Characterization and Clinical Significance of EIF1AX Mutations and Co-Mutations in Cytologically Indeterminate Thyroid Nodules: A 5-Year Retrospective Analysis

Stacey M. Gargano, Nitika Badjatia, Yanina Nikolaus, Stephen C. Peiper, Zi-Xuan Wang

Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University Hospital, Philadelphia, PA, USA

Correspondence: Stacey.Gargano@jefferson.edu; Tel.: + 1 215 955 4404; Fax.: + 1 215 923 1969

Received: 3 February 2021; Accepted: 8 April 2021

Abstract

Objective. Mutations in the EIF1AX gene have been recently detected in a small percentage of benign and malignant thyroid lesions. We sought to investigate the prevalence and clinical significance of EIF1AX mutations and co-mutations in cytologically indeterminate thyroid nodules at our institution. Materials and Methods. A 5-year retrospective analysis was performed on thyroid nodules with a cytologic diagnosis of Bethesda category III or IV, which had undergone testing by our in-house next generation sequencing panel. Surgically resected nodules with EIF1AX mutations were identified, and mutation type and presence of co-mutations were correlated with histopathologic diagnosis. Results. 41/904 (4.5%) cases overall and 26/229 (11.4%) surgically resected nodules harbored an EIF1AX mutation. The most common histologic diagnoses were follicular thyroid carcinoma and follicular variant of papillary thyroid carcinoma. 11/26 (42.3%) of nodules had isolated EIF1AX mutation. Co-mutations were found in RAS (12/26; 46.2%), TERT (5/26; 19.2%) and TP53 (2/26; 7.7%). EIF1AX mutation alone conferred a 36.4% risk of malignancy (ROM) and 54.5% ROM or noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), while the ROM was significantly higher in nodules with concurrent RAS (71.4%), TERT, TP53 and RAS+TERT (100%) mutations. Conclusion. EIF1AX mutations occur in benign and malignant follicular thyroid neoplasms. In our cohort, the majority of mutations occurred at the splice acceptor site between exons 5 and 6. Importantly, the coexistence of EIF1AX mutations with other driver pathogenic mutations in RAS, TERT and TP53 conferred a 100% ROM or NIFTP, indicating that such nodules require surgical removal.

Key Words: EIF1AX • Thyroid Nodule • Cytopathology • Next-Generation Sequencing.

Introduction

Up to 30% of biopsied thyroid nodules are classified as either “atypia of undetermined significance (AUS)/follicular lesion of undetermined significance (FLUS)” or “follicular neoplasm (FN)/suspicious for follicular neoplasm (SFN)”, Categories III and IV respectively in The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) (1). Because these diagnoses are not straightforward benign or malignant, and the reported risks of malignancy are highly variable for these categories, clinicians often struggle with the decision of whether or not to recommend surgical removal of these indeterminate nodules. Molecular analysis, via multi-gene sequencing assays and gene expression classifiers, has emerged as an important supplemental tool for evaluating thyroid nodules (1, 2). At our institution, thyroid fine needle aspiration (FNA) specimens with an indeterminate cytopathologic diagnosis are routinely tested via an in-house next generation sequencing (NGS) panel, in order to provide clinicians with more information that may help with risk stratification and guide clinical management.

The most commonly known mutated genes associated with thyroid malignancies are BRAF and RAS. The majority of BRAF mutations are found in classical papillary thyroid carcinoma (PTC), while RAS mutations are seen in follicular variant of papillary thyroid carcinoma (FVPTC) and
other malignant and benign follicular-patterned thyroid lesions (3). More recently, mutations in the EIF1AX (Eukaryotic translation initiation fac-

tor 1A, X-chromosomal) gene have been detected in a small percentage of various types of thyroid cancer as well as benign thyroid nodules. The like-

lihood of malignancy in an EIF1AX-mutated thy-

roid nodule is thought to correlate with the pres-

ence or absence of co-existing mutations and with

the position of the mutations within EIF1AX (4).

We sought to investigate the prevalence and clinical significance of EIF1AX mutations and co-

mutations by examining histologic outcomes of a large cohort of cytologically indeterminate thyroid nodules that underwent molecular testing and subsequent surgical resection.

Materials and Methods

Case Selection and Histopathologic Correlation

A database search was performed to identify all thyroid nodules with a cytologic diagnosis of Bethesda category III or IV, which had undergone testing by our in-house NGS panel over a 5-year period. Samples with EIF1AX mutations were identified, and the electronic medical record was searched to determine which nodules had undergone surgical resection. EIF1AX mutation type and presence of co-mutations were correlated with final histopathologic diagnosis.

Molecular Specimen Information

At the time of the FNA procedure, a separate need-

dle pass was collected in a vial containing metha-

nol/acetic acid (3:1 ratio). Of the 987 clinical FNA

specimens, 904 (91.6%) passed sample quality control and obtained molecular results.

Molecular Analysis

Mutations in EIF1AX were identified by NGS with a custom designed Thyroid cancer panel using Illumina's TruSeq Custom Amplicon version 1.5 reagents as previously described (5) or CTL Vari-

antPlex Assay for Illumina Platform (ArcherDx) following the manufacturer's protocol. Besides EIF1AX both TruSeq and Archer panels contained primers to amplify targeted regions of interest covering hotspots from additional thyroid cancer-related genes such as BRAF, GNAS, HRAS, NRAS, PIK3CA, PTEN, RET, TERTpro, TP53 and TSHR. Libraries were sequenced on the Illumina MiSeq or NextSeq 500 instrument with paired end 150-bp sequencing. Data analysis was performed as previously described (5) for all TruSeq libraries. For all libraries prepared using Archer VariantPlex methodology, fastq files were generated using a custom script and data analyses were performed using the CTL target region file and Archer analysis software version 6.2. Annotated variants were filtered and reported using an in-house, Web-based reporting application ClinMutReporter (Thomas Jefferson University Hospitals, Philadelphia, Pennsylvania).

Results

Patients

Among the 904 thyroid FNAs with an indetermi-

nate cytopathologic diagnosis (Bethesda Category III or IV) that were characterized by NGS, 41 cases (4.5%) had mutations in the EIF1AX gene. Histopathologic confirmation of the diagnosis was available in a surgical follow up specimen in 26 of the 41 cases (63.4%).

Clinical & Pathologic Features

The clinical and pathologic features of the 26 pa-

tients with full diagnostic characterization are shown in Table 1. The mean patient age was 64 (range: 43-81 years) and with a female preponder-
ance (female:males ratio=3.3). The mean size of the nodules was 2.8 cm (range: 1.1-6.3 cm). The cy-

topathologic diagnosis was Bethesda Category III (AUS/FLUS) in 12 (46.2%) and Bethesda Category IV (SFN/FN) in 14 (53.8%). As shown in Table 1 and Figure 1, the histopathologic analysis on follow up excisions included 6 neoplasms with a be-

nign diagnosis (follicular adenoma (FA)), 3 with
Table 1. Summary of All EIF1AX-Mutated Surgically Removed Nodules

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Nodule size (cm)</th>
<th>Cytologic diagnosis (Bethesda category)</th>
<th>Histopathologic diagnosis</th>
<th>EIF1AX mutation type</th>
<th>Alternate allele frequency (AAF)</th>
<th>EIF1AX mutation location</th>
<th>EIF1AX mutation significance</th>
<th>Co-existing mutation(s), AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>F</td>
<td>1.4</td>
<td>FA</td>
<td>c.370_371delinsTT; p.G124L</td>
<td>26.6%</td>
<td>Exon 6</td>
<td>Unknown</td>
<td>Pathogenic</td>
<td>KRAS p.A59del, 5.6%</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>F</td>
<td>4.1</td>
<td>FA</td>
<td>c.338_1_338delinsTT; p.?</td>
<td>3.3%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>43</td>
<td>F</td>
<td>1.1</td>
<td>FA</td>
<td>c.16G&gt;C; p.G6R</td>
<td>9.7%</td>
<td>N terminus</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>M</td>
<td>2.7</td>
<td>FA</td>
<td>c.338-2A&gt;C; p.?</td>
<td>7.1%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>F</td>
<td>2.5</td>
<td>FA</td>
<td>c.44G&gt;A; p.G15D</td>
<td>9.7%</td>
<td>N terminus</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>F</td>
<td>2.6</td>
<td>(Hurthle cell type) FA</td>
<td>c.338-2A&gt;T; p.?</td>
<td>14.1%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>F</td>
<td>5.2</td>
<td>NIFTP</td>
<td>c.338-4_355del; p.?</td>
<td>16.5%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>70</td>
<td>F</td>
<td>1.1</td>
<td>NIFTP</td>
<td>c.5C&gt;G; p.P2R</td>
<td>4.6%</td>
<td>N terminus</td>
<td>Pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>59</td>
<td>F</td>
<td>1.5</td>
<td>NIFTP</td>
<td>c.210G&gt;C; p.W70C</td>
<td>40.0%</td>
<td>Exon 4</td>
<td>Likely pathogenic</td>
<td>KRAS p.Q61K, 41.9%</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>51</td>
<td>M</td>
<td>1.8</td>
<td>FTC</td>
<td>c.338-1G&gt;C; p.?</td>
<td>19.5%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>KRAS p.Q61R, 8.7%</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>54</td>
<td>F</td>
<td>1.5</td>
<td>FTC</td>
<td>c.338-1G&gt;C; p.?</td>
<td>35.2%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>NRAS p.Q61R, 39.2%; TERT c-146C&gt;T, 39.7%</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>46</td>
<td>F</td>
<td>2.3</td>
<td>FTC</td>
<td>c.3_5dup; p.P2dup</td>
<td>4.2%</td>
<td>N terminus</td>
<td>Likely pathogenic</td>
<td>TERT c-146C&gt;T, 7.3%</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>69</td>
<td>F</td>
<td>2.2</td>
<td>FTC</td>
<td>c.28A&gt;G; p.K10E</td>
<td>31.2%</td>
<td>N terminus</td>
<td>Pathogenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>71</td>
<td>M</td>
<td>5.5</td>
<td>FTC</td>
<td>c.338-2A&gt;G; p.?</td>
<td>40.6%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>HRAS p.Q61R, 20.3%; TERT c-124C&gt;T, 11.4%</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>57</td>
<td>F</td>
<td>3</td>
<td>FTC</td>
<td>c.338-1G&gt;C; p.?</td>
<td>2.1%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>NRAS p.Q61K, 2.4%</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>60</td>
<td>F</td>
<td>6.3</td>
<td>FTC</td>
<td>c.338-2A&gt;T; p.?</td>
<td>12.7%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>KRAS p.Q61R, 12.8%; TERT c-124C&gt;T, 15.1%</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>69</td>
<td>F</td>
<td>3</td>
<td>(Hurthle cell type) HCC</td>
<td>c.338-1G&gt;C; p.?</td>
<td>26.6%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>81</td>
<td>M</td>
<td>3.7</td>
<td>(Hurthle cell type) HCC</td>
<td>c.338-1G&gt;A; p.?</td>
<td>19.1%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>71</td>
<td>F</td>
<td>3.5</td>
<td>(Hurthle cell type) HCC</td>
<td>c.338-1G&gt;T; p.?</td>
<td>40.3%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>TPS3 p.H179R, 66.4%</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>63</td>
<td>M</td>
<td>3.1</td>
<td>EFVPTC</td>
<td>c.429+1G&gt;A; p.?</td>
<td>39.5%</td>
<td>D143_splice site</td>
<td>Likely pathogenic</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>59</td>
<td>F</td>
<td>1.8</td>
<td>EFVPTC</td>
<td>c.338_1_338delinsACA; p.?</td>
<td>6.7%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>NRAS p.Q61K, 8.2%</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>68</td>
<td>F</td>
<td>1.3</td>
<td>EFVPTC</td>
<td>c.338-2A&gt;T; p.?</td>
<td>4.5%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>NRAS p.Q61R, 4.3%</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>58</td>
<td>M</td>
<td>4.5</td>
<td>EFVPTC</td>
<td>c.338-2A&gt;T; p.?</td>
<td>23.50%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>HRAS p.Q61R, 12.7%; TPS3 p.Q311R+14, 10.1% &amp; p.S240R, 10.9%</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>65</td>
<td>F</td>
<td>2.7</td>
<td>EFVPTC</td>
<td>c.26G&gt;T; p.G9V</td>
<td>22.2%</td>
<td>N terminus</td>
<td>Pathogenic</td>
<td>NRAS p.Q61R, 28.9%</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>68</td>
<td>F</td>
<td>2.6</td>
<td>IFVPTC</td>
<td>c.338_8_338_1del; p.?</td>
<td>26.3%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>HRAS p.Q61R, 25.5%; TERT c-124C&gt;T, 21.6%</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>74</td>
<td>F</td>
<td>1.5</td>
<td>IFVPTC</td>
<td>c.338-1G&gt;C; p.?</td>
<td>16.7%</td>
<td>A113_splice site</td>
<td>Pathogenic</td>
<td>YWHAG-BRAF Fusion</td>
<td></td>
</tr>
</tbody>
</table>

EFVPTC=Encapsulated follicular variant of papillary thyroid carcinoma; FA=Folicular adenoma, FTC=Follicular thyroid carcinoma; HCC=Hurthle cell carcinoma; IFVPTC=Infiltrative follicular variant of papillary thyroid carcinoma; M=Male, NIFTP=Noninvasive follicular thyroid neoplasm with papillary-like nuclear features.
a noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) diagnosis and 17 with a malignant diagnosis.

Malignant neoplasms included follicular thyroid carcinoma (FTC) (N=7), encapsulated follicular variant of papillary thyroid carcinoma (EFVPTC) with capsular invasion (N=5), Hurthle cell carcinoma (HCC) (N=3), and infiltrative follicular variant of papillary thyroid carcinoma (IF-VPTC) (N=2). The Bethesda Category III and IV cytopathologic diagnoses did not distinguish between benign and malignant thyroid neoplasms, as shown in Table 1.

**Molecular Characterization**

These 26 cases were characterized by mutations in the EIF1AX gene. Two distinct types of mutations were observed, as shown in Figure 2 and Table 1. A mutation at the 3’ end of the exon 6 splice site was observed in 17 cases and missense mutations in the N-terminal of the protein containing basic amino acids was present in 5 cases. In 4 patients the mutations did not fit neatly into this distribution. A duplication of the proline residue encoded by codon 2 was also detected, but it differs from the missense mutations more commonly seen in the N-terminal. In addition, the p.W70C mutation occurs in a “mini-cluster” of mutations that have been seen in non-thyroid malignancies, but have not been reported in thyroid neoplasia in the COSMIC (6) or cBioPortal (7) databases. The p.G124L and c.429+1G>A mutations have not been previously reported in thyroid malignancies. Single mutations in the exon 6 splice site or the basic amino terminal tail occurred in both benign and malignant thyroid neoplasms. In addition to the abnormal EIF1AX gene, mutations in significant tumor-associated genes occurred in 15 of the 26 cases, and 13 of the 15 were detected in malignant neoplasms. The additional mutated genes, shown in Table 1, included NRAS (N=6), HRAS (N=3), KRAS (N=3), the common TERT promoter mutation (N=5), and TP53 (N=2). In 4 cases the TERT promoter mutation occurred with a RAS gene mutation and in one patient a TP53 mutation occurred with a mutant RAS gene. One case also harbored a novel YWHAG-BRAF fusion.

*EIF1AX* mutation alone conferred a 36.4% (4/11) risk of malignancy (ROM) and 54.5% (6/11) risk of malignancy or NIFTP, while ROM was significantly higher in nodules with concurrent RAS (71.4%; 5/7), TERT, TP53 and RAS+TERT (100%) mutations (Table 2).
EIF1AX encodes the essential, ubiquitously-expressed eukaryotic translation initiation factor EIF1A with 144 amino acids. As a part of the 43S pre-initiation complex (PIC), it binds to the capped mRNA, aids in localizing the start codon and initiates translation. EIF1A protein contains a core RNA binding domain spanning the residues 32-95 which is flanked on either side by highly charged unstructured N- and C-terminus tails (8). Originally identified in uveal melanomas, EIF1AX mutations have now also been detected in a variety of other tumor types, including low-grade gliomas, lung adenocarcinoma, endometrial carcinoma, and various neoplastic and nonneoplastic thyroid lesions (4, 9-11). Missense mutations of the first 2-15 amino acids at the N-terminus of EIF1AX have been identified in several cancers (9, 12-14). An additional hotspot splice-site mutation (A113_splice) in the C-terminus of the protein is thought to be exclusive to thyroid carcinoma (9, 11, 15).

In our study, 4.5% of cytologically indeterminate thyroid nodules (41 out of 904 total cases) and 11.4% (26 out of 229) of those that were surgically resected harbored an EIF1AX mutation. Of these cases, the histopathologic diagnoses were benign neoplasm (FA) in 6/26 (23.1%), NIFTP in 3/26 (11.5%) and malignant neoplasm (FTC, HCC or FVPTC) in 17/26 (65.4%) (Table 3A). Karunamurthy et al. (4) found EIF1AX mutations in 27/647 (4.2%) of indeterminate cytology samples, of which 5 had surgical follow-up information (1 EFVPTC, 1 hyperplastic nodule (HN), and 3 FA) yielding an estimated ROM of 20%. Our overall ROM is much higher (65.4%), and even in our nodules with EIF1AX mutation alone and no other coexisting mutations, the ROM is 36.4% (4/11) and ROM/NIFTP is 54.5% (6/11) (Table 2). All of our nodules were neoplastic, though other studies have found mutations in one case of HN (4). Of note, the estimated ROM calculated in studies such as this one are overestimations of the actual

Discussion

EF1AX encodes the essential, ubiquitously-expressed eukaryotic translation initiation factor EF1A with 144 amino acids. As a part of the 43S pre-initiation complex (PIC), it binds to the capped mRNA, aids in localizing the start codon and initiates translation. EF1A protein contains a core RNA binding domain spanning the residues 32-95 which is flanked on either side by highly charged unstructured N- and C-terminus tails (8). Originally identified in uveal melanomas, EF1AX mutations have now also been detected in a variety of other tumor types, including low-grade gliomas, lung adenocarcinoma, endometrial carcinoma, and various neoplastic and nonneoplastic thyroid lesions (4, 9-11). Missense mutations of the first 2-15 amino acids at the N-terminus of EF1AX have been identified in several cancers (9, 12-14). An additional hotspot splice-site mutation (A113_splice) in the C-terminus of the protein is thought to be exclusive to thyroid carcinoma (9, 11, 15).
ROM, due to the impact of selection bias. Nodules that undergo surgical resection are more likely to have suspicious pre-operative clinical findings (e.g. worrisome radiologic features, interval growth), increasing the likelihood of malignancy regardless of the FNA diagnosis and molecular result.

Similar to other studies, the most common type of EIF1AX mutation identified in our study was the A113_splice mutation at the intron 5/exon 6 splice site of EIF1AX (17/26), followed by the missense mutations in N-terminus of the protein (5/26). Previous studies have concluded that the A113_splice mutation, especially with co-existing RAS mutation, is more frequently observed in thyroid cancer than isolated EIF1AX mutations or mutations at the N-terminus hotspot of the gene (4, 11, 14). In our study, 13/17 (76.5%) surgically resected nodules with the A113_splice mutation and 3/5 (60%) nodules with an N-terminal missense mutation were malignant. Of the 13 malignant nodules with the A113_splice mutation, 10 harbored co-mutations (5 harbored one co-mutation and 5 harbored two co-mutations). Of the 3 malignant nodules with N-terminus mutation, 2 harbored co-mutations. Please refer to Table 1 for details.

Four cases harbored EIF1AX mutations that did not belong to the 2 common types discussed above. The N-terminal duplication (c.3_5dup, p.P2dup) was not in the COSMIC database, but is likely similar to the missense mutations in the same region. The EIF1AX p.W70C identified in the NIFTP case had a concurrent RAS mutation. In the COSMIC database, 4 cases with this mutation and 3 additional missense mutations at this amino acid position had been reported in non-thyroid cancer specimens, which makes the p.W70C mutation likely pathogenic. The mutation p. G124L (c.370_371delinsTT), detected in a FA, has been reported once in the COSMIC database, in a small cell carcinoma of the lung. Mutations affecting the same amino acid, p.G124* and p.G124V, were reported in a single thyroid carcinoma specimen (cBioPortal, sample ID TCGA-EM-A3ST-01). Lastly, the mutation c.429+1G>A, exon 6/intron 6 splice site mutation, detected in one of our EFVPTC cases, has never been reported, but 7 malignant cases with mutations affecting the D143_splice site were documented in the COSMIC database. This mutation most likely affects splicing and hence the function of the protein.

Other studies have reported EIF1AX mutations co-occurring with several other driver mutations. Karunamurthy et al. (4) found co-mutations in 3/11 cases (2 with NRAS and 1 with NRAS, TP53 and TERT), all of which were malignant neoplasms. The TCGA study found co-mutations (KRAS and BRAF) in one of their 6 cases of EIF1AX-mutated PTC (9). Our study showed that EIF1AX mutations can co-occur with mutations in RAS, TP53 and TERT promoter (TERTp), and these co-mutations are associated with a very high ROM. Co-existing EIF1AX and a hotspot RAS mutation conferred an 85.7% ROM and 100% ROM or NIFTP, compared to EIF1AX mutation alone that conferred a 36.4% ROM. Malignancy risk was 100% for nodules containing co-mutations in TERTp, TP53, RAS+TERTp, and BRAF fusion. Clinicians must be aware of this and should strongly consider surgical intervention, at least a lobectomy, for patients with multiple such mutations in a thyroid nodule.

Key published studies on the EIF1AX mutations in thyroid nodules/cancer specimens are summarized in Table 3. The histopathologic diagnoses and ROM for EIF1AX mutation positive specimens identified from FNA with indeterminate cytology (Bethesda III and V), including our current study, are shown in Table 3A. The frequencies of the EIF1AX mutations in different categories of thyroid nodules from surgically resected specimens are presented in Table 3B.

While EIF1AX mutations were identified in HN, FA, and differentiated thyroid cancer (PTC and FTC), the percentage of specimens containing the EIF1AX mutations was enriched in advanced thyroid cancer. By combining the case numbers of the studies listed in Table 3B, there were 11 positive specimens for EIF1AX mutations in 97 PDTC (11.3%) and 9 positive in 97 ATC cases (9.3%). Additional co-existing mutations in PDTC and ATC include RAS, RAF, TERTp, TP53 mutations.
Table 3. Prevalence of EIF1AX Mutations in A) Fine Needle Aspirates (FNA) and B) Surgically Removed Nodules, in Present and Previously Published Studies

A. Characterization of EIF1AX Mutation-Positive Fine Needle Aspirates (FNA) with Indeterminate Cytology

<table>
<thead>
<tr>
<th>Study</th>
<th>EIF1AX-mutated FNA</th>
<th>Surgical pathology follow-up of EIF1AX-mutated FNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cases HN FA NIFTP PTC FTC HCC</td>
<td></td>
</tr>
<tr>
<td>Karunamurthy et al, 2016 (4)</td>
<td>27/647; 4.2%</td>
<td>5/27 1/5; 20% 3/5; 60% - 1/5; 20% - -</td>
</tr>
<tr>
<td>Present Study</td>
<td>41/904; 4.5%</td>
<td>26/41 6/26; 23.1% 3/26 11.5% 7/26 26.9% (FVPTC) 7/26 26.9% 3/26 11.5%</td>
</tr>
</tbody>
</table>

B. Frequency of EIF1AX Mutations in Surgically Removed Nodules

<table>
<thead>
<tr>
<th>Study</th>
<th>HN FA</th>
<th>Differentiated TC</th>
<th>Undifferentiated TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCGA, 2014 (9)</td>
<td>- -</td>
<td>6/402; 1.5% (5 FVPTC, 1 mix of follicular &amp; classical variant)</td>
<td>- - -</td>
</tr>
<tr>
<td>Kunstman et al, 2015 (16)</td>
<td>- -</td>
<td>-</td>
<td>3/22; 13.6%</td>
</tr>
<tr>
<td>Karunamurthy et al, 2016 (4)</td>
<td>1/80; 1.3% 2/27; 7.4% 2/86 2.3%</td>
<td>0/53; 0% 0/4; 0% 1/4 25%</td>
<td></td>
</tr>
<tr>
<td>Landa et al, 2016 (11)</td>
<td>- - -</td>
<td>-</td>
<td>9/84; 11% 3/33; 9%</td>
</tr>
<tr>
<td>Nicolson et al, 2018 (17)</td>
<td>- -</td>
<td>-</td>
<td>2/39; 5.1% - -</td>
</tr>
<tr>
<td>Pozdeyev et al, 2018 (15)*</td>
<td>- -</td>
<td>1/89; 1.1%</td>
<td>2/5; 40% -</td>
</tr>
<tr>
<td>Simoes-Pereira et al, 2019 (14)†</td>
<td>0/7; 0%</td>
<td>1/12; 8.3% (FVPTC)</td>
<td>2/9; 22.2% 0/7; 0%</td>
</tr>
</tbody>
</table>

ATC=Anaplastic thyroid cancer; FA=follicular adenoma; FTC=Hurthle cell carcinoma; HCC=Hurthle cell carcinoma; HN=Hyperplastic nodule; NIFTP=Noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PDTC=Papillary thyroid carcinoma; TC=Thyroid cancer; *Only those specimens that were sequenced on MSK-IMPACT panels were included in this table since EIF1AX is not tested by the FoundationOne Panels; †9 surgical specimens in this study had distinct histological type and were counted separately based on the histology. Please see the study by Simoes-Pereira et al. (14) for details.

Krishnamoorthy et al. (18) demonstrated EIF1AX A113_splice mutation increases protein synthesis globally and cooperates with RAS mutations to increase the stability of c-MYC protein to drive thyroid tumorigenesis.

Among the 26 cases carrying the EIF1AX mutations in our cohort, 11 cases had EIF1AX mutations alone and 15 had co-existing mutations detected by our NGS panel. Within the 15 co-mutation cases, 13 were in the RAS/RAF pathway (including 1 case with a non-hotspot KRAS mutation, p.A59del with unknown significance), and 2 cases had a mutation either in the TERT promoter or the TP53 gene. When we compared the alternate allele frequencies (AAF) of the EIF1AX and other co-existing mutations, the AAFs for the EIF1AX mutations were greater than (N=4) or similar to (N=10) that of other driver mutations (Table 1). In the case with a co-existing TP53 mutation, the AAF for the TP53 mutation was 66% and AAF for the EIF1AX mutation was 40%. The apparent higher AAF of TP53 is likely due to a loss of heterozygosity (LOH) event in this case, given the AAF of TP53 was well over 50%. The high AAFs of EIF1AX mutations in comparison to that of co-existing mutations suggests that EIF1AX mutations represent an early event, at least in some cases, that promotes initiation of the thyroid tumors and malignant transformation.

Conclusion

EIF1AX mutations can occur in both benign and malignant thyroid neoplasms. The type of EIF1AX mutation, and even more importantly, the presence or absence of co-mutations, has an impact on the likelihood of malignancy in an EIF1AX-mutat-
ed thyroid nodule. Therefore, in the face of a cyto-
logically indeterminate thyroid nodule with an
EIF1AX mutation detected upon molecular ana-
lysis, a more detailed look at the molecular profile,
in conjunction with the clinical and imaging find-
ings, may be helpful in predicting malignancy risk
during and determining optimal patient care.

What Is Already Known on This Topic:
EIF1AX mutations occur in wide variety of thyroid lesions, including
HN, FA, NIFTP, well differentiated carcinomas (FTC and FVPTC) and
poorly differentiated carcinomas (PDTC and ATC). Previous studies
examining surgically resected nodules have concluded that the A113-
nonsense mutation, especially with co-existing RAS mutation, is more fre-
cently observed in thyroid cancer than are isolated EIF1AX mutations
or mutations at the N-terminus hotspot of the gene. However, there is
limited data characterizing EIF1AX mutations in cytology samples
from indeterminate thyroid nodules.

What This Study Adds:
The ROM in an EIF1AX-mutated, cytologically indeterminate thyroid
nodule may be higher than previously reported, for in our study, 36.4%
of thyroid nodules with isolated EIF1AX mutation identified in the FNA
sample were diagnosed as malignant neoplasms upon surgical removal.
Furthermore, our data suggests that the presence of co-mutations, re-
gardless of the position of the EIF1AX mutation, has a significant influ-
ence on malignancy risk. The coexistence of EIF1AX mutations with
other pathogenic driver mutations in RAS, TERT and TP53 conferred a
100% ROM or NIFTP in our cohort, indicating that such nodules war-
rant surgical resection.

Authors’ Contributions: Conception and design: SG, YN
and ZW; Acquisition, analysis and interpretation of data: SG,
NB, YN, SP and ZW; Drafting the article: SG, NB and ZW;
Revising it critically for important intellectual content: SP and
ZW; Approved final version of the manuscript: SG, NB, YN,
SP and ZW.

Conflict of Interest: The authors declare that they have no
conflict of interest.

References
1. Rao SN, Bernet V. Indeterminate thyroid nodules in the
2. Kumar N, Gupta R, Gupta S. Molecular testing in diag-
nosis of indeterminate thyroid cytology: trends and driv-
Online ahead of print.
Comprehensive analysis of the transcriptional and muta-
tional landscape of follicular and papillary thyroid cancer.
4. Karunamurthy A, Panebianco F, Hsiao SL, Vorhauer J,
Nikoforova MN, Chiosea S, et al. Prevalence and pheno-
typic correlations of EIF1AX mutations in thyroid nod-
5. Simen BB, Yin L, Goswami CP, Davis KO, Bajaj R, Gong
JZ, et al. Validation of a next-generation-sequencing cancer
panel for use in the clinical laboratory. Arch Pathol Lab
6. Catalogue of Somatic Mutations in Cancer [database on
the Internet]. v92, released Aug 2020 Aug. [Cited 2021 Jan
7. cBioPortal for Cancer Genomics [database on the Inter-
net]. [cited 2021 Jan 29]. Available from: http://cbiopor-
tal.org.
8. Fekete CA, Applefield DJ, Blakely SA, Shirokikh N, Pesto-
tav T, Lorsch JR, et al. The eIF1A C-terminal domain pro-
motes initiation complex assembly, scanning and AUG
9. Cancer Genome Atlas Research Network. Integrated ge-
nomic characterization of papillary thyroid carcinoma.
10. Johnson DB, Roszik J, Shoustari AN, Eroglu Z, Balko
JM, Higham C, et al. Comparative analysis of the GNAQ,
GNA11, SF3B1, and EIF1AX driver mutations in melanoma
and across the cancer spectrum. Pigment Cell Mela-
11. Landa I, Ibrahimipasic T, Boucai L, Sinha R, Knauf JA,
Shah RH, et al. Genomic and transcriptomic hallmarks of
poorly differentiated and anaplastic thyroid cancers. J
12. Martin M, Masshofer L, Temming P, Rahmann S, Metz C,
Bornfeld N, et al. Exome sequencing identifies recurrent
somatic mutations in EIF1AX and SF3B1 in uveal melano-
DW, Kennedy CJ, et al. EIF1AX and NRAS mutations co-
occur and cooperate in low-grade serous ovarian carcino-
14. Simoes-Pereira J, Moura MM, Marques IJ, Rito M, Cabre-
ra RA, Leite V, et al. The role of EIF1AX in thyroid can-
15. Pozdeyev N, Gay LM, Sokol ES, Hartmaier R, Deaver KE,
Davis S, et al. Genetic analysis of 779 advanced differen-
tiated and anaplastic thyroid cancers. Clin Cancer Res.
2018;24(13):3059-68. doi 10.1158/1078-0432.CCR-18-
0373.
A, Healy JM, et al. Characterization of the mutational
landscape of anaplastic thyroid cancer via whole-exome