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Identification of a novel *HRAS* variant and its association with papillary thyroid carcinoma

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Abstract. HRas proto-oncogene (*HRAS*) is one of the most commonly mutated genes in thyroid cancer, with mutations frequently occurring in the follicular and Hurthle cell subtypes. However, the contribution of mutations in *HRAS* to papillary thyroid carcinoma (PTC) progression and the tall-cell variant (TCV) is poorly understood. The aim of the present study was to investigate the somatic genetic variants present in *HRAS* in patients with PTC, and to investigate the association of these mutations with PTC. The present study is a retrospective case-control study using tumor samples collected from 139 patients with PTC and blood samples from 195 healthy individuals. All patient samples were screened for mutations in 'hotspot' regions of *HRAS* and B-raf proto-oncogene (*BRAF*) by single-stranded conformational polymorphism analysis, followed by direct sequencing. A novel variant (IVS1-82del gctgggcctggg) in the *HRAS* 5'-untranslated region was identified. There was no difference in age or sex of patients with PTC and the healthy controls; however, the *HRAS* variant was more frequently detected in PTC tissue than in the healthy control samples (37 vs. 26%, P=0.04). There was no association between the *HRAS* variant and age, sex, tumor size, encapsulation, multifocality/intra-thyroidal spread, Tumor-Node-Metastasis stage, history of Hashimoto's disease, *BRAF* V600E mutation or PTC subtype (all P>0.05). There were differences of *BRAF* V600E distribution among different subtypes (χ^2=6.390, P=0.041). *HRAS* variant co-occurring with the *BRAF* V600E mutation accounted for 31.6% of the total number (P=0.196). Therefore, this novel variant of *HRAS* (IVS1-82del gctgggcctggg) may be associated with PTC; however, larger scale studies are required to assess the contribution of this novel *HRAS* variant to PTC progression.

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Introduction

Carcinoma of the thyroid gland is the most common malignant tumor of the endocrine system (1). Papillary thyroid cancer (PTC) is the most common histological type of thyroid cancer, representing 80% of all cases of thyroid cancer and 85% of cases of differentiated thyroid cancer (2,3). PTC is 2.9-3.8 times more common in women than in men (4), and is more common in regions associated with a high dietary intake of iodine (5). In the United States, the incidence of PTC is 1.56-3.58/100,000 men and 4.9-10.96/100,000 women (4). PTC is usually associated with a more positive prognosis than follicular thyroid cancer; however, certain subtypes are more aggressive than others (5). Of the PTC subtypes, the tall-cell variant (TCV) is among the most aggressive (6). The 2004 World Health Organization (WHO) classification defined TCV as PTC containing ≥50% tall cells (7). Other characteristics of TCV include an eosinophilic tall cell cytoplasm and nuclear features characteristic of PTC (7). However, the molecular mechanisms that cause TCV differentiation are unclear.

In recent years, B-raf proto-oncogene (*BRAF*) mutation has been demonstrated to be the most common genetic alteration in PTC (8). It is a molecular marker associated with aggressive tumor behaviors, including size, extra-thyroidal extension, multifocality, lymph node metastasis, tumor recurrence and advanced disease stage (9,10).

The rat sarcoma viral oncogene homolog (*RAS*) genes, which include the isoforms *HRAS, KRAS* and *NRAS*, are crucial effectors in a number of signaling cascades. The mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways, which mediate cell differentiation, proliferation, and survival, are affected by *RAS* genes (11,12). *RAS* activity is regulated by GTP-bound hydrolysis, and any mutation that results in the dysregulation of this hydrolysis results in aberrant MAPK and PI3K/(RAC serine/threonine-protein kinase (Akt) signaling, which are critical events in thyroid carcinogenesis (13).

HRAS is one of the most commonly mutated genes in PTC, particularly in variants identified in follicular (14-17)

and Hurthle cells (18), reflecting its key regulatory functions. The contribution made by *HRAS* to PTC progression is poorly understood. Therefore, the present study aimed to investigate the presence of somatic variants in *HRAS* exhibited by patients with PTC as well as by healthy individuals, and to investigate their association with PTC development. The results of the present study provide an improved understanding of PTC pathogenesis and may provide novel insight for the advancement of PTC treatment.

Materials and methods

Study design and subjects. The present study involves the retrospective investigation of tumor samples collected from patients with PTC who underwent thyroidectomy at the Beijing Friendship Hospital, Capital Medical University (Beijing, China) between January 2011 and February 2016. A total of 139 PTC patients (106 females and 33 males), age (48.7±9.3) years old. The final diagnoses were made by pathological examination of the specimens. The following inclusion criteria were applied: i) No treatment for PTC prior to the surgery; ii) the absence of any other type of malignant tumor; iii) tumor size >0.5 cm; iv) no distant metastasis identified prior to surgery; v) clear results from lymph node dissection; and vi) sufficient DNA extractable from the tissue for analysis.

A total of 195 blood samples from asymptotic people undergoing routine health examinations were acquired as healthy controls. The following exclusion criteria were applied: i) Any symptom of thyroid cancer, and ii) the identification of any biochemical abnormality.

The present study was approved by the Clinical Research Ethics Committee of Beijing Friendship Hospital, Capital Medical University.

Pathological evaluation. Following surgery, formalin-fixed paraffin-embedded (FFPE) tumor-rich tissue areas were dissected from unstained 4-μm sections under the guidance of stained slides which was stained by undiluted hematoxylin and eosin (Merck KGaA, Darmstadt, Germany) for 330 sec at room temperature, with the tumor area marked under the guidance of light microscope (original magnification x200). All histological slides were reviewed independently by experienced pathologists specialized in thyroid pathology (from the Peking University Third Hospital, Beijing, China). Diagnoses were performed according to the WHO classification (7). The tumors were classified into histological subtypes: Classic variant of papillary thyroid carcinoma (CVPTC), follicular variant of papillary thyroid carcinoma (FVPTC), and TCV.

DNA extraction. Tumor-rich areas were scraped from the paraffin sections, added to 500 μl xylene (concentration ≥99.0%; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and centrifuged 27,400 x g at room temperature for 15 min. The supernatant was discarded and 500 μl of anhydrous ethanol was used to disperse the pellet prior to centrifugation twice more 27,400 x g at room temperature for 10 min. The supernatant was discarded and 50 μl acetone was used to disperse the pellet prior to further centrifugation 27,400 x g at room temperature for 5 min. Subsequent to air drying, the pellet was suspended in 309 μl DNA extraction buffer (300 μl digestion buffer and 9 μl proteinase K; E.Z.N.A® FFPE DNA Kit; Omega Bio-Tek, Inc., Rockville, MD, USA), and incubated at 55˚C for 3-5 h. The DNA was extracted from the 195 control samples using Blood DNA kits (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions. The DNA concentration was determined by spectrophotometric absorption (A) at 230, 260, and 280 nm, and the DNA quality was evaluated by calculating the ratio of optical density (OD) value at 260 and 280 nm or 260 and 230 nm measured by a BioSpec-nano spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Single-stranded conformational polymorphism analysis (SSCP) and direct DNA sequencing for HRAS mutations. SSCP analysis was performed to prescreen for mutations in the *HRAS* and *BRAF* exons, in which hotpoint mutations can be identified (19). Primers for SSCP-polymerase chain reaction (PCR) were designed using the Primer 3.0 software (Premier Biosoft International, Palo Alto, CA, USA; Table I. PCR was performed in a total volume of 10 μl, consisting of 1 μl DNA solution (100 ng/μl), 0.5 units of Platinum Taq DNA polymerase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.1 μCi [a-^{33}P] deoxycytidine triphosphate (ICN Biomedicals, Irvine, CA, USA; specific activity of 3,000 Ci/mmol), 1-4 mmol/l $MgCl_2$, 0.1-0.2 mmol/l deoxynucleotide triphosphate, 0.2-0.4 mmol/l each primer, 10 mmol/l Tris-HCl (pH 8.3) and 50 mmol/l KCl in a thermal cycler (Biometra GmbH, Göttingen, Germany). The thermocycling conditions were as follows: 95˚C for 5 min, 37-40 cycles of 95˚C for 50 sec, 45-54˚C for 60 sec and extension 72˚C for 60 sec, and 72˚C for 5 min. Subsequent to PCR amplification, 10 μl PCR product was mixed with 20 μl loading buffer (0.02 M NaOH, 95% formamide, 20 mmol/l EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue) and denatured at 95˚C for 10 min, prior to quenching on ice. A total of 5.5 μl sample mixture was loaded onto a 12.5% polyacrylamide non-denaturing gel containing 10% glycerol. Electrophoresis was performed at 45 W for 3.5-4.5 h at room temperature with fan cooling. Gels were performed silver staining according to our previous study (20). Samples exhibiting mobility shifts in SSCP analysis were re-amplified using the same primers and PCR conditions as for SSCP analysis and sequenced to determine the *HRAS* and *BRAF* genotypes (Beijing Tianyi Huiyuan Co., Ltd., Beijing, China; Table I) (21).

Statistical analysis. χ^2 test was used to identify the association between *HRAS* and *BRAF* variants, the different subtypes of PTC, and lymph node metastasis. All statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Subject characteristics. Table II presents the clinical characteristics of the subjects. There were no differences in age and sex between the 139 patients with PTC and the 195 healthy individuals. However, *HRAS* variants were more frequent in patients with PTC compared with healthy individuals (37 vs. 26%, P=0.04).

Table I. Primers used for *HRAS* variant screening in PTC.

Gene (exon)	Forward sequence, 5'-3'	Reverse sequence, 5'-3'	Product size, bp
HRAS (1)	cagtccttgctgcctggc	atggttctggatcagctgga	264
HRAS (2)	cctgtctcctgcttcctctag	tggcaaacacacacaggaag	298
BRAF (15)	aactcttcataatgcttgctctga	agtaactcagcagcatctcagg	251

HRAS, HRas proto-oncogene; PTC, papillary thyroid carcinoma; *BRAF*, B-raf proto-oncogene.

Table II. Characteristics of the subjects.

Variable	PTC samples, n (%)	Control samples, n (%)	P-value
Total	139	195	
Age, years			0.14
≤45	78 (56.1)	126 (64.6)	
>45	61 (44.9)	69 (35.4)	
Sex			0.25
Female	106 (76.3)	136 (69.7)	
Male	33 (23.7)	59 (30.3)	
HRAS variant	51 (36.7)	51 (26.2)	0.04

PTC, papillary thyroid carcinoma; *HRAS*, HRas proto-oncogene.

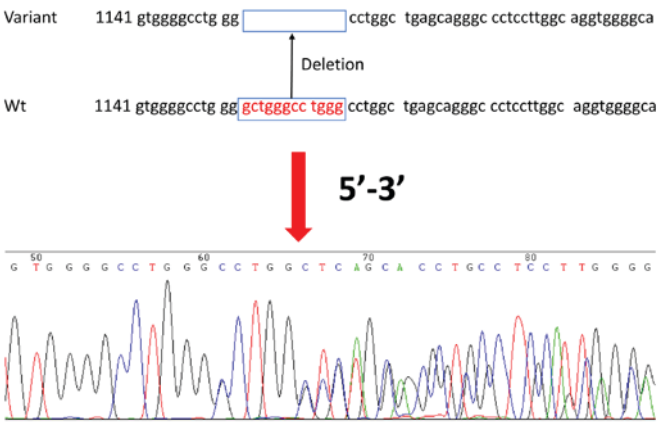

Figure 1. Identification and analysis of a novel HRAS variant in papillary thyroid carcinoma. (A) heterozygous variant with 12 bp deletion in the 5'-untranslated region (IVS1-82del gctgggcctggg) in the HRAS gene. Wt, wild-type.

Molecular analysis of HRAS and BRAF. A novel variant of *HRAS* (IVS1-82del gctgggcctggg; Fig. 1) was identified to the best of our knowledge for the first time in PTC and adjacent non-tumor tissue: 51/139 (37%) patients with PTC were heterozygous for the IVS1-82del gctgggcctggg variant, compared with 51/195 (26%) healthy controls (P=0.04; Table II). The *HRAS* variant was not specific to PTC but occurred more frequently in patients with PTC compared with healthy individuals. The frequency of the *HRAS* variant did not differ among PTC subtypes (P=0.95). There were no associations between the *HRAS* variant and age, sex, tumor size, encapsulation, multifocality/intrathyroidal spread, Tumor-Node-Metastasis stage (22), thyroid nodule status, Hashimoto history, *BRAF* mutation or PTC subtype (all P>0.05; Table III). There were significant differences in the number of *BRAF* mutations among the different subtypes (P=0.041; Table IV). The presence of the BRAF V600E mutant was not associated with that of the HRAS variant (P=0.196; Table V).

Discussion

HRAS is one of the most commonly mutated genes in thyroid cancer, particularly the follicular and Hurthle cell subtypes. However, its contribution to PTC and the TCV is poorly understood. Therefore, the present study aimed to investigate the presence of somatic variants in *HRAS* in patients with PTC and healthy controls, and to investigate their association with PTC development. The results demonstrated that a novel *HRAS* variant (IVS1-82del gctgggcctggg) could be associated with PTC. Larger studies are required to assess the distribution of this novel *HRAS* variant and to validate the results of the present study.

PTC is the most common form of thyroid cancer (2,3). In the present study, the percentage of TCV samples harboring the *HRAS* variant was 49.6%. The most common etiological factor associated with onset of PTC is radiation; however, other factors, including genetic susceptibility, have been demonstrated to be associated with PTC development (23), as have predispositions such as Hashimoto's thyroiditis (HT) (24). HT has been recognized as a common autoimmune thyroid disorder associated with various antibodies, including thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TgAb) (25). If patients present with diffuse goiter (Graves disease), and their TPOAb and TgAb levels are simultaneously increased, an HT diagnosis can be made. However, in the present study, no significant association between HT and the novel *HRAS* variant was identified.

Mutations associated with phenotypic susceptibility are popular in oncology research; however, such research often requires a large sample size to obtain reliable results. Furthermore, the identification of novel variants often requires DNA sequencing, which is an expensive technology with limited availability in certain countries. The most commonly used method is SSCP (26,27), which is an efficient and sensitive technique used for the identification of single-base mutations.

Mutations in the genes of the *RAS* family members are known to be associated with thyroid carcinogenesis; *RAS* mutations have been identified in PTC, follicular

Table III. Association of *HRAS* variant, IVS1-82del gctgggcctggg, with clinical features in patients with PTC.

Variable	Patients with PTC, n (%)	*HRAS* status, n (%)		P-value
		Wild-type	Variant	
Total	139	88	51	
Sex				0.68
Female	106 (76.3)	66 (75.0)	40 (78.4)	
Male	33 (23.7)	22 (25.0)	11 (21.6)	
Age, years				0.11
≤45	78 (56.1)	54 (31.4)	24 (47.1)	
>45	61 (43.9)	34 (68.6)	27 (52.9)	
Tumor size, mm				0.11
≤10	79 (56.8)	55 (62.5)	24 (47.1)	
>10	60 (43.2)	33 (37.5)	27 (52.9)	
Encapsulation	54 (38.9)	32 (63.6)	22 (53.1)	0.47
Multifocality/intrathyroidal spread	32 (23.0)	19 (21.6)	13 (25.5)	0.68
Lymph node metastasis	66 (47.5)	39 (44.3)	27 (72.7)	0.40
TNM stage				0.56
I/II	99 (71.2)	61 (69.3)	38 (74.5)	
III/IV	40 (28.8)	27 (30.7)	13 (25.5)	
Hashimoto's disease	77 (55.3)	51 (57.9)	26 (50.9)	0.49
PTC subtype				0.95
CVPTC	34 (24.5)	22 (25.0)	12 (23.5)	
FVPTC	36 (25.9)	22 (25.0)	14 (27.5)	
TCV	69 (49.6)	44 (50.0)	25 (49.0)	

HRAS, HRas proto-oncogene; TNM, Tumor-Node-Metastasis; PTC, papillary thyroid carcinoma; CVPTC, classic variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; TCV, tall-cell variant.

Table IV. *BRAF* V600E mutation occurrence in different subtypes of papillary thyroid carcinoma.

Subtype	Mutation, n (%)	Wild-type, n (%)	χ^2	P-value
CVPTC	25 (73.5)	9 (26.4)		0.041
FVPTC	24 (66.7)	12 (33.3)	6.390	
TCV	60 (86.9)	9 (13.0)		

BRAF, B-raf proto-oncogene; CVPTC, classic variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; TCV, tall-cell variant. [a]χ^2 test.

Table V. Association between *BRAF* mutation and the novel *HRAS* variant.

	HRAS$^-$, n	*HRAS*$^+$, n	Total, n	χ^2	P-value
BRAF$^-$	20	7	27	1.672	0.196
BRAF$^+$	68	44	112		
Total	88	51	139		

BRAF, B-raf proto-oncogene; *HRAS*, HRas proto-oncogene; *HRAS*$^-$, *HRAS* wild-type; *BRAF*$^-$, *BRAF* wild-type; *HRAS*$^+$, *HRAS* variant (IVS1-82del gctgggcctggg); *BRAF*$^+$, *BRAF* mutation exhibited. [a]χ^2 test.

carcinoma, follicular adenoma, and medullary thyroid carcinoma (17,28-32). Previous studies have demonstrated that various types of thyroid carcinoma, particularly FVPTC, harbor somatic mutations in *HRAS* (15,16,33). The *HRAS* gene is also often activated in urinary tract tumors (34). The 81T>C polymorphism in the *HRAS* gene is associated with increased risk of skin (35), oral (36), bladder (37), and gastric (38) cancer. It has been demonstrated that the 81T>C polymorphism, which increases protein expression without changing its function, was associated with aneuploidy in thyroid cancer (39). Previous studies have reported that the frequency of *RAS* variants was 10-43% in PCT (40-43).

The *BRAF* V600E mutation has been demonstrated to be the most common genetic alteration in PTC (8). *BRAF* is a member of the *RAF* family and is involved in the MAPK pathway (28). Briefly, the MAPK cascade is initiated upon *RAS* activation, which recruits *BRAF* to the plasma membrane. The present study demonstrated that *BRAF* mutations were more frequent in TCV than in other subtypes, and that the *HRAS* variant occurred concomitantly with the *BRAF* mutation in

31.6% of PTC samples (P=0.196). The concomitant mutations are typically present in the CVPTC and TCV subtypes (29.4 vs. 30.4%). This indicates that the concomitant mutations may be associated with aggressive disease behavior and poor prognosis; however, further studies are required to confirm this.

Two different mechanisms may be responsible for the carcinogenic effect of *HRAS* mutations: Modified protein function or increased protein expression (43,44-46). As RAS proteins are involved in cell differentiation, proliferation, and survival, increased expression or activity of *HRAS* may enhance these activities, which are associated with carcinogenesis. Indeed, increased *RAS* activation leads to constitutive activation of the downstream targets of RAS proteins, i.e., the MAPK and PI3K/Akt signaling pathways (13). The novel *HRAS* variant identified in the present study occurs at the 5' end of the sequence, which may affect the selective splicing of *HRAS* and could be associated with tumor pathogenesis. However, the exact effect of this variant on protein expression remains to be determined.

Concomitant *BRAF* and *RAS* mutations may allow simultaneous activation of the MAPK and PI3K/Akt signaling pathways in cancer cells, providing a growth advantage (47,48). Long-term follow-up revealed that patients with concomitant mutations had a poorer response to treatment and reduced disease-free survival times (49), indicating that activation of the two genes may have a synergistic effect on disease progression (50).

One previous study revealed no association between *HRAS* variants and tumor biology (51), whereas other studies have reported associations between *HRAS* variants and poorly differentiated tumors (51,52). In the present study, *HRAS* mutations were demonstrated to be associated with follicular thyroid lesions (32). *HRAS* has been demonstrated to be frequently mutated in Hurthle cells, which are believed to represent a common metaplastic change in damaged thyroid follicular epithelium (53). Hurthle cells can often develop into Hurthle cell cancer, which is categorized as an oncocytic variant of follicular carcinoma (54). The present study did not include follicular carcinoma or Hurthle cell cancer clinical cases; however, it is possible that the *HRAS* variant arises from follicular or Hurthle cells in PTCs. In addition, the results of the present study indicated that the novel *HRAS* variant tends to occur in the TCV. Additional studies are required to fully elucidate the role of the novel *HRAS* variant in tumor biology.

The present study is limited by the number of patients, retrospective nature, and constrained follow-up information. Furthermore, the potential cellular mechanisms of mutation functions in PTC were not determined. In conclusion, a novel variant of *HRAS* (IVS1-82del gctgggcctggg) was associated with PTC. Further studies are required to assess the distribution of this novel *HRAS* variant and to validate the results of the present study.

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