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Analysis of *RAS* mutation in thyroid nodular hyperplasia and follicular neoplasm in a Korean population

Sun Hye Jeong[1] | Hyun Sook Hong[1] | Eun Hye Lee[1] | Jeong Ja Kwak[2] | Ji Ye Lee[1]

[1]Department of Radiology, Soonchunhyang University Bucheon Hospital, Bucheon, Korea

[2]Department of Pathology, Soonchunhyang University Bucheon Hospital, Bucheon, Korea

Correspondence
Hyun Sook Hong, Department of Radiology, Soonchunhyang University Bucheon Hospital, Bucheon-si, Korea.
Email: hshong@schmc.ac.kr

Funding information
Soonchunhyang University

Summary

Background: To investigate the difference in frequency of *RAS* mutations between nodular hyperplasia (NH), follicular thyroid adenomas (FTAs) and follicular thyroid carcinomas (FTCs) in a Korean population.

Methods: *RAS* mutations in 50 NH, 57 FTAs and 39 FTCs between January 2002 and May 2015 were analysed by pyrosequencing.

Results: Nine nodules of 50 NHs (18%), 18 nodules of 39 FTCs (46.2%) and 19 nodules of 57 FTAs (33.3%) harboured *RAS* mutations. Three FTCs and three FTAs showed two point mutations simultaneously. *N-RAS* codon 61 (n = 6 of 9, 66.7%) and *H-RAS* codon 61 (n = 3 of 9, 33.3%) were found in NHs. *K-RAS* codons 12-13, *K-RAS* codon 61, *N-RAS* codons 12-13 and *H-RAS* codons 12-13 were not found in NHs. *N-RAS* codon 61 (n = 7 of 21, 33.3%), *K-RAS* codons 12-13 (n = 6 of 21, 28.6%), *H-RAS* codon 61 (n = 4 of 21, 19.0%), *K-RAS* codon 61 (n = 3 of 21, 14.3%) and *N-RAS* codons 12-13 (n = 1 of 21, 4.7%) were found in FTCs, and *N-RAS* codon 61 (n = 10 of 22, 45.5%), *K-RAS* codons 12-13 (n = 5 of 22, 22.7%), *H-RAS* codon 61 (n = 5 of 22, 22.7%), *K-RAS* codon 61 (n = 1 of 22, 4.5%) and *N-RAS* codons 12-13 (n = 1 of 22, 4.5%) were observed in FTAs.

Conclusions: The frequencies of RAS mutations among our Korean population were 18% in NHs, 46.2% in FTC and 33.3% in FTAs. N-RAS codon 61 was the most frequent mutation in NHs, FTCs and FTAs, and the frequency was not significantly different among the three groups. K-RAS codons 12-13 were the second most commonly involved site in FTCs and FTAs, whereas no mutation was detected at this site in NHs.

KEYWORDS

follicular, human genes, RAS mutations, thyroid cancer

1 | INTRODUCTION

Similar to other forms of differentiated thyroid cancer, follicular thyroid cancer most commonly presents as an asymptomatic mass that cannot be distinguished from a benign follicular neoplasm based on cytological, ultrasonography or clinical features alone. The diagnosis of follicular thyroid carcinoma (FTC) is made based on morphology, depending on the presence of capsular and/or vascular invasion in thyroidectomy specimens.[1-3] Therefore, molecular markers that can help distinguish between FTCs and benign follicular neoplasms, and can also identify life-threatening FTCs, have been investigated.

The *BRAF* V600E mutation is the most common genetic alteration in thyroid tumorigenesis and has been observed in 29%-83% of papillary thyroid carcinomas (PTCs).[4-6] *RAS* mutations are the

second most common genetic alteration in thyroid tumours. Recent studies have reported that 10%-20% of PTCs and 40%-50% of FTCs harbour *RAS* mutations.[3,7] These mutations have been associated with poor prognoses and tumour dedifferentiation.[8,9]

The *RAS* genes consist of three families: *N-RAS*, *H-RAS* and *K-RAS*. *RAS* point mutations mostly occur in codons 12, 13 and 61.[10,11] However, the implications for diagnostic detection of *RAS* mutations by fine-needle aspiration (FNA) or surgical specimens are not clear because *RAS* mutations occur not only in thyroid cancers, but also in histologically benign nodules including follicular thyroid adenomas (FTAs) and nodular hyperplasia (NH).[12] The frequency of *RAS* mutations in FTCs and FTAs is controversial, probably because a small number of cases were evaluated in previous studies, which also used different methodologies.

Some reports have suggested that *RAS* mutations are more prevalent in FTCs than FTAs.[13-15] However, our preliminary study found no significant difference in the prevalence of any *RAS* mutation subtype between FTCs and FTAs.[16] After finishing that study, we wondered whether a *RAS* mutation would be helpful to distinguish NHs from FTCs and FTAs and eventually reduce unnecessary thyroid surgery. There is no reliable report describing a *RAS* analysis of NH to date.

Therefore, the aims of this study were (a) to compare the frequency of *RAS* mutations among follicular thyroid nodules, (b) to determine differences in *RAS* mutations between benign NH and follicular neoplasm and (c) to determine if *RAS* analysis can help reduce unnecessary surgery, if benign NH can be differentiated from follicular neoplasm by *RAS* analysis.

In the present study, we analysed the clinical significance and diagnostic utility of *RAS* mutations for differentiating NHs from follicular neoplasms.

2 | MATERIALS AND METHODS

2.1 | Materials

Our institutional review board approved the present retrospective study, and the requirement for informed consent was waived. We analysed surgically confirmed NH (n = 50), FTAs (n = 57) and FTCs (n = 39). The material was retrieved from the files of the Pathology Department, Soonchunhyang University Bucheon Hospital, from January 2002 to May 2015. We included 56 FTAs and 35 FTCs, all of which were evaluated in our previous study, and we additionally evaluated 50 NH, 1 FTA and 4 FTCs. Haematoxylin and eosin (H&E)-stained sections were evaluated histologically by a pathologist (JJ Kwak) to classify the tumours according to the 2004 World Health Organization histological classification of thyroid tumours. *RAS* mutations were investigated in all 146 samples.

2.2 | DNA isolation

Follicular thyroid carcinoma or FTA areas were marked using the H&E-stained sections as a guide. Each marked area was prepared from four to five sections (10 μm thick) using a microtome and transferred to an Eppendorf tube. Microdissected specimens from the paraffin-embedded blocks were subjected to treatment with a deparaffinization solution (Qiagen, Hilden, Germany) to remove the paraffin. DNA was isolated using a QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's protocol.

2.3 | Detection of K-, N- and H-RAS mutations by pyrosequencing

Primers were designed using PyroMark Assay Design software (ver. 2.0; Qiagen). Polymerase chain reaction (PCR) was performed using a PyroMark PCR Kit (Qiagen) after initial denaturation at 95°C for 15 minutes, followed by 45 cycles of a three-step PCR protocol that included 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C and 30 seconds of elongation at 72°C. This was followed by a final 10-minute extension phase at 72°C.

Briefly, 5 μL genomic DNA was amplified using template-specific PCR primers (including one biotin-labelled primer) and template-specific PCR conditions. Next, the PCR products were immobilized on streptavidin Sepharose beads, and single-stranded DNA was prepared to allow subsequent annealing of the sequencing primer to the template DNA. Then, the primed single-stranded DNA was released from the streptavidin surface and transferred to a PyroMark Q24 system (Qiagen) for pyrosequencing.

2.4 | Statistical analysis

The sex and age of the patients who were analysed for *RAS* mutations were tabulated (Table 1). The three groups were compared using Student's *t* test (for patient age) or the chi-square test (for sex). To compare the frequency of *RAS* mutations among NHs, FTCs and FTAs, we used Fisher's exact test or Pearson's chi-square test, as appropriate. *P*-values ≤0.05 were considered significant. The statistical analysis was performed using MedCalc (ver. 12.7.4.0; MedCalc Software, Ostend, Belgium) and SPSS statistical software (ver. 21.0; IBM Corp, Armonk, NY, USA).

3 | RESULTS

3.1 | Patients

The age of the patients ranged from 12 to 82 years (mean, 43.5 years). The mean age of the patients with NH, FTC and FTA was 41.9, 49.3 and 40.9 years, respectively. The patients with FTA were significantly younger than the patients with FTC ($P = 0.046$). There were 134 female and 12 male patients in this study.

3.2 | RAS point mutations

We checked for the presence of *N-RAS*, *H-RAS* and *K-RAS* gene mutations in 50 NHs, 57 FTAs and 39 FTCs (Table 2). In all, 52 mutations in the 46 nodules of the 146 NHs and follicular neoplasms were detected: nine nodules of 50 NHs (18%), 18 nodules of 39 FTCs (46.2%)

and 19 nodules of 57 FTAs (33.3%). Six nodules showed two point mutations (three FTCs: *K-RAS* 12-13 and *H-RAS* 61 [n = 1], *K-RAS* 12-13 and *N-RAS* 61 [n = 1] and *K-RAS* 61 and *H-RAS* 61 [n = 1]; and three FTAs: *K-RAS* 12-13 and *N-RAS* 61 [n = 2], *K-RAS* 12-13 and *H-RAS* 61 [n = 1]).

N-RAS codon 61 (n = 6 of 9, 66.7%; Figure 1) and *H-RAS* codon 61 (n = 3 of 9, 33.3%) were found in NHs. *K-RAS* codons 12-13, *K-RAS* codon 61, *N-RAS* codons 12-13 and *H-RAS* codons 12-13 were not found in NHs.

N-RAS codon 61 (n = 7 of 21, 33.3%), *K-RAS* codons 12-13 (n = 6 of 21, 28.6%; Figure 2), *H-RAS* codon 61 (n = 4 of 21, 19.0%), *K-RAS* codon 61 (n = 3 of 21, 14.3%) and *N-RAS* codons 12-13 (n = 1 of 21, 4.7%) were found in FTCs, and *N-RAS* codon 61 (n = 10 of 22, 45.5%), *K-RAS* codons 12-13 (n = 5 of 22, 22.7%; Figure 3), *H-RAS* codon 61 (n = 5 of 22, 22.7%), *K-RAS* codon 61 (n = 1 of 22, 4.5%) and *N-RAS* codons 12-13 (n = 1 of 22, 4.5%) were observed in FTAs.

Of the 52 mutations, 23 (44.2%) were in *N-RAS* codon 61 (observed in 6 NHs, 7 FTCs and 10 FTAs), 11 (21.2%) were in *K-RAS* codons 12-13 (observed in 6 FTCs and 5 FTAs), 12 (23.1%) were in *H-RAS* codon 61 (observed in 3 NHs, 4 FTCs and 5 FTAs), 4 (7.7%) were in *K-RAS* codon 61 (observed in 3 FTCs and 1 FTAs), and the remaining two mutations (3.8%) were in *N-RAS* codons 12-13 (observed in 1 FTC and 1 FTA). We did not detect mutations in *H-RAS* codons 12-13.

A significant difference in the frequency of the *K-RAS* codon 12-13 mutation was observed between NHs and FTCs ($P = 0.017$), whereas no other *RAS* mutation subtypes were significantly different among the three groups.

TABLE 1 Demographics of nodular hyperplasia, follicular carcinoma and follicular adenoma

Variables	NH (n = 50)	FTC (n = 39)	FTA (n = 57)	Total (n = 146)	*P*-value[a]	Post hoc comparison[b]		
						NH vs FTA	NH vs FTC	FTA vs FTC
Age (y), mean ± SD	41.9 ± 10.5	49.3 ± 18.2	40.9 ± 13.1	43.5 ± 14.3	**0.022**	1	0.083	**0.046**
Sex, n (%)								
Male	3 (6.0)	1 (2.6)	8 (14.0)	12 (8.2)	0.124			
Female	47 (94.0)	38 (97.4)	49 (86.0)	134 (91.8)				
Volume (mm^3), mean ± SD	6.0 ± 6.2	11.9 ± 11.0	9.8 ± 11.3	8.8 ± 9.9	**0.007**	0.1	**0.032**	1

FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; NH, nodular hyperplasia; SD, standard deviation.
[a]*P*-values were calculated by one-way analysis of variance or Kruskal-Wallis test for continuous variables and chi-square test or Fisher's exact test for categorical variables.
[b]Post hoc comparison was conducted by Bonferroni's correction.
Bold values indicates significant differences at a significance level of .05.

TABLE 2 Frequency of RAS mutation between nodular hyperplasia, follicular carcinoma and follicular adenoma

Variables	NH (n = 50)	FTC (n = 39)	FTA (n = 57)	Total (n = 146)	*P*-value[a]	Post hoc comparison[b]		
						NH vs FTA	NH vs FTC	FTA vs FTC
RAS mutation (%)								
(–)	41 (82.0)	21 (53.8)	38 (66.7)	100 (68.5)	**0.017**	0.342	**0.025**	0.875
(+)	9 (18.0)	18 (46.2)	19 (33.3)	46 (31.5)				
RAS mutation per site (%)								
K12/13	0 (0.0)	6 (28.6)	5 (22.7)	11 (21.2)	**0.009**	0.178	**0.017**	1
K61	0 (0.0)	3 (14.3)	1 (4.5)	4 (7.7)	0.093			
N12/13	0 (0.0)	1 (4.7)	1 (4.5)	2 (3.8)	0.731			
N61	6 (66.7)	7 (33.3)	10 (45.5)	23 (44.2)	0.712			
H12/13	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA			
H61	3 (33.3)	4 (19.0)	5 (22.7)	12 (23.1)	0.805			
RAS mutation frequency in total	9/300 (3.0)	21/234 (9.0)	22/342 (6.4)	52/876 (5.94)	**0.013**	0.197	**0.016**	0.984

FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; NA, not available; NH, nodular hyperplasia.
[a]*P*-values were calculated by one-way analysis of variance or Kruskal-Wallis test for continuous variables and chi-square test or Fisher's exact test for categorical variables.
[b]Post hoc comparison was conducted by Bonferroni's correction.
Bold values indicates significant differences at a significance level of .05.

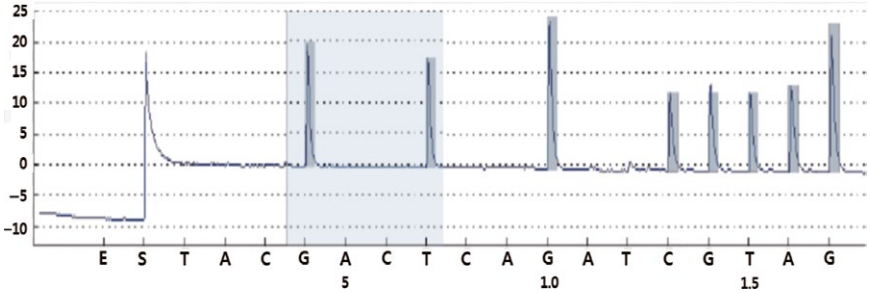

FIGURE 1 The result of pyrosequencing of *N-RAS* codon 61 mutation found in nodular hyperplasia

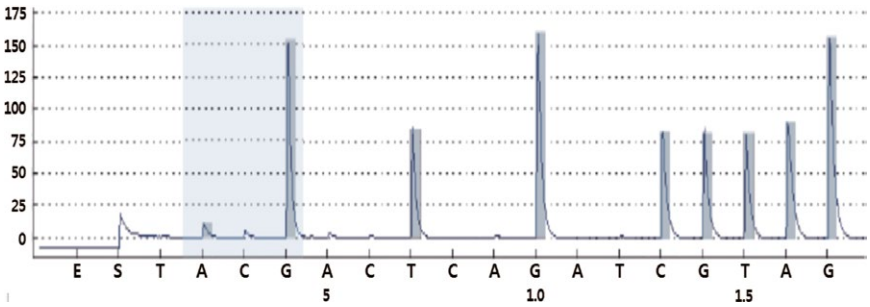

FIGURE 2 The result of pyrosequencing of *K-RAS* codon 12-13 mutation found in follicular thyroid carcinoma

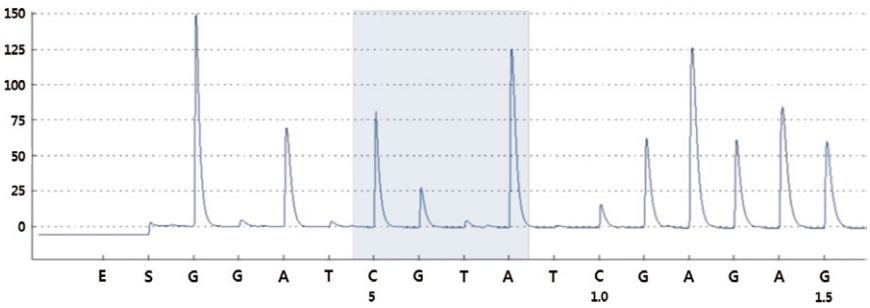

FIGURE 3 The result of pyrosequencing of *K-RAS* codon 12-13 mutation found in follicular thyroid adenoma

4 | DISCUSSION

The presence of *RAS* mutations suggests the possibility of a broad spectrum of tumours, from benign follicular adenoma to FTC, follicular variants (FVs) of PTC, anaplastic carcinoma and poorly differentiated thyroid carcinoma.[8,17-20]

H-RAS, *N-RAS* or *K-RAS* participation in the *RAS-RAF*-MAPK pathway is essential for controlling the proliferation, differentiation and survival of eukaryotic cells.[21] The *H-RAS*, *N-RAS* and *K-RAS* oncogenic mutations found frequently in human tumours disrupt the normal outcome of those signalling pathways, thus leading to tumour development.[21]

Somatic mutations in codons 12-13 and 61 of one of the three *RAS* genes have been found in 18%-52% of FTCs[13,15,22-25] and 24%-53% of FTAs.[13-15,22,23] The reported incidence of *RAS* mutations in both FTCs and FTAs probably varies because the number of cases is typically small. Our previous study targeting the Korean population with a relatively large number of surgically proved cases (35 FTCs and 56 FTAs) showed a similar *RAS* mutation frequency between FTCs (45.7%) and FTAs (33.9%).[16] According to our previous study, the incidence rate of *RAS* mutations was not so helpful to differentiate FTCs from FTAs.[16]

Several reports have suggested that *RAS* mutations are most frequently detected on codon 61 of *N-RAS* in FTCs.[3,8,12,13,26-28] On the other hand, other studies have shown that mutations in *N-RAS* codon 61 predominate in various kinds of follicular thyroid lesion relative to other *RAS* mutations.[26,29,30] We found no significant differences in *RAS* mutation frequency between FTCs and FTAs for any *RAS* mutation subtype. Nikiforova et al[3] suggested that *RAS*-initiated FTCs develop through a benign follicular adenoma stage because both typically express Hector Battifora mesothelial-1 and galectin-C. Thus, activating *RAS* alone appears insufficient to detect malignant growth, but may be a predisposing factor for the acquisition of additional genetic or epigenetic alterations that lead to a fully transformed phenotype.[3]

The present study may be the first to include a relatively large number of cases (n = 50) and analyse subtypes of *RAS* mutation in NHs. In the present study, *N-RAS* codon 61 was the most frequent not only in FTCs and FTAs but also in NHs. The frequency was not different among the three groups. *K-RAS* codons 12-13 were the second most commonly involved site in FTCs and FTAs, whereas no mutation was detected in NH. A significant difference was observed in the frequency of the *K-RAS* codon 12-13 mutation between NHs

and FTCs (P = 0.02), although the frequency of the *K-RAS* codon 12-13 mutation was not different between NHs and FTAs or FTCs and FTAs.

Because we included only surgically proven NHs or follicular neoplasms, we could not evaluate the long-term prognosis or *RAS* (+) benign nodules. We do not know whether NHs presenting with a *RAS* mutation have a different prognosis compared to NHs without a *RAS* mutation. However, one study[31] showed that benign thyroid nodules harbouring *RAS* mutations had a larger mean volume (P = 0.017) and RAS mutation-positive nodules displayed a mean 27.6% yearly volume increase, during RAS mutation-negative nodules remained unchanged after a 25-month follow-up.

Although no nodules displayed clinical features suspicious of malignant conversion during follow-up in that study, the authors suggested that more careful follow-up and timely surgical management are needed for thyroid nodules presenting with *RAS* mutations and benign cytology. A prospective study with a large number of cases and a much longer follow-up would be necessary to assess the prognosis of benign nodules harbouring a *RAS* mutation.

Several limitations of this study should be mentioned. First, there were significant differences in the volumes of NHs and FTCs and in the mean ages of the patients with FTAs and FTCs. However, this was inevitable because we included all surgically confirmed NHs, FTAs and FTCs during the study period. However, we presumed that this demographic difference was not important when investigating the incidence of *RAS* mutations.

Second, we did not evaluate whether the FNA results were concordant with the surgical specimen results. Because this study investigated the incidence of *RAS* mutations in NH, FTAs and FTCs, surgical specimens provided more accurate information. Although several studies have demonstrated that pyrosequencing can easily detect point mutations in *RAS* on FNA results,[7,29,32] further study using FNA results is needed.

5 | CONCLUSIONS

The frequencies of *RAS* mutations among our Korean population were 18% in NHs, 46.2% in FTC and 33.3% in FTAs. *N-RAS* codon 61 was the most frequent mutation in NHs, FTCs and FTAs, and the frequency was not significantly different among the three groups. *K-RAS* codons 12-13 were the second most commonly involved site in FTCs and FTAs, whereas no mutation was detected at this site in NHs. A significant difference was observed in the frequency of *K-RAS* codon 12-13 mutation between NHs and FTCs (P = 0.017).

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CONFLICT OF INTEREST

Nothing to declare.

AUTHOR CONTRIBUTION

Sun Hye Jeong and Hyun Sook Hong conceived the presented idea. Sun Hye Jeong, Hyun Sook Hong, Jeong Ja Kwak, Eun Hye Lee and Ji Ye Lee carried out the experiment and analysed the data. Sun Hye Jeong and Hyun Sook Hong wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

ORCID

Sun Hye Jeong http://orcid.org/0000-0002-3309-0693
Ji Ye Lee http://orcid.org/0000-0002-3929-6254
Hyun Sook Hong http://orcid.org/0000-0003-3210-9740

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