Determining the Prevalence of RET/PTC Mutation in Cases Where Thyroid Nodules in American Thyroid Association (ATA) Ultrasonography (USG) Guidance According to Risk Category is Determined and investigating the Relation of Malignancy

Mehmet Erdogan (drmerdogan61@hotmail.com)
Ege University Medical School https://orcid.org/0000-0002-0300-3923
Muammer Karadeniz
Gedik University: T C Istanbul Gedik Universitesi
Yesim Ertan
Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi
Ahmet Gokhan Ozgen
Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi
Sadık Tamsel
Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi
Timur Kose
Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi
Afig Berdeli
Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi
Murat Ozdemir
Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi

Research Article

Keywords: Thyroid nodules, fine needle aspiration, RET/PTC mutations

Posted Date: June 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-585707/v1

Abstract

OBJECTIVE

RET/PTC in FNA (Fine needle aspiration) biopsy smears could improve the FNA diagnosis. The aim of study determining the prevalence of RET/PTC mutation in cases where thyroid nodules in ATA USG according to risk catogory is determined and investigating the relation of malignancy.

METHODS

RNA was extracted from 60 routine FNA. RET/PTC rearrangements were detected by real-time quantitative polymerase chain reaction, and, in parallel, RT-PCR is used to detect the chimeric RET/PTC1, RET/PTC2 and RET/PTC3 transcripts in RNA extracted from FNA.

RESULTS

A total of 75% FNA RET rearrangements were detected in the 20 PTC patients. RET/PTC positive was detected in 35% of cases without cancer (sensitivity 75%, specificity 65%) (p = 0,003). Significant correlation was found between A-high suspicion USG group (80%), B- intermediate suspicion USG group (45%) and C- low suspicion USG group (20%) in of RET/PTC rearrangements (p = 0.001). A significant correlation was found between the USG groups and the PTC (respectively group A,B,C: 60%, 30%, 10%)(p = 0.003). There was a statistical difference related to in the USG groups frequency between Classic type PTC (70%) and Follicular type PTC (30%)(p = 0.019). There was a statistical difference related to between RET/PTC and PTC types in the USG groups (p = 0.012). There was a statistical difference related to between usg groups tumor diameter A and C (P = 0.010).

CONCLUSİON

Molecular testing of FNA samples improves presurgical diagnosis. RET/PTC was found to be significantly higher in the diagnoses of the PTC with 75% sensitivity and 65% specificity

Introduction

More than 20% of the general population has a palpable thyroid nodule and the percentage rises to 70% based on ultrasound identifications. In 95 % of the cases, the nodule is simply a hyperplastic or benign lesion [1]. Thyroid cancer is the most common malignancy of the endocrine system [2]. The best reliable diagnostic test for thyroid nodules is fine needle aspiration (FNA). Cytological analysis is now, almost routinely, being combined with molecular genetics to enable the pathologist to make a more objective diagnosis [1,3]. The molecular mechanism of thyroid cancers is now properly well cognised. Molecular research might be used as a rule-in test for analysis of cancer in thyroid nodules [4,5]. Nowadays there is

a better identification of the process implicated in the start and progression of thyroid cancer. It is actually now recognised that BRAF and RAS genetic abnormality and RET/PTC and PAX8/PPARγ rearrangements account for the majority of molecular alterations detected in differentiated thyroid cancers [6]. The most common genetic mutations in papillary and follicular thyroid cancers are point mutations of the BRAF or RAS genes, while the well known chromosomal alterations are RET/PTC and PAX8/PPARγ rearrangements[2,7]. The RET/PTC rearrangement and the BRAF(V600E) mutation are the two prevalent molecular alterations associated with papillary thyroid carcinoma (PTC), and their recognition is gradually being used as an assistant to cytology in diagnosing PTC. On the other hand, there are warnings related with the use of the molecular method in fine-needle aspiration (FNA), particularly for RET/PTC, that should be taken into consideration. It has been declared that a clonal or sporadic existence of this pathology in follicular cells can distinguish between malignant and benign nodules [8]. There are many factors for thyroid cancer etiology one of these is rearranged during transfection (RET) mutations. The RET receptor is fundamentally a tirosinkinase membran receptor that is controlled at the 10th chromosome [9]. Rearrangement of the RET gene, also known as RET/PTC rearrangement, is the most common genetic alteration identified to date in thyroid papillary carcinomas [10]. There are now at least 15 types of RET/PTC rearrangements involving RET and 10 different genes [11]. Conflicting results on the frequency and type of RET/PTC rearrangements have been reported in relation to age, radiation exposure, and histological tumor variant [12].

This study aims to determine the prevalence of RET/PTC mutation in cases where thyroid nodules in ATA USG according to risk category is determined and investigating the relation of the malignancy.

Materials And Methods

Patients

Medical records of patients were prospectively evaluated. 102 patients met inclusion criteria and were assessed for eligibility. 42 patients were excluded. We exclude; 2 patients who had previously been exposed to high levels of radiation, 13 patients who had Hashimoto’s thyroiditis and Graves’ disease, 18 patients who do not want to undergo surgery and 9 patients who had disappeared on follow-up. Sixty patients with thyroid nodules were included in this study. The study protocol was approved by the ethics committee of the Ege University Medical School. Informed consent had been obtained before the study in all patients. The study was carried out in the department of endocrinology and metabolism diseases at the Ege University Faculty of Medicine.

Physical examination, thyroid profiles, thyroid ultrasonography and FNA (Ret/PTC) were performed before surgical procedure. Sonographic images were classified according to the American Thyroid Association guidelines [13]. 3 USG groups: A- high suspicion, B- intermediate suspicion and C- low suspicion were included in the study according to ATA nodule sonographic patterns and risk of malignancy.

In the sonographic pattern; High suspicion (groups A) features are solid hypoechoic nodule or solid hypoechoic component of a partially cystic nodule with one or more of the following features: irregular margins (infiltrative, microlobulated), microcalcifications, taller than wide shape, rim calcifications with small extrusive soft tissue component, evidence of extrathyroidal extension (ETE). Intermediate suspicion (groups B) features are hypoechoic solid nodule with smooth margins without microcalcifications, ETE, or taller than wide shape. Low suspicion (groups C) features are isoechoic or hyperechoic solid nodule, or partially cystic nodule with eccentric solid areas, without microcalcification, irregular margin or ETE, or taller than wide shape.

In the thyroid nodules, indications for surgical treatment included suspicious lesions detected by fine needle aspiration biopsy and growing nodules. Primary surgical treatment was Total Tyroidectomy (TT) or neartotal tyroidectomy when preoperative diagnosis was available.

Thyroid FNA samples were provided from the Department of Endocrinology for molecular examinations of the patients with thyroid nodules. RET/PTC analyses from FNA samples were carried out at Molecular Medicine Research Laboratory of Ege University.

RET-PTC genes rearrangement method from fine needle aspiration samples.

manufacturer’s instructifine

The Fine Needle biopsy sample is placed in the tube containing Ambion RNAlater Stabilization solution (Thermo Fisher Scientific Waltham, MA USA) and the needle is washed several times.

The RNA Stabilization Solution is an aqueous tissue storage reagent that rapidly permeates most tissues to stabilize and protect RNA in fresh specimens. It eliminates the need to immediately process or freeze samples; the specimen can simply be submerged in RNAlater Solution and stored for analysis at a later date. Samples stored in RNAlater Solution have been used successfully with TRI Reagent® Solution kit according to the manufacturer’s instructions.

Dissolved samples at room temperature were rotated for 5 minutes at a high speed of 14,000 rpm in a refrigerated centrifuge and the top was thrown. The Pellet is washed with PBS and centrifuged again. Isotaion of mRNA from 200 mkl PBS suspended sample is performed according to prospectus using the QIAmp RNAlater mini kit.

The final elution product is kept in 50µl RNAse-free Water. RNA concentration is measured in NanoDrop digital spectrophotometer and standardized by dilating to 10ng/mkl. Diluted and standardized RNA samples were kept in -80^{o}C cabinets or 196^{o}C nitrogen tank until run.

CDNA SYNTHESIS WITH REVERSE TRANSCRİPTASE PCR

CDNA synthesis was initiated from RNA obtained with Tagman RT Reagents kit (Applied Byosistems, CA, USA). For this, the components written in the protocol and their quantities were adjusted.

10xRT Buffer: 2,5µl

$MgCl_2$: 5,5µl

dNTP: 1,25µl

10xRandom Hexamer: 1,25µl

RNase inhibitör: 1,5µl

Multiscribe RT 0,8µl

dH_2O: 9,5µl

RNA (örnek) 2,5µl

The mixture obtained is added in Veriti Thermal Cycler (Applied Biosystems) by adjusting the following temperature and time:

+ 25^0C 10 minute a cycle

+ 48^0C 60 minute a cycle

+ 4^0C ∞

cDNA material can be kept at + 4^0C or -20^0C for a long time.

The integrity of the RNA and efficiency of the RT reaction in each sample was confirmed for the housekeeping genes GAPDH or PGK1 whereas the presence of follicular epithelial cells was assessed by the presence of thyroglobulin (Tg) mRNA on RT-PCR as previously described by Rebelo S et al (Rebelo S Domingues R, Catarino AL et al. Immunostaining and RT-PCR: different approaches to search for RET rearrangements in patients with papillary thyroid carcinoma [14]. PCR for ret/PTC-1, -2, and – 3 was performed as described previously. The analysis of ret/PTC gene rearrangements refines the fine needle aspiration diagnosis of thyroid cancer [15]. Negative controls performed with each RT-PCR reaction omitted either template or reverse transcriptase. The products were resolved on a 2 % agarose gel containing ethidium bromide and visualized under UV light.

Statistical Analyses

Statistical tests were performed using the IBM SPSS Statistics for Windows, Version 25.0. (Released 2017, Armonk, NY: IBM Corp.) Whether the numerical variables showed a normal distribution or was not examined by Shapiro Wilk Test in each subgroup. An independent Two-Group Student-t Test was used for two-group comparisons, and One-Way Analysis of variance (One-Way ANOVA) was used for multiple-group comparisons. After variance analysis, Bonferroni Test was used as Post HOC method.

Relationships between categorical variables were examined using the Chi-Square Test. All statistical hypothesis checks were carried out at the 0.05 significance level.

Results

Demographic data, various clinic and laboratory characteristics of the patients with thyroid nodules that were taken to the study are given at Table 1. In our study, 60 nodules were analyzed. A total of 29 RET rearrangements were detected in the 60 nodules examined (48.3%). FNA RET rearrangements results were PTC 80%(4/5), suspect citology 75%(15/20), atypical nodule 40% (4/10), groving benign nodule 20% (3/15), recurrent nondiagnostic nodule 30%(3/10).

Table 1
Demographic, clinical and laboratory characteristics of patients with thyroid nodules

	Patients with Thyroid nodules (n = 60) Mean ± Standard deviation
Age (year)	41.60 ± 5.95
Sex (man/women) ***	35/65% (21/39)
Region where patients live	Aegean region
Ethnic difference	None
Exposure to radiation history	None
Presence of familial thyroid cancer	None
Hashimoto's thyroiditis	None
Graves' disease	None
Anti-Thyroglobulin	-
Anti-TPO	-
Calcitonin	-
(FNAB) RET/PTC +	48.3% (29/60)
(FNAB) RET/PTC 1	20% (12/60)
(FNAB) RET/PTC 3	16.7% (10/60)
(FNAB) RET/PTC 1–2	6.7% (4/60)
(FNAB) RET/PTC 2	3.3% (2/60)
(FNAB) RET/PTC1-3	1.7% (1/60)

Specifically, RET/PTC-1, RET/PTC-3, RET/PTC1 + RET/PTC2, RET/PTC2 and RET/PTC1 + RET/PTC3 transcripts were detected in 12 of 60 (20%), 10 of 60 (16.7%), 4 of 60 (6.7%), 2 of 60 (3.3%) and 1 of 60 (1.7%) of all nodules examined, respectively (Table 1). When all patients with RET/PTC rearrangements (n = 29) evaluated between them, the positiveness was found as followings: RET/PTC1 12 of 29 (41.4%) RET/PTC3 10 of 29(34.5%), RET/PTC1 + RET/PTC2 4 of 29 (13.8%), RET/PTC2 2 of 29 (6.9%), RET/PTC1 + RET/PTC3 1 of 29 (3.4%).

A total of 75%(15/20) FNA RET rearrangements were detected in the 20 PTC. RET/PTC positive was detected in 35%(14/40) of cases without cancer (sensitivity 75%, specificity 65%). The RET/PTC rearrangements was found to be statistically significant in PTC (p = 0,003). Significant correlation was found between A-high suspicion USG group (80%), B- intermediate suspicion USG group (45%) and C- low suspicion USG group (20%) in of RET/PTC rearrangements (p = 0.001) (Table 2). Significant correlation was found between USG groups and PTC (respectively group A,B,C: 60%, 30%, 10%)(p = 0.003)(Table 3). There was a statistical difference related to in the USG groups frequency between Classic type PTC (70%) and Follicular type PTC (30%)(p = 0.019)(Table 4). There was statistical difference related to between RET/PTC and PTC types in the USG groups (p = 0.012)(Table 4). There was no statistical difference related to between RET/PTC and PTC subtypes (P = 0.613) (Table 5). There was no statistical difference related to between PTC type (Classic type and Follicular type) and TSH, age and tumor diameter (respectively p = 0.740, p = 0.138, p = 0.875) (Table 6). There was no statistical difference related to between RET PTC type (1,3) and TSH, age and tumor diameter (respectively p = 0.626, p = 0.241, p = 0.752) (Table 7).

Table 2
The relation between USG Groups and RET/PTC types of the patients with thyroid nodules

USG	Group A	Group B	Group C
***RET/PTC +	80% (16/20)	45% (9/20)	20% (4/20)
RET/PTC1	7	4	1
RET/PTC3	6	3	1
RET/PTC1-2	2	1	1
RET/PTC2	0	1	1
RET/PTC1-3	1	0	0

*** Significant correlation was found between A-high suspicion USG group,

B- intermediate suspicion USG group and C- low suspicion USG group in of RET/PTC rearrangements (p = 0.001)

Table 3
The relation between USG and RET/PTC types of the patients with PTC

USG	Group A	Group B	Group C
***PTC	60% (12/20)	30% (6/20)	10% (2/20)
RET/PTC +	83% (10/12)	67% (4/6)	50% (1/2)
RET/PTC1	6/7	2/4	1/1
RET/PTC3	4/6	2/3	0/1
RET/PTC1-2	0/2	0/1	0/1
RET/PTC2	0	0/1	0/1
RET/PTC1-3	0/1	0	0

*** Significant correlation was found between USG groups and PTC (p = 0.003)

Table 4
The relation between USG and pathological subtipe of the patients with PTC

USG	Group A	Group B	Group C
*Classic type 70% (14/20)	64% (9/14)	29% (4/14)	7% (1/14)
Follicular type 30% (6/20)	50% (3/6)	33% (2/6)	17% (1/6)

* There was a statistical difference related to in the USG groups frequency between Classic type PTC (70%) and Follicular type PTC (30%) P = 0.019 (A, B, C)

*There was statistical difference related to between RET/PTC and PTC types in the USG groups (P = 0.012) (C/F)

Table 5
The relation between RET/PTC types and pathological subtipe of the PTC patients

RET/PTC + 75% (15/20)	Classic type 73% (11/15)	Follicular type 27% (4/15)
RET/PTC 1	55% (6/11)	75% (3/4)
RET/PTC 3	45% (5/11)	25% (1/4)

There was no statistical difference related to between RET/PTC and PTC subtypes (P = 0.613)

Table 6
The relation between PTC type (Classic type and Follicular type) and TSH, Age and tumor diameter

	Classic type (n = 14)	Follicular type (n = 6)	
TSH (mU/L)	2.04 ± 0.97	1.88 ± 0.96	p = 0.740
Age(year)	39.5 ± 4.09	42.50 ± 3.61	p = 0.138
Diamater (mm)	12.71 ± 3.49	13.00 ± 4.09	p = 0.875

Table 7
The relation between RET PTC type (1,3) and TSH, Age and tumor diameter

	RET/PTC 1 PTC	RET/PTC 3 PTC	
TSH (mU/L)	1.83 ± 0.72	2.31 ± 0.91	P = 0.626
Age(year)	39.83 ± 6.91	39.30 ± 4.11	p = 0.241
Diamater (mm)	12.22 ± 4.38	12.83 ± 2.78	p = 0.752

There was no statistical difference related to between sex and usg groups and PTC and RET/PTC (respectively p = 1, p = 1, p = 0.935) (Table 8). There was no statistical difference related to between age and usg groups and PTC and RET/PTC (respectively p = 0.274, p = 0.185, p = 0.092) (Table 8). There was no statistical difference related to between TSH and usg groups and PTC and RET/PTC (respectively p = 0.512, p = 0.675, p = 0.870) (Table 8). There was no statistical difference related to between RET/PTC positive (12.46 ± 3.71mm) and RET/PTC negative (13.80 ± 3.27mm) tumor diameter (p = 0.485). There was statistical difference related to between usg groups tumor diameter A and C (P = 0.010) (Table 8).

Table 8
The relation between USG groups and Sex, Age, TSH, tumor diameter

	Group A	Group B	Group C	p
Sex (Male/Female) 35/65% (21/39)	7/13	7/13	7/13	P = 1
PTC (Male/Female) 35/65% (7/13)	4/9	2/4	1/0	P = 1
RET/PTC (Male/Female) 48% (10/21) / 49% (19/39)	6/10	3/6	1/3	P = 0.935
Age(year)	40.05 ± 4.2	41.80 ± 8.2	43.10 ± 4.2	P = 0.274
TSH (mU/L)	1.85 ± 0.87	2.45 ± 0.91	1.90 ± 0.72	P = 0.512
* TM (mm)	11.25 ± 3.01	14.00 ± 2.82	18.50 ± 0.70	P = 0.010

* There was statistical difference related to between USG groups tumor diameter A and C (P = 0.010)

Discussion

Recently, RET/PTC rearrangements have been shown not only in PTC but also in benign thyroid lesions. In recent years, thyroid cancer has been at the forefront of molecular pathology as a result of the consequences of the Chernobyl disaster and the recognition of the role of RET/PTC rearrangements in papillary thyroid carcinomas (PTCs). The evolution of molecular tests for thyroid nodules followed the discovery of various diagnostic and prognostic molecular markers of thyroid cancer that can be applied to thyroid FNA [16].

The most frequent initial manifestation of thyroid cancer is the appearance of a nodüle, most of which are benign nodules. Estimating, less than 5% are malignant nodules. In reality, some cases are misdiagnosed, and many patients undergo unnecessary surgery. Because of this, an accurate pre-surgery evaluation is required. In most cases, the most reliable diagnostic test for thyroid nodules is fine needle aspiration (FNA) cytology.

In our study, we performed the molecular analysis using a procedure that involves RET/PTC gene mutation in easily obtainable FNA samples. We aimed to attempt to improve the efficacy of the FNA diagnosis of thyroid nodules and thus patient management.

In our previous study, RET/PTC in tumor tissue was determined positive in 67(66,3%) of totally 101 patients and RET/PTC determined negative in 34(33,7%) [17]. In our recent newer study, A total of 75% FNA RET rearrangements were detected in the 20 PTC. RET/PTC positive was detected in 35% of cases without cancer (sensitivity 75%, specificity 65%). RET/PTC rearrangements was found to be statistically significant in PTC (p = 0,003). In first years, RET/PTC was considered a PTC specific. Later times, it was also found sporadically in benign thyroid lesions [18,19]. Sapio MR and all. investigated whether a search

for the oncogenes RET/PTC, TRK and BRAF(V600E) in thyroid aspirates could refine an uncertain diagnosis. On final analysis, no false-positive results were reported in 131 samples and five out of seven carcinomas (71%) were correctly diagnosed [20]. In our study, the FNA RET/PTC results were (80%) PTC, (75%) suspect citology, (40%) atypyc nodule, (20%) benign nodule, (30%) nondiagnostic nodule.

In a multicenter study in Italy, the rate of mutation-positive FNAs were found related to the risk of malignancy of in each Bethesda diagnostic categories class [21]. On the other hand, in a study in Germany, their data suggest that the application of the current seven-gene panel in a routine primary referral setting does not improve the presurgical diagnosis of thyroid FNAs [22].

For the first time in literature, our study investigated RET/PTC in according to ATA USG risk groups (A-high suspicion, B- intermediate suspicion and C- low suspicion). Significant correlation was found between A-USG group (80%), B-USG group (45%) and C-USG group (20%) in of RET/PTC rearrangements ($p = 0.001$) (Table 2). Similarly with the literature, in our study significant correlation was found between USG groups and PTC (respectively group A,B,C: 60%, 30%, 10%)($p = 0.003$)(Table 3) [13]. When we considered patients with PTC according to histological types, they were composed of classical type and follicular variant (FV). The FV is the most common type of PTC after classic PTC [23]. In our study, classical type and FV of PTC prevalence was similar with literature [24]. In our study, in frequency between Classic type and Follicular varient PTC significant correlation was found with USG groups ($p = 0.019$) (Table 4).

The prevalence of RET rearrangements have been examined in cohorts of papillary carcinomas in many countries. The prevalence has shown a marked geographic discordance [17,25–28]. RET/PTC1 and RET/PTC3 are the most encountered types [29]. Similarly, RET/PTC1 and RET/PTC3 the most type of RET/PTC was found in our study.

The relationship between RET/PTC prevalence and types of PTC were investigated. No correlation difference was found between RET/PTC and types of PTC (Table 5).

In a study Basolo F at al. investigated the prognostic meaning of RET/PTC rearrangement on the long term outcome of PTC. No correlation was found between RET expression and other parameters such as age at diagnosis, sex, histological variant and tumor class [30].

The relationship between prognostic factors and RET/PTC prevalence and types in patients with PTC were investigated in our study. There was no statistical difference related to TSH, age and tumor diameter in both PTC type (Classic and Follicular) and RET PTC type (1,3) (Table 6 and Table 7). No correlation was found with RET/PTC releated to multifocality, bilaterality, soft tissue invasion and lymph node metastases.

In the beginning of the literature firstly, our study investigated the prevalence of RET/PTC mutation in thyroid nodules according to risk category ATA USG. On the other hand, we were determined the relation of the between RET/PTC and malignancy. No differant was found between USG groups and TSH, sex,

age at diagnosis and tumor diameter (Table 8). In addition, No difference was found with sex in USG groups neither RET/PTC nor PTC. There was a statistical difference related tumor to diameter with groups A and C (Table 8).

The weaknesses of our study were the absence of other molecular markers and the number of patients. Strengths of the study were the cytological evaluation and histopathological confirmation of RET/PTC according to the ATA USG risk category for the first time.

As a result, Cytological analysis is now, practically routinely, being joined with molecular analysis to enable the patholog to make a more actual diagnosis. Molecular testing of FNA samples improves presurgical diagnosis. RET/PTC frequency in FNA significant difference was found in ATA USG risk category. RET/PTC was found to be significantly higher in the diagnoses of the PTC with 75% sensitivity and 65% specificity. However, a larger clinical study will be required to verify this resuls.

Declarations

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval Consent forms and protocols were approved by the ethics committee of the Ege University Medical School. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/ or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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