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Detection of *RET* rearrangements in papillary thyroid carcinoma using RT-PCR and FISH techniques - A molecular and clinical analysis

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ABSTRACT

Introduction: Oncogenic *BRAF* and *RAS* mutations as well as multiple known (and yet unknown) *RET* fusion oncogenes comprise the majority of causative molecular alterations in papillary thyroid carcinoma (PTC). Apparently "mutation-negative" PTCs encompass a heterogenous group impeding analysis of prognostic significance of underlying genetics.
Material and methods: *BRAF* wild type PTC tissue of 56 patients was analyzed using two established methods: hybrid-specific RT-PCR for the predominant rearrangement RET/PTC1 and fluorescent in situ hybridization (FISH). Clinical features of the cases with and without *RET* rearrangement were compared (patient age, gender, tumor size, focality, lymph node affection, and iodine avidity).
Results: RT-PCR revealed RET/PTC1 rearrangements in five of 56 tumors (9%). FISH confirmed these, and identified four additional *RET* rearrangements (9/56; 16%). Loss of the iodine avidity only occurred in cases of RET/PTC hybrids (7/9 tumors), but not in RET/PTC-negative PTCs (0/41 tumors with available uptake information; $p = 0.029$). The risk to develop lymph node metastases was eight times higher in presence of *RET* rearrangements ($p = 0.010$).
Conclusions: FISH analysis, in contrast to hybrid-specific RT-PCR, revealed infrequent and unknown *RET* fusion genes. The presence of *RET* rearrangements was associated with a significantly elevated risk to develop iodine refractory disease and lymph node metastases. Of note, significant clinical discrimination was only achievable when taking the FISH results into account; differences would have been missed when using the RT-PCR method only. Increasing evidence of the clinical impact of RET/PTC-positivity may influence the decision on the extent of surgical resection, especially on lymph node dissection, in PTCs.

Introduction

Within the category of differentiated thyroid carcinoma, papillary thyroid carcinoma (PTC) is represented with a frequency of 80–85%, whereas follicular thyroid carcinoma (FTC) is relatively rare (10–15%) [1]. The following genetic alterations were discovered to be highly relevant for the development of thyroid carcinoma: point mutations of *BRAF*, in particular BRAFV600E, RAS (*HRAS*, *NRAS*, *KRAS*), *TERT*, *TP53*, *EIF1AX* and *PIK3CA* [2–6]. In addition, gene fusions hold importance: *NTRK1*, *NTRK3*, *ALK*, *THADA* and *BRAF* can be involved in rearrangement processes driving PTC development [4,6–8]. The *PAX8-PPARγ* fusion was detected primarily in FTC, follicular differentiated PTC, and follicular thyroid adenoma [9]. The most common genetic rearrangement in PTC

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involves the *RET* gene [10–13]. Moreover, epigenetic alterations such as DNA methylation, histone modifications and miRNA changes were reported to be influencing factors of biological behavior in differentiated thyroid carcinoma [14].

The small remaining percentage of apparently "mutation-negative" PTCs, carrying none of the above mentioned known genetic alterations when examined with routine laboratory methods, comprise a heterogeneous tumor subset whose clinical behavior or prognostic relevance cannot be determined. Elucidation of these unknown genetic alterations in PTCs will aid in classification of these RET/PTC-, *BRAF*, and RAS-negative tumors. There is evidence that in PTCs, genetic alterations such as RET/PTC rearrangements, *BRAF* and *RAS* mutations do not simultaneously exist [2]; BRAFV600E mutations almost exclusively occur in tumors lacking RET/PTC rearrangements [15–17]. In this study, therefore, we exclusively analyze PTC tissues, which do not harbor *BRAF* mutations, to search for yet unknown genetic alterations.

The proto-oncogene *RET* ("rearranged during transfection") is located on chromosome 10q11.21 and encompasses 21 exons [18,19]. It encodes a receptor tyrosine kinase located at the cell membrane (Fig. 1). There are two main isoforms: RET9 and RET51. These are generated by alternative 3'splicing and hence are defined by the C-terminal domain [20–22]. In summary, signal transduction is effected as follows: glial cell line-derived neurotrophic factor (GDNF)-family ligands, 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 [20,23]. Thereby, autophosphorylation of selected tyrosine residues at the intracellular kinase region of RET is induced. Depending on the phosphorylation site, different pathways are activated, partly mediated by the binding of adaptor proteins and enzymes including SHC, FRS2, SRC and PLCG. These pathways comprise RAS-MAPK and PI3K-AKT cascades, which are involved in the regulation of cell survival and proliferation [20,24–27].

Being relevant for neuronal cell differentiation and growth, within the thyroid gland, *RET* is primarily expressed in thyroid C-cells [28], whereas in follicular cells it is virtually inactive [29]. Chromosomal rearrangement (by inversions, translocations, insertions or deletions) combining the *RET* gene to another, primarily unrelated gene, results in the development of a fusion gene, which receives the acronym RET/PTC [30]. However, due to the multiplicity of RET/PTC rearrangements, numerous authors refrain from the use of the acronyms, denominating the fusion according to the genes involved [13]. The rearrangement leads to the development of a protein which comprises the C-terminal kinase region of RET and the N-terminal end deriving from a partner gene carrying a promoter for expression in thyroid follicular cells and possibly further regulatory elements [24]. More than 19 possible oncogenic RET/PTC rearrangements have been described to date [13,31–34]. Of these, RET/PTC1 and RET/PTC3 (both effected by intrachromosomal, paracentric inversion) were described to have the highest frequency of up to 90% of all *RET* rearrangements [10–13]. In sporadic thyroid cancer, the frequency of RET/PTC1 is 2 times higher than of RET/PTC3 [13]. These rearrangements are responsible for the loss of the transmembrane domain and the extracellular regulatory region (Fig. 2). RET/PTC1 results from a rearrangement of the *RET* gene to *CCDC6* (*H4*) with an original distance of 30Mb ("CCDC6-RET"), whereas RET/PTC3 originates from rearrangement between *RET* and *NCOA4* (*ELE 1*) with a distance of at least 500Mb ("NCOA4-RET") [11,33–37]. The resulting alterations in the hybrid protein allow for ligand-independent RET-dimerization, thereby inducing uncontrolled autophosphorylation processes at the kinase region. Constitutive activation of the downstream signaling pathways finally contributes to tumorigenesis [20,30,38–41].

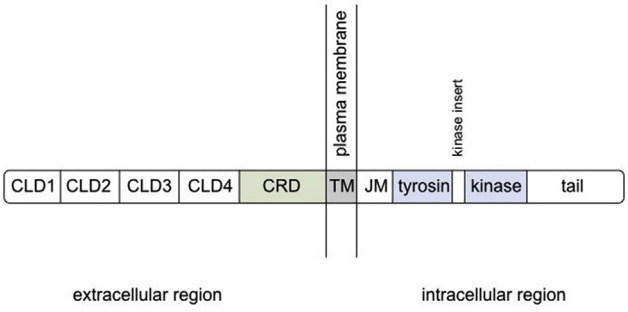

Fig. 1. RET protein (modified from Arighi et al. [20]). The RET protein encompasses the following regions (from extracellular to intracellular region): the ligand-binding domain contains 4 cadherin-like structures (CLD1-4). Between CLD3 and CLD3, there is a Ca^{2+}-binding region. A cysteine-rich domain (CRD) connects CLD4 to the transmembrane region (TM). The intracellular part contains a juxtamembrane region (JM) connecting the TM to the tyrosine kinase region [20].

This study focuses on the assessment of different rearrangements involving the *RET* gene in *BRAF* wild type PTC. However, compared to the straightforward *BRAF* testing, the identification of *RET* rearrangements in PTCs is hampered by the fact that multiple oncogenic fusions exist. Laboratory testing with reverse transcription polymerase chain reaction (RT-PCR) and mutant-specific amplification designed for the predominant rearrangement RET/PTC1 is therefore destined to underestimate the actual frequency of *RET* rearrangements. In addition, the recently established panels for an encompassing characterization of PTC, based on next-generation sequencing, restrict the analysis to the most prevalent *RET* fusions [6].

Preoperatively assessed genetic alterations as tumor biomarkers (*BRAFV600E* or *TERT* mutation) - detected in FNAB-derived cell materials -, are nowadays already taken into consideration for the indication for thyroid surgery [42,43]. In addition, an increasing number of studies examines the impact of molecular genetic alterations on the prognosis of thyroid malignancies. Results of these studies were already included in risk-stratifications that guide individualized surgical as well as the post-surgical treatment [44]. Moreover, new classifications of differentiated thyroid carcinomas (DTCs) based on molecular genetic studies were proposed that discriminate BRAF-like, RAS-like and non-BRAF-non-RAS-like tumors [34]. However, results of molecular genetic studies can be misleading, if a considerable number of tumors is actually inaccurately categorized as "mutation-negative", because rare mutations and gene fusions are missed by the applied detection method (e.g., mutation/hybrid-specific RT-PCRs). Therefore, our genetic analysis with the FISH method (detection of any – known or unknown – RET/PTC rearrangements) on apparently "mutation-negative" PTCs aims to support optimized, more differentiated treatment algorithms in these thyroid tumors, based on the prognostic impact of underlying mutations and gene fusions.

Material and methods

Patients

BRAF wild type PTC tissue (exclusion of *BRAF* mutation by routine diagnostic molecular testing) from 56 patients (18 male, 38 female, median age: 49 years), who underwent thyroidectomy for suspicious thyroid nodules or previously assessed PTC at the Section of Endocrine Surgery, Department for General-, Visceral- and Transplantation Surgery of the University Medical Center, Johannes Gutenberg University Mainz, was analyzed by both RT-PCR with

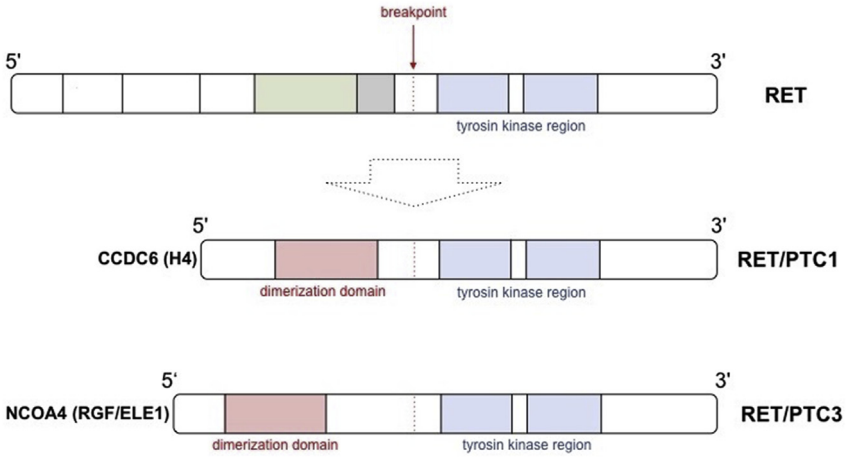

Fig. 2. Genetic alterations of the *RET* gene (modified from Arighi et al. [20]). The red arrow represents the breakpoint where the tyrosine kinase domain of the *RET* gene is rearranged to another gene. As examples RET/PTC1 ("CCDC6-RET"), combining *CCDC6* (*H4*) to the kinase domain and RET/PTC3 ("NCOA4-RET"), combining *NCOA4* (*RFG*,"*RET-activating gene ELE1*") to the kinase region [20] are shown. With this genetic alteration, ligand-independent dimerization of the RET/PTC protein results, leading to constitutive tyrosine kinase activation.

RET/PTC1-specific amplification (including Sanger sequencing) and fluorescence in situ hybridization (FISH).

Methods

RT-PCR based amplification

Nucleic acid was isolated from formalin-fixed paraffin-embedded (FFPE) tissue slices (4 μm). Tissue deparaffinization was performed using xylene and ethanol (SAV Liquid Production GmbH, Flintsbach am Inn, Germany). For RT-PCR, the concentration of 100 ng/μL total nucleic acid in 5mM Tris, pH8 (AppliChem, Darmstadt, Germany) was produced.

RT-PCR:. RNA was used to create cDNA, which allows for an amplification of the concise genetic information, avoiding the interruption by introns. An amplification of the RET fusion on DNA level was not possible because of the strands' exceeding length. Random primers (Promega, Fitchburg, Wisconsin, USA) were added to 11 μL of RNA template for annealing at 70 °C. M-MLV Reverse transcriptase (Promega, Fitchburg, Wisconsin, USA) was used for creation of cDNA, according to the manufacturer's instructions. The quality of the cDNA preparations was confirmed by cDNA-specific TaqMan GAPDH "inventoried" assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

RET/PTC1 nested PCR. For amplification, RET/PTC1 nested PCR was performed. Primers for CCDC6 (*H4*) (forward) and the tyrosine kinase region (reverse) (Table 1) were used, according to Sapio et al. [45]. A total volume of 20μL was processed comprising template, forward/reverse primers and master mix (Qfast Master mix, Qiagen Fast Cycling PCR-Kit, Qiagen, Hilden, Germany). 35 cycles of denaturation (96 °C for 5s), annealing (60 °C for 5s) and extension (68 °C for 7s) were performed and PCR products analyzed on 2% agarose gel (Fig. 3). DNA migration towards the anode was photo-documented.

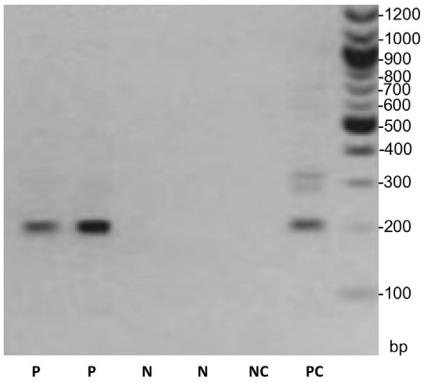

Fig. 3. Gel electrophoresis displaying results of hybrid-specific RT-PCR-based analysis of the RET/PTC1 rearrangement. N: negative for RET/PTC1, P: positive for RET/PTC1, NC: negative control, PC: positive control.

Sanger sequencing. For determination of the nucleotide sequences to confirm the results from gel electrophoresis, Sanger sequencing was performed using GenomeLab DTCS Quick Start Kit (Beckman Coulter Life Sciences, Brea, USA), CEQ8000 analyzer and software (Beckman Coulter Life Sciences, Brea, USA).

FISH analysis

Tissue preparation (from FFPE tissue slides, 4 μm) including deparaffinization and proteolysis was performed using the ZytoLight FISH-Tissue Implementation Kit (ZytoVison GmbH, Bremerhaven, Germany) according to the manufacturer's instructions. Denaturation and hybridization (hybridizer: Dako, Hamburg, Germany) was carried out using the kit "ZytoLight SPECT RET Dual Color Break Apart Probe" (ZytoVison GmbH, Bremerhaven, Germany). The fluorescent dyes used were ZyOrange (excitation: 547nm, emission: 572 nm, chr10:43,340,888–43,510,171) for

Table 1
Primer sequences for hybrid-specific RET/PTC1 amplification.

RET/PTC1	forward	RET/PTC1-F	5′-AGCGCCAGCGAGAGCGACACG-3′
	reverse	RET/PTC-R	5′-TACCCTGCTCTGCCTTTCAGATGG-3′
RET/PTC nested	forward	RET/PTC1-nF	5′-GTCGGGGGGCATTGTCATCT-3′
	reverse	RET/PTC-nR	5′-AGTTCTTCCGAGGGAATTCC-3′

proximal marking of the RET gene and ZyGreen (excitation: 503 nm, emission: 528 nm, chr10:43,626,274–43,902,346) for distal marking. To detect *RET* rearrangements, a fluorescence microscope with double-pass filter (orange/green) was used (Olympus, Tokio, Japan), including photo documentation (Fig. 4). The rearrangement pattern showed one separated orange and green signal (distance: at least two signal strengths) and one fused signal per nucleus. For analysis, for each sample 50 nuclei were counted and considered 100%. As a cut-off for positivity, >15% of FISH-positive nuclei were set (10–14% = borderline positivity) [46].

Statistical analysis

Data was registered in Excel tables (Microsoft Corporation, Redmond, USA) and analyzed with Stata/SE, version 12.1. An eight-field-table visualized the agreement between FISH and RT-PCR regarding presence versus absence of RET/PTC rearrangements. A rearrangement was present when one or both results were positive. Explorative characteristics of the participants and their tumor entities compared cases with and without RET/PTC rearrangements. The comparison of FISH with RT-PCR and the summary of characteristics included counts, percentages, means, and standard deviations. Univariate odds ratios with 95% confidence intervals and p-values compared cases with and without rearrangements by group for binary and by unit increase (logistic regression) for continuous characteristics.

Results

BRAF wild type PTC tissues from 56 patients (18 male, 38 female, median age: 49 years) underwent analysis. Hybrid-specific RT-PCR-based amplification revealed the expression of RET/PTC1 rearrangements in five of 56 cancer cases (9%). FISH confirmed them and identified four additional non-RET/PTC1 rearrangements, - that is, yet uncharacterized *RET* hybrids - resulting in a total of nine of 56 cases (16%, Table 2). These were five females and four males.

The suitability of the chosen FISH technique and result interpretation - implementing a cut-off value of rearrangement positivity in at least 15% of analyzed nuclei (cells) per tumor – to discriminate between truly RET-hybrid-positive and -negative PTCs is reflected in the results obtained: 43/51 (84%) tumors demonstrated FISH counts below 10%, while only a minority of 4/51 (8%) of tumors displayed counts of 11–14%; only three of the nine PTCs deemed positive for RET hybrids were detected with counts of 15–19%, while the majority of 6/9 (66%) displayed counts above 20% (Table 2).

In the statistical analysis, the most striking differences between cases with and without RET/PTC rearrangements were the number of affected lymph nodes and the iodine uptake. Patients with RET/PTC rearrangements harbor a risk of lymph node affection, which is eight times higher than for patients without *RET* rearrangement. For each 10% additional affected lymph nodes, the odds of RET/PTC rearrangements doubled. A loss of the iodine uptake ability of the malignant PTC tissue only occurred in cases with RET/PTC rearrangements - namely in seven of nine tumors (78%) -, while all of the 41 cases with available information on the iodine uptake status demonstrated iodine avidity (Table 3). The age of PTC patients ranged from 23 to 81 years and the tumor size ranged between 0.7 and 6.5 cm. There was no association of *RET* rearrangement and tumor size or multifocality, and patient age or gender in the cohort analyzed.

Discussion

With 16% of the cases, *RET* rearrangements are frequent in *BRAF* wild type PTCs. Of note, our applied FISH technique - using a cut-off of 15% FISH-positive nuclei (cells) - identified almost twice as many rearrangements as hybrid-specific RT-PCR in the present study: 9 versus 5 RET/PTC-positive tumors; 4 yet unidentified RET/PTC

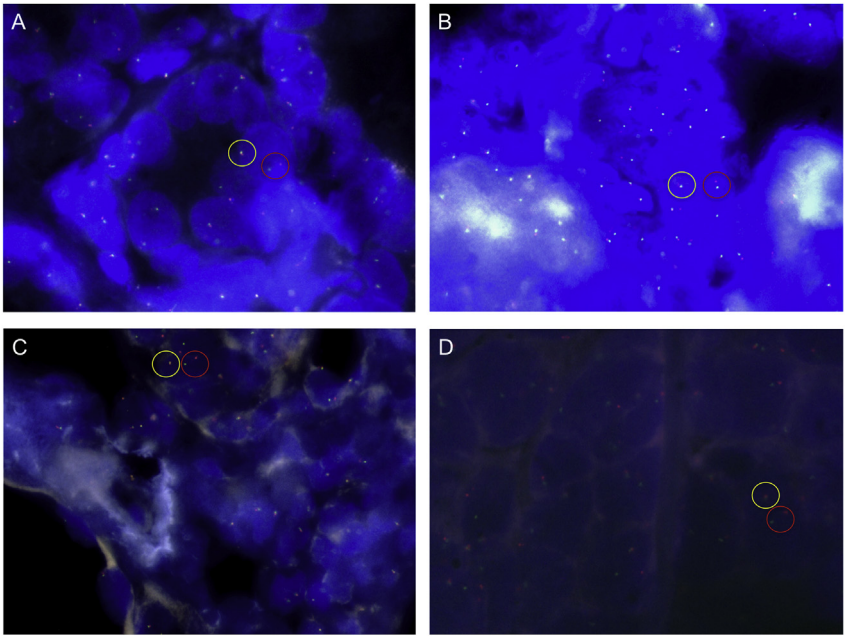

Fig. 4. A–D: *RET* gene rearrangement in fluorescence in situ hybridization (FISH). *RET* gene rearrangement pattern: one break-apart (exemplarily indicated by red circle) and one intact fusion signal (exemplarily indicated by yellow circle), magnification ×100. In all cases shown, the RET/PTC1-specific RT-PCR analysis remained negative. 3A: FISH-positive sample displaying a *RET* rearrangement pattern in 18% of tumor cells (9/50 nuclei). 3B: FISH-positive sample displaying a *RET* rearrangement pattern in 30% of tumor cells (15/50 nuclei). 3C: FISH-positive sample displaying a *RET* rearrangement pattern in 22% of tumor cells (11/50 nuclei). 3D: FISH-positive sample displaying a *RET* rearrangement pattern in 40% of tumor cells (20/50 nuclei).

Table 2
Intersecting set FISH and RT/PCR results for RET/PTC rearrangement.

		FISH: counts (percentage of 56 tumors examined)				
		Positive		Negative		Total
		≥20%	15–19%	10–14%	<10%	
RT-PCR	Positive	3 (5)	2 (4)	0 (0)	0 (0)	5 (9)
	Negative	3 (5)	1 (2)	4 (7)	43 (77)	51 (91)
	Total	6 (11)	3 (5)	4 (7)	43 (77)	56

Table 3
Participant and cancer characteristics by FISH results for RET/PTC rearrangement.

	FISH ≥ 15% vs. < 15%		Odds ratio		
	Positive (n = 9)	Negative (n = 47)	Size	95% confidence	p-value
Age in years (mean ± SD)	51 ± 21	47 ± 15	1.0	1.0–1.1	0.539
Female gender (%/cases)	56/9	70/47	0.5	0.1–3.1	0.448
Tumor size in centimeters	2.4 ± 1.9	2.0 ± 1.8	1.1	0.8–1.6	0.540
Lymph nodes:					
examined dozens[a]	3.4 ± 2.6	1.2 ± 1.4	1.8	1.2–2.7	0.004
lymph node affection	**78/9**	**30/47**	8.2	1.3–87.3	**0.010**
1/10 affected[a]	2.5 ± 2.1	0.7 ± 1.1	2.0	1.2–3.4	0.008
Iodine uptake (%/valid cases)	**78/9**	**100/41**	0.0	0.0–0.4	**0.029**
Cancer free, after operation	78/9	96/46	0.2	0.0–2.6	0.121
Recurrence	0/7	7/44	0.0	0.0–8.7	1.000
Multifocal cancer	11/9	11/47	1.0	0.0–11.4	1.000

[a] The odds ratios are also for a one dozen and for a 10% increase, respectively. Statistically significant results given in bold letters are further discussed in the paper.

hybrids in addition to 5 confirmed RET/PTC1 hybrids. Previously, we detected a frequency of 15% of *RET* rearrangements in a cohort of 137 German PTC patients, which is concordant with the present findings [3]. In the literature, the reported frequency of *RET* rearrangements in papillary carcinomas ranges from 0 to 87% [47]. These differences are partly explained by geographical diversity, pre-selection of the analyzed cohort, and different sensitivities of the assays used for detection. In this study, we have analyzed a pre-selected cohort, as *BRAF* mutant tumors were a priori excluded from analysis (since current knowledge states that they do not carry synchronous *RET* rearrangements). Therefore, the expected percentage of *RET* hybrids in an unselected German PTC cohort would be lower (i.e. minus the percentage of *BRAF*-positive tumors).

Our study underlines that RT-PCR with mutant-specific amplification is limited to the detection of *RET* rearrangements known prior to analysis. The sensitivity of RT-PCR is restricted by its high specificity for RET/PTC1, or other defined RET/PTC variants, for which the RT-PCR-based amplification is designed. PTC cases harboring yet unidentified oncogenic *RET* fusions cannot be assessed, and the exact frequency of rearrangements hence remains underestimated. FISH appears to have the better suitability as a method for screening for rearrangements, as rare and yet unidentified fusions of the *RET* gene can be assessed. FISH is characterized by a high sensitivity, but a low specificity, as any present RET/PTC rearrangement can be identified by this method. Even non-malignant rearrangements are made visible using this approach, potentially leading to an incorrect attribution of oncogenic potential to a rearrangement [48]. The presence of a *RET* rearrangement does not strictly indicate malignancy, since it can also be present in low frequency in benign thyroid nodules [48,49] and in follicular cells of patients with Hashimoto's thyroiditis [50]. The cut-off for the rearrangement-positive cell fraction that indicates malignancy – 15% in this FISH study – is of central importance. However, the clinical aggressiveness of RET/PTC-derived thyroid carcinoma might still depend on the actual subgroup, defined by the specific fusion gene [51]. As a perspective, the four tumors identified in this study, which reliably demonstrated *RET* rearrangements other than RET/PTC1, will undergo further investigation, to identify the exact fusion genes (ongoing research).

With regard to clinical significance of underlying molecular genetic alterations in PTCs, our study illustrates that presence of RET/PTC rearrangements affect the iodine avidity, as the majority of PTC cases losing ability of iodine uptake harbored RET/PTC rearrangements (7 of 9 tumors) whereas none of the cases without rearrangements were involved (0/41 tumors; $p = 0.029$). In the literature it was described that constitutive activation of the MAPK pathway promotes aberrant silencing of iodine-handling genes [52,53]. Furthermore, in vitro experiments illustrated that the inhibition of the PI3K/Akt pathway led to an increased iodine avidity [54]. As these pathways were shown to be upregulated in patients harboring RET/PTC rearrangements, the present observation is in line with published data. The inhibition of the MEK/ERK pathway in RET/PTC1 cell lines was shown to have a stimulating effect on the expression of the sodium/iodide symporter gene in tumor cells [55,56]. In patients harboring a RET/PTC rearrangement - assuming that an increased activity of the aforementioned pathways is prevalent - a therapy using tyrosine kinase inhibitors appears as a plausible treatment option. Loss of iodine avidity was primarily described in association with BRAFV600E mutation, which also causes an upregulation of MAPK-signaling due to the loss of response to the negative feedback from ERK to RAF [57].

The number of affected lymph nodes in the present analysis is also higher in patients harboring RET/PTC gene fusions, compared to the group without *RET* rearrangements (both groups *BRAF* wild type, 25% versus 7% on average). In the literature, a potential role of *RET* rearrangements favoring the development of metastatic disease was discussed [3,58,59].

We did not detect significant differences concerning the risk of recurrence of disease in patients with or without RET/PTC rearrangements (*BRAF* wild type). An analysis of tumor-specific survival following diagnosis-adapted surgery suggested an advantage for patients carrying RET/PTC rearrangements over a cohort harboring BRAFV600E mutations [3].

The present data does not support the hypothesis that PTC patients harboring *RET* rearrangements are significantly younger than those carrying other genetic alterations. The mean age of *RET*-positive patients in this study was 51 years, whereas in our preceding study from 2010, a mean age of 41.85 years was observed [3].

In our approach, we solely used PTC tissue previously confirmed to harbor malignancy by histopathological examination. PTCs with *BRAF* mutations were excluded, since the literature suggests that there is no significant overlap of this alteration with RET/PTC rearrangements [15–17]. After all, it is possible that the RET/PTC-positive tumors identified in the present study carry concomitant genetic alterations other than *BRAF* mutations. For further elucidation, and to validate the findings in the presently available data, large-scale comprehensive gene panel analyses on *BRAF* wild type PTC tumors need to be carried out, including use of FISH analysis, possibly complemented by modern next-generation sequencing assays.

Conclusions

This comparison study confirmed the suitability of FISH analysis to detect less frequent and unknown *RET* fusion genes as an advantage over RT-PCR with RET/PTC1-specific amplification.

This analytical advantage was found to be of clinical impact, since the presence of RET/PTC gene fusions in the *BRAF* wild type series was associated with a significantly elevated risk of loss of iodine avidity in the respective carcinomas during the course of disease. Furthermore, the risk to develop lymph node metastases was eight times higher for the group harboring *RET* rearrangements than in the remaining cohort. Of note, these statistically significant clinical differences due to the molecular genetic background of the affected malignancies would have not been identifiable using the common hybrid-specific RT-PCR method alone, without additional FISH analysis. Taking our results into account, the evidence increases that a more aggressive surgical management with focus on lymph node dissection might be justified in patients with *RET* rearrangement-positive (*BRAF* wild type) PTCs.

Further comprehensive clinical studies accompanied by modern gene panel analysis will be needed to sustain the present findings, and to exclude once for all the presence of overlapping mutations responsible for PTC tumorigenesis and progression.

Declaration of interest

None.

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All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from the participants included in this study. The local ethical committee has approved of this study.

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