Pathology & Oncology Research (2020) 26:507–513
https://doi.org/10.1007/s12253-018-0540-3

ORIGINAL ARTICLE

RET/PTC Gene Rearrangements in Thyroid Carcinogenesis: Assessment and Clinico-Pathological Correlations

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Received: 4 October 2018 / Accepted: 12 November 2018 / Published online: 22 November 2018

Abstract

Rearranged during transfection (*RET*) is a proto oncogene implicated in thyroid carcinogenesis of papillary type (PTC). The *RET* proto-oncogene in PTC is constitutively activated by fusion of its tyrosine kinase domain with the 5 ´region of another gene thereby generating chimeric products collectively named *RET/PTCs. RET/PTC1* and *RET/PTC3* are best characterized among all *RET/PTC* rearrangements. Kashmir valley has witnessed an alarming increase in thyroid cancer incidence in young women. Therefore, we investigated the occurrence of *RET/PTC* 1 & 3 rearrangements by semi quantitative and qPCR in thyroid cancer patients ($n = 48$) of Kashmiri population and interrelated results with various clinicopathological characteristics. We observed that all the *RET/PTC* rearrangements were confined to PTC cases (10/40). Presence of *RET/PTC* rearrangement significantly correlated with gender, elevated TSH levels and lymph node metastasis. Overall, our study advocates that *RET/PTC3* rearrangement is a frequent event in the carcinogenesis of thyroid gland in Kashmiri population although a study with a larger sample size is needed to get a clear scenario.

Keywords Thyroid Cancer · TSH · Papillary thyroid cancer · Kashmir Valley · RET/PTC rearrangement

Introduction

Thyroid cancer is a very common malignancy of endocrine system with increasing incidence rate over the previous two decades. However, the mortality rates are not too high due to good screening techniques and early diagnosis [1]. The disease which is more common in women than in men could become the fourth most common cancer by 2030 in the United States [2]. Several risk factors have been attributed to the development of thyroid carcinomas such as exposure to ionizing radiations, iodine uptake, Hashimoto's thyroiditis, diabetes, reproductive factors [3]. Main histotypes of thyroid cancers include Papillary thyroid cancer (PTC), Follicular thyroid cancer (FTC) and Anaplastic thyroid cancer (ATC). of which PTC accounts for 80% of all the cases worldwide [4]. In thyroid tumours the difference between benign and malignant disease is largely based on a cytological assessment of thyroid cells obtained by fine needle aspiration cytology (FNAC). However many benign thyroid nodules show morphological features of PTC making FNAC inconclusive. Genetic markers especially pathogenic oncogenes known for thyroid cell transformation improve FNAC precision [5].

One of the first oncogenes revealed to have a role in PTC was *RET/PTC*. It is one of the established genetic markers exclusively of PTC. The *RET* (rearranged during transfection) is a proto-oncogene which encodes a cell membrane receptor tyrosine kinase. The RET protein encompasses an ligand-binding extracellular domain followed by a transmembrane region (a cysteine-rich loop) and an intracellular domain (containing the juxtamembrane domain and the tyrosine kinase domain) [6]. Activation of *RET* stimulates mitogen-activated protein kinase

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(*MAPK*) and PI3K pathways that promote cell growth, differentiation, proliferation and cellular survival [7]. In PTC, the fusion of the Receptor tyrosine kinase domain with the 5′ end sequence of one of the different heterologous genes via rearrangements creates a series of at least 12 chimeric oncogenes jointly branded as *RET/PTC*. *RET/PTC1* and *RET/PTC3* being most common variants result from the fusion of tyrosine kinase domain of *RET* with *H4* (D10S170) and *ELE1* gene respectively [8].

Kashmir valley has witnessed an alarming increase in PTC over the last few years with 75% of the patients being young females. In our previous studies we have elucidated the downstream gladiators of RET such as *BRAF* and *RAS* genes in our population, wherein, the *BRAF* mutation accumulated to 25% with absence of any *RAS* Mutation [9, 10]. Therefore, in this study, we determined the prevalence of *RET/PTC1* and *PET/PTC3* rearrangements as a possible cause for PTC in Kashmiri population and interrelated results with various clinicopathological factors.

Materials and Methods

Sample Collection

Forty-eight ($n = 48$) thyroid tumour and their adjacent normal tissues were collected from the Department of General and Minimal Invasive Surgery, SKIMS, Srinagar and Department of ENT, SMHS, Govt. Superspeciality Hospital, Srinagar. All the samples were histopathologically confirmed. Tissue samples were collected and immediately stored at −80 °C till further use. Chemo or radiotherapy was not received by any patient.

RNA Extraction, cDNA Synthesis and Semiquantitative PCR

RNA was extracted using trizol reagent (Invitrogen Inc.) (Fig. 1) and cDNA was synthesized using single strand cDNA synthesis kit (Thermo Scientific Ltd.) according to manufacturer's guidelines. Briefly, 1 μg RNA was reverse-transcribed using AMV Reverse transcriptase and oligo dT primers in a final volume of 20 μl. For semi-quantitative PCR the cDNA synthesized was subjected to routine endpoint PCR. The amplification reaction was carried out in 25 μl reaction volume which included 50 mM $Mgcl_2$, 10 mM dNTP mix, 1 U Taq Polymerase, 10 mM primers specific for *RET/PTC1*, *RET/PTC3* and *β-actin.* Primer sequences were; *RET/PTC1* Forward: 5'-ATTGTCATCTCGCCGTTC-3′, Reverse 5'-TGCTTCAGGACGTTGAAC-3′; *RET/PTC3* Forward: 5'-TGGAGAAGAGAGGCTGTATC-3′, Reverse 5'-CGTTGCCTTGACTTTTC-3′; *β-actin* Forward: 5'-TGCGTGACATTAAGGAGAAG-3′, Reverse: 5'-GCTCGTAGCTCTTCTCCA-3′. PCR Conditions were: 95 °C for 10 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 30 s, and 72 °C for 10 min. The amplicons were resolved using 2% agarose gels containing ethidium bromide and finally visualized by gel documentation system (Fig. 2).

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed on samples which were positive for *RET/PTC* rearrangements by semi quantitative PCR. qRT-PCR assay was performed by *SYBR Green master mix qRT-PCR kit* (Thermo Scientific Ltd.) as per maker's instruction. Briefly 02 μl of cDNA was mixed with distilled water, SYBR Green Master Mix and primers and reaction mixture brought to a final total volume of 25 μl. PCR and data acquisition was performed by *Piko Real* Real-Time PCR (Thermo Scientific Ltd.). qRT-PCR data were quantitated using the relative quantification by ΔΔCT method. One positive sample of *RET/PTC3* rearrangements identified by semi-quantitative PCR served as a reference standard (calibrator). β-Actin mRNA was used as an internal control. qRT-PCR reactions were carried out in triplicates, and no template control (NTC) was

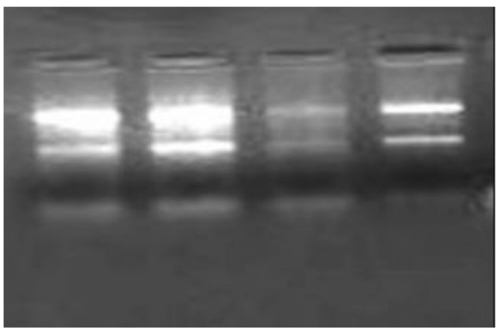

Fig. 1 Representative gel picture of RNA extracted from frozen tissue specimen. Lane 1&2 shows RNA extracted from normal and tumor tissues respectively

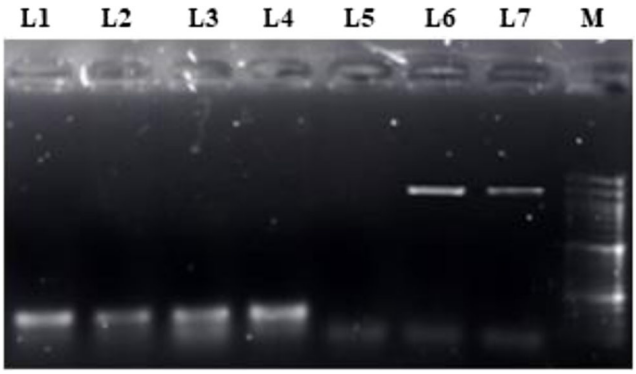

Fig. 2 Representative gel picture for semi quantitative PCR. L1, L2, L3, L4 shows amplification of B-actin; L5 shows RET/ PTC1 (−) ive sample; L6 & L7 show RET/PTC 3 (+) ive samples. Lane M shows bands for 50 bp ladder

included each time. For ensuring single product formation *Melt curve analysis* was performed (Fig. 3).

Statistical Analysis

Pearson's χ^2 test, Fisher's exact test or χ^2 test (trend) were used for discrete variables; independent t-test and paired t-test for continuous variables. Bivariate logistic regression analysis was used to obtain odds ratios (ORs) and 95% confidence intervals (CIs). $P \leq 0.05$ was marker of significance. Statistical tests were performed using the software SPSS 16.0 (SPSS Inc., Chicago, Illinois).

Results

Patient Features

In this study thyroid cancer cases ($n = 48$) which histologically confirmed and their adjacent normal tissues were analyzed for *RET/PTC* rearrangements. The cases included 40 females and 08 males. 40 of 48 (83.3%) subjects were < 45 years and 8 of 48 (16.7%) were ≥ 45 years had a mean age of 24 ± 38. No patient had a history of exposure to any kind of ionizing radiations.at the time of diagnosis. Table 1 contains the clinicopathological characteristics of the study subjects.

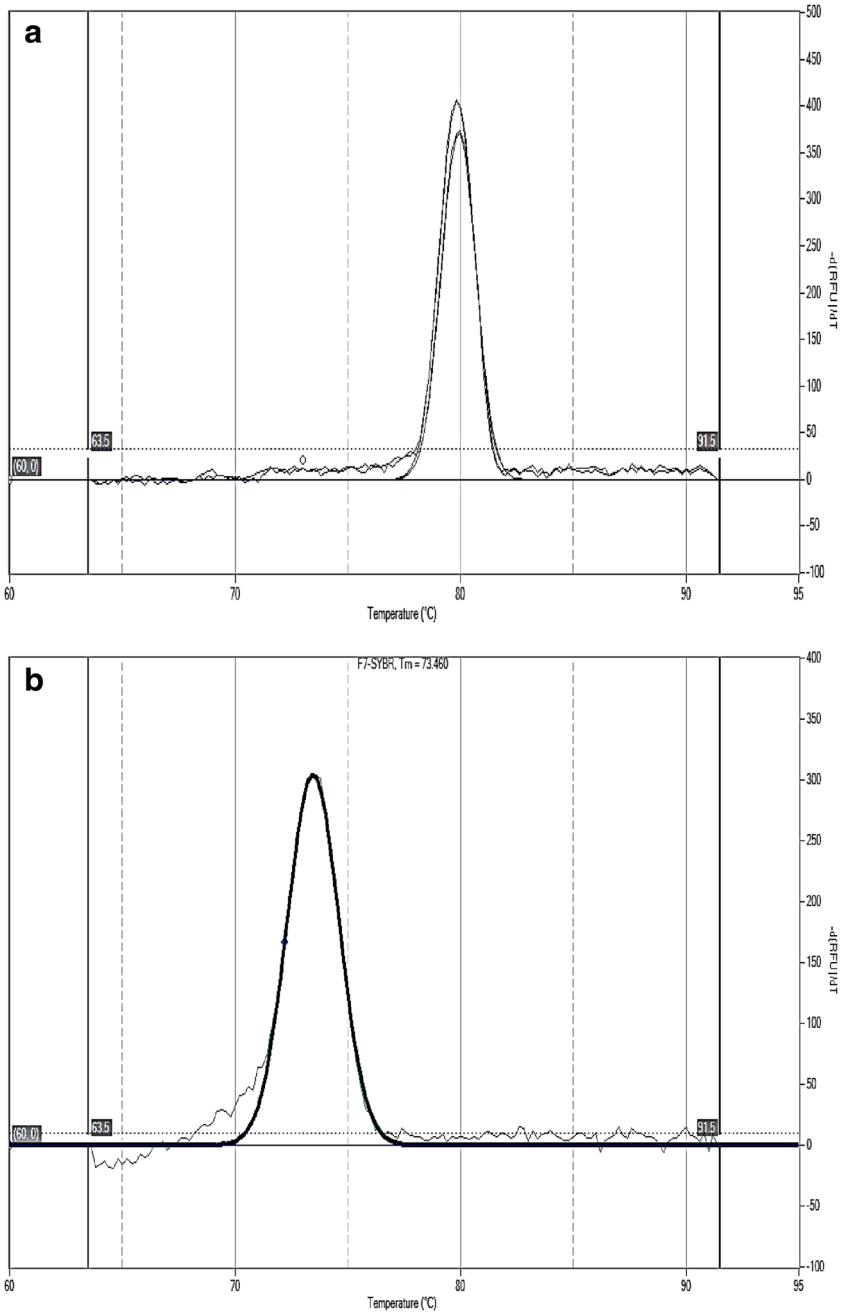

Fig. 3 **a** Melt curve for the amplification of RET/PTC3 **b** Melt curve for the amplification of beta Actin

Table 1 Clinico-epidemiological and clinico-pathological variables of thyroid cancer patients used for analysis of RET/PTC rearrangements

Variable	Parameter	Cases (n = 48)	
		n	% n
Sex	Female	40	83.3
	Male	08	16.7
Age in years	<45	40	83.3
	≥45	08	16.7
Dwelling	Rural	38	79.1
	Urban	10	20.9
Smoking status	Non-smoker	40	83.3
	Smoker	08	16.7
BTD	Yes	28	58.4
	No	20	41.6
TSH levels	Elevated	18	37.5
	Normal	30	62.5
Histological types	Papillary	38	79.1
	Follicular	04	8.3
	Others	06	12.6
Grade	WD	40	83.3
	PD	08	16.7
Stage, < 45 years	Stage I	16	33.4
	Stage II	19	39.6
Stage, ≥ 45 years	Stage I&II	08	16.6
	Stage III/above	05	10.4
LN metastasis	Present	30	62.5
	Absent	18	37.5
V/C Invasion	Present	25	52.0
	Absent	23	48.0

n; Number, BTD; Benign Thyroid Disease, TSH; Thyroid stimulating hormone, LN; Lymph node, V/C; Vascular/Capsular, WD; Well differentiated, PD; Poorly differentiated

RET/PTC1 & 3 Detection

We investigated *RET/PTC* rearrangements in 40 PTC, 03 FTC and 05 other types of thyroid cancer cases and their histologically verified adjacent normal tissues. *RET/PTC1* rearrangement was absent in our population. Only *RET/PTC3* rearrangements were observed confined to 20.83% (10/48) of the cases (Fig. 1). *RET/PTC3* rearrangement was not observed in any of the normal tissues. qPCR analysis was performed for all the samples which were positive for *RET/PTC3* rearrangements by semi-quantitative PCR. All the samples found positive for rearrangements by semi quantitative PCR gave positive signal for qRT-PCT. Further, the relative quantification of the data showed that there was no significant difference between the expression levels of *RET/PTC3*-positive samples and positive control (calibrator). Melt curve analysis showed that there was no production of any nonspecific template (Fig. 2).

RET/PTC3 Rearrangement and Correlation with Clinicopathological Characteristics

The relationship between *RET/PTC3* rearrangements and clinicopathological characteristics is given in Table 2. A statistical significance was observed with gender [Adj OR = 5.6, 95% CI (1.1–19.1) (P = 0.04)], elevated TSH levels [Adj OR = 0.09, 95% CI (0.01–0.5) (P = 0.003)] and lymph node metastasis [Adj OR = 0.2, 95% CI (0.03–1.08) (P = 0.049)]. But, the association of *RET/PTC3* rearrangements with age, dwelling, smoking status, the presence of benign thyroid disease, histological types, grade, stage and vascular invasion was not statistically significant.0.

Discussion

The identification of various molecular mechanisms in the thyroid cell transformation is critical for understanding the pathogenesis of this disease. Several genetic alterations have been reported in thyroid cancers such as mutations in *RAS, BRAF, P53*, *TSHR* genes [4, 11]. However, *RET/PTC* oncogene is particularly important for its specificity in thyroid neoplasms. *RET/PTC* rearrangements have been hallmarks of PTCs [12, 13]. The RET/PTC oncoproteins are constitutively phosphorylated receptors produced by rearrangements of *RET* with a range of activating genes. These rearrangements in PTC have been strongly associated with ionizing radiations as seen in atomic bomb children survivors of post-Chernobyl nuclear disaster [14–16].

In the present study we investigated the prevalence of *RET/PTC1* and *RET/PTC3* rearrangements in thyroid cancers in northern India (Kashmir) and interrelated the results with their various clinicopathological features. To our knowledge this is the first study conducted in Kashmir valley regarding the occurrence of *RET/PTC* rearrangements in thyroid carcinogenesis. Out of 48 thyroid cancer cases *RET/PTC1* rearrangement was absent but *RET/PTC3* rearrangement was found in 20.83% (10/48) of cases which were confined to PTC only. The study conducted on German population showed low frequency of *RET/PTC* rearrangements in PTC with only 7% *RET/PTC1* and 1% *RET/PTC3* positivity [17]. Similar low frequency results have been observed in France (11%) and Saudi Arabia (3%) [6, 18]. Studies conducted from Taiwan showed a high frequency of *RET/PTC1* & 2 rearrangements in PTC (55%) [19]. Similar high frequency results have been observed in New Caledonian (70%) and Australian population (85%) and Italy (33–35%) [6, 20]. Studies done on Korean population found *RET/PTC1, RET/PTC2* and *RET/PTC3* rearrangements to be 6.5%, 6.5% and 0% respectively whereas studies from Japanese population showed prevalence of *RET/PTC* rearrangements to be 30% in PTC [21, 22]. *RET/PTC* rearrangements have been reported to be very high (69% to

Table 2 Association of RET/PTC Rearrangement with different variables of thyroid cancer patients

Variable	Cases n = 48 (%)	*RET/PTC* Rearrangement (n = 48)		OR (95% CI)	*P* value
		Positive *n* = 10 (20.8%)	Negative *n* = 38 (79.2%)		
Gender					
Female	40(83.3)	06 (15.0)	34 (85.0)	5.6(1.1–19.1)	0.04
Male	08(16.7)	04 (50.0)	04 (50.0)		
Age					
< 45	40(83.3)	07(17.5)	33(82.5)	2.8(0.5–14.1)	0.2
≥ 45	08(16.7)	03(37.5)	05(62.5)		
Dwelling					
Rural	38(79.1)	08(21.0)	30(79.9)	0.9(0.1–5.3)	0.6
Urban	10(20.9)	02(20.0)	08 (80.0)		
Smoking status					
Non-Smoker	40(83.3)	09(20.0)	31(80.0)	0.5(0.05–4.5)	0.5
Smoker	08(16.7)	01(50.0)	07(50.0)		
BTD					
Yes	28(58.4)	08(29.0)	20(69.0)	0.2(0.05–1.5)	0.1
No	20(41.6)	02(10.0)	18(90.0)		
TSH levels					
Elevated	18 (44.8)	08(44.4)	10(55.6)	0.09(0.01–.0.5)	0.003
Normal	30(55.2)	02(6.6)	28(93.7)		
Histological types					
Papillary	40(83.4)	10(25.0)	30(75.0)		
Follicular	03(6.2)	00(00.0)	03(100)	0.7 (0.07–.7.0)	0.4
Others	05(10.4)	00(00.0)	05(100)	0.4 (0.05–4.3)	0.4
Grade					
WD	40(83.3)	07(17.5)	33(82.5)	2.8(0.5–14.6)	0.2
PD	08(16.7)	03(37.5)	05(62.5)		
Stage, < 45 years					
Stage I	16(33.4)	06(37.5)	10(62.5)	1.9(0.6–6.5)	0.2
Stage II	19(39.6)	02(10.5)	17(89.5)		
Stage, ≥ 45 years					
Stage I&II	08(16.6)	00(0.0)	08(100.0)	6.7(0.5–86.5)	0.1
Stage III & above	05(10.4)	02(40.0)	03(60.0)		
V/C Invasion					
Present	30 (62.5)	08(26.6)	22(73.4)	0.34(0.06–1.8)	0.2
Absent	18 (37.5)	02(11.1)	16(88.9)		
LN metastasis					
Present	25(52.0)	08(32.0)	17(68.0)	0.2 (0.03–1.08)	0.049
Absent	23(48.0)	02(8.6)	21(91.4)		

n; Number, BTD; Benign Thyroid Disease, TSH; Thyroid stimulating hormone, LN; Lymph node, V/C; Vascular/ Capsular, WD; Well differentiated, PD; Poorly differentiated

83%) in areas exposed to radiations [16, 23]. This heterogeneity in *RET/PTC* rearrangements may be due to ethnicity, different geographical location, environmental exposure and techniques employed to detect *RET/PTC* rearrangements. Although majority of studies support the fact that *RET/PTC* rearrangements occurs due to double-stranded break in each gene participating in rearrangement but there are studies who have shown that in addition to ionizing radiations, other recognized carcinogens, such as caffeine, ethanol, hypoxia, can also induce double-stranded DNA breaks and generate *RET/PTC* rearrangements [24] which are in consistency with our study where all the patients with *RET/PTC3* rearrangements had no history of exposure to any kind of radiations.

In our study we also correlated *RET/PTC3* positivity with various clinicopathological characteristics. *RET/PTC3* rearrangements were significantly associated with gender, lymph node metastasis and elevated TSH levels ($P \leq 0.05$). Our results are in coherence with Adeniran et al. who reported that

lymph node metastasis is significantly associated with *RET/PTC* rearrangements [25]. Su et al. reported that high prevalence of *RET/PTC* rearrangements is associated with female gender and younger age [26]. Although statistically insignificant, the *RET/PTC3* rearrangements were restricted only to PTC ($P > 0.05$) in uniformity with the majority of studies [14, 15, 27] but not in full agreement with few studies who established that *RET/PTC* rearrangements are present in other variants such as FTC, MTC, hoshimotos thyroiditis, adenomas and goiter in addition to PTC [28–31]. In agreement with our observation several other studies did not show any link between age, tumor size, staging, history of benign thyroid disease histological subtype smoking, alcohol consumption and *RET/PTC* rearrangements [32, 33].

In summary, this is the first study regarding the quantitative analysis of rearranged forms of *RET* in thyroid carcinoma from this part of the world which is very imperative for understanding the role of *RET* activation in thyroid carcinogenesis. Our results indicate that *RET/PTC3* rearrangements were restricted to PTC. There was a significant association between *RET/PTC3* rearrangements and the clinicopathological parameters like, female gender, elevated TSH levels and lymph node metastasis. Although the observations are quite promising but a study with a larger sample size is needed to validate our results.

Acknowledgments The authors acknowledge the surgical staff of the Department of General Surgery, SKIMS and ENT, SMHS hospital who facilitated the tissue sampling and the scientific staff of Department of Biotechnology, University of Kashmir who helped in standardizing the qPCR conditions.

Funding The Study was supported by the Department of Biotechnology, Ministry of Science and Technology, Govt. of India (BT/PR4626/MED/30/702/2012 Dated: 07.08.2013).

Compliance with Ethical Standards

Conflict of Interest There is no conflict of interest.

Ethical Approval The study was approved by the Ethical Clearance Committee of SKIMS.

Informed Consent All the samples were collected after taking written informed consent from the patients and proper ethical procedures were followed.

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