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Original Article

ANKRD26-RET - A novel gene fusion involving *RET* in papillary thyroid carcinoma

Julia Isabelle Staubitz[a,*], Thomas Johannes Musholt[a], Arno Schad[b], Erik Springer[b], Hauke Lang[c], Krishnaraj Rajalingam[d], Wilfried Roth[b], Nils Hartmann[b]

[a] *Section of Endocrine Surgery, Department of General, Visceral and Transplantation Surgery, University Medical Center, Johannes Gutenberg University Mainz, Langenbeckstraße 1, D-55131 Mainz, Germany*
[b] *Institute of Pathology, University Medical Center, Johannes Gutenberg-University Mainz, Langenbeckstraße 1, D-55131 Mainz, Germany*
[c] *Department of General, Visceral and Transplantation Surgery, University Medical Center, Johannes Gutenberg University Mainz, Langenbeckstraße 1, D-55131 Mainz, Germany*
[d] *Cell Biology Unit, University Medical Center, Johannes Gutenberg-University Mainz, Langenbeckstraße 1, D-55131 Mainz, Germany*

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ABSTRACT

Background: Rearrangements of *RET* are drivers of oncogenesis, traceable in different cancer types as papillary thyroid carcinoma (PTC), non-small cell lung cancer, colorectal or breast cancer. Anchored multiplex PCR based next-generation sequencing (NGS) can detect *RET* rearrangements involving previously unknown partner genes.
Methods: A sample of PTC underwent NGS, following detection of *RET* rearrangement by fluorescence in situ hybridization. Expression analysis of *ANKRD26* and *RET* was performed for the tumor harboring *ANKRD26-RET*, for corresponding normal thyroid tissue and PTC tumors with representative genetic alterations (*BRAFV600E, CCDC6-RET*), complemented by a comparative search in the "UniProt" database.
Results: NGS analysis resulted in the discovery of the fusion *ANKRD26-RET. ANKRD26* mRNA was expressed in all PTC tumors (*ANKRD26-RET, BRAFV600E, CCDC6-RET*) and in normal thyroid tissue, whereas *RET* mRNA was detected only in the tumors with *RET* rearrangement. On protein level, *ANKRD26-RET* combines the RET tyrosine kinase to ankyrin repeat and coiled-coil domains.
Conclusions: *ANKRD26-RET* is a novel rearrangement of the *RET* gene, associated with *RET* expression in thyroid tissue. The result is a fusion of the RET tyrosine kinase to prominent protein-protein interaction motifs. Further studies are required to investigate the influence of different *RET* rearrangements on metastasis and disease-free survival in PTC.

Introduction

Since the 1990s, rearrangements of the *RET* gene were discovered as molecular alterations responsible for tumorigenesis in different cancer types. In approximately one third of papillary thyroid carcinoma cases, *RET* rearrangements are detectable, with a variation depending on geographical background and the applied method of assessment [1–4]. In 1–2% of non-small cell lung cancer, *RET* rearrangements are detected [5]. Moreover, *RET* rearrangements were reported in breast cancer, colorectal cancer and other tumor entities (Table 1).

The proto-oncogene *RET* ("rearranged during transfection") encompasses 21 exons. It is located on chromosome 10q11.21. *RET* encodes for a receptor tyrosine kinase [6,7]. Activation of the tyrosine kinase influences the regulation of RAS-MAPK and PI3K-AKT cascades, which are essentially involved in the regulation of cell survival, differentiation and proliferation [8–12]. The physiological activation of the RET tyrosine kinase is transacted by glial cell line-derived neurotrophic factor (GDNF)-family ligands, which - in presence of GDNF-family receptor-α proteins - bind to the extracellular receptor domain of RET, leading to the formation of a symmetric dimer complex. In brief, an autophosphorylation of selected tyrosine residues at the intracellular kinase region of RET occur, which contributes to the activation of this kinase. Depending on the phosphorylation site, different pathways can be activated, partly mediated by the binding of adaptor proteins and enzymes including SHC, FRS2, SRC and PLCG [8]. In case of *RET* rearrangement, regulatory regions, deriving from partner genes, can exert a significant influence on the expression of *RET* and its tyrosine kinase activity [13]. Oncogenic rearrangement can result in

* Corresponding author.
E-mail address: julia.staubitz@unimedizin-mainz.de (J.I. Staubitz).

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Table 1
Fusion partner genes of *RET*-rearrangements in different cancer types.

	Partner gene	Cancer type	Year	First Description
1	*CCDC6 (H4)*	Thyroid carcinoma		
		Papillary thyroid carcinoma	1990	Grieco et al. [15]
		Huerthle cell carcinoma	2002	Chiappetta et al. [56]
		Anaplastic thyroid carcinoma	2013	Guerra et al. [57]
		Non-small cell lung cancer	2012	Takeuchi et al. [24]
				Wang et al. [5]
		Colon adenocarcinoma	2014	Stransky et al. [13]
2	*PRKAR1A*	Papillary thyroid carcinoma	1992	Lanzi et al. [17]
3	*NCOA4 (RFG, ELE)*	Thyroid carcinoma		
		Papillary thyroid carcinoma	1994	Santoro et al. [18]
				Bongarzone et al. [16]
		Huerthle cell carcinoma	2003	Musholt et al. [58]
		Non-small cell lung cancer	2012	Wang et al. [5]
		Colorectal cancer	2015	Le Rolle et al. [59]
		Salivary gland intraductal carcinoma	2018	Skalova et al. [60]
4	*GOLGA5*	Papillary thyroid carcinoma	2000	Rabes et al. [19]
		Spitzoid neoplasm	2014	Wiesner et al. [32]
5	*TRIM24 (TIF1, HTIF)*	Papillary thyroid carcinoma	2000	Rabes et al. [19]
6	*TRIM33 (RFG7)*	Papillary thyroid carcinoma	2000	Rabes et al. [19]
		Non-small cell lung cancer	2013	Drilon et al. [25]
7	*KTN1*	Papillary thyroid carcinoma	2000	Salassidis et al. [21]
8	*RFG8*	Papillary thyroid carcinoma	2000	Klugbauer et al. [61]
9	*PCM1*	Papillary thyroid carcinoma	2000	Corvi et al. [62]
10	*TRIM27 (RFP)*	Papillary thyroid carcinoma	2003	Saenko et al. [63]
		Salivary gland intraductal carcinoma	2018	Skalova et al. [60]
11	*ELKS*	Papillary thyroid carcinoma	2005	Liu et al. [64]
12	*HOOK3*	Papillary thyroid carcinoma	2007	Ciampi et al. [65]
13	*KIF5B*	Non-small cell lung cancer	2012	Kohno et al. [23]
				Takeuchi et al. [24]
		Spitzoid neoplasm	2014	Wiesner et al. [32]
14	*ACBD5*	Lung cancer	2012	Lipson et al. [66]
15	*FGFR1OP*	Chronic myelomonocytic leukemia	2012	Ballerini et al. [30]
		Acute myeloid leukemia	2014	Bossi et al. [31]
16	*BCR*	Chronic myelomonocytic leukemia	2012	Ballerini et al. [30]
17	*TBL1XR1*	Papillary thyroid carcinoma	2014	Stransky et al. [13]
18	*FKBP15*	Papillary thyroid carcinoma	2014	Stransky et al. [13]
19	*ERC1*	Breast cancer	2014	Stransky et al. [13]
20	*AKAP13*	Papillary thyroid carcinoma	2014	Stransky et al. [13]
21	*SPECC1L*	Papillary thyroid carcinoma	2014	Stransky et al. [13]
21	*KIAA1468*	Non-small cell lung cancer	2014	Nakaoku et al. [27]
		Papillary thyroid carcinoma	2018	Staubitz et al. [43]
22	*RUFY2*	Non-small cell lung cancer	2014	Zheng et al. [67]
		Papillary thyroid carcinoma	2018	Staubitz et al. [43]
23	*CUX1*	Non-small cell lung cancer	2014	Lira et al. [26]
24	*MYH13*	Medullary thyroid carcinoma	2015	Grubbs et al. [68]
25	*MYO5C*	Non-small cell lung cancer	2016	Lee et al. [69]
26	*ANK3*	Thyroid carcinoma	2016	Chai et al. [70]
27	*CLIP1*	Non-small cell lung cancer	2016	Drilon et al. [71]
28	*KIAA1217*	Non-small cell lung cancer	2016	Lee et al. [28]
29	*AFAP1L2*	Papillary thyroid carcinoma	2017	Iyama et al. [72]
30	*PPFIBP2*	Papillary thyroid carcinoma	2017	Iyama et al. [72]
31	*PICALM*	Non-small cell lung cancer	2017	Gautschi et al. [73]
32	*EPHA5*	Non-small cell lung cancer	2017	Gautschi et al. [73]
33	*RRBP1*	Colorectal Cancer	2017	Kloosterman et al. [74]
34	*UEVLD*	Papillary thyroid carcinoma	2017	Lu et al. [75]
35	*SQSTM1*	Papillary thyroid carcinoma	2017	Kato et al. [76]

a constitutive activation of the encoded *RET* receptor tyrosine kinase.

In the field of papillary thyroid carcinoma, *RET* rearrangements received the acronym "RET/PTCx", which were numbered consecutively according to their first description in the literature [14]. The rearrangement "RET/PTC1" involves the 5′ donor gene *CCDC6* [15]. "RET/PTC2" describes a rearrangement of *RET* to *PRKAR1A*, whereas "RET/PTC3" and "RET/PTC4" refer to rearrangements of *RET* to *NCOA4* [16–18]. Similarly, in "RET/PTC5", *RET* is fused to *GOLGA5*, in "RET/PTC6" to *TRIM24*, in "RET/PTC7" to *TRIM33*, in "RET/PTC8" to *KTN1* and in "RET/PTC9" to *RFG8* [19–21]. Nowadays, due to the multiplicity of RET/PTC rearrangements and their appearance in cancer types other than PTC, a nomenclature according to the involved genes is applied [22]. In the field of non-small cell lung cancer, rearrangements of *RET* to *KIF5B, CCDC6, NCOA4, TRIM33, CUX1, KIAA1468* and *KIAA1217* were reported [5,23–28]. In colon adenocarcinoma, *RET* fusions involving *CCDC6* and *NCOA4* were observed, whereas in breast cancer the rearrangement *ERC1-RET* was detected [13,29]. Also, in other cancer types as leukemia (*BRC-RET, FGFR1OP-RET*) and tumors of the skin (*GOLGA5-RET, KIF5B-RET*), *RET* fusion oncogenes were described as the underlying molecular alterations [30–32]. With the use of modern next-generation sequencing methods becoming more frequent in the last years, an increasing number of novel *RET* rearrangements was reported (Table 1).

For the treatment of *RET* rearrangement positive cancer, different multikinase inhibitors were applied in the clinic - so far, however, with limited long-term success [33,34]. More specific inhibitors are subject of ongoing developments as represent possible tools for targeted therapies in the future. The success of these

future therapies will depend on a comprehensive understanding of the landscape of *RET* rearrangements and their effect on protein-protein interactions.

Material and methods

Material

Tumor tissue was collected from a 78-year-old male patient, who underwent total thyroidectomy and central lymph node dissection for papillary thyroid carcinoma, follicular variant (Fig. 1).

The tumor sample was selected after previous exclusion of *BRAFV600E* mutation and RET/PTC1 (*CCDC6-RET*) fusion, as described by Musholt et al. [35]. Further, the presence of a *RET/PTC* rearrangement was indicated by fluorescence in situ hybridization (FISH), "ZytoLight SPECT RET Dual Color Break Apart Probe" (ZytoVison GmbH, Bremerhaven, Germany) [36].

Methods

Tumor tissue was retrieved from 4 µm formalin-fixed paraffin-embedded (FFPE) tissue slices (80% tumor cells). Extraction of DNA/RNA was performed using Maxwell RSC FFPE Kit (Promega, Wisconsin, USA) after manual microdissection.

Next-generation sequencing

Targeted next-generation sequencing was performed using Archer VariantPlex and Archer FusionPlex solid tumor kit (Archer, Boulder, Colorado, USA) according to the manufacturer's instructions. For VariantPlex, DNA libraries are generated by two polymerase chain reactions (PCR). Libraries are uniquely tagged with a specific combination of two indices during library preparation. The FusionPlex library preparation protocol includes the generation of cDNA from RNA prior to the aforementioned steps. The combination of the VariantPlex and FusionPlex protocol allows for an analysis of potential mutational events in over 60 genes. Furthermore, unknown gene fusions involving one known gene partner (e. g. *RET*) included in the gene panel can be identified. The MiSeq System (Illumina, San Diego, California, USA) was used for library sequencing. Data was demultiplexed and aligned to the human reference genome hg19. The output (FASTQ format) was processed using Archer Analysis Software (Archer, Boulder, Colorado, USA). To eliminate false positive results, filters for the detection of variants were used as follows: depth ≥ 100; allele frequency ≥10%. In-frame fusions were considered only. Called variants from the Cosmic (v65) scientific database were integrated by the software "Archer Analysis".

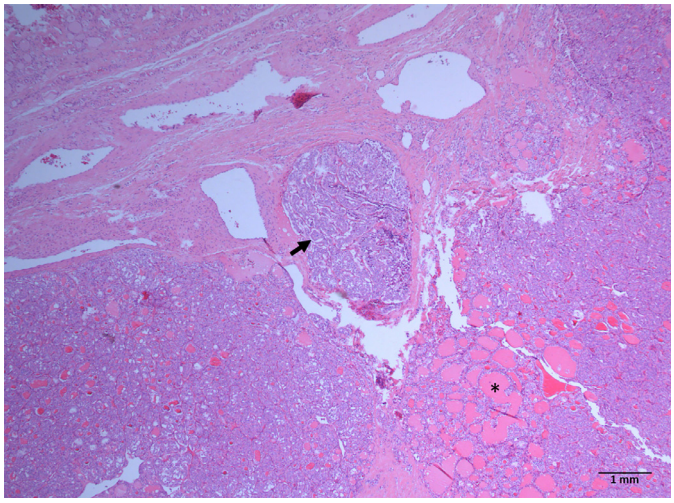

Fig. 1. Histological image of the index patient tissue harboring *ANKRD26-RET*. Histological image of the index patient harboring *ANKRD26-RET*: a follicular variant papillary thyroid carcinoma. Condensed colloid in irregularly developed follicular structures is apparent (*). The partly atrophic epithelium forms typical papillae (arrow). Capsular invasion is evident. Hematoxylin and eosin stain.

Confirmation of ANKRD26-RET

The newly identified fusion gene *ANKRD26-RET* was analyzed in the extracted tumor RNA and in the RNA derived from corresponding normal thyroid tissue of the same patient (Maxwell RSC FFPE Kit, Promega), using reverse transcription polymerase chain reaction (RT-PCR). cDNA was generated via reverse transcription (Random primers (C1181, Promega, Wisconsin, USA), 70 °C for 5 min; Mastermix (M-MLV Reverse Transcriptase M1701, Promega, Wisconsin, USA; RNAsin Plus RNAse inhibitor N2615, Promega, Wisconsin, USA; dNTP set 100 mM, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 40° for 60 min). For fusion-specific amplification, the following primers were used: ANKRD26_Ex29_fw2 (TCCATGGAGATTTAAAAACTAGTCAA) and RET_Ex12_rv1 (TGTACCCTGCTCTGCCTTTC; biomers.net GmbH, Ulm, Germany). 2.0 µl of cDNA template was processed with the Fast Cycling PCR Mix (Qiagen, Venlo, Netherlands). The PCR program encompassed the following temperatures: 95 °C 5 min, 96° 5 s, 52° 5 s, 68° 7 s, 72° 1 min, 35 cycles, reaction volume 20 µl. For this combination of primers, a sequence length of 211 bp was expected and analyzed in gel electrophoresis (2% agarose gel with ethidium bromide).

Finally, Sanger sequencing was carried out by GenomeLab DTCS Quick Start Kit (Beckman Coulter life sciences, Brea, USA) using the primer RET_Ex12_rv2: TGTACCCTGCTCTGCCTTTC (biomers.net GmbH, Ulm, Germany); PCR program 96 °C 20 s, 50° 20 s, 60° 4 min, 30 cycles. Beckman Coulter CEQ8000 analyzer and software was used for analysis.

Expression analysis of ANKRD26 and RET

Three PTC samples were analyzed: the tumor tissue of the present case harboring *ANKRD26-RET* ("T1"), and tumor samples harboring typical genetic alterations in PTC (*BRAFV600E* ("T2") and *CCDC6-RET* ("T3")). Also, normal thyroid tissue of these 3 patients underwent expression analysis.

RNA extraction and generation of cDNA was performed as described above. The protocol for analysis was based on primer combinations ANKRD26ex32fw1, GCATGGAGAACTACTTGAGCA + ANKRD26ex32rv1, CCAGATTCCAATTCAGCAGCA (expected amplicon length 91 bp, expression of *ANKRD26*), RETex14fw1, GGCAGATCTCACAGGGGATG + RETex15rv1, CTTCATCTTCCGCCCCTCAG (resulting amplicon length 101 bp, expression of *RET*) and ANKRD26ex29fw2, TCCATGGAGATTTAAAAACTAGTCAA + RETex12rv2, TGTACCCTGCTCTGCCTTTC (resulting amplicon length 186 bp, expression of breakpoint region in *ANKRD26-RET*). For amplification, the following cycling protocol was used: 95 °C for 15 min; 94 °C for 30 s, 52° for 90 s and 72° for 45 s, 6 cycles; 94 °C for 30 s, 56° for 30 s, 29 cycles. A total of 2.1 µl of cDNA was processed, 1 µl of each primer for aforementioned combinations (biomers.net GmbH, Ulm, Germany), 6.4 µl of H_2O and 10.5 µl of Mastermix (Qiagen Multiplex-Mix, Qiagen, Hilden, Germany).

PCR products were analyzed by gel electrophoresis in 2% agarose gels (ethidium bromide).

Prediction of protein expression

The "Ensembl" platform was used to retrieve the common transcripts of ANKRD26 and RET (http://ensemblgenomes.org, [37]). The full sequence of the fusion gene, based upon "Ensembl" results, was reconstructed. A translation to the amino acid sequence was performed using "ExPASy" platform (https://www.expasy.org, [38]). Protein domains expressed by the fusion gene were analyzed using "UniProt" database (https://www.uniprot.org, [39]).

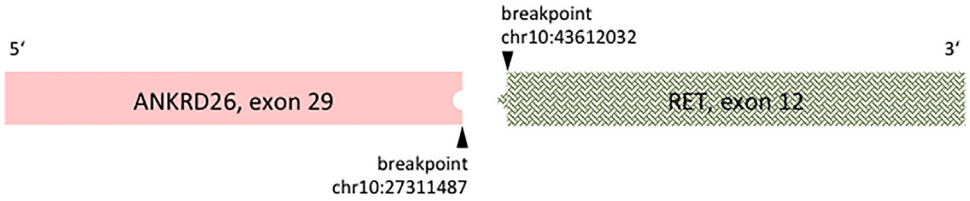

Fig. 2. ANKRD26-RET fusion gene.
In *ANKRD26-RET, ANKRD* exon 29 is fused to exon 12 of *RET* (RNA-based illustration). Results are shown according to next-generation sequencing "Archer Analysis" software.

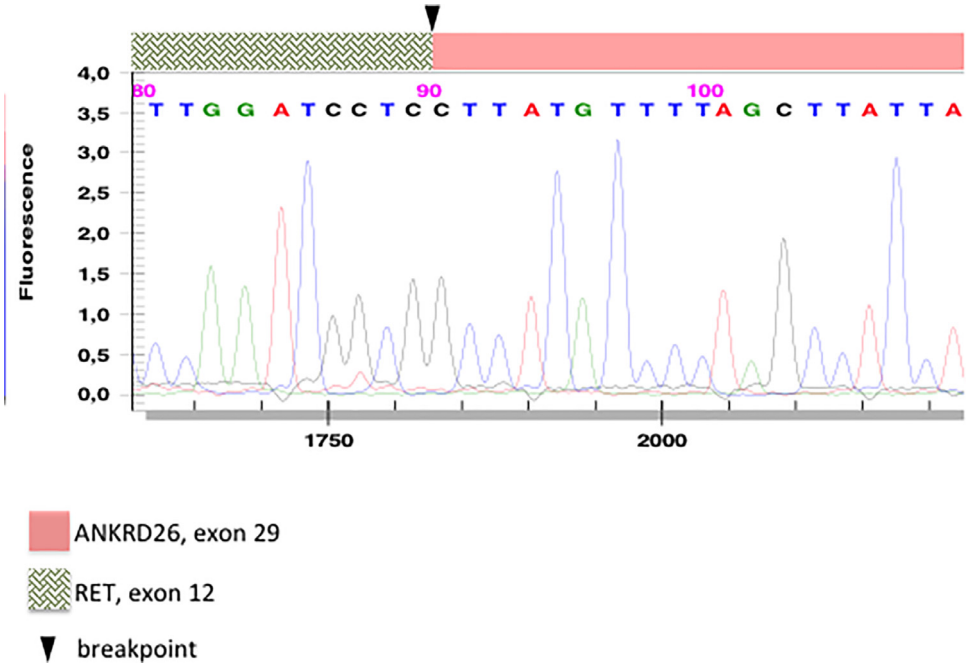

Fig. 3. Sanger sequencing.
Sanger sequencing confirmed the presence of the fusion *ANKRD26-RET*, showing the breakpoint on RNA level (primer: RET_rv2). Note that the reverse complementary strand is shown for *ANKRD26-RET* fusion transcript. Arrows indicate breakpoint.

Results

Next-generation sequencing: discovery of ANKRD26-RET

Targeted next-generation sequencing using Archer FusionPlex revealed the expression of *ANKRD26-RET* in the selected papillary thyroid carcinoma (Reads: 42,%Reads: 93.3). The breakpoint within *RET* on RNA level was at the initial sequence of exon 12, chr10:43612032, whereas in *ANKRD26*, it was in exon 29, chr10:27311487 (Fig. 2).

The mutational profile was analyzed by targeted next-generation sequencing with Archer VariantPlex and FusionPlex. The method, which allows for a simultaneous analysis of over 60 genes, including typical spots of genetic alterations in PTC (*BRAF, NTRK1/2/3, TERT, PPARG, HRAS, KRAS, NRAS* and others), did not reveal any other concomitant genetic rearrangement or variants in the tumor tissue sample.

Confirmation of ANKRD26-RET *by PCR and Sanger sequencing*

Gel electrophoretic analysis of the fusion-specific amplification product confirmed the existence of the novel fusion gene *ANKRD26-RET* in the tumor tissue. In normal thyroid tissue of the same patient, the fusion was not detectable. Sanger sequencing of the PCR product again confirmed the existence of the novel RET/PTC fusion of tumor c-DNA (Fig. 3).

Semi-quantitative expression analysis of ANKRD26 *and* RET

The semi-quantitative expression analysis illustrated that in all types of tumor (T1 (*ANKRD26-RET* positive), T2 (*BRAFV600E* positive), T3 (*CCDC6-RET* positive) as well as in normal thyroid tissue derived from the same patients, *ANKRD26* was expressed (Fig. 4).

In contrast, significant *RET mRNA* expression was restricted to the tumors T1 (*ANKRD26-RET* positive) and T3 (*CCDC6-RET* positive). In the *BRAFV600E* mutation positive tumor, T2, as well as in the normal thyroid tissue samples, expression of *RET* was almost absent and may be related to expression to non-follicular cells (Fig. 4). As expected, the breakpoint region *ANKRD26-RET* was expressed singularly by the index tumor T1.

Prediction of protein expression ANKRD26-RET

Based on the results of the "UniProt" database research for Ankyrin repeat domain-containing protein 26 (ANKRD26-201 ENST00000376087.4, 6Q9UPS8) the presence of two coiled-coil domains (positions 529–566 and 743–873), and an incomplete coiled-coil region (905–1405, originally 905–1472) in ANKRD26-RET was predicted. The ankyrin repeats (ANK1-ANK5) at positions 45–75, 79–108, 112–141, 145–174 and 178–207 in ANKRD26 are thoroughly included in the ANKRD26-RET protein. At position 1405, the ANKRD26 portion is fused to RET, which contains the RET tyrosine kinase (positions 1417–1709 in ANKRD26-RET, originally positions 724–1016 in RET, Fig. 5).

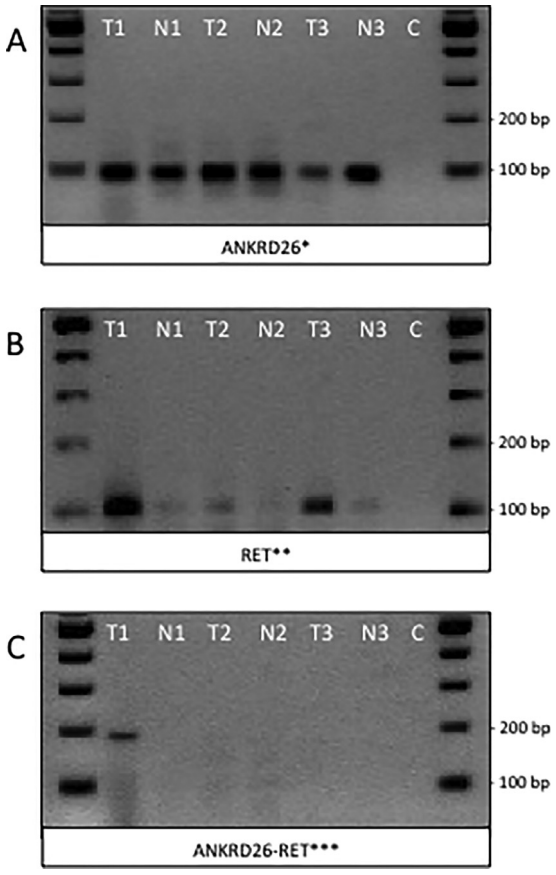

Fig. 4. Expression analysis of ANKRD26 and RET.
The expression of the genes *ANKRD26* and *RET*, as well as the fusion gene *ANKRD26-RET* are presented in Figure 4A-C:
A (*ANKRD26**): primers: ANKRD26ex32fw1 + ANKRD26ex32rv1 (amplicon length: 91 bp), ubiquitous expression of *ANKRD26* in all types of PTC and normal thyroid tissue.
B (*RET***): RETex14fw1 + RETex15rv1 (amplicon length: 101 bp), significant *RET* expression singularly in tumors T1 (*ANKRD26-RET*) and T3 (*CCDC6-RET*).
C (*ANKRD26-RET****): ANKRD26ex29fw2 + RETex12rv2 (amplicon length: 186 bp), expression of the breakpoint region of *ANKRD26-RET* singularly in T1.
T1: *ANKRD26-RET*, N1: corresponding normal tissue for T1, T2: *BRAFV600E*, N2: corresponding normal thyroid tissue for T2, T3: *CCDC6-RET*, N3: corresponding normal thyroid tissue for T3, C: control, H_2O.

Discussion

The discovery of novel RET/PTC rearrangements is facilitated by the use of modern next-generation sequencing techniques, which - by targeting one gene partner - can reveal the identity of the second gene involved. However, the presence of fusion genes in papillary thyroid carcinoma is ranging from 6–46%, and the landscape of gene fusions in PTC has been scrutinized largely, mapping rearrangements of *RET, NTRK1, NTRK3, ALK, PPARG, BRAF* and *THADA* [22,40]. The most common form of genetic rearrangement involves *RET* (approximately one third of sporadic PTC cases). The most prevalent rearrangements involve the partner genes *CCDC6* and *NCOA4* [22]. Consequently, in PTC, the likelihood to discover novel oncogenic RET rearrangements - even when using modern next-generation sequencing techniques - is relatively low. Yet, a pre-selected cohort allows for an increased probability to detect rare genetic rearrangements. In this case, a tumor sample was analyzed, which was previously discovered to harbor a rearrangement of the *RET* gene by fluorescence in situ hybridization, and was negative for its most common rearrangement RET/PTC1 (*CCDC6-RET*), excluded by fusion-specific RT-PCR amplification [36].

Tumor tissue was retrieved from a 78-year-old patient who suffered from the follicular variant of papillary thyroid carcinoma (pT3, pN0 (0/2), M0, R0). For the follicular variant of PTC, *BRAF* mutations were described to have a relatively high frequency [41]. Moreover, *RAS* mutations were discovered primarily in encapsulated follicular variant PTC [41,42]. In addition, *RET* rearrangements can play a role for the follicular variant of PTC, as we previously detected *RUFY2-RET* in this PTC subtype [43]. In the present case, neither BRAF mutations, nor *RAS* (*HRAS, NRAS, KRAS*) mutations were detected. Yet, the novel rearrangement *ANKRD26-RET* was identified. The analysis of normal thyroid tissue of the same patient did not show the specific rearrangement, underlining the relevance of the fusion in association with tumor development.

Analyses of the *RET* gene in HTori-3 human thyroid cells showed that within intron 11, there is a fragile site, which facilitates DNA breakage [44]. Similarly, the two most common rearrangements of the RET gene, RET/PTC1 (*CCDC6-RET*) and RET/PRC3 (*NCOA4-RET*), are constituted by the breakpoint within intron 11 of RET [45,46]. In the present analysis on RNA basis, the breakpoint resulted at the initial sequence of exon 12, implying that on DNA level, the common fragile site - intron 11 - is affected.

ANKRD26 is a newly discovered fusion gene partner to *RET*. We observed a ubiquitous expression of *ANKRD26* in normal thyroid tissue, as well as in the analyzed samples of papillary thyroid carcinoma harboring *ANKRD26-RET* rearrangement, *CCDC6-RET* rearrangement and *BRAFV600E* mutation. In the literature, *ANKRD26* (*KIAA1074*) mutations were described as causative alterations for hematological disorders as inherited thrombocytopenia and acute myeloid leukemia [47–49]. A research in the murine model (3T3-L1 cells) identified interaction partners of ANKRD26 to be the triple functional domain protein (TRIO), the G protein pathway suppressor 2 (GPS2), the delta-interacting protein A (DIPA) and the hyaluronan-mediated motility receptor (HMMR). ANKRD26, as well as the identified interaction partners, were shown to have a regulating influence on adipogenesis [50].

The ankyrin repeats and coiled-coil domains of the fusion protein (Fig. 5) derive from the *ANKRD26* portion of *ANKRD26-RET*. Coiled-coil domains represent recognized dimerization motifs [51,52]. The ankyrin repeat, a module among the most common structural motifs in proteins, was reported to specifically mediate protein-protein interactions. Serial ankyrin repeats can form ankyrin repeat domains, usually comprising four to six repeats

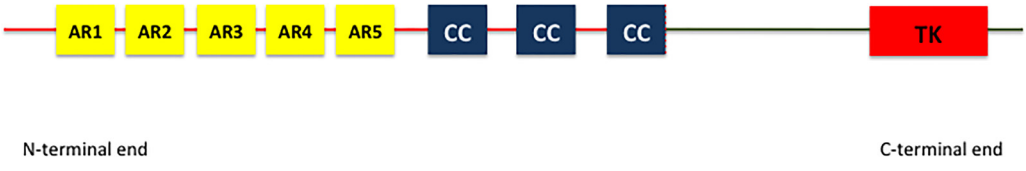

Fig. 5. Protein interaction motifs in ANKRD26-RET.
In ANKRD26-RET, there are 5 ankyrin repeats (ANK1-ANK5) at positions 45–75, 79–108, 112–141, 145–174, 178–207), 2 coiled-coil domains (positions 529–566 and 743–873), and an incomplete coiled-coil domain (905–1405, originally 905–1472 in ANKRD26). At position 1405, the ANKRD26 portion is fused to RET, which contains the RET tyrosine kinase (positions 1417–1709, originally positions 724–1016 in RET). ANKRD26-RET comprises 1807 amino acids.
AK: ankyrin repeat, CC: coiled-coil domain, TK: tyrosine kinase domain.

[53]. In the ANKRD26-RET protein, five sequent ankyrin repeats are included.

Physiologically, RET activation is distinctly regulated by (GDNF)-family ligands, which - in presence of GDNF-family receptor-α proteins - bind to the extracellular receptor domain of RET, leading to the formation of a symmetric dimer complex. An autophosphorylation of selected tyrosine residues at the intracellular kinase region of RET results. The fusion of the RET tyrosine kinase to dimerization motifs deriving from ANKRD26, however, could lead to ligand independent dimerization. Tumorigenesis can be effectuated by a constitutive activation of the downstream pathways. To closely analyze the impact on subsequent signaling cascades, further analyses of the proteome are required.

Generally, *RET* rearrangement appears in different cancer types, and the use of modern next-generation sequencing methods uncovers the variety of genes, which can be fused to *RET* (Table 1). It appears plausible, that a rearrangement connecting *RET* to a gene, which is expressed in the affected tissues, is the basis to unfold its oncogenic potential. As this study underlines, in thyroid tissue, *RET* usually is transcriptionally silent, unless it is fused to genes, which are expressed in thyroid tissue (*ANKRD26, CCDC6,* Fig. 4). The presence of dimerization motifs, contributed by the fusion partner genes of *RET,* facilitates tumorigenesis, as a ligand independent, abbreviated activation pathway of the tyrosine kinase can occur. Several studies describe coiled-coil domains, sterile alpha motif (SAM) domains, LisH domains and tryptophan-aspartate repeats (WDR), which enable ligand-independent dimerization, and thereby an activation of the chimeric RET protein [13,33]. Yet it is unclear, whether different *RET* rearrangements, or different subgroups of *RET* rearrangements defined by the regulatory regions contributed by the partner genes, play a role in the clinical development of disease. In addition, the disruption of the fusion partner gene might play an important role, as e.g. a loss of the tumor suppressor function of *PRKAR1A* in RET/PTC2 and an impaired proapoptotic effect of *CCDC6* in RET/PTC1 [22,54,55]. Further large-scale studies of the different rearrangements in relation to the clinical development, regarding the development of metastases and disease-free survival, are required.

So far, treatment approaches with tyrosine kinase inhibitors showed modest long-term results in the field of *RET* rearrangement driven cancers, which might be explained by a relatively low specificity for the primary target, that sets a limit to the maximum drug dose [33,34]. The development of more specific RET inhibitors could facilitate a targeted approach for the multiplicity of rearrangement positive cancers. In addition, a combined inhibition of downstream targets in the MAPK pathway was described as a promising strategy [33]. With a more profound knowledge of the regulatory regions and protein-protein interaction motifs influencing the RET tyrosine kinase activity, also an upstream intervention might be taken into consideration for a future biological armamentarium against *RET* driven cancers.

Conclusion

ANKRD26-RET is a novel fusion of the *RET* gene. It is first described in the follicular variant of papillary thyroid carcinoma. The present research suggests that the fusion of *RET* to the partner gene *ANKRD26* enables its expression in thyroid tissue. On protein basis, *ANKRD26-RET* leads to the fusion of the tyrosine kinase region of RET to prominent protein-protein interaction motifs (coiled-coil and ankyrin repeat domains), potentially facilitating a constitutive tyrosine kinase activation.

Generally, the use of modern next-generation sequencing methods uncovers the variety of genes, which can be fused to *RET* in different cancer types. It is still unclear whether different *RET* rearrangements are responsible for a diverse course of disease in PTC. Large-scale studies of the different rearrangements in relation to the clinical development, e.g. development of metastases and disease-free survival, are required.

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There is nothing to be declared.

Ethics statement

This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). We have obtained an informed consent from all patients included in this study. The local ethics committee approved this study.

Declaration of interest

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Supplementary materials

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References

[1] Zhu Z, Ciampi R, Nikiforova MN, Gandhi M, Nikiforov YE. Prevalence of RET/PTC rearrangements in thyroid papillary carcinomas: effects of the detection methods and genetic heterogeneity. J Clin Endocrinol Metab 2006;91:3603–10.
[2] Nikiforova MN, Caudill CM, Biddinger P, Nikiforov YE. Prevalence of RET/PTC rearrangements in Hashimoto's thyroiditis and papillary thyroid carcinomas. Int J Surg Pathol 2002;10:15–22.
[3] Tallini G, Santoro M, Helie M, Carlomagno F, Salvatore G, Chiappetta G, et al. RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. Clin Cancer Res 1998;4:287–94.
[4] Lam AK, Montone KT, Nolan KA, Livolsi VA. Ret oncogene activation in papillary thyroid carcinoma: prevalence and implication on the histological parameters. Hum Pathol 1998;29:565–8.
[5] Wang R, Hu H, Pan Y, Li Y, Ye T, Li C, et al. RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. J Clin Oncol 2012;30:4352–9.
[6] Ishizaka Y, Itoh F, Tahira T, Ikeda I, Sugimura T, Tucker J, et al. Human ret proto-oncogene mapped to chromosome 10q11.2. Oncogene 1989;4:1519–21.
[7] Takahashi M, Ritz J, Cooper GM. Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell 1985;42:581–8.
[8] Arighi E, Borrello MG, Sariola H. RET tyrosine kinase signaling in development and cancer. Cytokine Growth Factor Rev 2005;16:441–67.
[9] Santoro M, Carlomagno F. Central role of RET in thyroid cancer. Cold Spring Harb Perspect Biol 2013;5. doi:10.1101/cshperspect.a009233.
[10] Freche B, Guillaumot P, Charmetant J, Pelletier L, Luquain C, Christiansen D, et al. Inducible dimerization of RET reveals a specific AKT deregulation in oncogenic signaling. J Biol Chem 2005;280:36584–91.
[11] Sariola H, Saarma M. Novel functions and signalling pathways for GDNF. J Cell Sci 2003;116:3855–62.
[12] Encinas M, Crowder RJ, Milbrandt J, Johnson EM Jr. Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem 2004;279:18262–9.
[13] Stransky N, Cerami E, Schalm S, Kim JL, Lengauer C. The landscape of kinase fusions in cancer. Nat Commun 2014;5:4846. doi:10.1038/ncomms5846.
[14] Marotta V, Guerra A, Sapio MR, Vitale M. RET/PTC rearrangement in benign and malignant thyroid diseases: a clinical standpoint. Eur J Endocrinol 2011;165:499–507.
[15] Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, et al. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell 1990;60:557–63.
[16] Bongarzone I, Butti MG, Coronelli S, Borrello MG, Santoro M, Mondellini P, et al. Frequent activation of ret protooncogene by fusion with a new activating gene in papillary thyroid carcinomas. Cancer Res 1994;54:2979–85.

[17] Lanzi C, Borrello MG, Bongarzone I, Migliazza A, Fusco A, Grieco M, et al. Identification of the product of two oncogenic rearranged forms of the RET proto-oncogene in papillary thyroid carcinomas. Oncogene 1992;7:2189–94.
[18] Santoro M, Dathan NA, Berlingieri MT, Bongarzone I, Paulin C, Grieco M, et al. Molecular characterization of RET/PTC3; a novel rearranged version of the RETproto-oncogene in a human thyroid papillary carcinoma. Oncogene 1994;9:509–16.
[19] Rabes HM, Demidchik EP, Sidorow JD, Lengfelder E, Beimfohr C, Hoelzel D, et al. Pattern of radiation-induced RET and NTRK1 rearrangements in 191 post-chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. Clin Cancer Res 2000;6:1093–103.
[20] Klugbauer S, Rabes HM. The transcription coactivator HTIF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas. Oncogene 1999;18:4388–93.
[21] Salassidis K, Bruch J, Zitzelsberger H, Lengfelder E, Kellerer AM, Bauchinger M. Translocation t(10;14)(q11.2:q22.1) fusing the kinetin to the RET gene creates a novel rearranged form (PTC8) of the RET proto-oncogene in radiation-induced childhood papillary thyroid carcinoma. Cancer Res 2000;60:2786–9.
[22] Yakushina VD, Lerner LV, Lavrov AV. Gene fusions in thyroid cancer. Thyroid 2018;28:158–67.
[23] Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, et al. KIF5B-RET fusions in lung adenocarcinoma. Nat Med 2012;18:375–7.
[24] Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. RET, ROS1 and ALK fusions in lung cancer. Nat Med 2012;18:378–81.
[25] Drilon A, Wang L, Hasanovic A, Suehara Y, Lipson D, Stephens P, et al. Response to Cabozantinib in patients with RET fusion-positive lung adenocarcinomas. Cancer Discov 2013;3:630–5.
[26] Lira ME, Choi YL, Lim SM, Deng S, Huang D, Ozeck M, et al. A single-tube multiplexed assay for detecting ALK, ROS1, and RET fusions in lung cancer. J Mol Diagn 2014;16:229–43.
[27] Nakaoku T, Tsuta K, Ichikawa H, Shiraishi K, Sakamoto H, Enari M, et al. Druggable oncogene fusions in invasive mucinous lung adenocarcinoma. Clin Cancer Res 2014;20:3087–93.
[28] Lee MS, Kim RN, I H, Oh DY, Song JY, Noh KW, et al. Identification of a novel partner gene, KIAA1217, fused to RET: functional characterization and inhibitor sensitivity of two isoforms in lung adenocarcinoma. Oncotarget 2016;7:36101–14.
[29] Kim SY, Oh SO, Kim K, Lee J, Kang S, Kim KM, et al. NCOA4-RET fusion in colorectal cancer: therapeutic challenge using patient-derived tumor cell lines. J Cancer 2018;9:3032–7.
[30] Ballerini P, Struski S, Cresson C, Prade N, Toujani S, Deswarte C, et al. RET fusion genes are associated with chronic myelomonocytic leukemia and enhance monocytic differentiation. Leukemia 2012;26:2384–9.
[31] Bossi D, Carlomagno F, Pallavicini I, Pruneri G, Trubia M, Raviele PR, et al. Functional characterization of a novel FGFR1OP-RET rearrangement in hematopoietic malignancies. Mol Oncol 2014;8:221–31.
[32] Wiesner T, He J, Yelensky R, Esteve-Puig R, Botton T, Yeh I, et al. Kinase fusions are frequent in Spitz tumours and spitzoid melanomas. Nat Commun 2014;5:3116. doi:10.1038/ncomms4116.
[33] Drilon A, Hu ZI, Lai GGY, Tan DSW. Targeting RET-driven cancers: lessons from evolving preclinical and clinical landscapes. Nat Rev Clin Oncol 2018;15:151–67.
[34] Fallahi P, Ferrari SM, Mazzi V, Vita R, Benvenga S, Antonelli A. Personalization of targeted therapy in advanced thyroid cancer. Curr Genomics 2014;15:190–202.
[35] Musholt TJ, Schonefeld S, Schwarz CH, Watzka FM, Musholt PB, Fottner C, et al. Impact of pathognomonic genetic alterations on the prognosis of papillary thyroid carcinoma. ESES Vienna presentation. Langenbecks Arch Surg 2010;395:877–83.
[36] Musholt TJ, Staubitz JI, Cámara RJA, Musholt PB, D H, Springer E, et al. Detection of RET rearrangements in papillary thyroid carcinoma using RT-PCR and FISH techniques - A molecular and clinical analysis. Eur J Surg Oncol 2018. doi:10.1016/j.ejso.2018.11.009.
[37] Kersey PJ, Allen JE, Allot A, Barba M, Boddu S, Bolt BJ, et al. Ensembl Genomes 2018: an integrated omics infrastructure for non-vertebrate species. Nucleic Acids Res 2018;46:D802–D8d8. doi:10.1093/nar/gkx1011.
[38] Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 2003;31:3784–8.
[39] The UniProt Consortium UniProt: the universal protein knowledgebase. Nucleic Acids Res 2017;45:D158–DD69. doi:10.1093/nar/gkw1099.
[40] Agrawal N, Akbani R, Aksoy BA, Ally A, Arachchi H, Asa SL, et al. Integrated genomic characterization of papillary thyroid carcinoma. Cell 2014;159:676–90.
[41] Rivera M, Ricarte-Filho J, Knauf J, Shaha A, Tuttle M, Fagin JA, et al. Molecular genotyping of papillary thyroid carcinoma follicular variant according to its histological subtypes (encapsulated vs. infiltrative) reveals distinct BRAF and RAS mutation patterns. Mod Pathol 2010;23:1191–200.
[42] Nikiforov YE, Seethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LDR, et al. Nomenclature revision for encapsulated follicular variant of papillary thyroid Carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. JAMA Oncol 2016;2:1023–9.
[43] Staubitz JI, Schad A, Springer E, Rajalingam K, Lang H, Roth W, et al. Novel rearrangements involving the RET gene in papillary thyroid carcinoma. Cancer Genet 2018;230:13–20.
[44] Gandhi M, Dillon LW, Pramanik S, Nikiforov YE, Wang YH. DNA breaks at fragile sites generate oncogenic RET/PTC rearrangements in human thyroid cells. Oncogene 2010;29:2272–80.
[45] Nikiforov YE, Koshoffer A, Nikiforova M, Stringer J, Fagin JA. Chromosomal breakpoint positions suggest a direct role for radiation in inducing illegitimate recombination between the ELE1 and RET genes in radiation-induced thyroid carcinomas. Oncogene 1999;18:6330–4.
[46] Smanik PA, Furminger TL, Mazzaferri EL, Jhiang SM. Breakpoint characterization of the ret/PTC oncogene in human papillary thyroid carcinoma. Hum Mol Genet 1995;4:2313–18.
[47] Marconi C, Canobbio I, Bozzi V, Pippucci T, Simonetti G, Melazzini F, et al. 5′UTR point substitutions and N-terminal truncating mutations of ANKRD26 in acute myeloid leukemia. J Hematol Oncol 2017;10:18. doi:10.1186/s13045-016-0382-y.
[48] Ferrari S, Lombardi AM, Putti MC, Bertomoro A, Cortella I, Barzon I, et al. Spectrum of 5′UTR mutations in ANKRD26 gene in patients with inherited thrombocytopenia: c.-140C>G mutation is more frequent than expecteditor. Platelets 2017;28:621–4.
[49] Perez Botero J, Dugan SN, Anderson MW. ANKRD26-Related thrombocytopenia. In: Adam M.P., Ardinger H.H., Pagon R.A., Wallace S.E., Bean L.J.H., Stephens K., Amemiya A., editors GeneReviews((R)). Seattle (WA): University of Washington, Seattle;1993.
[50] Liu XF, Bera TK, Kahue C, Escobar T, Fei Z, Raciti GA, et al. ANKRD26 and its interacting partners TRIO, GPS2, HMMR and DIPA regulate adipogenesis in 3T3-L1 cells. PLoS ONE 2012;7:e38130. doi:10.1371/journal.pone.0038130.
[51] Apgar JR, Gutwin KN, Keating AE. Predicting helix orientation for coiled-coil dimers. Proteins 2008;72:1048–65.
[52] Lu Q, Ye F, Wei Z, Wen Z, Zhang M. Antiparallel coiled-coil–mediated dimerization of myosin x. Proc Natl Acad Sci 2012;109:17388–93.
[53] Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY. The ankyrin repeat as molecular architecture for protein recognition. Protein Sci 2004;13:1435–48.
[54] Sandrini F, Matyakhina L, Sarlis NJ, Kirschner LS, Farmakidis C, Gimm O, et al. Regulatory subunit type I-alpha of protein kinase a (PRKAR1A): a tumor-suppressor gene for sporadic thyroid cancer. Genes Chromosomes Cancer 2002;35:182–92.
[55] Celetti A, Cerrato A, Merolla F, Vitagliano D, Vecchio G, Grieco M. H4(D10S170), a gene frequently rearranged with RET in papillary thyroid carcinomas: functional characterization. Oncogene 2004;23:109–21.
[56] Chiappetta G, Toti P, Cetta F, Giuliano A, Pentimalli F, Amendola I, et al. The RET/PTC oncogene is frequently activated in oncocytic thyroid tumors (Hurthle cell adenomas and carcinomas), but not in oncocytic hyperplastic lesions. J Clin Endocrinol Metab 2002;87:364–9.
[57] Guerra A, Di Crescenzo V, Garzi A, Cinelli M, Carlomagno C, Tonacchera M, et al. Genetic mutations in the treatment of anaplastic thyroid cancer: a systematic review. BMC Surg 2013;13(Suppl 2):S44. doi:10.1186/1471-2482-13-s2-s44.
[58] Musholt PB, Imkamp F, von Wasielewski R, Schmid KW, Musholt TJ. RET rearrangements in archival oxyphilic thyroid tumors: new insights in tumorigenesis and classification of Hurthle cell carcinomas? Surgery 2003;134:881–9.
[59] Le Rolle AF, Klempner SJ, Garrett CR, Seery T, Sanford EM, Balasubramanian S, et al. Identification and characterization of RET fusions in advanced colorectal cancer. Oncotarget 2015;6:28929–37.
[60] Skalova A, Vanecek T, Uro-Coste E, Bishop JA, Weinreb I, Thompson LDR, et al. Molecular profiling of salivary gland intraductal carcinoma revealed a subset of tumors harboring NCOA4-RET and novel TRIM27-RET Fusions: a report of 17 cases. Am J Surg Pathol 2018;42:1445–55.
[61] Klugbauer S, Jauch A, Lengfelder E, Demidchik E, Rabes HM. A novel type of RET rearrangement (PTC8) in childhood papillary thyroid carcinomas and characterization of the involved gene (RFG8). Cancer Res 2000;60:7028–32.
[62] Corvi R, Berger N, Balczon R, Romeo G. RET/PCM-1: a novel fusion gene in papillary thyroid carcinoma. Oncogene 2000;19:4236–42.
[63] Saenko V, Rogounovitch T, Shimizu-Yoshida Y, Abrosimov A, Lushnikov E, Roumiantsev P, et al. Novel tumorigenic rearrangement, Delta rfp/ret, in a papillary thyroid carcinoma from externally irradiated patient. Mutat Res 2003;527:81–90.
[64] Liu RT, Chou FF, Wang CH, Lin CL, Chao FP, Chung JC, et al. Low prevalence of RET rearrangements (RET/PTC1, RET/PTC2, RET/PTC3, and ELKS-RET) in sporadic papillary thyroid carcinomas in Taiwan Chinese. Thyroid 2005;15:326–35.
[65] Ciampi R, Giordano TJ, Wikenheiser-Brokamp K, Koenig RJ, Nikiforov YE. HOOK3-RET: a novel type of RET/PTC rearrangement in papillary thyroid carcinoma. Endocr Relat Cancer 2007;14:445–52.
[66] Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med 2012;18:382–4.
[67] Zheng Z, Liebers M, Zhelyazkova B, Cao Y, Panditi D, Lynch KD, et al. Anchored multiplex PCR for targeted next-generation sequencing. Nat Med 2014;20:1479–84.
[68] Grubbs EG, Ng PK, Bui J, Busaidy NL, Chen K, Lee JE, et al. RET fusion as a novel driver of medullary thyroid carcinoma. J Clin Endocrinol Metab 2015;100:788–93.
[69] Lee SH, Lee JK, Ahn MJ, Kim DW, Sun JM, Keam B, et al. Vandetanib in pretreated patients with advanced non-small cell lung cancer-harboring RET rearrangement: a phase II clinical trial. Ann Oncol 2017;28:292–7.
[70] Chai L, Li J, Lv Z. An integrated analysis of cancer genes in thyroid cancer. Oncol Rep 2016;35:962–70.
[71] Drilon AE, Hellmann MD, Wang L, Cho EJ, Ladanyi M, Rizvi NA. Clinicopathologic features of advanced RET fusion-positive lung cancers and outcomes in

comparison to other fusion-positive lung cancers. J Clin Oncol 2014;32:8076. doi:10.1200/jco.2014.32.15_suppl.8076.
[72] Iyama K, Matsuse M, Mitsutake N, Rogounovitch T, Saenko V, Suzuki K, et al. Identification of three novel fusion Oncogenes, SQSTM1/NTRK3, AFAP1L2/RET, and PPFIBP2/RET, in thyroid cancers of young patients in Fukushima. Thyroid 2017;27:811–18.
[73] Gautschi O, Milia J, Filleron T, Wolf J, Carbone DP, Owen D, et al. Targeting RET in patients with RET-Rearranged lung Cancers: results from the global. Multicent RET Registry J Clin Oncol 2017;35:1403–10.
[74] Kloosterman WP, Coebergh van den Braak RRJ, Pieterse M, van Roosmalen MJ, Sieuwerts AM, Stangl C, et al. A systematic analysis of oncogenic gene fusions in primary colon cancer. Cancer Res 2017;77:3814–22.
[75] Lu Z, Zhang Y, Feng D, Sheng J, Yang W, Liu B. Targeted next generation sequencing identifies somatic mutations and gene fusions in papillary thyroid carcinoma. Oncotarget 2017;8:45784–92.
[76] Kato S, Subbiah V, Marchlik E, Elkin SK, Carter JL, Kurzrock R. RET aberrations in diverse Cancers: next-Generation sequencing of 4,871 patients. Clin Cancer Res 2017;23:1988–97.