

Impact of NRAS Q61R Mutations on Papillary Thyroid Carcinoma: A Correlation with Clinicopathological Parameters

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ABSTRACT

Background: RAS gene mutations are found in a variety of malignancies throughout the body. NRAS mutations have been identified in several types of thyroid cancer. Although concurrent RAS mutations have recently been reported in papillary thyroid carcinoma (PTC), their impact on tumor progression and patient survival remains unclear.

Objectives: To investigate the significance of NRAS mutations in PTC and their correlation with clinicopathological characteristics.

Materials and methods: Formalin-fixed paraffin-embedded tissue blocks were obtained from 60 histologically confirmed PTC cases, referred between June 2022 and June 2023 to the National Center for Education Laboratories, Medical City Campus, and AL-Kimma Private Hospital in Baghdad, Iraq. The study investigated the presence of the NRAS Q61R mutation in these samples and analyzed its association with various clinical and pathological parameters, including age, sex, PTC variants, tumor size, capsular invasion, multifocality, extrathyroidal extension, and lymph node metastasis.

Results: The NRAS Q61R mutation was detected in 11 out of 60 PTC specimens (18.3%), with no statistically significant difference between patients with and without the mutation (P-value = 0.073). However, the presence of the NRAS mutation was significantly associated with encapsulated tumors (63.6%, P-value = 0.007). No significant associations were found between NRAS mutation status and other clinicopathological variables, including age, sex, PTC subtype, tumor size, multiplicity, extrathyroidal extension, or lymph node metastasis (P-value > 0.05).

Conclusion: The NRAS Q61R mutation was detected at a low frequency in PTC and was significantly associated only with encapsulated tumors. No correlation was found with aggressive features, suggesting a limited role in tumor progression. Further studies with larger sample sizes are recommended to clarify its clinical significance.

Keywords: Papillary thyroid carcinoma; NRAS *q61R* mutation; Clinicopathological parameters.

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INTRODUCTION

Thyroid cancer is the most common endocrine malignancy worldwide. Based on the Philippine 2020 Cancer Registry and Research Annual Report, it is the ninth most common malignancy [1]. According to the latest GLOBOCAN data, it currently ranks as

the 7th most common cancer [2, 3]. Papillary thyroid cancer (PTC) is the most frequent and treatable kind of thyroid malignancy [4]. It accounts for 70-90% of thyroid cancers [5]. The prevalence of PTC has been growing as the ultrasonic examination has become more popular [6, 7]. The incidence of PTC has dramatically grown in various nations since the 1970s [8]. The majority of PTC patients have an indolent course as compared to other malignant tumors [9, 10]. However, a subset of PTC patients develops extremely aggressive metastatic illness [11].

Gene changes contribute to tumor aggressiveness and re-

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currence. One of these genes is the RAS gene [12]. Genetic changes in the mitogen-associated protein kinase (MAPK) pathway play a significant role in the development and progression of PTC [13, 14]. RAS genes such as NRAS, HRAS, and KRAS are classified as proto-oncogenes. A solitary point mutation in the RAS gene impairs this finely organized signaling in approximately one-third of all human malignancies. The majority of these mutations impact amino acids G12, G13, or Q61, resulting in a reduction of intrinsic and/or RAS GAP-stimulated GTPase activity. Mutations that occur in G13 and Q61, unlike G12, have been demonstrated to enhance intrinsic and GEFs (guanine nucleotide exchange factors)-mediated current exchange rates. RAS predominantly remains in the GTP-bound, active state, recognized for its oncogenic properties.

NRAS is by far the most frequently changed RAS isoform, with 84% of mutations altering codon 61. Thyroid cancer has a comparable tendency for codon 61 mutations, although other cancer types don't [15]. Studies observed that the RAS mutation pattern of FVPTC (follicular variant PTC) was more similar to that of FTC (follicular thyroid carcinoma) than to that of PTC, demonstrating a relationship between RAS mutation and follicular differentiation of thyroid tumors [16]. Specific clinicopathological features, such as older age, male sex, tumor size ≥ 4 cm, presence of extrathyroidal extension, and cervical lymph node metastasis, are considered poor prognostic markers in PTC [17]. Lymph node involvement and metastases > 3 cm, and distant metastases [18, 19], Which distinctly alter disease-free survival (DFS) and disease-specific survival (DSS) [20].

The incidence of PTC in Iraq has notably increased in recent years [21]. According to the 2020 Iraq Cancer Registry, thyroid carcinoma is the second most common malignancy among females, with PTC comprising approximately 83.5% of thyroid cancer cases. This trend highlights a growing public health concern, especially among women. Genetic mutations, including NRAS codon 61 alterations, are known to influence tumor behavior and prognosis. While NRAS Q61R mutations are well-documented in follicular thyroid carcinoma, their role in PTC remains unclear. This study aimed to determine the frequency of NRAS Q61R mutations in PTC and assess their association with aggressive histopathological features. To our knowledge, this is the first Iraqi study focusing on this specific mutation in PTC.

MATERIALS AND METHODS

A retrospective cross-sectional study that utilized formalin-fixed, paraffin-embedded (FFPE) tissue blocks obtained from histologically confirmed cases of PTC. The samples were collected from patients referred to the National Center for Educational Laboratories at the Medical City Campus and AL-Kimma Private Hospital, Baghdad, Iraq, over the period from June 2022 to June 2023. The current study obtained approval from the Institutional Review Board of Teaching Laboratories of Baghdad Medical City (Reference number 254, dated December 26, 2024). Informed consent from the participants was waived owing to the retrospective nature of the study. Moreover, the study complies with the principles of Helsinki of the latest updated version in October 2024.

A total of 60 PTC samples (12 males and 48 females) were included in the study. The actual number of collected cases was lower than the initially estimated sample size, which was

calculated using the GeoPoll formula:

$$\begin{aligned}\text{Sample size} &= (Z\text{-score})^2 \times SD(1 - SD)/(CI)^2 \\ &= (1.96)^2 \times 0.5 \times 0.5/(0.1)^2 = 96.04\end{aligned}$$

This shortfall was mainly attributed to challenges in obtaining well-preserved tissue samples and the high cost of testing materials. Inclusion criteria encompassed all cases with a final diagnosis of PTC, regardless of patient age and sex. Exclusion criteria involved papillary microcarcinoma variants, due to their typically low-risk clinical behavior, as well as any samples that were damaged, inadequately preserved, or lacking complete clinical or pathological data. All other histological variants of PTC were included.

Data collected for each case included demographic variables (age and sex), histopathological subtype of PTC, tumor size (based on the maximum diameter), tumor multifocality, encapsulation status, lymph node involvement, and the presence of extrathyroidal extension. FFPE tissue blocks were sectioned and stained with hematoxylin and eosin (H&E). All slides were re-examined by pathologists, and the findings were documented accordingly.

Immunohistochemical (IHC) staining was performed in paraffin-embedded sections that were cut into 4 μm sections to obtain optimum resolution after staining. The anti-NRAS antibody (catalogue No. BSB 2664, BIO SB, USA) is a rabbit monoclonal antibody (clone RBT-NRAS, IgG isotype) specifically developed to detect the NRAS protein with a point mutation at codon 61 (Q61R). It exhibits membranous staining specificity and is optimized for IHC applications. diluted in a pH 7.5 buffer. It is provided in a ready-to-use, prediluted format and is suitable for use on FFPE tissues, cell preparations, and frozen tissue sections.

Tissue sections were prepared for IHC analysis by first air-drying, followed by deparaffinization using xylene. The sections were then dehydrated and rehydrated through a graded series of alcohol solutions. Antigen retrieval was performed using *ImmunoDNA Retriever* solution containing either citrate or EDTA, applied after heat-induced epitope retrieval. Endogenous peroxidase activity was blocked using a peroxidase blocking reagent. Subsequently, 50 μL of the prediluted primary anti-NRAS antibody was applied to each section. After incubation, a suitable secondary antibody was added, followed by visualization with a DAB (3,3'-diaminobenzidine) substrate chromogen solution. Finally, the sections were counterstained with hematoxylin and examined under a light microscope for evaluation.

The staining intensity of NRAS Q61R was rated as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), and 3+ (high staining). The NRAS Q61R IHC was considered positive if it revealed cytoplasmic and/or membranous immunoreactivity with a staining intensity of 2+ or 3+, regardless of the number of tumor cells stained [22].

The collected data were coded, entered, presented, and analyzed by computer using the available database software program IBM SPSS Statistics (IBM Statistical Package for the Social Sciences, Chicago, IL, USA), version 29. Data has been provided using basic statistical measures, including frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance and importance of differences between various means (quantitative data) were assessed using the Student's *t-test* for comparisons between two independent means. The importance of variations in distinct

percentages (qualitative data) was evaluated using the Pearson Chi-square test (χ^2 -test), with variations in distinct percentages (qualitative data) Yates' adjustment or the Fisher Exact test as appropriate. Statistical significance was deemed present when the P-value was less than 0.05.

RESULTS

The mean age of the patients was 46.6 ± 14.0 (Range 23-85) years. The vast majority (n = 47, 78.3%) of participants were females. The highest (n = 19, 31.7%) age group affected was 30-39 years. The classical variant is evident in 43 (71.7%). Infiltrative tumors are seen in 42 samples (70%). The highest tumor size is 1.5-2.0 cm (n = 14, 23.3%). Multifocal tumors are present in 30 (50%). Extrathyroidal extension is established in only 3 (5%), while lymph node metastasis is 17 (28.3%), as shown in Table 1.

The variants of PTC are shown in Figure 1.

Table 1. The clinicopathological parameters in 60 patients with papillary thyroid carcinoma (PTC).

Variables	Frequency	Percent
Age in years		
< 30	3	5.0
30–39	19	31.7
40–49	12	20.0
50–59	18	30.0
60–69	3	5.0
≥ 70	5	8.3
Sex		
Male	13	21.7
Female	47	78.3
Type of PTC		
Classical	43	71.7
Follicular	16	26.7
Sclerosing	1	1.7
Capsule		
Encapsulated	18	30.0
Infiltrative	42	70.0
Size (cm)		
1.0	11	18.3
1.5	14	23.3
2.0	13	21.7
2.5	5	8.3
3.0	6	10.0
3.5	5	8.3
4.0	6	10.0
Multiplicity		
Unifocal	30	50.0
Multifocal	30	50.0
Extra-thyroid extension		
Positive	3	5.0
Negative	52	86.7
Not identified	5	8.3
Lymph node metastasis		
Positive	17	28.3
Negative	34	56.7
Not identified	9	15.0

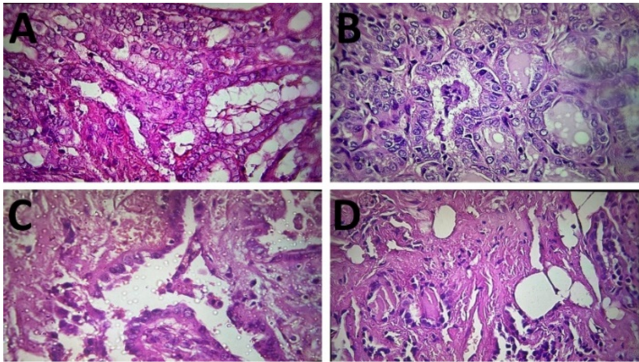


Figure 1. Pathological variants of papillary thyroid carcinoma (PTC). A: Classical, characterized by papillary architecture with nuclear features of ground glass nucleus, pseudo-inclusion and nuclear grooving X400. B: Follicular variant, predominant follicular pattern with PTC nuclear features X400. C: Hobnail variant, papillary or micropapillary architecture and tumor cells have apically placed nuclei X400. D: Sclerosing variant: Dense fibrosis (sclerosis) throughout the tumor with nuclear features of PTC X100 (H&E stain).

NRAS mutation was detected in 11 (18.3%) samples of PTC (Table 2). The intensity of the immune stain in the NRAS mutation detected by immunostaining is shown in Table 3 and Figure 2.

The age group below 50 years is the most common age group with NARS mutation (n = 7, 63.6%). The vast majority of mutations are seen in females (n = 10, 90.9%). The mutation is more evident in the follicular variant PTC (n = 6, 54.5%). Encapsulated form PTC showed more association with mutation (n = 7, 63.6%). The mutation is more associated with a size of more than 2 cm (n = 7, 63.8%). Multifocal tumors are more associated with mutation than unifocal tumors (n = 6, 54.5%). All samples that showed extra thyroid extension did not show the mutation. Finally, lymph node metastasis samples showed the mutation in only two samples (18.2%). There are no statistically significant differences (P-value > 0.05) between NRAS mutation and the above-mentioned variables as mentioned earlier. Around 2/3rd of mutations is found in encapsulated tumors (P-value = 0.007), as shown in Table 4.

Table 2. NRAS mutation expression in 60 patients with papillary thyroid carcinoma.

NRAS	Frequency	Percent	P-value
Positive	11	18.3	0.073
Negative	49	81.7	

Table 3. The intensity of the immune stain in the NRAS mutation detected by immunostaining.

NRAS intensity of immunostaining	Number	Percent
+1	4	26.7
+2	7	46.6
+3	4	26.7

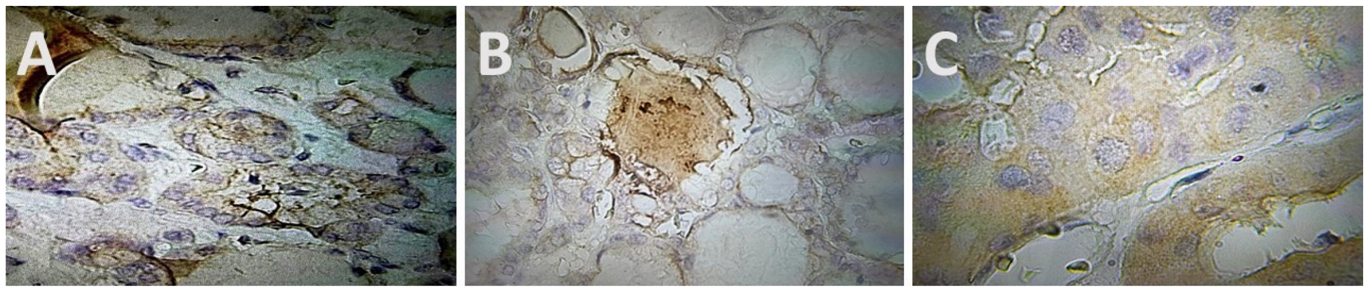


Figure 2. The intensity of immunohistochemical staining of NRAS in papillary thyroid carcinoma (PTC). A: The intensity of immunostaining +3 X400, B: The intensity of immunohistochemical staining +2 X400. C: Immunohistochemical staining of NRAS Q61R in PTC X1000 with oil immersion (NRASQ61R immunohistochemical stain).

Table 4. Correlation of NRAS mutation with clinicopathological parameters in 60 patients with papillary thyroid carcinoma (PTC)*.

Variable	NRAS Positive (n = 11)		NRAS Negative (n = 49)		P-value
	No.	%	No.	%	
Age (years)					0.203
<30	2	18.2	1	2.0	
30–39	4	36.4	15	30.6	
40–49	1	9.1	11	22.4	
50–59	4	36.4	15	30.6	
60–69	–	–	2	4.1	
≥70	–	–	5	10.2	
Sex					0.317
Male	1	9.1	11	22.4	
Female	10	90.9	38	77.6	
Type of PTC					0.066
Classical	5	45.5	38	77.6	
Follicular	6	54.5	10	20.4	
Sclerosing	–	–	1	2.0	
Presence of a capsule					0.007*
Infiltrative	4	36.4	38	77.6	
Encapsulated	7	63.6	11	22.4	
Size (cm)					0.335
1.0	2	18.2	9	18.4	
1.5	2	18.2	12	24.5	
2.0	3	27.3	10	20.4	
2.5	1	9.1	4	8.2	
3.0	–	–	6	12.2	
3.5	–	–	5	10.2	
4.0	3	27.3	3	6.1	
Multiplicity					0.739
Unifocal	5	45.5	25	51.0	
Multifocal	6	54.5	24	49.0	
Extra-thyroid extension					0.355
Positive	–	–	3	6.1	
Negative	11	100	41	83.7	
Not identified	–	–	5	10.2	
Lymph node metastasis					0.492
Positive	2	18.2	15	30.6	
Negative	8	72.7	26	53.1	
Not identified	1	9.1	8	16.3	

* Significant difference between percentages using Pearson Chi-square test (χ^2 -test) at the 0.05 level.

DISCUSSION

RAS genes participate in the RAS-RAF-MAPK and PI3K-AKT cell signaling pathways, influencing functions such as cell cycle control, cell growth, cellular migration, and induction of apoptosis. In typical cells, the functions of RAS genes and proteins are monitored and equilibrated within the molecular regulation mechanism to provide the required cellular responses for growth and differentiation. RAS gene mutations, on the other hand, cause GTPase activity to be lost, trapping RAS proteins in a GTP-bound conformation that is always active. This activates the pathways of MAPK and PI3K-AKT, potentially causing cancer. Following the identification of the RAS gene over three decades ago, RAS mutations have been linked to over 40 different forms of neoplasms. Mutations in NRAS frequently involve alterations in 12, 13, or 61 amino acid residues, resulting in constitutive activation of NRAS [23], thereby transforming a normal cell into a malignant one in a different form of human tumors. So, NRAS mutation in thyroid carcinoma, particularly in follicular carcinoma, has been clarified; however, it remains controversial in the case of PTC. Additionally, the rate of presence of the NRAS Q61R mutation in PTC and its correlation with aggressive features have not yet been tested in the Iraqi population.

Our result for the presence of mutation in PTC samples was 18.3%. There was a variation in results in different studies, which may be related to the method of sample selection, the size of the samples, and the process of mutation detection. Geographical variation could potentially influence the findings. Our result was comparable to the results of the study by Harahap et al. (NRAS mutation was detected in 22/172 (12.8%) through polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing analysis) [24] as well as in Fakhruddin et al. study [25], NRAS mutation was examined in 194 samples and was positive in 21 samples. Despite the notable mutation detected by various methods (PCR and reverse hybridization), the P-value remains below 0.0001. At the same time, other investigations showed low expression or no expression, like in the study by Vidinova et al. [26]. The study was done on 50 PTC samples of the Bulgarian population; the mutation was tested by Sanger sequencing. In another study by Eng et al. [27], the NRAS mutation was detected in 28% of the PTC patient samples in Malaysia. Therefore, there are variations in results among studies. This variation may be related to study design (as the present study excluded papillary microcarcinoma, which shows a relatively high rate of mutation in comparison to classical variants in other studies). Another issue is the testing technique of the study; Sanger sequencing and some other mutation detection techniques may exhibit greater sensitivity. Sample size may interfere with the rate of significance of mutation, and, not to forget, geographical factors also play a role. Overall, these studies, including our own, have not shown a high mutation rate of NRASQ61R in PTC.

There was no significant correlation with increasing age, although the mutation was more evident in individuals under 50 years of age. This suggests that NRAS mutations may occur more frequently in middle-aged patients, although no strong age-related link was found. This finding is consistent with a recent study [24] Among those under 55 years old, the mutation was found in 14 out of 22 (53.8%).

There was a connection between being female and having the mutation. We found more NRAS mutations in females (90.9%) than in males (9.1%), but this significant difference

didn't reach statistical significance (P-value = 0.317). The higher occurrence in females matches what is generally seen in PTC. The female predominance aligns with the known gender distribution in PTC overall. While in Fakhruddin et al. study [25] It is equivocal in both males and females, as noted in a recent study by Harahap et al. [24], the female sex was 66.7%; these variations may be related to the method of sample selection.

NRAS mutations were more frequent in the follicular variant (54.5%) compared to the classical type (45.5%). The FVPTC has a greater frequency of KRAS mutations relative to the classical form owing to its unique molecular characteristics. Encapsulated FVPTC displays a molecular profile that more closely aligns with follicular adenomas, characterized by a greater prevalence of NRAS mutations and a lower prevalence of BRAF mutations. In contrast, the infiltrative version demonstrates an opposite trend [28]. However, the P-value was 0.066, indicate lack of significance, the mutation rate in this variant remained higher compared to other subtypes, this trend supports prior studies suggesting a higher NRAS mutation rate in follicular-patterned PTC, and this result is comparable with many studies, like Harahap et al. study [24], which revealed that the follicular variant evident in 14/22, as the finding of NRAS mutation at codon 61 was the most prevalent genetic change in FVPTCs [29]. There was a significant link between the existence of a mutation and encapsulated tumors. This suggests that NRAS-mutated tumors may follow a more indolent growth pattern, consistent with a comparable result found in a study by Harahap et al. (12/22 samples) [24]. Tumor size showed no significant association with NRAS mutation status (P-value = 0.335). The mutation was relatively evenly distributed across the tumor size groups and was detected in tumors measuring ≥ 4 cm in only 3 cases. The current study showed comparable results with a recent study by Harahap et al. (tumours ≥ 4 cm, 7/22) [24]. There was no significant correlation between tumor focality and NRAS status. Both NRAS-positive and negative groups showed a nearly equal distribution between multifocal and unifocal tumors. Multifocal shows an increasing rate, while in the study by Harahap et al., multifocality is evident in only 4/22 samples [24]. The mutation did not show a significant correlation with extrathyroidal extension. This again may suggest a less aggressive behavior in NRAS-mutated tumors, or it may relate to the fact that many samples did not include the extrathyroidal extension feature.

There was no significant association between NRAS mutation and lymph node involvement (P-value = 0.492). The findings may suggest that the presence of the NRAS mutation is not related to lymph node metastasis that mean not associated with aggressive features of PTC, additionally, the number of cases harboring the mutation was limited, as was the number of samples that presenting with lymph node metastasis, which may have impacted the ability to establish a statistically significant association. A comparable finding in Fakhruddin et al. [25], there was no significant correlation with lymph node metastasis. In Eng et al. [27]. The study focuses on three clinicopathological parameters: age, sex, and lymph node metastasis. Notably, these parameters were not significant (P-value > 0.9999, 0.1130, and > 0.9999, respectively).

The number of PTC cases analyzed may be inadequate to generalise the findings to broader populations or tumour subtypes, partly due to cost constraints and difficulties in sample collection. All samples were FFPE, but some suffered from

degradation and suboptimal preservation, complicating IHC processing and reducing image resolution. These limitations restricted the study to a single diagnostic modality and prevented the use of more advanced, sensitive techniques such as next-generation sequencing (NGS) or digital PCR.

CONCLUSION

The NRAS Q61R mutation was detected at a low frequency in cases of PTC. Among the clinicopathological features analyzed, the mutation showed a significant correlation only with encapsulated tumors, suggesting a possible association with a more indolent or less invasive histological subtype. However, no significant association was observed between the presence of this mutation and indicators of tumor aggressiveness, such as extrathyroidal extension, or lymph node metastasis. These findings suggest that the NRAS Q61R mutation may not play a major role in promoting aggressive behaviour in PTC. Nevertheless, due to the limited sample size and low mutation frequency, further studies with larger cohorts are recommended to validate these observations and to better understand the potential clinical relevance of NRAS mutations in PTC.

ETHICAL DECLARATIONS

Acknowledgments

None.

Ethics Approval and Consent to Participate

The present study was approved by the Institutional Review Board of Teaching Laboratories of Baghdad Medical City

(Reference number 254, dated December 26, 2024). Informed consent from the participants was waived owing to the retrospective nature of the study. Moreover, the study complies with the principles of Helsinki of the latest updated version in October 2024.

Consent for Publication

Not applicable.

Availability of Data and Material

Data generated during this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contributions

Ibrahim WK was responsible for data collection, data analysis, formatting the references, and manuscript drafting. AL-Khafaji KR conceptualized the study, supervised the work, and critically reviewed the manuscript. Both authors reviewed and approved the final version of the manuscript.

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