *SLITRK5* in 67 PTC compared with normal thyroid tissues, thus, suggesting an impact of the genes in thyroid tumourigenesis in particular for *TP73* and *SLITRK5* for which the de-regulated gene expression (up-regulation for *TP73*; down-regulation for *SLITRK5*) was in accordance with the genomic CNA (gain of *TP73*; loss of *SLITRK5*). For a deeper mechanistic insight we have compared genomic copy number data and relative gene expression of the ten candidate genes in individual cases (Supplementary Figure 1). The resulting bean plots show that for individual cases the copy number status is in accordance with a de-regulated gene expression. However, in a sub-group of cases the de-regulated expression cannot be explained by a genomic CNA. Thus, from this comparison it is apparent that an inter-individual variation in the regulation of gene expression may exist.

The observation that there is no absolute correlation of mRNA expression with the observed CNA is not surprising because it is known from a study by Jarvinen *et al*. (2008) that the extent of correlation between CNA and corresponding mRNA expression is highly variable due to other factors regulating gene expression such as miRNA expression. A surprising result from our study is that genomic CNA are very similar between thyroid neoplasms from *Trk-T1* transgenic mice and human PTC with predominant involvement of *RET/PTC* rearrangements or *BRAF* mutations (Table 3). It has been shown in an earlier study (Unger *et al*. 2008) that major differences in CNA in PTC depending on the involvement of alterations in the MAP kinase pathway exist. The detected CNA in our study might either interact with the MAP kinase pathway or in PTC cases negative for *BRAF* mutations and *RET/PTC* rearrangements indicate alternative routes of tumour development involving different pathways.

Another interesting finding of the present study is the involvement of specific CNA in different age groups of human PTC. While the DNA gains on 2q37.1–37.3 and 1p36.32–35.3 and the DNA loss on 4q22.3 are exclusively observed in PTC from young patients (Unger *et al*. 2008, Hess *et al*. 2011) the DNA gain on 5q21.1 is more frequently ($P<0.001$) observed in PTC from adults (Unger *et al*. 2008) and thus pointing to different age-related genetic routes of tumour development in these patients.

The most prominent alterations in mice were copy number gain on murine chromosome 1C4–E2.3 and the copy number loss on murine chromosome 14E4–E5 because the syntenic regions on 2q37.1–37.3 and 13q31.2–33.1 were most frequently altered in human PTC from young patients. The genes *TWIST2*, *PTMA* and *PDE6D* map to chromosomal band 2q37. An association with cancer has been reported for prothymosin-α (*PTMA*) that is involved in chromatin de-condensation (Karetsou *et al*. 2004). An up-regulation of this gene has been observed in human gastric cancer (Leys *et al*. 2007) and has been described as a prediction marker for radiosensitivity in rectal cancer (Ojima *et al*. 2007). Phosphodiesterase 6D (*PDE6D*) interacts with *HRAS* (Hanzal-Bayer *et al*. 2002, Nancy *et al*. 2002). *HRAS* itself is frequently involved in thyroid carcinogenesis (Nikiforov 2008). *TWIST2* over-expression overcomes pre-mature senescence of epithelial cells and cooperates with oncoproteins (such as *RAS* and *ERBB2*) thereby promoting epithelial–mesenchymal transition and invasiveness (Ansieau *et al*. 2008). GO-term enrichment analysis also revealed a significant association of *TWIST2* with biological processes such as cell differentiation and cellular development that are assumed to be important in tumour development. Of note, the highly related *TWIST1* was recently found to be up-regulated in anaplastic thyroid cancer (Salerno *et al*. 2011). As far as the copy number loss on 13q31.2–33.1 is concerned, the *SLITRK5* gene and the linked *SLITRK* gene cluster appear to be the most promising candidates because no other known gene is located within this chromosomal band. Thus, several *SLITRK* family members may be involved in human PTC development while only *SLITRK5* seems to be involved in mouse tumours, because it is the only member located within murine 14E4–E5, while *SLITRK1* and *SLITRK6* are located on the chromosomal band 14E3 which is not affected by the observed deletion in *Trk-T1* transgenic thyroid tumours. Members of the *SLITRK* family are integral membrane proteins with homology to the *SLIT* family in the N-terminal extracellular domain and to the *TRK* neurotrophin receptors in the C-terminal intracellular domain (Aruga & Mikoshiba 2003, Aruga *et al*. 2003). While the biological function of *SLITRK* genes is not elucidated so far, it is interesting to note that these genes are expressed in haematopoietic stem cells and leukaemia and are novel markers of haematopoietic stem and progenitor cells (Milde *et al*. 2007).

In conclusion, molecular cytogenetic analyses of thyroid tumours from the *Trk-T1* transgenic mouse model have revealed four recurrent CNA on murine chromosomes 1C4–E2.3, 3H1–H3, 4D2.3–E2 and 14E–E5 that point to *Ptma*, *Pde6d*, *Twist2*, *Taf12*, *Ythdf2*, *Srm*, *Tp73* and *Slitrk* genes as novel candidate genes in thyroid tumourigenesis. Syntenic human chromosomal regions are frequently affected by CNA in human PTC indicating a role of these candidate genes in human carcinogenesis as well. Moreover, some specific CNA can be attributed to different age groups of PTC patients indicating age-dependent routes of tumour development. Thus, this mouse model provides a powerful tool to unravel the molecular mechanisms of thyroid tumourigenesis.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0387.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the EU grant FI6R-CT-2006-36495 (GENRISK-T). This study has also been in part supported by a grant of the Italian Association for Cancer Research (AIRC) to M Santoro.

Acknowledgements

We thank the International Pathology Panel of the Chernobyl Tissue Bank for confirmation of diagnosis: Dr A Abrosimov, Prof. T I Bogdanova, Prof. V LiVolsi, Prof. M Ito, Prof. J Rosai and Prof. E D Williams. We further thank F Basolo for non-radiation associated thyroid tumour RNAs and J Rothstein for the *Tg-Trk-T1* mice. We thank A Greco and M A Pierotti for the *Trk-T1* molecular construct. The excellent technical assistance of E Konhäuser is gratefully acknowledged.

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Received in final form 21 March 2012
Accepted 23 March 2012
Made available online as an Accepted Preprint
26 March 2012