NRAS Codon 61 Mutation Is Associated with Distant Metastasis in Patients with Follicular Thyroid Carcinoma

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Background: Known factors related to distant metastases in follicular thyroid carcinoma (FTC) included age, primary tumor size, and invasiveness. Distant metastasis is a main cause of death in FTC patients. Several studies showed that the presence of *RAS* mutations is also associated with poor clinical outcomes. We analyzed *RAS* mutations in FTC with distant metastases, FTC without a distant metastasis, follicular adenoma (FA), and nodular hyperplasia (NH). Furthermore, we elucidated the relationship between *RAS* mutations and clinical outcomes in FTC patients.

Methods: We selected patients who underwent a thyroidectomy for FTC with distant metastases ($n = 28$), size matched FTC specimens without a distant metastasis ($n = 28$), FA ($n = 17$), and NH ($n = 12$). *NRAS, HRAS,* and *KRAS* mutations were assessed using direct sequencing.

Results: Among 85 patients, 39 patients (46%) had *RAS* mutations. The *NRAS* codon 61 mutation ($n = 21$; 25%) was the most common point mutation. *HRAS* codon 61, *KRAS* codon 12/13, and *KRAS* codon 61 mutations were found in 7, 6, and 4 patients, respectively. A *NRAS* codon 12/13 mutation was found in only 1 patient, and a *HRAS* codon 12/13 mutation was not found. *RAS* mutations were significantly more common in the FTC than FA or NH groups. Especially, the *NRAS* codon 61 mutation was associated with distant metastasis in patients with FTC.

Conclusions: The presence of a *RAS* mutation, especially a *NRAS* codon 61 mutation, was significantly associated with the distant metastasis. The *NRAS* codon 61 mutation status might be a potential prognostic factor in FTC patients.

Introduction

RAS IS A CLASSICAL DUAL ACTIVATOR of the mitogen activated protein kinase and the phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathways. The *RAS* genes including *NRAS, HRAS,* and *KRAS* are known as proto-oncogenes. Many kinds of cancers, including thyroid, pancreas, biliary, colon, and melanoma, carry *RAS* mutations. The frequency of *RAS* mutations in thyroid cancer is reported to be about 14%, and many thyroid cancers carry mutations in all three isoforms (1). Several histologic types of malignant thyroid cancer (follicular thyroid carcinoma, encapsulated follicular variant papillary thyroid carcinoma, anaplastic thyroid cancer, and poorly differentiated thyroid cancer), as well as benign thyroid tumors carry *RAS* mutations with variable frequency (2,3). The higher rates of mutation in malignant thyroid tumors in comparison with benign tumors suggest that a *RAS* mutation is an early event in follicular thyroid carcinogenesis (4,5). Several studies reported that *RAS* mutations are also associated with

aggressive thyroid cancer behavior, distant metastases, and poor clinical outcome (6–10). Garcia *et al.* have reported that *RAS* mutations are associated with loss of tumor differentiation, large tumor size, vascular invasion, distant metastasis, and survival. However, that study cohort included various histologic types of tumors other than follicular thyroid carcinomas (FTC) (10). A recent study retrospectively analyzed 58 FTC patients, and found that 14 of these cases had a distant metastasis, and 33 harbored a *RAS* mutation (9). That study reported that *RAS* mutations are associated with not only distant metastasis but also high mortality in FTC patients.

FTC is considered a more aggressive disease with poorer prognosis than papillary thyroid carcinoma (PTC). It is less likely to have lymph node metastases but more likely to hematogenously disseminate to distant organs in comparison with PTC (11). Some FTCs even initially present with a distant metastasis. According to previous reports, the percentage of patients who present with distant metastasis at the initial presentation varies from 3.1% to 21% of FTC patients

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(12–16). Distant metastasis is the most important prognostic factor, and patients with a distant metastasis demonstrate an approximately five-fold higher mortality than patients without distant metastasis (13). Known factors related to distant metastases included age, primary tumor size, and invasiveness (16,17).

In our present study, we analyze the frequency of *RAS* mutations in patients who present with FTC with or without distant metastasis, follicular adenomas (FA), and nodular hyperplasia (NH) to elucidate the roles of *RAS* mutations in follicular thyroid carcinogenesis and metastasis. In addition, we assessed the relationship between *RAS* mutations and clinical outcomes in FTC patients including survival and distribution of metastatic sites.

Material and Methods

Patients

We selected patients who underwent a thyroidectomy and were diagnosed with FTC and distant metastases (FTC M1) between 1998 and 2012 in the Asan Medical Center. We identified 28 patients with FTC M1 and 28 size matched FTC specimens without a distant metastasis (FTC M0). We also identified FA ($n = 17$), and NH ($n = 12$). For the mutational analysis, all tissue specimens were independently reviewed by two expert pathologists (DES and GG), who specialize in thyroid pathology. Pathological diagnoses were made according to the latest World Health Organization classifications for thyroid cancer. FTCs were further diagnosed as either minimally invasive or widely invasive according to the World Health Organization classification for capsular invasion and vascular invasion. We excluded the oncocytic subtype of FTCs and solid growth patterned tumors after reviewing the specimens. This study protocol was approved by the institutional review board of Asan Medical Center.

Definitions

The diagnosis of distant metastasis was confirmed by pathological examination of metastatic site(s) of the FTC, assessment of ^{131}I uptake in distant organs using ^{131}I whole body scan following radioactive iodine (RAI) ablation, and/or assessment of nonradioactive iodine imaging including computed tomography, ^{18}F-fluorodeoxyglucose positron emission tomography with computed tomography, or magnetic resonance imaging. FTC M1 was defined as both synchronous and metachronous metastasis. We classified FTC M1 according to the number of metastatic sites. Metastatic disease at one site was metastases in single organ (e.g., lung or bone only). Both lung and bone metastases were classified as metastatic disease at two sites and metastases at three or more sites were classified as metastatic disease at multiple sites (e.g., lung, bone, and brain). FTC M0 patients were defined as patients who did not demonstrate any evidence of metastasis at initial surgery or during the median follow up period (10.1 years; range, 2.0–14.8 years).

Mutational analysis of the RAS genes

We used DNA from primary thyroid tumors for polymerase chain reaction (PCR) to compare the *RAS* mutation status in all patients. Surgical removal of metastases was performed in only eight patients with FTC M1. However, we could not extract the DNA from the metastatic specimens because those samples were too old. Eighty-five paraffin-embedded thyroid specimens were microdissected and sectioned to 5–10 slices of 10 μm thickness. Thirty-five patients (41%) presented with multiple tumors, including 20 patients with FTC and NH, 7 patients with multiple FTCs, 5 patients with FTC and micro-PTC, 2 patients with FTC and FA, and 1 patient with multiple NHs. We extracted DNA from dominant tumors when the specimens contained multiple FTCs or NHs.

Genomic DNA from formalin-fixed paraffin-embedded (FFPE) tissues was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Purified DNA was directly subjected to PCR with primers designed to detect each mutation site. *NRAS*, *HRAS*, and *KRAS* mutations were examined using PCR and amplified using appropriate primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/thy).

DNA was used as template after quantification by Nano-Drop (Thermo scientific, Wilmington, DE). PCR was performed using a BioMix kit (Bioline, Taunton, MA). Each PCR reaction (50 μL) contained 10 pmol/μL primer set with 1× PCR buffer, 2.5 mM $MgCl_2$, and 1 μg genomic DNA. Most of the specimens used were old, so we used a large amount of template DNA to obtain an adequate PCR product. PCR reactions were performed using the following settings: 95°C initial denaturation for 5 minutes, denaturation at 94°C for 30 seconds, annealing of the primer to the template at 55–65°C for 30 seconds, and primer extension at 72°C for 30 seconds. Denaturation, annealing, and primer extension were repeated for 35 cycles followed by a final extension at 72°C for 5 minutes. The PCR reaction was then cooled to 4°C. The final PCR products were confirmed using electrophoresis. To determine any genetic mutation, DNA sequencing was performed using the antisense primers. The DNA sequences were then read using a DNA analyzer (Bioedit version 7.2.0, Carlsbad, CA), and *RAS* mutations were thereby identified. Each DNA sample was assayed at least twice in order to confirm the *RAS* mutation status.

Statistical analysis

R version 3.0 (R Foundation for Statistical Computing, Vienna, Austria, www.R-project.org) was used to analyze all data. Associations between the *RAS* mutation status and the four study groups (FTC M1, FTC M0, FA, and NH) were evaluated using the Kruskal-Wallis test. Correlations between *RAS* mutation, distant metastasis, and metastastic sites were analyzed using the chi-squared or Fisher's exact test. The predictive factors for distant metastases in FTC were assessed using univariate and multivariate logistic regression models. Overall survival was evaluated at the end of follow up using the Kaplan-Meier analysis. In this study, p-value < 0.05 was considered to indicate statistical significance.

Results

Baseline characteristics of study subjects

Baseline characteristics of the 28 FTC M1 patients in our study cohort are listed in Supplementary Table S2. The mean age of patients in the FTC M1 group was 63.5 years (range;

42–82), and 22 patients were female (79%). All FTC M1 patients received a total thyroidectomy. The mean tumor size was 4.6 ± 2.9 cm (range, 0.5–15.0 cm). Twenty-three (82%) and 22 of specimens (79%) of FTC M1 had capsular or vascular invasion, respectively. Lymph nodes dissection was performed in 15 patients (53%), and 3 of these patients (11%) had cervical lymph nodes metastasis. Twenty-seven patients received remnant RAI ablation after surgery, and one FTC patient with brain metastasis could not receive RAI ablation. Fifteen patients received external beam radiation therapy and/or systemic chemotherapy. Metachronous metastases were found in 7 patients (lung only in 3 patients, both lung and bone in 3 patients, and metastatic disease at multiple sites in 1 patient). The median time between initial surgery and detection of metastasis was 3.8 years in these patients.

The mean age of the FTC M0 group was 50.2 years (range, 34–71). Twenty-six patients in the FTC M0 group were female (93%). The mean tumor size was 4.1 ± 2.2 cm (range, 1.0–12.0). Twenty- six (93%) and 3 specimens (11%) of the FTC M0 had capsular invasion or vascular invasion, respectively. Lymph node dissection was performed in 6 patients (21%), but no patient with lymph node metastasis was identified. Twenty-one (75%) patients in the FTC M0 group received remnant RAI ablation.

In the FA group, the mean age was 49.9 years (range, 38–64). All FA patients were female. The mean age of the NH group was 52.8 years (range, 34–74), and 10 patients were female (83%).

Frequency of RAS gene mutations in study patients

Among 85 patients, 39 patients (46%) had RAS mutations. The NRAS codon 61 mutation ($n = 21$, 25%) was the most common point mutation (Table 1). HRAS codon 61, KRAS codon 12/13, and KRAS codon 61 mutations were found in 7, 6, and 4 patients, respectively. A NRAS codon 12/13 mutation was found in only 1 patient, and a HRAS codon 12/13 mutation was not found. The point mutations identified in our study are summarized in Supplementary Table S3.

RAS mutations were detected more frequently in the FTC group than in the FA or NH groups (Fig. 1). We performed analyses of the presence of a mutation among 6 RAS codons ("six codons"; NRAS codon 61, NRAS codon 12/13, HRAS codon 61, HRAS codon 12/13, KRAS codon 61, and KRAS codon 12/13), presence of a mutation among 3 specific RAS codons ("three codons"; NRAS codon 61, HRAS codon 61,

and KRAS codon 12/13), and presence of the NRAS codon 61 mutation ("single codon"). Three kinds of RAS mutation analyses demonstrated a significant association between RAS mutations and FTC. Especially, the NRAS codon 61 mutation was more frequently detected in the FTC M1 than in the FTC M0 group (46% vs. 18%; $p = 0.04$, Fig. 1). However, analyses of "six codons" ($p = 0.59$) and "three codons" ($p = 0.59$) showed no statistically significant difference between the FTC M1 and FTC M0 groups (Fig. 1).

A comparison of the clinicopathological features in FTC patients according to NRAS codon 61 mutation status

The presence of a NRAS codon 61 mutation was significantly associated with tumor size and presence of distant metastases (Table 2). Although tumor size was smaller in NRAS codon 61 mutant tumors than in NRAS codon 61 wild type tumors, NRAS codon 61 mutant tumors were more likely to have distant metastases. The univariate model showed that age 45 years or older and the presence of a NRAS codon 61 mutation were significant factors associated with distant metastases. In the multivariate model, the presence of a NRAS codon 61 mutation was the only independent predictor of distant metastases (Table 3).

RAS mutation and FTC invasion

We subcategorized FTCs as minimally invasive FTC ($n = 45$; 80%) or widely invasive FTC ($n = 11$; 20%). All patients with widely invasive FTC demonstrated distant metastases, so there was a significant association between widely invasiveness and distant metastases ($p < 0.001$). RAS mutation frequencies were similar in both groups (minimally invasive FTC group, 24 of 45 patients, 53%; widely invasive FTC group, 7 of 11 patients, 64%).

Survival in FTC patients according to RAS mutation status

The mean survival time in patients with FTC was 13.3 ± 0.3 years. There was no significant difference in mean survival time between the RAS mutation positive FTC and RAS mutation negative FTC patients at the end of follow up (13.9 ± 0.7 and 13.0 ± 0.7 years respectively; Fig. 2A). NRAS codon 61 mutation positive FTC patients also demonstrated no difference in mean survival time compared with NRAS

TABLE 1. THE FREQUENCY OF RAS MUTATIONS IN STUDY GROUPS

| | Total | NH | FA | FTC | |
				FTC M0	FTC M1
Any RAS mutations	39/85 (46)*	1/12 (8)	7/17 (41)	14/28 (50)	17/28 (61)
NRAS codon 61	21/85 (25)	0/12 (0)	3/17 (18)	5/28 (18)	13/28 (46)
HRAS codon 61	7/85 (8)	1/12 (8)	1/17 (6)	3/28 (11)	2/28 (7)
KRAS codon 12/13	6/85 (7)	0/12 (0)	2/17 (12)	4/28 (14)	0/28 (0)
KRAS codon 61	4/85 (5)	0/12 (0)	1/17 (6)	1/28 (4)	2/28 (7)
NRAS codon 12/13	1/85 (1)	0/12 (0)	0/17 (0)	1/28 (4)	0/28 (0)
HRAS codon 12/13	0/85 (0)	0/12 (0)	0/17 (0)	0/28 (0)	0/28 (0)

*RAS mutation positive patients /total patients (%).

NH, nodular hyperplasia; FA, follicular adenoma; FTC, follicular thyroid carcinoma; FTC M0, follicular thyroid carcinoma without distant metastasis; FTC M1, follicular thyroid carcinoma with distant metastases.

FIG. 1. *RAS* mutation frequencies in study groups using three kinds of *RAS* mutation analyses (six codons, three codons, and single codon). [a]Analysis of *RAS* mutation frequency between FTC group and FA or NH groups. FTC had significantly more *RAS* mutations than FA or NH groups in all three kinds of *RAS* mutation analyses (six codons, three codons, and single codon). [b]Analysis of *RAS* mutation frequency between FTC M1 and FTC M0 groups. Only *NRAS* codon 61 mutation had a significant association with FTC M1 group. NH, nodular hyperplasia ($n = 12$); FA, follicular adenoma ($n = 17$); FTC, follicular thyroid carcinoma; FTC M0, FTC without a distant metastasis ($n = 28$); FTC M1, FTC with distant metastases ($n = 28$).
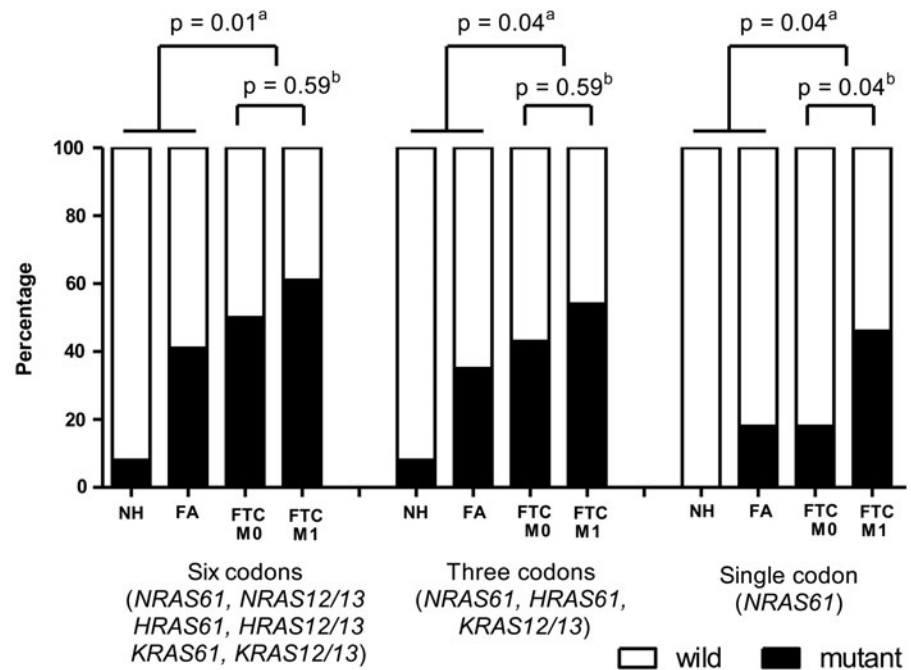
codon 61 mutation negative FTC patients (12.2 ± 1.2 and 13.6 ± 0.7 years respectively; Fig. 2B). *RAS* mutation positive FTC and *RAS* mutation negative FTC patients demonstrated similar clinicopathological features including age, sex, tumor size, FTC invasion, lymph node metastasis, and distant metastasis (Supplementary Table S4).

Subgroup analysis in FTC patients with distant metastases

There was also no significant difference in mean survival time during the follow-up between *RAS* mutation positive FTC M1 and *RAS* mutation negative FTC M1 patients (12.8 ± 2.9 years and 7.2 ± 0.5 years respectively, $p = 0.067$).

We analyzed the *RAS* mutation status according to the sites of metastases. Metastatic sites included lung only ($n = 8$), bone only ($n = 5$), both lung and bone ($n = 9$), and multiple sites ($n = 6$; Fig. 3). Metastases at multiple sites involved brain, muscle, liver, para-aortic lymph nodes, or kidney in addition to lung and bone. Patients with metastatic disease at one site (lung or bone only) were more likely to carry a *RAS*

mutation than those with metastatic disease at two or more sites ($p = 0.02$, odds ratio $= 8.25$ [95% C.I. 1.33-51.26]; Fig, 3).

Discussion

In this study, we investigated the *RAS* mutation status according to the presence of a distant metastasis in a large number of FTC patients. This is the largest study addressing the role of *RAS* mutations in FTC patients with distant metastases. Our study shows that the presence of a *RAS* mutation is associated with FTC, and that a *NRAS* codon 61 mutation is significantly associated with distant metastases in FTC patients. This result suggests that, the presence of a *NRAS* codon 61 mutation could be a factor predicting distant metastases.

FTC frequently metastasizes to distant organs. Some FTCs initially present with distant metastasis, and the percentage of FTC patients with distant metastasis at initial diagnosis varies between 3.1% and 21% in the literature (12–16). Distant metastasis is the most important prognostic factor in FTC. Sugino *et al.* reported that 10-year cancer-specific survival in

TABLE 2. A COMPARISON OF CLINICOPATHOLOGICAL FEATURES IN FTC PATIENTS
ACCORDING TO THE *NRAS* CODON 61 MUTATION STATUS

	Total	NRAS 61 wild type	NRAS 61 mutant	p-Value
Patient, *n*/total (%)	56/56 (100)	38/56 (68)	18/56 (32)	
Age, mean (range)	56.8 (34–82)	56.1 (34–80)	58.4 (42–82)	0.42
Sex, female	48/56 (86)	32/38 (84)	16/18 (89)	0.99
Tumor				
Mean size ± SD, cm (range)	4.4 ± 2.5 (0.5–15.0)	4.7 ± 2.9 (0.5–15.0)	3.6 ± 1.2 (1.7–6.0)	0.03
Widely invasive FTC	11/56 (20)	7/38 (18)	4/18 (22)	0.73
Lymph node metastasis	3/56 (5)	2/38 (5)	1/18 (6)	0.99
Distant metastasis	28/56 (50)	15/38 (39)	13/18 (72)	0.04
RAI treatment	48/56 (86)	32/38 (84)	16/18 (89)	0.99
Mortality	9/56 (16)	6/38 (16)	3/18 (17)	0.99

NRAS 61, *NRAS* codon 61; SD, standard deviation; RAI, radioactive iodine.

TABLE 3. THE PREDICTIVE FACTORS FOR DISTANT METASTASES IN FOLLICULAR THYROID CARCINOMAS

		Univariate		Multivariate	
	n (%)	OR [95% CI]	p-Value	OR [95% CI]	p-Value
Age ≥45 years[a]	26 (93)	8.41 (1.66–42.76)	0.01	4.43 (0.75–25.98)	0.1
Sex, female	22 (79)	0.28 (0.05–1.54)	0.14		
Tumor size[b]		1.08 (0.87–1.34)	0.48	1.12 (0.82–1.53)	0.47
Widely invasive FTC[c]	11 (39)	NA	NA	NA	>0.99
Lymph node metastasis[c]	3 (11)	NA	NA		
NRAS codon 61 mutation[a]	13 (46)	3.99 (1.18–13.5)	0.026	5.75 (1.32–25.13)	0.02

[a]Covariates that were significant factors in the univariate analysis (age ≥45 years, NRAS codon 61 mutation), tumor size, and invasiveness were included in the multivariate model for distant metastases.
[b]Tumor size was included in the model as a continuous variable.
[c]Univariate analysis was not performed for widely invasive and lymph node metastasis due to low number of patients. All widely invasive follicular thyroid carcinomas included in FTC M1.
CI, 95% confidence interval; NA, not applicable; OR, odds ratio.

FTC patients with distant metastasis was 41% in comparison with 96% in patients without distant metastasis. Age 45 years old or more, tumor size over 4 cm, and widely invasive FTC are significant factors related to distant metastasis (16). A NRAS codon 61 mutation could also be an important factor to predict distant metastasis in FTC patients according to the result of our study. As our study cohort was larger than previous studies, we could demonstrate a clear association between the RAS mutation and distant metastases (9,10).

RAS mutations seem to be not only involved in early carcinogenesis but also in FTC progression according to the results of several studies (7–10). The neoplastic proliferation of thyroid follicular cells develops by altering multiple molecular pathways such as the PI3K/AKT pathway involving the RAS, RAF, RET, and NTRK1 genes (7,18). RAS, which is a dual activator of the mitogen activated protein kinase and PI3K/AKT pathways, predominantly activates the PI3K/AKT pathway in follicular thyroid carcinogenesis (19). Previous studies showed that the frequency of RAS gene mutations is higher in FTC (23.8–56.9%) than in FA patients (18.0–47.8%) (8,9,20–22). The higher rate of mutation in malignant thyroid tumors suggests that RAS mutations are an early event in follicular thyroid carcinogenesis (4,5). Fur-

thermore, several in vivo and in vitro studies have reported that the introduction of mutant RAS to normal thyroid cells causes colonies to demonstrate well differentiated phenotypes and cell proliferation profiles that are consistent with FA (23–25). Other mutations, such as PTEN promoter hypomethylation, in addition to a RAS mutation could be needed to develop a FTC from a FA (26,27). More study is needed to find the genetic differences in FA and FTC patients.
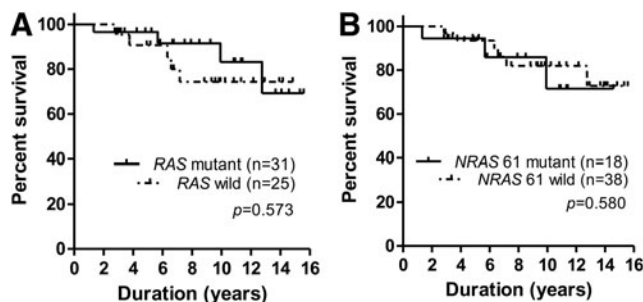

FIG. 2. Survival analysis of FTC patients. (A) Overall survival in the FTC patients according to the RAS mutation status. There was no difference in the overall survival between RAS mutant FTC and wild-type FTC patients. (B) Overall survival in the FTC patients according to NRAS codon 61 mutation status demonstrated no difference between NRAS codon 61 mutant-FTC and NRAS codon 61 wild-type FTC patients.
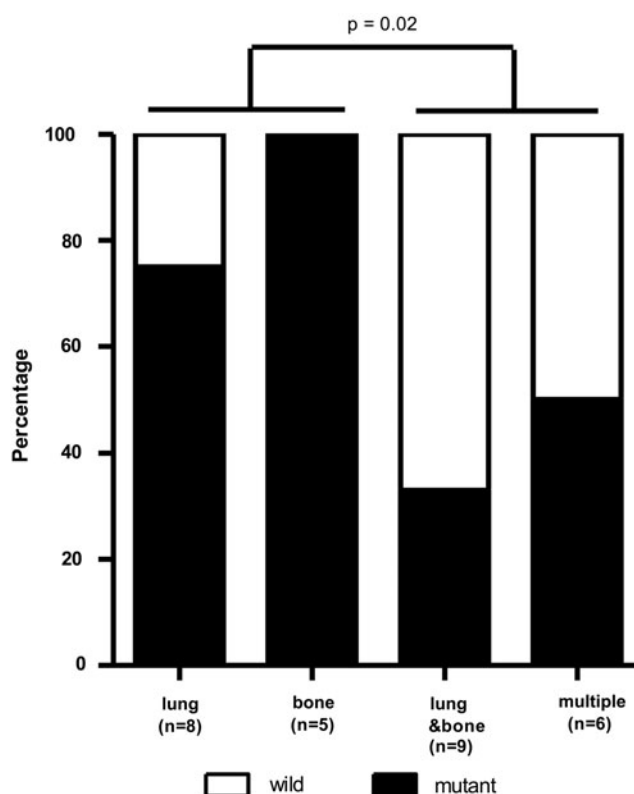

FIG. 3. Distribution of metastatic sites in follicular thyroid carcinoma with distant metastases. Metastatic disease at one site (lung or bone only) was more significantly associated with RAS mutations than metastatic disease at two or more sites (both lung and bone, or multiple sites).

Distant metastases of differentiated thyroid cancers usually show long-term stable or slowly progressive disease. Metastatic dormancy could explain the features of long-term stable distant metastases of thyroid cancer. Metastatic dormancy is defined as the ability of small clusters of cancer cells to migrate to distant sites and then survive in a quiescent state for long periods without significant growth (28,29). Although not significant, *RAS* mutation positive FTC M1 patients had longer survival time than *RAS* mutation negative FTC M1 patients. *RAS* mutation negative FTC with distant metastases demonstrated more extensive metastases than their *RAS* mutation positive counterparts, and this might have led to the offsetting of survival difference in FTC. *RAS* mutation positive FTC might gradually metastasize and in a stepwise manner unlike *RAS* mutation negative FTC. After metastasis develops, *RAS* mutations might not play a major role in cancer progression and dedifferentiation. Additional mutations might be needed to promote metastastatic progression in *RAS* mutation positive FTC with distant metastases. *RAS* mutation negative FTC with distant metastases might express undiscovered oncogenes that promote aggressive behavior. It is crucial to define the processes by which cancer metastasizes, and how metastatic dormancy is gained or lost in primary and metastatic settings in the future.

Fine needle aspiration cytology (FNAC) is the standard tool for detecting thyroid cancer (30). Recently, Gupta *et al.* reported that 83% of nodules with indeterminate and preoperative *RAS* mutation positive FNAC were diagnosed as cancer after surgery (31). If the thyroid nodules are diagnosed as a follicular neoplasm, and have a *RAS* mutation in a FNAC specimen, these tumors are expected to show more aggressive behavior (such as distant metastases) than those without a mutation based on our data. Therefore, preoperative *RAS* mutation analysis on FNAC samples diagnosed as follicular neoplasm will be useful for clinical decision making including the determination of the extent of surgical intervention. However, we need further studies based on long-term follow up data for elucidation of clinical usefulness of *RAS* mutation examination on FNAC.

In conclusion, the presence of a *RAS* mutation was significantly associated with FTC and a *NRAS* codon 61 mutation was especially associated with distant metastases in FTC patients. Our findings in this study suggest that *NRAS* codon 61 mutation status might be a potential prognostic factor in FTC patients.

Acknowledgment

Author Disclosure Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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