Variations in MAP kinase gladiators and risk of differentiated thyroid carcinoma

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Received March 27, 2021; Accepted October 27, 2021

Abstract. Thyroid carcinoma (TC) accounts for ~2.1% of newly diagnosed cancer cases. Mutations in KRAS, HRAS, NRAS and BRAF are primary participants in the development and progression of various types of malignancy, including differentiated TC (DTC). Therefore, the present prospective cohort study aimed to screen patients with DTC for variations in RAS gene family and BRAF gene. Exon 1 and 2 of KRAS, HRAS, NRAS and exon 15 of BRAF gene were screened for hotspot mutations in 72 thyroid tumor and adjacent normal tissue samples using di-deoxy Sanger sequencing. HRAS T81C mutation was found in 21% (15 of 72) of DTC tissue samples, therefore this mutation was investigated in blood samples from patients with DTC and controls as a genetic polymorphism. In addition, HRAS T81C genotypes were determined in 180 patients with DTC and 220 healthy controls by performing restriction fragment length polymorphism. BRAFV600E mutation was confined to classical variant of papillary thyroid cancer (CPTC; 44.4%) and was significantly associated with multifocality and LNM metastasis. No mutation was found in exons 1 and 2 of KRAS and NRAS and exon 2 of HRAS genes, however, mutation was detected in exon 1 of HRAS gene (codon 27) at nucleotide position 81 in 21% (15 of 72) of DTC tumor tissue samples. Furthermore, HRAS T81C single nucleotide polymorphism was significantly associated with the risk of DTC with variant genotypes more frequently detected in cases compared with controls (P<0.05). Moreover, frequency of variant genotypes (TC+CC) was significantly higher among DTC cases with no history of smoking, males, greater age, multifocality and LN metastasis compared with healthy controls (P<0.05). BRAFV600E mutation was primarily present in CPTC and associated with an aggressive tumor phenotype but mutations in RAS gene family were not present in patients with DTC. HRAS T81C polymorphism may be involved in the etiopathogenesis of DTC in a Pakistani cohort. Furthermore, testing for the BRAFV600E mutation may be useful for selecting initial therapy and follow-up monitoring.

Introduction

Thyroid cancer (TC) has become a global concern due to its increasing incidence rate and was ranked 9th among all cancers in 2020, with ~586,000 cases globally and 3 times higher incidence in women compared with men (1). Due to more stringent diagnostic practices, TC incidence has begun to decline in women (2,3). TC generally derives from epithelial follicular cells, also known as C cells, and ~90% of TC cases are well-differentiated (WD). DTC is further classified as papillary (PTC) or follicular (FTC), depending on histopathological criteria (4). DTCs frequently have genetic alterations in the gladiators, molecules associated with the mitogen activated protein kinase (MAPK) signaling pathway, including RET/PTC rearrangement and point mutations in RAS and BRAF genes, leading to activation of the MAPK pathway (5).

BRAF serine-threonine kinase belongs to the family of RAF proteins. BRAF mutations are oncogenic driver mutations associated with solid tumors, including thyroid carcinoma. Among all BRAF mutations identified, BRAFV600E accounts for >90% (5). Missense mutation results in T>A transversion at nucleotide position 1799 (c.T1799A), leading to substitution of valine (V) into glutamic acid (E) at codon 600, which disrupts interactions between the activation loop and ATP binding site and allows formation of new interactions that keep the protein in a catalytically active conformation, resulting in continuous phosphorylation of MEK (6,7). The prevalence of BRAFV600E in TC is more heterogeneous in Asian populations, spanning 28.2±90.0% (8). BRAF mutations are highly prevalent in papillary carcinoma with classical histology and in the tall cell variant, but are rare in the follicular variant (9). In many studies, the presence of BRAF mutation has been associated

DOI: 10.3892/mco.2021.2478
with aggressive tumor characteristics, such as extrathyroidal extension, advanced tumor stage at presentation, tumor recurrence and lymph node (LN) or distant metastasis (9,10). Mutations in RAS and BRAF genes rarely overlap in the same tumor and are mutually exclusive (5, 11).

Mammalian cells contain three functional poteonco
genomes of the RAS family known as KRA (Kristan), HRAS (Harvey) and NRAS (Neuroblastoma) (12). These genes encode small GTPases, which are primary participants in the transmission of growth signals from cell membrane receptors to the nucleus (13). Gain of function mutations in the RAS gene family result in continuous stimulation of cell growth and proliferation, even in the absence of extracellular signals (14), resulting in tumorigenesis. Point mutations in the RAS gene (exon 1; codons 12 and 13) increase its affinity for GTP or inactivate its autocatalytic GTPase function (exon 2; codon 61) (15) thereby, permanently activating the MAPK and PI3K-AKT pathways. Point mutations of RAS occur variably in all types of thyroid follicular cell-derived tumors. In FTC, RAS mutations are found in 40-50% of tumors (16) and may also correlate with tumor dedifferentiation and less favorable prognosis (17). In PTC, RAS mutations are relatively infrequent and occur in ~10% of tumors (18). Papillary carcinoma with RAS mutations almost always exhibit follicular variant histology; this mutation also correlates with significantly less prominent nuclear features of papillary carcinoma, more frequent encapsulation and lower rate of LN metastasis (19). Studies have reported an association between RAS mutations and more aggressive behavior of PTC and higher frequency of distant metastasis (20,21).

Besides the mutation hotspots of KRAS, HRAS and NRAS, inherited single nucleotide polymorphism (SNP) in exon 1 of HRAS T81C (rs12628) is associated with risk of different types of human cancer. HRAS T81C homozygous variant genotype (CC) has been associated with bladder cancer, chronic myeloid leukemia and TC (22,23). This SNP, located at codon 27 of exon 1, does not disturb the p21 protein structure and function as codons CAC and CAT both encode histidine (His27His).

This variation instead disturbs expression of HRAS by inducing overexpression (24) and may be associated with additional polymorphic loci inside regulatory sections of HRAS. Earlier studies also investigated the role of HRAS T81C SNP in TC and associated it with aneuploidy in follicular tumors of thyroid (22, 24).

The present study hypothesized that mutations in hotspot regions of RAS (KRAS, HRAS, NRAS); BRAF and presence of HRAS T81C variation may modulate susceptibility to TC. To verify this hypothesis, these mutations and polymorphisms in TC were assessed to ascertain their association and functional role in thyroid carcinogenesis of Pakistani population.

Materials and methods

Study design. The present investigation was a prospective cohort study conducted by the Department of Biological Sciences, International Islamic University, Islamabad; Department of Biochemistry, Pakistan Institute of Medical Sciences (PIMS) Islamabad and Combined Military Hospital (CMH) Muzaffarabad, Pakistan. Ethical approval was obtained from Ethical Review Board of PIMS. All participants enrolled in the study provided written informed consent allowing the use of their tissue and blood samples. Patients with any genetic disorder, other type of cancer or receiving chemotherapy were excluded from the study.

Study subjects and sample collection for analysis of BRAF and RAS mutations. Thyroid tumor and adjacent normal tissue (n=72; distance, 5-10 mm) samples were obtained from historically confirmed patients with DTC who underwent total or hemi-thyroidectomy in the Department of General Surgery, PIMS and CMH, Pakistan between 2016 and 2018. Tissue samples were collected in sterile vials and immediately stored at -80°C until further processing.

Study subjects and sample collection for HRAS T81C genotyping. A total of 180 peripheral blood samples from patients with DTC were collected from Department of General Surgery, PIMS and CMH, Pakistan, between 2017 and 2019. In addition, 220 blood samples were collected from ethnicity-matched healthy controls free from any type of malignancy, who visited hospitals for routine checkup. A total of ~3 ml each blood was collected in EDTA-coated vials from patients with DTC and healthy controls and stored at -80°C until further processing.

DNA extraction. DNA from fresh tumor and adjacent normal tissue was isolated using PureLink Genomic DNA Mini kit (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 5 formalin-fixed, paraffin embedded (FFPE) tissue samples were retrieved from Department Pathology, PIMS and CMH, Pakistan, which had not been immediately collected following resection of thyroid gland. DNA was isolated from FFPE tissue using QiAamp DNA FFPE Tissue kit (Qiagen GmbH). Furthermore, DNA was isolated from blood samples of cases and controls using QiAamp DNA Blood Mini kit (Qiagen GmbH). Quantification of isolated DNA was performed using NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific, Inc.).

PCR. The primer sequences used to amplify exon 15 of BRAF; exon 1 and 2 of each KRAS, HRAS and NRAS gene along with their annealing temperatures and amplicon size are given in Table I. Each PCR reaction was executed in a final volume of 50 µl containing 2 each forward and reverse primers (20 pM/µl), 3 genomic DNA, 18 sterile water and 25 µl GoTaq 2X Green Master mix (Promega Corporation). PCR reaction was performed using the following thermocycling conditions: Initial denaturation for 5 min at 95°C, followed by denaturation of template DNA for 35 sec at 94°C, annealing for 35 sec and primer extension for 35 sec at 72°C. Denaturation, annealing and primer extension steps were repeated for 35 cycles. Final extension was performed for 5 min at 72°C as previously described (22,25). The PCR product was run on 2% agarose gel and analysed using AlphaImager™ Gel Imaging System (ProteinSimple). Double distilled water (ddH2O) was used as a negative control.

Di-deoxy Sanger sequencing. Gene JET PCR Purification kit (Thermo Fisher Scientific, Inc.; cat. no. K0702) was used to purify PCR products according to the manufacturer's instructions. The purified PCR products of different DNA samples
were subjected to Sanger sequencing using SeqStudio Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

**PCR-restriction fragment length polymorphism (RFLP).** For HRAS T81C genotyping, exon 1 of HRAS was amplified by PCR as previously described (15,22), yielding a 186 bp product (Table I). For RFLP, PCR product was subjected to digestion with DraIII (Thermo Fisher Scientific, Inc.) restriction enzyme at 37˚C for 16 h. The homozygous variant genotype (CC) was cut into fragments of 128 and 58 bp; homozygote wild genotype (TT) yielded a single fragment of 186 bp while heterozygous variant (TC) yielded 186, 128 and 58 bp fragments. Digestion products were subjected to 3% agarose gel electrophoresis and documented using AlphaImager™ Gel Imaging System (ProteinSimple).

**Statistical analysis.** Data are presented as the mean ± SD of three independent repeats. $\chi^2$ test was used to compare cases and controls in terms of categorical variables, such as age, sex, histological type, thyroid stimulating hormone (TSH) levels, residence and smoking status using multiple logistic regression analysis. A goodness of fit test was applied to assess whether polymorphisms between cases and controls were present in Hardy-Weinberg equilibrium. Estimation of the relative risk and degree of association between genotypes and risk factors of TC were determined by calculation of the odds ratio (OR) with 95% confidence interval (CI). P≤0.05 was considered to indicate a statistically significant difference. All statistical analysis was performed using SPSS V. 23.0. software (IBM Corp.).

**Results**

**Characteristics of patients with TC for tissue analysis.** For mutation analysis of BRAF, HRAS, KRAS and NRAS genes, a total of 72 DTC and adjacent normal tissue samples were taken. Table II shows the frequency distribution of selected socio-demographic and clinicopathological characteristics of DTC cases for mutational analysis. Among DTC cases, 30.6% (22 of 72) were male and 69.4% (50 of 72) were female. A total of 54 of 72 (75%) patients were <55 years of age and 18 of 72 (25%) were ≥55 years of age. The number of non-smokers and smokers were 65 (90%) and 7 (10%) respectively. Furthermore, TSH levels were normal and elevated in 58.4% (42 of 72) and 41.6% (30 of 72) of cases, respectively. The normal reference range for TSH was taken as 0.35‑6.0 µIU/ml. History of benign thyroid disease (BTD; including thyroid adenoma, goitre and thyrotoxicosis) was found in 80% of patients. WDTC was present in 94.0% (68 of 72) of patients. Vascular/capsular invasion and lymph node metastasis was positive in 43.1 and 55.8% of patients, respectively. Other clinicopathological details of TC cases are shown in Table II.

**Mutational analysis of BRAF and RAS genes.** Exon 15 of BRAF gene was screened for presence of hotspot mutations in DTC tumor and adjacent normal tissue. T to A transversion was noted at nucleotide position 1,799 (c.T1799A) in 28% (20 of 72) of patients with DTC, resulting in substitution of V into E at codon 600. BRAF mutation was not found in adjacent normal tissue samples. Partial electropherograms showing T to A tranversion in BRAF V600E mutation are depicted in Fig. 1. BRAF V600E mutation was not found in any patients with follicular variant of PTC (FPTC). BRAF V600E mutation was confined to classical variant of PTC (CPTC) (P=0.0001). A higher frequency of BRAF V600E mutation (41%) was significantly associated with higher tumour focality and LN metastasis (P=0.03 and 0.005; Table III). Table III shows the association between BRAF V600E mutation status with demographic and clinicopathological features of patients with DTC.

Table I. Primer sequences, annealing temperature and product size of exons for PCR amplification.

| Gene   | Exon | Primer Sequence, 5'→3' | Annealing temperature, ˚C | Product size, bp |
|--------|------|------------------------|---------------------------|-----------------|
| BRAF   | 15   | F: TCATAATGCTTGCTCTGATAGGA  
R: GGCCAAAAATTTAATCAGTGGA | 58                         | 224                          |
| NRAS   | 1    | F: AGTACTGTAGTGGCTGCCC  
R: CCTACCTCTATGGTGTTGGGATC | 60                         | 185                          |
| NRAS   | 2    | F: CCCCTTACCCTCCACAC  
R: AGGTTAATATCGCAAAATGAC | 55                         | 196                          |
| HRAS   | 1    | F: CAGGAGACCTGTAGGGAAGA  
R: GGCACCTGAGCGGCGGCTAG | 60                         | 186                          |
| HRAS   | 2    | F: TCTGCGAGATTTCCAGGG  
R: GGTTCACCTGTACTGTTGGA | 55                         | 194                          |
| KRAS   | 1    | F: GTACTGTGGATGGATTTGTGAT  
R: TGAAAATGGTCAGAAACC | 55                         | 285                          |
| KRAS   | 2    | F: CTTCTCAGGATTTGCTACAG  
R: TTATTTATGCCAATAACACAAATA | 55                         | 1585                         |

F, forward; R, reverse.
Exons 1 and 2 of \textit{KRAS}, \textit{NRAS} and \textit{HRAS} genes were screened for presence of hotspot mutations in codons 12, 13 and 61 of a RAS gene family. No mutation was found in any of these codons in DTC tumor or adjacent normal tissue samples. Following DNA sequencing of \textit{HRAS}, a frequent substitution of T to C was found in exon 1 at codon 27 (cDNA position 81), which was present in wobble base position (Fig. 2). \textit{HRAS T81C} mutation was found in 21% (15 of 72) of DTC tumor tissue. Therefore, this mutation was investigated in blood samples of DTC cases and controls as a potential genetic polymorphism.

\textit{Characteristics of patients with TC for blood analysis.} A total of 180 patients with DTC, along with 220 healthy controls, were selected for the study of \textit{HRAS T81C} polymorphism. Out of 180 DTC cases, 72.8% (131 of 180) were <55 years of age and 27.2% (49 of 180) were ≥55 years of age; 37.7% (68 of 180) were male and 62.3% (112 of 180) were female. The proportion of non-smokers was 68.8% (124 of 180) and that of smokers was 31.2% (56 of 180). The cases and controls were matched with respect to sex, age, dwelling and smoking status (P=0.18; 0.91; 0.46 and 0.83). Socio-demographic and clinicopathological characteristics of DTC cases and controls are listed in Table IV.

\textit{Analysis of HRAS T81C SNP.} PCR amplified product of \textit{HRAS} exon 1 and fragment digestion of PCR product by \textit{DraIII} restriction enzyme is shown in Fig. 3. Frequency distribution of \textit{HRAS T81C} genotypes TT, TC and CC among cases were 37.7, 46.1 and 16.1%, respectively, in patients, compared with 54.5, 34.1 and 11.4%, respectively, in controls. Furthermore, the allele frequency of T and C among cases was 60.8 and 39.2%, respectively, and 71.5 and 28.5%, respectively, in controls. The difference in genotypic and allele frequency between cases and controls was statistically significant (P≤0.05; Table V). As the frequency of homozygous mutant (CC) genotype was low,
the combined variant (TC+ CC) genotype was compared with homozygous wild genotype (TT) between cases and controls to investigate the increased cancer risk associated with variant genotypes. Overall frequency of TC + CC was significantly greater in cases compared with controls (62.2 vs. 45.4%) with OR of 2.0 (95% CI, 1.3-2.9; P=0.0009; Table V).

Genotype frequencies of TT and TC + CC were compared between DTC cases and controls with respect to different socio-demographic and clinicopathological parameters (Table VI). The results indicated significantly higher frequency of variant genotype (TC + CC) in male patients with DTC compared with males in the control group (61.7 vs. 40.8%; P=0.01). Age was a strong risk factor for DTC as the difference in the frequency of TC + CC genotype between cases and controls ≥55 years of age was significant (59.27 vs. 19.3%; P=0.00002; OR=6.0). Similarly, patients with DTC living in rural areas had significantly higher frequency of variant genotype (TC + CC) compared with controls (62.57 vs. 42.1%; P=0.001). Furthermore, combined TC and CC genotype was significantly greater in non-smoker DTC patient compared with non-smoker control group (61.37 vs. 44.9%; P=0.007). TC and CC were frequently observed in patients with DTC without history of BTD compared with patients with BTD (76.07 vs. 52.3%; P=0.002). A high frequency of variant genotype (TC + CC) was found in patients with DTC with multifocal disease (70.27 vs. 55.2%; P=0.04) and LN metastasis (84.37 vs. 48.2%; P=0.00001) compared with patients with unifocal disease and without LN metastasis. Association of rare variants (TC + CC) with other socio-demographic and clinicopathological parameters of DTC cases and controls is shown in Table VI.

Patient follow-up. The patients were followed until the end of radioiodine therapy (data not shown). In patients with DTC lacking BRAF V600E mutation, low doses of I-131 (2.5-3 mCi) were given and patients responded well with high uptake. In addition, patients with DTC with BRAF V600E mutation exhibited decreased uptake of I-131 at low doses; therefore high doses of I-131 were given (75-80 mCi) for proper uptake and subsequent response to radio-iodine therapy.

Discussion

The MAP kinase pathway serves as a signal transducer between the extracellular environment and the nucleus (26). Extracellular signals, such as hormones and growth factors, interact with RET to activate small G-proteins of the RAS family, which activate and recruit RAF protein to the cell membrane where it is activated (27). Active BRAF signals via MEK to activate ERK, which activates downstream transcription factors to induce cell differentiation, proliferation, growth and apoptosis (28).

Triggering kinase activity makes BRAF a potent activator of MEK. BRAF V600E mutation increases the kinase activity of BRAF by nearly 700-fold, thereby stimulating constitutive activation of MEK/ERK signaling in tumor cells in the absence of extracellular stimuli, allowing the cell to become self-sufficient in growth signals within this pathway (28). Here, BRAF V600E...
mutation was found in 28% of patients with DTC. **BRAF**<sup>V600E</sup> mutation has been detected in 40-70% of malignant melanoma, 45-55% of DTC and 10% of colorectal cancer (29). In addition, the **BRAF**<sup>V600E</sup> mutation has also been identified in ovarian, breast and lung cancer (9,30,31). Clinically, FPTC metastasizes to cervical lymph nodes less frequently than CPTC but has a similar survival rate (29,32). In the present study, the **BRAF**<sup>V600E</sup> mutation was present in 44.4% of CPTC cases but absent in FPTC cases. The prevalence of **BRAF**<sup>V600E</sup> mutation in CPTC is 50‑60% (9,10,33). Studies have shown that FPTC molecular profile may be different from that of CPTC (34,35). Additionally, it has been reported that FPTC has lower **BRAF** but higher **RAS**
mutation rate compared with CPTC (36). The present study demonstrated an association between \textit{BRAF}^{V600E} mutation and multifocality and LN metastasis in DTC. Accumulating data have shown that \textit{BRAF}^{V600E} mutation is associated with unfavourable clinicopathological characteristics, such as extra-thyroidal extension, LN metastasis, recurrence and advanced disease stage in DTC (37,38). \textit{BRAF}^{V600E} is associated with silencing of multiple thyroid-specific iodine-metabolizing genes such as sodium/iodide symporter and apical iodide transporter, responsible for transportation of inorganic iodine into thyroid cells (39,40). Consequently, tumors harbouring the mutation are, to an extent, resistant to radio iodine ablation.

### Table IV. Frequency distribution of socio-demographic and clinicopathological characteristics of DTC cases and controls for \textit{HRAS T81C} genotyping

| Characteristic                  | Cases, n=180 (%) | Controls, n=220 (%) | \(\chi^2\) | P-value |
|--------------------------------|------------------|---------------------|-----------|---------|
| Sex                            |                  |                     |           |         |
| Male                           | 68.0 (37.7)      | 98.0 (44.5)         | 1.87      | 0.18    |
| Female                         | 112.0 (62.3)     | 122.0 (55.5)        |           |         |
| Age, years                     |                  |                     |           |         |
| <55                            | 131.0 (72.8)     | 158.0 (71.8)        | 0.05      | 0.91    |
| ≥55                            | 49.0 (27.2)      | 62.0 (28.2)         |           |         |
| Dwelling                       |                  |                     |           |         |
| Rural                          | 120.0 (66.6)     | 138.0 (62.7)        | 0.67      | 0.46    |
| Urban                          | 60.0 (33.4)      | 82.0 (37.2)         |           |         |
| Smoking status                 |                  |                     |           |         |
| Non-smoker                     | 124.0 (68.8)     | 149.0 (67.7)        | 0.06      | 0.83    |
| Smoker                         | 56.0 (31.2)      | 71.0 (32.3)         |           |         |
| BTD                            |                  |                     |           |         |
| Absent                         | 75.0 (41.6)      |                     |           |         |
| Present                        | 105.0 (58.3)     |                     |           |         |
| Histological type              |                  |                     |           |         |
| PTC                            | 151.0 (83.9)     |                     |           |         |
| FTC                            | 29.0 (16.1)      |                     |           |         |
| TSH levels                     |                  |                     |           |         |
| Normal                         | 52.0 (28.9)      |                     |           |         |
| Elevated                       | 128.0 (71.1)     |                     |           |         |
| Grade                          |                  |                     |           |         |
| WD                             | 176.0 (97.8)     |                     |           |         |
| PD                             | 4.0 (2.2)        |                     |           |         |
| Tumor focality                 |                  |                     |           |         |
| Unifocal                       | 96.0 (53.3)      |                     |           |         |
| Multifocal                     | 84.0 (46.7)      |                     |           |         |
| Stage, <55 years               |                  |                     |           |         |
| I                              | 65.0 (36.1)      |                     |           |         |
| II                             | 66.0 (36.7)      |                     |           |         |
| Stage, ≥55 years               |                  |                     |           |         |
| I+II                           | 26.0 (14.4)      |                     |           |         |
| ≥III                           | 23.0 (12.8)      |                     |           |         |
| V/C invasion                   |                  |                     |           |         |
| Absent                         | 93.0 (51.7)      |                     |           |         |
| Present                        | 87.0 (48.3)      |                     |           |         |
| LN metastasis                  |                  |                     |           |         |
| Absent                         | 110.0 (61.1)     |                     |           |         |
| Present                        | 70.0 (38.9)      |                     |           |         |

DTC, differentiated thyroid cancer; BTD, benign thyroid disease; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; TSH, thyroid stimulating hormone; WD, well-differentiated; PD, poorly differentiated; V/C, vascular/capsular; LN, lymph node.
Table V. Distribution of HRAS T81C genotypes and its allele frequency in DTC cases and controls for HRAS T81C genotyping.

| Type          | DTC cases, n=180 (%) | Controls, n=220 (%) | OR (95% CI)   | P-value |
|---------------|----------------------|---------------------|---------------|---------|
| Genotype      |                       |                     |               |         |
| TT            | 68.0 (37.7)           | 120.0 (54.5)        | 1.00 (Ref)    |         |
| TC            | 83.0 (46.1)           | 75.0 (34.1)         | 1.90 (1.30-3.00) | 0.0020* |
| CC            | 29.0 (16.1)           | 25.0 (11.4)         | 2.05 (1.10-3.80) | 0.0300* |
| TC + CC       | 112.0 (62.2)          | 100.0 (45.4)        | 2.00 (1.30-2.90) | 0.0009* |
| Allele        |                       |                     |               |         |
| T             | 219.0 (60.8)          | 315.0 (71.5)        | 1.00 (Ref)    |         |
| C             | 141.0 (39.2)          | 125.0 (28.5)        | 1.60 (1.20-2.20) | 0.0010* |

*P<0.05. DTC, differentiated thyroid cancer; TC, genotype formed by combination of thymine and cytosine residues; TT, genotype with combination of two thymine residues; CC genotype formed by combination of two cytosine residues.

used for the treatment of TC, which may explain the more aggressive phenotype exhibited by DTC harboring BRAFV600E mutation (41).

RAS is the most commonly mutated gene family in TC that contributes to cancer initiation and progression via inhibition of GTP hydrolysis by diminishing GTPase activity (42). Gain of function mutations in the hotspot regions of the RAS gene family affect codons 12 and 13 in exon 1 and codon 61 in exon 2. No mutation in any of the RAS genes was found in the present study, which supports previous studies indicating that mutations in BRAF and RAS generally occur in a mutually exclusive manner (42,43). Mutual exclusiveness suggests that MAP kinase pathway is controlled at different levels to regulate TC pathogenesis. Certain studies observed an increase in RAS mutation in dietary iodine-deficient countries, such as eastern Hungary and Japan (44,16) whereas, Vuong et al (45) reported no difference in frequency of RAS mutations between iodine-rich and -deficient countries.

To the best of our knowledge, the present study is the first multicentric study from Pakistan exploring the utility of HRAS T81C SNP analysis in TC risk, which may help in future treatment modalities. HRAS T81C SNP was found to be a strong risk factor for TC (22,24). The HRAS T81C variant (TC + CC) and heterozygous genotype (TC) were found in 62.2 and 46.1% of patients with TC compared with 45.4 and 34.1% of controls, respectively; these rates are higher in comparison with other ethnic groups (21,46). The reason for the higher frequency of variant genotype in DTC cases compared with controls may be attributed to geographical differences and relatively small sample size (21). In addition, HRAS T81C was significantly associated with the risk of DTC in the present study (P=0.0009). Earlier studies have also reported significant association of HRAS T81C SNP with risk of gastric, colon and bladder cancer (47,48), although, the frequency of variant genotype reported by those studies was relatively less compared with the present study.

Following stratification of HRAS T81C genotypes with clinicopathological risk factors in patients with DTC, TC and CC variants have been significantly associated with higher age, which is in line with previous studies that identified higher age as a key risk factor for tumorigenesis in relation to HRAS T81C gene polymorphism (48,49). However, an earlier study demonstrated no significant association of age with risk of thyroid cancer in relation to HRAS T81C genotypes (22). Males and rural dwellers with DTC exhibited greater frequency of TC + CC compared with control males and rural dwellers, which differs from earlier studies (22,47). The present results demonstrated that variant genotype (TC + CC) was inversely associated to smoking status. Cigarette smoking may lower endogenous TSH levels in the body and hence lower the risk of TC (50). These results were different to earlier studies, in which no association of TSH level with smoking was found (51,52). Patients with DTC with no history of BTD had greater frequency of TC + CC genotype. Khan et al (22) found no association between HRAS T81C genotype and history of BTD. As BTD is a risk factor and molecular crosstalk occurs during the initiation and progression of cancer, there may be other molecular changes responsible for the development of BTD, which may serve a role in the development of cancer phenotype. Although previous history of BTD was recorded, the present study included historically confirmed patients with DTC rather than patients with any BTD. Investigation of MAP kinase pathway aberrations in Pakistani individuals with BTD will improve understanding of the etiopathogenesis of TC in this region. In the present study, HRAS T81C variant genotype was associated with multifocality and LN metastasis (P≤0.05). An earlier studies demonstrated no significant association of variant genotype with multifocality and LN metastasis (53). The present study did not observe any association between histological type, tumor grade, TSH levels, V/C invasion and tumor stage with HRAS T81C TC + CC genotype. However, Krishna et al (54) showed high expression of HRAS protein in WDTC and higher stage. A previous study has reported no association between HRAS T81C variants and histological type of TC (55).

Although the mechanism underlying the role of HRAS T81C SNP in cancer initiation is not completely known, this SNP may not be involved in delaying GTP-bound activated state (56) and alteration of the RAS protein structure (24), but rather affect cancer susceptibility via linkage with other polymorphic sites in functional intron regions of HRAS (57,58). HRAS T81C exon 1 may be linked to rs112587690 SNP in intron 1 and L-myc rs3134613 SNP in the development of cutaneous melanoma and colorectal cancer, respectively (57,58). The polymorphism may also be linked to a candidate region with variable tandem repeat present downstream of exon 4, exhibiting possible...
Table VI. Association of HRAS T81C genotypes with socio-demographic and clinicopathological parameters of DTC cases and controls for HRAS T81C genotyping.

| Parameter                   | Cases                                      | Controls                                   | OR (95% CI)               | P-value |
|-----------------------------|--------------------------------------------|--------------------------------------------|---------------------------|---------|
|                             | TT, n=180.0 (%)                            | TC + CC, n=112.0 (%)                       |                           |         |
|                             | n=68.0 (37.7%)                             | n=112.0 (62.3%)                            |                           |         |
| Sex                         |                                            |                                            |                           |         |
| Male                        | 68.0 (37.7)                                | 42.0 (61.7)                                | 2.30 (1.20-4.40)          | 0.01000 |
|                            | 112.0 (62.3)                               | 122.0 (55.5)                               | 1.70 (1.00-2.90)          | 0.06000 |
| Age, years                  |                                            |                                            |                           |         |
| <55                         | 131.0 (72.8)                               | 83.0 (63.4)                                | 1.40 (0.80-2.20)          | 0.20000 |
| ≥55                         | 409.0 (27.2)                               | 29.0 (59.2)                                | 2.30 (1.40-3.80)          | 0.00100 |
| Dwelling                    |                                            |                                            |                           |         |
| Rural                       | 120.0 (66.6)                               | 75.0 (62.5)                                | 1.40 (0.70-2.70)          | 0.30000 |
| Urban                       | 60.0 (33.4)                                | 37.0 (61.6)                                | 1.40 (0.70-2.70)          | 0.30000 |
| Smoking status              |                                            |                                            |                           |         |
| Non-smoker                  | 124.0 (68.8)                               | 76.0 (61.3)                                | 1.90 (1.20-3.10)          | 0.00700 |
| Smoker                      | 56.0 (31.2)                                | 36.0 (64.3)                                | 2.10 (1.00-4.20)          | 0.05000 |
| BTD                         |                                            |                                            |                           |         |
| No                          | 75.0 (41.6)                                | 57.0 (76.0)                                | 2.90 (1.50-5.50)          | 0.00200 |
| Yes                         | 105.0 (58.3)                               | 55.0 (52.3)                                |                           |         |
| Histological type           |                                            |                                            |                           |         |
| PTC                         | 151.0 (83.9)                               | 91.0 (60.3)                                | 0.60 (0.20-1.40)          | 0.29000 |
| FTC                         | 29.0 (16.1)                                | 21.0 (72.4)                                |                           |         |
| TSH levels                  |                                            |                                            |                           |         |
| Normal                      | 52.0 (28.8)                                | 35.0 (67.3)                                | 1.40 (0.70-2.70)          | 0.40000 |
| High                        | 128.0 (71.1)                               | 77.0 (60.1)                                |                           |         |
| Tumor grade                 |                                            |                                            |                           |         |
| WD                          | 176.0 (97.7)                               | 110.0 (62.5)                               | 1.70 (0.20-12.10)         | 100.000 |
| PD                          | 4.0 (2.2)                                  | 2.0 (50.0)                                 |                           |         |
| Tumor focality              |                                            |                                            |                           |         |
| Unifocal                    | 96.0 (53.3)                                | 53.0 (55.2)                                | 0.50 (0.30-0.97)          | 0.04000 |
| Multifocal                  | 84.0 (46.7)                                | 59.0 (70.2)                                |                           |         |
| Stage <55 years             |                                            |                                            |                           |         |
| I                           | 65.0 (36.1)                                | 38.0 (58.5)                                | 0.60 (0.30-1.30)          | 0.30000 |
| II                          | 66.0 (36.7)                                | 45.0 (68.1)                                |                           |         |
| Stage ≥55 years             |                                            |                                            |                           |         |
| I+II                        | 26.0 (14.4)                                | 10.0 (38.4)                                | 0.10 (0.03-0.50)          | 0.03000 |
| ≥III                        | 23.0 (12.8)                                | 19.0 (82.6)                                |                           |         |
Table VI. Continued.

| Parameter      | Cases                      | Controls                   | OR (95% CI) | P-value |
|----------------|----------------------------|----------------------------|-------------|---------|
|                | TT, n=68.0 (37.7%)         | TC + CC, n=112.0 (62.3%)   |             |         |
|                | n=180.0 (%)                | n=220.0 (%)                |             |         |
| V/C invasion   |                            |                            |             |         |
| No             | 93.0 (51.7)                | 50.0 (53.7)                | 0.50 (0.30‑0.90) | 0.02000 |
| Yes            | 87.0 (48.3)                | 62.0 (71.2)                |             |         |
| LN metastasis  |                            |                            |             |         |
| No             | 110.0 (61.0)               | 53.0 (48.2)                | 0.20 (0.10‑0.40) | 0.0001<sup>a</sup> |
| Yes            | 70.0 (38.9)                | 59.0 (84.3)                |             |         |

BTD, benign thyroid disease; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; TSH, thyroid stimulating hormone; WD, well-differentiated; PD, poorly differentiated; V/C, vascular/capsular; LN, lymph node; DTC, differentiated thyroid cancer. *P<0.05.

Table VII. Genetic association study of *HRAS T81C* polymorphism.

| Model          | Genotype  | Cases     | Controls   | OR (95% CI) | P-value |
|----------------|-----------|-----------|------------|-------------|---------|
| Co-dominant    | T/T       | 68.0 (37.8) | 120.0 (54.5) | 1.0 (Ref)   |         |
|                | T/C       | 83.0 (46.1) | 75.0 (34.1)  | 1.9 (1.3‑3.0) | 0.0020  |
|                | C/C       | 29.0 (16.1) | 25.0 (11.4)  | 2.0 (1.1‑3.8) | 0.0300  |
| Dominant       | T/T       | 68.0 (37.8) | 120.0 (54.5) | 1.0 (Ref)   |         |
|                | T/C + C/C | 112.0 (62.2) | 100.0 (45.4) | 2.0 (1.3‑3.0) | 0.0009  |
| Recessive      | T/T + T/C | 151.0 (83.9) | 195.0 (88.6) | 1.0 (Ref)   |         |
|                | C/C       | 29.0 (16.1) | 25.0 (11.4)  | 1.5 (0.8‑2.7) | 0.2000  |
| Over-dominant  | T/T + C/C | 97.0 (53.9)  | 145.0 (65.9) | 1.0 (Ref)   |         |
|                | T/C       | 83.0 (46.1) | 75.0 (34.1)  | 1.6 (1.1‑2.5) | 0.0200  |
| Additive       | T/T       | 68.0 (37.8) | 120.0 (54.5) | 1.0 (Ref)   |         |
|                | C/C       | 29.0 (16.1) | 25.0 (11.4)  | 2.0 (1.1‑3.8) | 0.0300  |

T/C, genotype formed by combination of thymine and cytosine residues; T/T, genotype with combination of two thymine residues; C/C genotype formed by combination of two cytosine residues.
transcriptional enhancer activity (59). Furthermore, reports have shown the association of \textit{HRAS T8I/C} SNP with hexanucleotide region present 80 bp upstream of 5’ exon 1 (55,60,61). Additionally, the \textit{HRAS T8I/C} SNP is also associated with polymorphic intron D2 (dopamine) region of \textit{HRAS} that may serve as a regulator of Intron D exon inclusion (24). \textit{HRAS T8I/C} polymorphism follows a dominant mode of inheritance, which assumes that carriers of wild genotypes are associated with lower cancer risk compared with heterozygous and rare genotypes (62).

As the sample size of the present study was modest, further studies with larger sample size and follow-up of patients are required to authenticate the association to better distinguish racial and ethnic differences affecting the pathogenesis and severity of DTC.

In summary, \textit{BRAF V600E} mutation may be implicated in the pathogenesis of DTC in a mutual exclusive manner with \textit{RAS} mutations in Kashmiri population. \textit{BRAF V600E} mutation was confined to CPTC variant and was significantly associated with multifocality and LN metastasis, suggesting that \textit{BRAF V600E} mutation may be useful for evaluation of prognosis of patients with DTC. These results indicated that \textit{BRAF} may be a promising target for pharmacological intervention in DTC. \textit{HRAS T8I/C} variant genotype was increased in DTC with dominant pattern of inheritance. \textit{HRAS T8I/C} variant genotype increased risk of DTC with no history of smoking, males, higher age, multifocality and LN metastasis. Further analysis of other genetic markers and long-term clinical follow-up may improve understanding of DTC.

Acknowledgements

Not applicable.

Funding

The present study was funded by International Islamic University, Islamabad (grant no. NRPU 8038).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

FAR, GHB and MSK conceptualized the study, collected data and wrote the manuscript. GHB and MSK analyzed the data and reviewed and edited the manuscript. FAR and ST performed the experiments. ST supervised the study and provided resources. FAR and ST visualized the data. MSK and GHB confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Review Board of PIMS (approval no.F-1-1/2015/ERB/SZABMU). All samples were collected with written informed consent from patients and proper ethical procedures were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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