

Study of BRAF and RAS Mutations in Thyroid Nodules with Indeterminate Cytology and Papillary Thyroid Cancer

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ABSTRACT

OBJECTIVES

Thyroid cancer Treatment decision-making is often guided by tumor tissue molecular analysis. The aim of this study was the detection of BRAF, NRAS and HRAS mutations in Georgian patients with thyroid cancer and determination of the frequency of these mutations in the respective populations.

SETTING

Diagnostic molecular laboratory located in Tbilisi, Georgia.

PARTICIPANTS

116 patients with thyroid cancer participated in the study.

PRIMARY AND SECONDARY OUTCOME MEASURES

Genetic change is the main force of thyroid tumor development, based on new methods of managing thyroid cancer. The latest significant genetic discovery in thyroid cancer is the BRAF-T1799A (V600E) transformation (the gene for B-type RAF kinase, BRAF). Since the initial report of this breakthrough in thyroid cancer years ago, rapid progress has been made. The BRAF mutation is the most common genetic change in thyroid cancer. BRAF and NRAS mutations are frequent genetic alterations found in thyroid nodules. These molecular markers establish a differential diagnosis and facilitate clinical decision-making. Prevalence of thyroid nodule-associated mutations has not been studied in Georgia. We evaluated BRAF, NRAS and HRAS mutations in Georgian patients with indeterminate cytology or diagnosed with papillary thyroid cancer (PTC).

RESULTS

BRAF (V600E), NRAS (G12C, G12D, Q61R, and Q61K) and HRAS (G12C, G13R, and Q61R) were determined in the DNA extracted from fine needle aspirate specimens. In total, 116 patient samples were analyzed using competitive-specific

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TaqMan PCR (Cast PCR TM). In these samples, 36 were diagnosed as papillary thyroid carcinoma, and 80 were indeterminate by Bethesda system for reporting thyroid cytopathology (BSRTC III-V).

BRAF (V600E) mutation was the most frequent genetic alteration found in 31% of all analyzed samples. Specifically, this mutation was present in 61% of PTC cases and 18% of cases classified as indeterminate (BSRTC III-V). NRAS mutations were present in 16% of PTC and 30% of indeterminate cytology samples. NRAS G12D and Q61R were most prevalent at 36.6% and 40% of all NRAS mutations. BSRTC IV category of indeterminate cytology had the highest frequency of NRAS mutations at 43%. From analyzed samples, HRAS (Q61R) mutation was present in only one PTC case.

KEYWORDS

BRAF (V600E); RAS mutations; Thyroid cancer; PTC

1. INTRODUCTION

Thyroid cancer is a tumor of thyroid similar to other cancers, the causes of the disease. Thyroid cancer is not well known so far, but research on this issue is currently underway. There Cases where thyroid cancer is related to pre-exposure to radiation. Cancer is known to be Thyroid sometimes develops after about 5-20 years of radiation therapy to the neck area, where it is given as a treatment for other cancers. It is also possible that a diet low in iodine increases the risk of glandular thyroid cancer. There is an increased risk of developing thyroid cancer if close relatives of the family are infected. The Different types of genetic mutations develop as a result of random processes such as DNA polymerase replication, radiation, chemical and oxidative impacts on DNA molecules, inefficiency of reparative system (Figure1).

The central role of genetic mutations in the formation and development of tumors

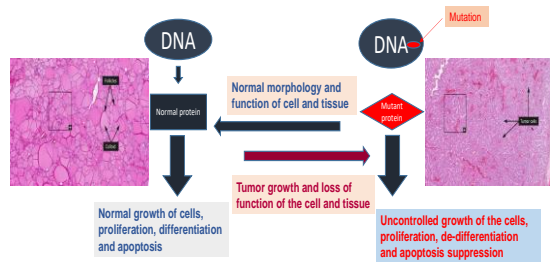


Figure1: Schematic illustration of the Different types of genetic mutations develop as a result of random processes such as DNA polymerase replication, radiation, chemical and oxidative impacts on DNA molecules, inefficiency of reparative system, etc.

BRAF and RAS's central role in the MAPK oncogene signaling pathway

At the quintessence of thyroid cancer pathogenesis are 2 classical signaling pathways, Both of MAPK and PI3K-AKT pathways are coupled to the receptor tyrosine kinase (RTK) at the cell membrane, which transduces an extracellular outgrowth stimulus that motivate downstream intracellular signaling (Figure 2). This may be a standout amongst the three isoforms of the RAF Serine-threonine kinase and the predominant isoform discovered over thyroid follicular units. When activated that B-RAF protein phosphorylates, the following protein in the sign cascade MEK and ERK Those Proteins capacity contributes of the RAS/MAPK pathway's Part in Mobile proliferation, migration, and separation [1]. That large portion normal B-RAF transformation discovered previously, thyroid Carcinomas is a purpose transformation in deposit 600 directing, including a substitution from valine to glutamate (V600E).

This Transformation brings about that constitutive actuation of the B-RAF Protein and consequently the RAS/MAPK pathway. The Actuation of the B-RAF protein appears with a chance to be brought on by an Interruption of the hydrophobic collaborations the middle of it.

Actuation circle and the ATP tying webpage in wild-type B-RAF, this hydrophobic cooperation assistance

administers that protein to a dormant conformity at disrupted B-RAF stays to an active, reactant conformity [2,3]. This brings about the Constitutive phosphorylation for its downstream focuses [2]. Those B-RAF V600E purpose change may be a large portion predominant for papillary thyroid carcinomas (PTC) [2]. However, it will be uncommon clinched alongside follicular variants about thyroid. Carcinoma, B-RAF is a Perfect hereditary marker to use previously Thyroid tumor sequencing board. It will be found on the whole manifestations of Thyroid carcinoma and appears to be on assuming an exceptionally imperative part [2-4].

BRAF's mutation excludes follicular carcinoma and is exclusively found in popular carcinoma. It is unambiguous to indicate the formation of the carnival. With various studies, this mutation is associated with unfavorable prognosis [5,6], namely, with a high tumor stage extraterritorial invasive, lymphatic, regional and distant metastases [7-9], tumor size, [10,11] with vague recurrence of tumors, calcification [12,13], iodine metabolism sodium and iodine serpent dysfunction and therefore less sensitive to radioactive iodine [14,15]. Patients with BRAF mutation may benefit more from extensive surgery, [16] with post-operative treatment with high doses of radioactive iodine, with less depression and frequent monitoring of the thyroid-stimulating hormone [17].

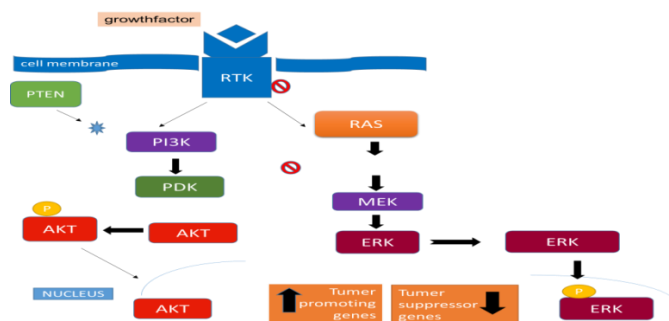


Figure 2: Schematic illustration of the MAPK pathway. Once RTK is activated by an extracellular signal, downstream activation of RAS, followed by BRAF, MEK, and then ERK, occurs. ERK then enters the nucleus to induce tumor-promoting genes and down regulate tumor suppressor genes and thyroid iodide-handling genes.

RAS mutations happen with variable recurrence altogether sorts for thyroid follicular derived tumors. RAS perspective mutations are mossy cup basic done follicular thyroid carcinoma (FTC) [18]. The predominance of RAS transformations in thyroid Carcinoma makes it a feasible hereditary marker and additionally a suitable prognostic tool, provided for that, investigations propose it might build that possibility for harmful change what's more tumor progression [19].

Oncogenic mutations of RAS include about 15 mutations per gene (H/N - RAS) each. From these mutations, in most cases of thyroid tumors, H and NRAS mutations are found namely, the codons 12/13/61 [20-22]. This group of mutations is the second most frequent mutation in thyroid tumors, it occurs in almost all the spectrum of these tumors, invasive and high potential for de-diversity [23]. These mutations are rare in papillary tumors, where their presence occurs frequently the follicular variants of the papillary tumor [24]. The latter types are quite difficult to determine by cytological analysis. Most often, RAS mutations are found in follicular, less differentiated and non-diagnosed tumors [25].

2. MATERIALS AND METHODS

Isolate gDNA, Measure DNA concentration, setup PCR mixes and plate, run PCR and finally analyze data.

Patients and Samples

One hundred and sixteen Tissue samples collected from patients with thyroid cancer. A fine needle aspiration biopsy (FNA) of thyroid nodules is the gold standard for diagnosing thyroid cancer and Formalin-Fixed Paraffin-Embedded (FFPE) samples.

DNA Extraction

DNA was extracted from collected samples Using DNA Tissue Kit by Qiagen Company (Qiagen QIAamp DNA Mini Kit).

Polymerase Chain Reaction (PCR)

116 patient samples were analyzed using Competitive allele - specific TaqMan PCR (Cast PCR TM). In these samples, 36 were diagnosed as papillary thyroid carcinoma, and 80 were indeterminate by Bethesda System for Reporting Thyroid Cytopathology (BSRTC III-V). We studied one Mutation, BRAF V600E for BRAF gene, four Mutations NRAS Q61K, NRAS Q61R, NRAS G12C and NRAS G12D for NRAS gene and one Mutations HRAS Q61R for HRAS gene by using TaqMan® Assay. We used 4 µL of gDNA per sample for the QuantStudioTM 5D Digital PCR assay. The digital PCR was run in a final volume of 20 µL, which included QuantStudioTM 5D Master Mix (Life Technologies) as shown in the following table 1.

Chemicals	Volume (µL)
TaqMan® Genotyping Master Mix, 2 ×	10
Prepared gDNA sample	4
(Optional) 50 × Exogenous IPC Template DNA	0.4
(Optional) 10 × Exogenous IPC Mix	2
TaqMan® Mutation Detection Assay	2
Nuclease-free water	1.6
Total volume of super mix	20

Table 1: Represent the chemicals for the reaction mixture and their volumes.

Thermal cycling conditions		
Stage	Temp (°C)	Time
Hold	95°C	10 min
Cycling (5 cycles)	92°C	15 sec
	58°C	1 min
Cycling (40 Cycles)	92°C	15 sec
	60°C	1 min

Table 2: TaqMan® mutation detection cycling protocol.

After all the additives were completed, the samples were expelled by the centrifugal (Galaxy Mini centrifuge) for half a minute to ensure homogeneity of all materials. Then put samples with a thermal cycler and fill the device according to the following program (table 2).

3. RESULTS AND DISCUSSION

Genetic change is the main force of thyroid tumor development, based on new methods of managing

thyroid cancer. The latest significant genetic discovery in thyroid cancer is the BRAF-T1799A (V600E) transformation (the gene for B-type RAF kinase, BRAF). Since the initial report of this breakthrough in thyroid cancer years ago, rapid progress has been made. The BRAF mutation is the most common genetic change in thyroid cancer. BRAF and NRAS mutations are frequent genetic alterations found in thyroid nodules.

These molecular markers establish a differential diagnosis and facilitate clinical decision-making. Prevalence of thyroid nodule-associated mutations has not been studied in Georgia. We evaluated BRAF, NRAS and HRAS mutations in Georgian patients with indeterminate cytology or diagnosed with papillary thyroid cancer (PTC) [2,5].

3.1 Mutation analysis

BRAF (V600E), NRAS (G12C, G12D, Q61R, and Q61K) and HRAS (G12C, G13R, and Q61R) were determined in the DNA extracted from FNA and FFPE specimens. In total, 116 patient samples were analyzed using Competitive-specific TaqMan PCR (Cast PCR TM) (table 3). Amplification plots of BRAF and RAS mutations. Identifying BRAF mutation with polymerase chain reaction in poster operative sample of thyroid (figure 3). In these samples, 36 were diagnosed as papillary thyroid carcinoma, and 80 were indeterminate by Bethesda System for Reporting Thyroid Cytopathology (BSRTC III-V). BRAF (V600E) mutation was the most frequent genetic alteration found in 31% of all analyzed samples (figure 4). Specifically, this mutation was present in 61% of PTC cases and 18% of cases classified as indeterminate (BSRTC III-V). BRAF (V600E) mutation was present in 61% of PTC cases and 67% of cases classified as BSRTC V. NRAS mutations were present in 17% of PTC and 43% of indeterminate cytology samples classified as BSRTC IV (figure 5). NRAS mutations were present in 16% of PTC and 30% of indeterminate cytology samples. NRAS G12D and

Q61R were most prevalent at 36.6% and 40% of all NRAS mutations (figure 6). BSRTC IV category of indeterminate cytology had the highest frequency of NRAS mutations at 43%. From analyzed samples, HRAS (Q61R) mutation was present in one PTC case. BRAF at 53%, NRAS G12D and Q61K (at 16% and 18%) mutations were most prevalent from all positive sample's analyses (figure 7).

Total Number of Patients	Positive	Negative
116	67	49

Table 3: Number of all patient and type of results.

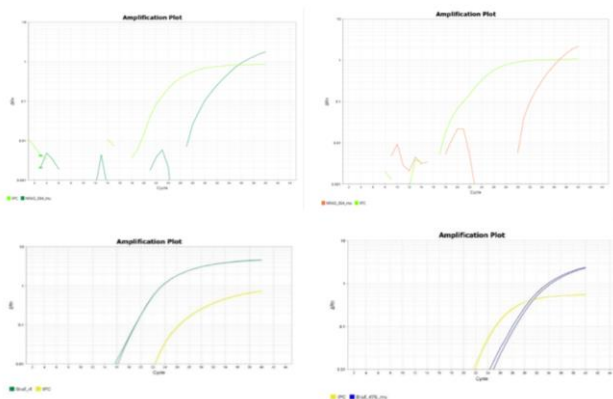


Figure 3: Amplification plots of BRAF and RAS mutations Identifying BRAF mutation with polymerase chain reaction in poster operative sample of thyroid.

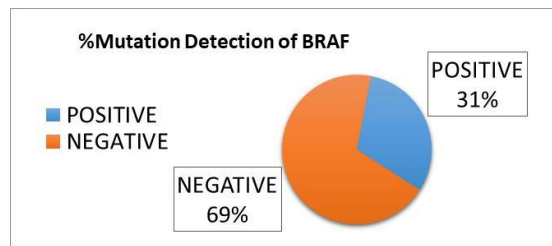


Figure 4: BRAF (V600E) mutation was the most frequent genetic alteration found in 31% of all analyzed samples.

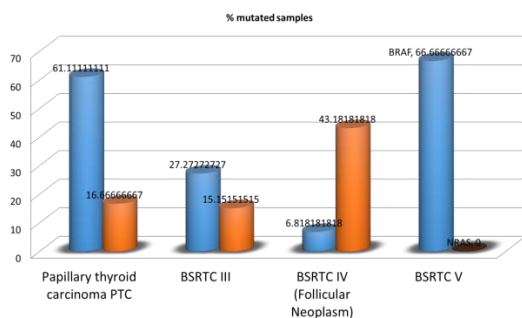


Figure 5: BRAF (V600E) mutation was present in 61% of PTC cases and 67% of cases classified as BSRTC V. NRAS mutations were present in 17% of PTC and 43% of indeterminate cytology samples classified as BSRTC IV.

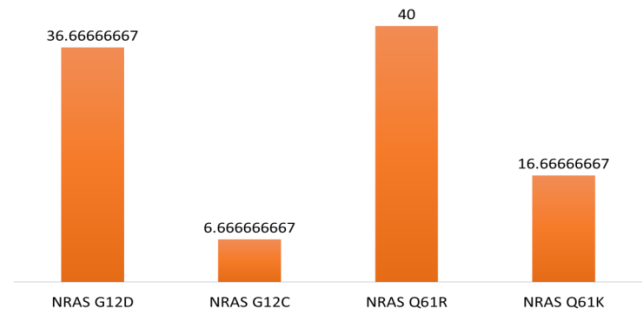


Figure 6: NRAS G12D and Q61R were most prevalent at 36.6% and 40% of all NRAS mutations.

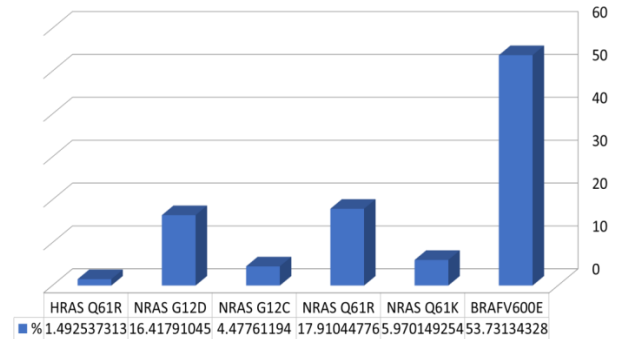


Figure 7: BRAF at 53%, NRAS G12D and Q61K (at 16 and 18%) mutations were most prevalent from all positive sample's analyses.

Statistical analysis

Statistical analyses were performed with SPSS software (version 11.0). All numeric data were expressed as mean ± SD and differences between means were compared by t-test. Probability values of <0.05 were considered statistically significant.

BRAF and RAS mutations According to age

In case of BRAF mutation we found 3 men and 6 women in age under 30 years, we found to 7 men and 11 women in age in between (30-40) years and found 5 men and 15 women more than 40 years in case of NRAS mutations were revealed under the age of 30 years 2 men and 3 women; 12 mutations in the ages of 30 years - 40 years was 3 men and 9 women and 14 mutations were 3 men and 11 women in the age more than 40. RAS mutations were increased after 40 years: 34 patients out of 67 as showed in the table 4.

		BRAF					
		p < 30 y		p < 30-40 > p		p > 40	
Type	AGE	M	F	M	F	M	F
Positive patients		3	6	7	11	5	15
Negative patients		7	13	4	15	8	36
TOTALS		10	19	11	26	13	51

		NRAS					
		p < 30 y		p < 30-40 > p		p > 40	
Type	AGE	M	F	M	F	M	F
Positive patients		3	3	3	9	3	11
Negative patients		8	18	9	15	12	25
TOTALS		11	23	13	27	15	41

		HRAS					
		p < 30 y		p < 30-40 > p		p > 40	
Type	AGE	M	F	M	F	M	F
Positive patients		1	3			2	2
Negative patients		9	18	12	24	13	36
TOTALS		10	25	11	30	17	42

Table 4: BRAF and RAS mutations according to age.

Dissection

The effect of BRAF mutation in PTC management is still unclear. Several researches have showed important relation between BRAF mutation and poor signs changes [13,26]. But this research results discrepancy with some articles like studies of BRAF mutation in an Italian and Japanese population [27,28]. In spite of this recent analyses which showed almost relationship between BRAF mutation and high risk of thyroid cancer especially PTC, that steps should be use to modify the first step treatments in BRAF - positive with PTC [29,30]. The main propose of our study to estimate the relation between BRAF and RAS mutations and high prognostic variables in thyroid cancer. Our results

showed that BRAF (V600E) mutation was present in 61% of PTC cases and 67% of cases classified as BSRTC V. NRAS mutations were present in 17% of PTC and 43% of indeterminate cytology samples classified as BSRTC IV. NRAS G12D and Q61R were most prevalent of all NRAS mutations. BSRTC IV category of indeterminate cytology had the highest frequency of NRAS mutations at 43%. From analyzed samples, HRAS (Q61R) mutation was present in one PTC case. Finally, 29 out of 36 PTC cases were positive for the mutations indicating 81% of diagnostic sensitivity of the test. This result means that there is high association between BRAF mutation status and all thyroidectomy and type of PTC. We also found that male sex has a poor prognostic factors in PTC but for women increased the rate of total thyroidectomy and classic PTC were associated with positive BRAF and RAS mutations analysis in our study. Many researches unable to make relationship between patient sex and BRAF mutation [20,25,31-34]. This significant association which we found in our study between BRAF V600E positivity and PTC and the causes for this are unclear.

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