Identification of Rare, Canonically Mutually Exclusive Variants in Thyroid FNAs

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INTRODUCTION

The most common genomic variants in thyroid nodules occur in the MAPK pathway and include *BRAF* and *N-/H-/K-RAS*. The co-occurrence of variants in these genes has so rarely been observed that they are generally considered “mutually exclusive”. One explanation is that no (or limited) selective advantage is imparted by an additional event in the same pathway (Figure 1). However, studies of these events have been relatively small, limiting estimates of their actual occurrence.

METHODS

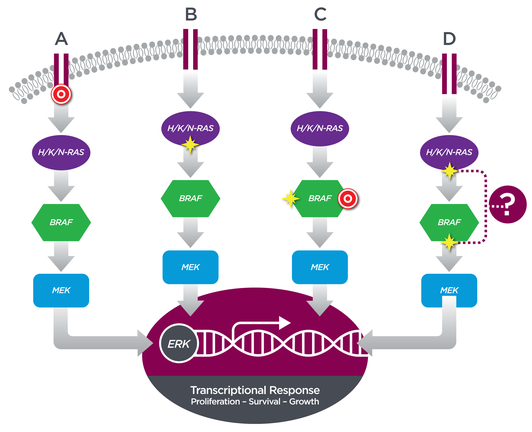
De-identified whole-transcriptome RNA-seq data from 47,695 consecutive Bethesda III-VI nodule samples submitted to Veracyte for molecular testing were analyzed. Samples with rare variant combinations were confirmed with a targeted DNA AmpliSeq assay.

RESULTS

Of the 47,695 FNAs analyzed, 12,077 (25.3%) had 1 genomic alteration, 263 (0.55%) had multiple variants: 229 had 2 variants, 28 had 1 fusion + 1 variant, 1 had 2 variants + 1 fusion, 2 had 2 fusions, and 3 had 3 variants (Figure 2). 7 FNAs (0.015%) contained canonically mutually exclusive variants (5 *RAS*+*RAS*, 1 *BRAF*+*RAS*, and 1 *BRAF*+*BRAF*) (Table 1). All were confirmed by AmpliSeq: 1 *BRAF* V600E + *BRAF* K601E in a Bethesda III 1.6 cm nodule, 3 *HRAS* Q61R + *NRAS* Q61R in unrelated cytologically indeterminate 1.8, 2.2, and 4.4 cm nodules, 1 *HRAS* Q61K + *KRAS* G12C in a 2.7 cm Bethesda III nodule, 1 *NRAS* Q61K + *NRAS* Q61R in a 2.7 cm Bethesda III nodule, and 1 *BRAF* V600E + *KRAS* Q61K in a 2.8 cm Bethesda V nodule. The *BRAF* V600E + *BRAF* K601E data showed the variants were present on different DNA molecules (Figure 3B). Thyroid surgery has not occurred in this patient. The smaller *HRAS* + *NRAS* was a follicular adenoma, while the largest nodule remains unoperated. The 2.2 cm *HRAS* + *NRAS* nodule and the *HRAS* + *KRAS* nodule were both encapsulated follicular variant of papillary thyroid carcinoma (fvPTC) with only capsular invasion. The *NRAS* + *NRAS* (Figure 3A) was a well circumscribed and encapsulated adenomatoid nodule, while the *BRAF* V600E + *KRAS* nodule was a multifocal fvPTC with extrathyroidal extension.

FIGURE 1.

Somatic alterations activate the MAP Kinase signaling pathway in the absence of signals.



The end result of the MAPK pathway is phospho ERK translocation into the nucleus and transcriptional activation.

(A) Receptor Tyrosine Kinase fusions (red circle) cause constitutive activation of the MAPK pathway. Inhibitors are available for some RTK fusions.

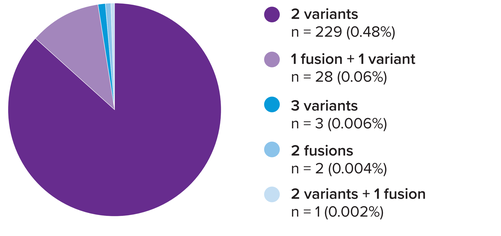
(B) Nucleotide variants (yellow star) in RAS-family genes can activate the MAPK pathway.

(C) Nucleotide variants in BRAF, such as V600E, activate the MAPK pathway. Inhibitors are available for BRAF V600E variants.

(D) Can multiple variants within the same pathway exist? Previous studies suggest that simultaneous variants are mutually exclusive.

FIGURE 2.

The Expression of multiple genomic alterations is rare among 47,695 consecutive Bethesda III-VI FNAs in the Veracyte Clinical Laboratory.



263 (0.55%) of samples expressed ≥2 genomic alterations. Of them, 229 (87.1%) expressed 2 variants, 28 (10.6%) expressed 1 variant and 1 fusion, 3 expressed 3 variants (1.1%), 2 expressed 2 fusions (0.76%), and 1 expressed 2 variants and 1 fusion.

TABLE 1.

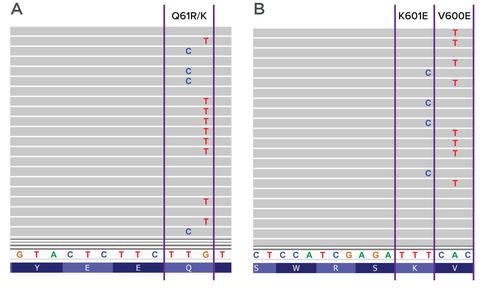
Canonically mutually exclusive variants are observed in the Veracyte Clinical Laboratory and confirmed by AmpliSeq.

Variant	Bethesda	Age (years)	Nodule Size	Surgical Histopathology	AmpliSeq Confirmation Status
BRAF:p.V600E (19.3%), *KRAS*:p.Q61K (18.2%)	Bethesda V	78	2.8 cm	Invasive multifocal fvPTC with extrathyroidal extension	Confirmed
NRAS:p.Q61R (20.1%), *HRAS*:p.Q61R (11.0%)	Bethesda III	30	2.2 cm	Encapsulated fvPTC with capsular invasion	Confirmed
BRAF:p.V600E (31.0%), *BRAF*:p.K601E (11.7%)	Bethesda III	47	1.6 cm	No surgery	Confirmed
NRAS:p.Q61R (17.6%), *HRAS*:p.Q61R (8.6%)	Bethesda IV	43	1.8 cm	Follicular adenoma	Confirmed
HRAS:p.Q61K (26.8%), *KRAS*:p.G12C (24.8%)	Bethesda III	72	2.7 cm	Encapsulated fvPTC with capsular invasion	Confirmed
NRAS:p.Q61R (28.1%), *HRAS*:p.Q61R (3.5%)	Bethesda III	69	4.4 cm	No surgery	Confirmed
NRAS:p.Q61R (26.5%), *NRAS*:p.Q61K (9.6%)	Bethesda III	17	2.7 cm	Adenomatoid nodule	Confirmed

7 of the FNAs with >1 alteration contained mutually exclusive variant combinations. All were Afirma GSC Suspicious. 2 patients were male and 5 patients were female. AmpliSeq-derived Variant Allele Frequencies (VAF) are in parentheses. Coverage ranged from 1,225x to 2,000x.

FIGURE 3.

Mutually exclusive variants within the same gene are present on different molecules.



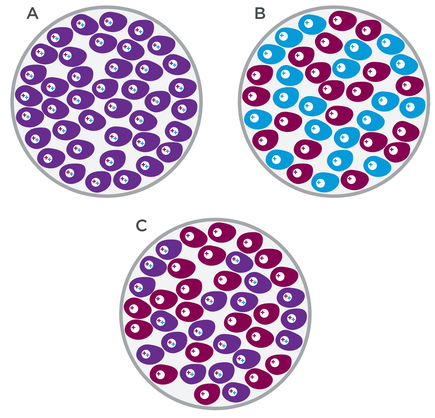
Integrated Genome Viewer plot showing individual sequence reads, with each line representing one read. Bases that do not match the reference genome (hg19) are displayed in bold.

(A) NRAS locus. Red T bases denote Q61R, while blue C bases denote Q61K. Note that Q61R and Q61K are not present in the same sequence.

(B) BRAF locus. Red T bases denote V600E, while blue C bases denote K601E. Vertical lines denote the boundaries of K601E and V600E. Note that V600E and K601E are not present in the same sequence. The BRAF gene is on the minus strand and is shown 3’ > 5’, oriented to the genomic sequence.

FIGURE 4.

Scenarios for canonically mutually exclusive variants in thyroid nodules.



(A) A clonal expansion of cells containing copies of both mutually exclusive variants in the nucleus. The purple cellular color reflects the presence of both variant 1 (Red +) and variant 2 (Blue +) in the nucleus.

(B) Two separate populations of cells are contained within a thyroid nodule. Red cells have a copy of variant 1 (Red +), while blue cells have a copy of variant 2 (Blue +).

(C) Cells with both variants arise from a cell with one single variant. A red cell with a copy of variant 1 (Red +) has acquired a copy of variant 2 (Blue +) and expanded. Purple cells reflect the presence of both variants.

These distinct possibilities could be distinguished via single cell sequencing.

CONCLUSION

Previous studies were underpowered to define the precise frequency, genomic constituents, and histopathological correlates of rare co-occurring “mutually exclusive” variants. We identified and confirmed them by an orthogonal method in a large set of thyroid FNAs. It is unknown if these events are occurring in the same cell or in different clonal populations (Figure 4). If in the same cell, it is unknown how downstream signaling impacts the tumor’s histology and clinical behavior.