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Case Report

Concomitant *BRAF* V600E and *NRAS* Q61R Mutations in the Same Thyroid Nodule: A Case Study

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ABSTRACT

Background: Papillary Thyroid Cancer (PTC) is the most common type of well differentiated endocrine malignancy. Generally thyroid nodules with multiple oncogenic mutations are uncommon with an occurrence which may be related to more aggressive biological behavior of tumors. Rearranged in Transformation/Papillary Thyroid Carcinomas (*RET*/PTC) rearrangement, *RAS* and BRAF mutations are considered to be mutually exclusive in PTC. Concomitant *RET*/PTC, RAS, or *BRAF* mutations have been documented, although the impact of these mutations for tumor growth and survival is debated.

Case presentation: Here, we present a rare case of woman 46 years old with a neck mass and thyroid nodule classified as Toll-Like Receptor 5 (TIR5) on cytological examination. We found contemporary *BRAF* p.(Val600Glu) (p. (V600E); c. 1799T>A) and *NRAS* p. (Gln61Arg) (p. (Q61R); c.182A>G) mutations in morphologically different areas within the same lobe (the right one). Two lesions show different morphology. The mutated *BRAF* lesion showed morphological characteristics compatible with classic papillary carcinoma while the mutant *NRAS* lesion shows morphological features compatible with follicular variant papillary carcinoma.

Results and conclusion: To the best of our knowledge, this is the first time that such mutations, which are normally mutually exclusive, have been detected at the same time. The findings of synchronous mutations are rare occurrence, suggesting for Intratumoral Heterogeneity (ITH) even in PTC. Patients with multiple mutations have a clinical worse prognosis, generally characterized by an aggressive thyroid cancer, which may influence the surgical treatment, chemotherapy, and *BRAF* V600E mutation-targeting therapy.

Keywords: Papillary thyroid cancer, Concomitant mutations, Intratumoral heterogeneity, Prognostic markers, Cytology

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INTRODUCTION

Thyroid cancer is the most common malignancy of the endocrine system (Al Hamad AM, *et al*., 2019). More than 95% of thyroid carcinomas originate from the follicular cells of the thyroid, while only a minority (~3%) originate from C-parafollicular cells, leading to the onset of medullary thyroid carcinomas (Abdullah MI, *et al*., 2019; Henderson YC, *et al*., 2009).

Differentiated carcinomas have been identified into 3 histological subtypes-

- Papillary Carcinoma (PTC) (80%-85%)
- Follicular Carcinoma (FTC) (10%-15%)
- Hürtle Cell Carcinoma (HCC) (3%-5%) whose development and prognosis is similar to that of follicular carcinoma

Despite most of PTCs and FTCs has a good prognosis with a 5-year survival rate of more than 90%, a low fraction might become more aggressive over time (Xing M, *et al*., 2014; Henderson YC, *et al*., 2009). PTC is a solid tumor that normally occurs inside the thyroid. Histologically, it is characterized by the presence of papillae, epithelial cells arranged around a fibrovascular stem (Abdullah MI, *et al*., 2019). Despite most of PTCs are well differentiated with a low rate of local invasion, recurrences, or metastases (regional or distant), there are several tumor variants, with distinct pathological, and molecular features. Because of their aggressive behavior, the latest American Thyroid Association (ATA) guidelines have classified these pathological subtypes as having an intermediate risk of recurrence (Sak SD, 2015; Coca-Pelaz A, *et al*., 2020).

The main variants of PTC with prognostical implications are the Follicular Variant PTC (FVPTC), Diffuse Sclerosing Variant (DSV), Tall Cell Variant (TCV), Columnar Cell Variant (CCV), Cribriform Variant (CV), Hurtle Cell Variant (HCV), and Hobnail Variant (HV). Their evaluation is usually histological due to the uncommon morphological features (Coca-Pelaz A, *et al*., 2020). The main molecular alterations are the same as in traditional PTC, with the exception of the hobnail variant, which has changes in the Telomerase Reverse Transcriptase (*TERT*) promoter (44.4%), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic subunit Alpha (*PIK3CA*) (28.8%), Catenin Beta 1 (*CTNNB1*) (16.7%), estimated Glomerular Filtration Rate (eGFR) (11.1%), Serine/Threonine Kinase 1 (*AKT1*) (5.5%), and Neurogenic locus notch homolog protein 1 (*NOTCH1*) (5.5%) and HCV, whose oncocytic features are linked to mitochondrial DNA mutations (Sak SD, 2015). Alterations in *BRAF* gene have been referred as absent in the Criobriforme variant and uncommon in DSV, where ALK gene rearrangement was recently identified (Sak SD, 2015; Coca-Pelaz A, *et al*., 2020).

The FVPTC is a well-circumscribed or encapsulated tumor with architecture that might be mistaken for follicular adenoma or follicular carcinoma, has also been widely characterized (Rivera M, *et al*., 2010). It is known that mutations in the *RAS* oncogene has been linked to encapsulated/well-circumscribed tumors, whereas invasive FVPTC has been related to both *BRAF* and *RAS* mutations (Rivera M, *et al*., 2010).

The gold standard approach for the diagnosis of PTC is Fine Needle Aspiration and Cytology (FNAC) which classifies thyroid biopsies as suspicious or malignant based on their cytological outcome (Bagga PK and Mahajan NC, 2010). Although FNAC can reveal papillary structures, preoperative diagnosis is primarily based on the detection of typical nuclear features such as "Orphan

Annie" nuclei (clear intranuclear pseudoinclusions) and nuclear grooves (folds in the nuclear membrane) (Shrestha RT, *et al*., 2015). PTC is confirmed by the presence of psammoma bodies, calcium salt deposits, in a cervical lymph node (Abdullah MI, *et al*., 2019; Krishnamurthy A and Vaidhyanathan A, 2011). The cytological examination allows the diagnosis of cancer in most cases. However, due to limited sampling or a lack of well-expressed PTC markers, several nodules later confirmed as malignant are indeed classified as indeterminate by cytology (Bagga PK and Mahajan NC, 2010). As a result, the use of molecular test is required in order to have a better understanding of the disease progression (Colombo C, *et al*., 2019). The most prevalent genetic mutation in papillary thyroid cancer is *BRAF* V600E, as well as the less common *RAS* mutations, or *RET* (*RET*/PTC traslocations) or NTRK rearrangements, which play a key role in the PTC pathogenesis (Al Hamad AM, *et al*., 2019; Zou M, *et al*., 2014; Henderson YC, *et al*., 2009; Wang YL, *et al*., 2008; Ren H, *et al*., 2020; Li XO, *et al*., 2014). Genetic changes in *RET*/PTC, *RAS*, and *BRAF* are thought to be mutually exclusive and their overlapping has been debated for a long time. However, concurrent mutations in PTC have recently been reported and regarded as proof of existence of Intratumoral Heterogeneity (ITH) also in PTC (Zou M, *et al*., 2014; Henderson YC, *et al*., 2009; Chmielik E, *et al*., 2018).

CASE PRESENTATION

A 46-year-old, female patient was admitted to our hospital in May 2020 with a neck lump that grew in size over the year. Previous history of thyroid disease in the family was not reported and laboratory test did not detect thyroid disorders such as hyperthyroidism and hypothyroidism. The right lobe presented in section, macroscopically, present at the upper/middle III a nodule of 1.5 cm and a further nodule of 4 mm at the III medium.

Fine needle aspiration from the right thyroid lobe was interpreted as TIR5, sign of aggressiveness and high level of tumor malignancy. Therefore the patient underwent to total thyroidectomy with central neck dissection.

For the right lobe, the surgical pathology examination revealed a size of 5 × 3 × 2 cm; for the left lobe, 4 × 3 × 1 cm. In addition, two nodules, one 1.5 cm in diameter and the other 4 mm, have been identified in the right lobe. Although both lesions were classified as PTC, morphological variances were detected (*Figure 1*).

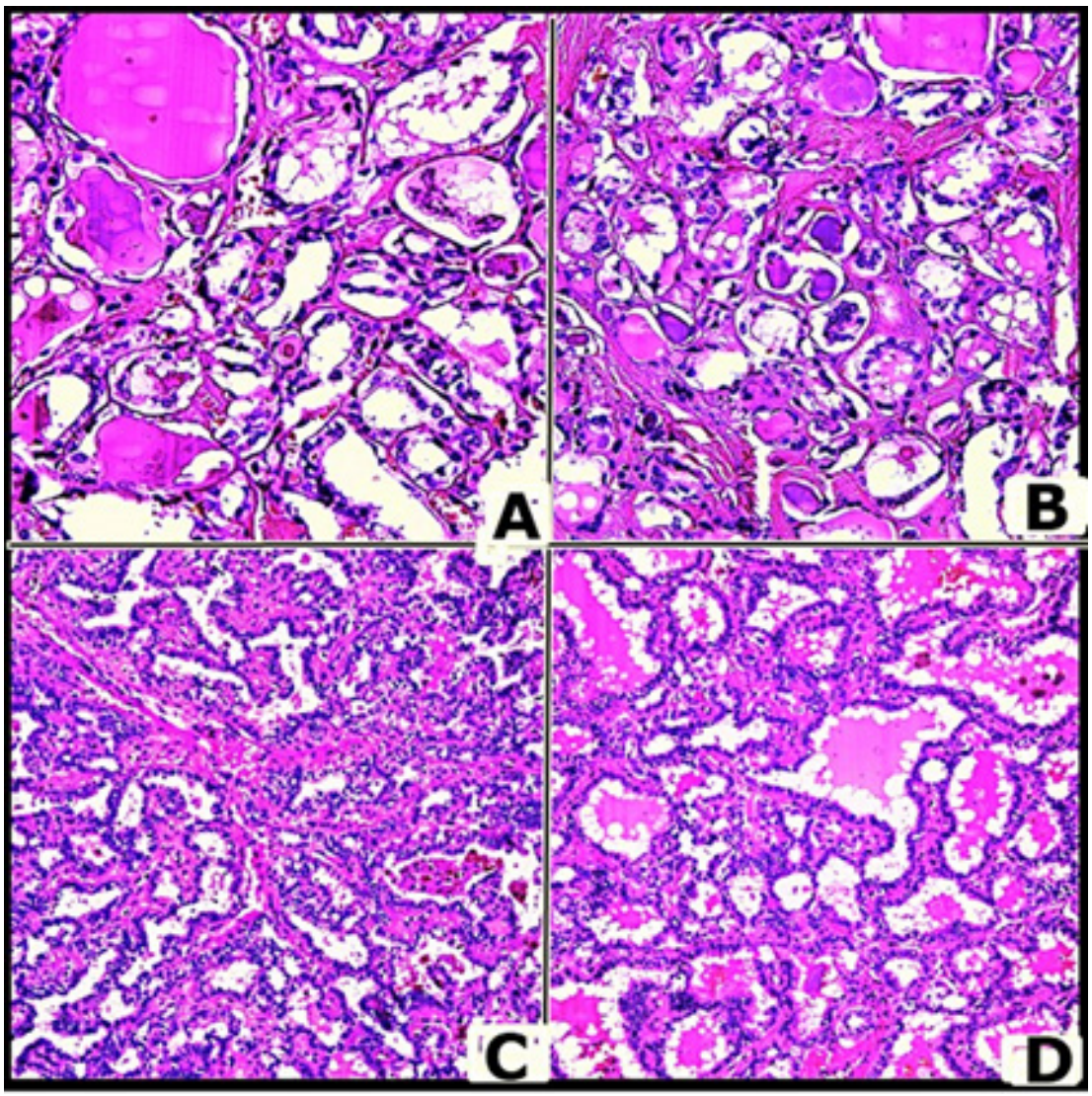

Figure 1: (A,B) High power magnification image demonstrates the carcinoma cells *BRAF* V600E positive, with marked nuclear features and psuedoinclusions in the context of classical PTC; (C,D) High power magnification image demonstrates the carcinoma cells *NRAS* positive of FVPTC

The largest lump had a classic papillary histology with well-developed nuclear features of PTC and inflammatory cells mixed in typical nuclear features such as orphan Annie nuclei (clear intranuclear pseudoinclusions) and nuclear grooves (folds in the nuclear membrane) suggestive of PTC have been described. The diagnosis has been confirmed by the presence of psammoma bodies (calcium salt deposits) in a cervical lymph node. Instead, microscopic examination of 4 mm nodule revealed morphological features of a variant follicular PTC, FVPTC, extracapsular type.

Formalin Fixed Paraffin Embedded tissue (FFPE) sample from surgical resection, was used for DNA extraction and each sample underwent molecular analysis to determine subclonality with regard to *BRAF* and *RAS* mutational status. The slides were reviewed by 2 expert pathologists; areas tumor displaying distinct histological pattern were separately micro-dissected to ensure high tumor tissue content and a minimum of 10% tumor purity cells was required for sample processing. DNA and RNA were extracted using respectively Qiagen QIAMP DNA FFPE KIT and RNeasy FFPE Kit according to manifacturer instructions and sample concentration was evaluated with nanodrop and Qubit.

BRAF and *RAS* mutational status was investigated by RealTime PCR using kit Thyroid Cancer Mutation Detection Kit (THDNA-RT64, Entrogen) intended for the detection of *BRAF*, *KRAS*, *NRAS* and *HRAS* somatic mutation in human genomic DNA. *RET*/PTC, *RET*/PTC2, *RET*/PTC3, *PAX8*/*PPARY* translocation analysis on RNA were investigated by Easy Pgx Thyroid Fusion kit and the one-step real-time PCR as amplification method. Neither area was confirmed rearranged in terms of gene fusions.

RESULTS AND DISCUSSION

The results highlighted *BRAF* p.(Val600Glu) for the 1.5 cm nodule while *NRAS* p. (Gln61Arg) for the 4 mm nodule. We enforced our findings because of several clinical cases with concurrent mutations in PTC which are referred in literature (Costa AM, *et al*., 2008; Zou M, *et al*., 2014, Xing M, *et al*., 2014).

Genetic changes in the *RAS* gene, as well as *RET*/PTC fusion or *TERT1* promoter alterations, were simultaneously observed in PTC *BRAF* V600E (Costa AM, *et al*., 2008). However, to the best of our knowledge, this is the first time that the *BRAF* V600E and *NRAS* Q61R mutations, which are normally mutually exclusive, have been detected at the same time. Papillary thyroid carcinoma is an indolent tumor with a low death rate (Shrestha RT, *et al*., 2015). Several studies have identified two types of genetic alterations in thyroid cancer-

Point mutations in *BRAF*, *KRAS*, *NRAS* or *HRAS*, and chromosomal translocations involving *RET*/PTC1, *RET*/PTC3, or PAX8/PPARY (Xing M, *et al*., 2014; Ieni A, *et al*., 2021). The detection of these genetic markers allows a definitive diagnosis of malignant tumor that is distinct from thyroid nodules, which is considered to be benign. Furthermore, these gene markers may provide important prognostic value for patients with various subtypes.

All genetic alterations are assumed to be mutually exclusive and their overlapping has been debated for a long time. However, concurrent mutations in PTC have been discovered and regarded as a rare occurrence (Zou M, *et al*., 2014). *RAS* mutation and *RET*/PTC1 fusion co-expression was reported by Di Cristofaro *et al*. in 1/24 follicular variant PTC (FVPTC) and *BRAF* mutation *RET*/PTC3 fusion were found in 1/26 of classic PTC patients (CPTC) (Di Cristofaro J, *et al*., 2006).

Henderson *et al*. reported 5/54 PTC patients with coexisting *BRAF* mutation *RET*/PTC fusion where the authors referred a correlation between mutation status and clinicopathological variables since patients with dual mutation were older and had more advanced tumor (80% in T4) than those with *BRAF* V600E mutation only (27% in T4) (Henderson YC, *et al*., 2009).

Xing *et al*. referred that *BRAF* V600E and *TERT* promoter mutations cooperatively identify an aggressive papillary thyroid cancer with the worst clinicopathological outcome (Xing M, *et al*., 2014).

In 8/15 (53%) subclonal or nonclonal PTC, Zhu *et al*. confirmed *RET*/PTC rearrangement and *RAS* or *BRAF* mutations, but none in clonal PTC. According to the authors concomitant mutations were considered to occur more frequently in advanced stages of disease, and long-term follow-up showed that patients with contemporary mutations had a poor response to treatment and a reduced disease-free survival rate (Zou M, *et al*., 2014).

Costa *et al*. observed concomitant *BRAF* V600E and KRASG13D+G12S mutations in 4/35 PTC (Costa AM, *et al*., 2008) focusing on the assumption that *BRAF* alone isn't a predictor of poor outcome. Nevertheless, when combined with other genetic alterations, it identifies a subset of PTC with higher risk of recurrence and decreased survival. In agreement with this, we have reported the first case of two distinct oncogenic driver mutations, respectively *BRAF* V600E and *NRAS* Q61R within the same thyroid nodule; this could enforce the evidence of a wide variety of biological behaviors in PTCs, ranging from the most indolent (well differentiated type) to the most aggressive malignancy. Only a few molecular markers linked to an increased risk of death are currently available, and their effectiveness in preoperative risk stratification and therapeutical planning remains unknown. As a result, a proper molecular characterization may be a useful tool for personalizing the initial surgical strategy, the follow up and ultimately to apply new therapies. Clinical trials using *BRAF* inhibitors for advanced thyroid cancer have revealed conflicting outcomes (Savvides P, *et al*., 2013; Ho AL and Sherman E, 2011). In the context of a *RAS* mutation, recent investigations have shown that *BRAF* inhibitors might paradoxically boost Mitogen-Activated Protein Kinase (MAPK) activation (Heidorn SJ, *et al*., 2010). As a result, patients who have both a *BRAF* and a *RAS* mutation may not be candidates for *BRAF* inhibitors treatment since the reactivation of MAPK is involved in the resistance mechanism (Lo RS, 2012). Moreover, the occurrence of concomitant mutations enforced the evidence of Intratumoral Heterogeneity (ITH) even in PTC. ITH refers to subclonal genetic variability within a tumor in contrast to the concept of a tumor as a clonal and homogeneous swarm (Finkel A, *et al*., 2016; Guerra A, *et al*., 2014). It is caused by genetic instability and the accumulation of genetic changes, both key factors in the growth of a tumor from an early stage to a more aggressive cancer.

The existence of ITH in PTC, its extension and biological impact is debated. However, several studies have shown that it is not a minor event in PTC, but a key factor for therapeutic failure and poor prognosis (Chmielik E, *et al*., 2018; Fugazzola L, *et al*., 2020). Nowadays, in the era of personalized medicine, the discovery that some tumors are heterogeneous in terms of individual mutations has significant therapeutic implications as well as translational value (Chmielik E, *et al*., 2018; Ieni A, *et al*., 2021). Tumors with a specific molecular alteration in only a minority of neoplastic cells are likely to have low sensitivity to targeted therapies; as a consequence the finding of this internal tumor-like complexity, was the starting point for the use of a drug combination to restrict tumor growth. As a result, this report supports the assumption that synchronous mutations in PTC are linked to more aggressive tumor behavior, which could affect surgical procedure selection as well as post-surgery care. It also shows the potential impact of molecular testing/screening in selecting patients for more aggressive treatment. However the benefits of such an approach have yet to be confirmed. Anyway, it would be useful to increase the understanding of ITH in order to properly advise therapy and improve survival of patients.

CONCLUSION

The identification of specific genomic alterations drivers for several malignancies has enabled the development of customized therapies with prom-

ising response rates. Unfortunately, most cancers are caused by a complex interaction of genetic, transcriptomic, and proteomic alterations, as well as anomalies in the tumor microenvironment and immune system.

The recent development of new technologies such as second generation sequencing, Next Generation Sequencing (NGS), and the numerous advances in the field of genomics have allowed a continuous and further evolution of "precision oncology". While recognizing the value of morphological and histological data, the new paradigm of "mutational oncology", has opened the era of genomic profiling tests, this would improve the selection of the anticancer drug based on the "driver" mutation and agnostic approval, namely the therapeutic indication regardless of the tumor site.

Due to the high levels of tumor heterogeneity and individual genomic complexity, customized drug combination is a key factor in therapeutic management optimization. This implies a patient-centered cancer therapy approach in order to get the correct drug(s) to the right patients at the right time and, as a result, overcome resistance mechanisms.

DECLARATIONS

All authors have read, edited, and contributed to the content of this manuscript. This work has not been previously published and has not been considered for publication elsewhere.

ETHICAL APPROVAL

All authors certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards

CONSENT TO PARTICIPATE

The participant has consented to the submission of the case study to the journal.

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