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ORIGINAL ARTICLE

Absence of *EIF1AX*, *PPM1D*, and *CHEK2* mutations reported in Thyroid Cancer Genome Atlas (TCGA) in a large series of thyroid cancer

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Abstract

Introduction The Thyroid Cancer Genome Atlas (TCGA) was a major project that significantly clarified the key underlying genetic aberrations in papillary thyroid cancer. It confirmed the previously known somatic mutations and gene fusions and disclosed additional genetic alterations that were previously unknown. Among the most significant novel genetic mutations were those in *EIF1AX*, *PPM1D*, and *CHEK2*.

Objectives We sought to determine the rates of these novel genetic alterations in a large sample of our patients to test the prevalence, reproducibility, and significance of these findings.

Patients and methods We studied thyroid cancer (TC) tumor tissues from 301 unselected patients using polymerase chain reaction (PCR) and direct Sanger sequencing. DNA was isolated from paraffin-embedded formalin-fixed tumor tissue. Exons and exon–intron boundaries harboring the previously reported mutations in TCGA were amplified using PCR and directly sequenced.

Results We found only one of the 301 tumors (0.3%) harboring A113_splice site mutation at the intron 5/exon 6 splice site of *EIF1AX* gene. Apart from this single mutation, none of the 301 tumors harbored any of the previously reported mutations in any of the three genes, *EIF1AX*, *PPM1D*, and *CHEK2*. A number of previously reported single nucleotide polymorphisms (SNP) were found in *CHEK2, PPM1D* but not in *EIF1AX*. These include *CHEK2* SNPs, rs375130261, rs200928781, rs540635787, rs142763740, and rs202104749. The *PPM1D* SNPs rs771831676 and rs61757742 were present in 1.49% and 0.74%, respectively. Each of these SNPs was present in a heterozygous form in 100% of the tumors. An additional analysis of these samples for the most frequently reported mutations in DTC such as $BRAF^{V600E}$, *TERT promoter*, and *RAS* showed a prevalence of 38.87% (117/301), 11.96% (36/301), and 7.64% (23/301), respectively.

Conclusions Except for a rare A113_splice site mutation in *EIF1AX*, other recently described somatic mutations in *EIF1AX, PPM1D*, and *CHEK2* were absent in this large series of patients with TC from a different racial group (Saudi Arabia). This might be related to the different techniques used (PCR and direct sequencing) or low density of the mutants. It might also reflect racial differences in the rate of these mutations.

Keywords Thyroid cancer · Mutations · TCGA · Thyroid Cancer Genome Atlas

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Introduction

Thyroid cancer (TC) is the most common endocrine malignancy. Its incidence has been steadily increasing over the last 4 decades [1, 2]. Most of this increase in incidence occurs in differentiated thyroid cancer (DTC). There has also been significant progress in our understanding of the molecular pathogenesis of DTC [3]. Using conventional methods, several characteristic genomic mutations have been identified in DTC [3]. Among the most important and

well-studied genetic aberrations are $BRAF^{V600E}$ mutation in papillary thyroid cancer (PTC) and *RAS* mutations in follicular thyroid cancer (FTC) [4–10]. Other genetic alterations include gene fusions such as *RET/PTC1* and *RET/PTC3* in PTC [11], *PAX8/PPARy* in FTC [12, 13], and single point mutations in other genes including *PTEN* [14], *PIK3CA* [15], and *TERT* promoter [5, 9, 16, 17]. Using several next-generation sequencing (NGS) platforms and other more advanced technologies, the Thyroid Cancer Genome Atlas (TCGA) confirmed the importance of the above-mentioned genetic alterations in DTC and described several other previously unknown genetic variations [18]. Indeed, the TCGA project has decreased the so-called "dark matters" in the molecular genetics of DTC from about 25% to 3.5% [18]. Although single point mutations in *BRAF* and *RAS* genes remained the most common mutations in DTC, mutations in other novel genes were found to be significantly present occurring in 1.2–2.7% of cases [18]. In this study, we have investigated the frequency of genetic alterations in three genes that have been listed in the TCGA data as the most common previously unknown genetic alterations in PTC. These genes are *EIF1AX, PPM1D*, and *CHEK2*.

Patients and methods

After obtaining an Institutional Review Board approval of the King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia, we studied tumor tissues of 301 unselected patients diagnosed with TC for mutations in *CHEK2, PPM1D*, and *EIF1AX*. An informed consent was waived as this research work was limited to archived pathology materials. The tumor tissue was carefully examined and dissected by an experienced endocrine pathologist (H.A.H.) and subjected to DNA extraction.

Genotyping

DNA was isolated from macrodissected formalin-fixed paraffin-embedded tumor tissues of a total of 301 cases of TC using the genomic DNA extraction kit (QIAamp DNA FFPE Tissue Kit, Catalog No. 56404) according to the manufacturer's instructions. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and DNA purity was assured by the A260/280 ratio, with a ratio of ≥1.8 indicating good purity. Using polymerase chain reaction (PCR), we amplified the exons and exon–intron boundaries that encompass the previously described mutations in the three genes in the TCGA database. These included exons 10, 12, and 14 of *CHEK2*; exons 2, 5, and 6 of *PPM1D*; and exons 1 and 2 and 6 of *EIF1AX*. The primers and the annealing temperatures are listed in Table 1 and the PCR conditions were 35 cycles of 94 °C for 30 s, with a specified annealing temperature (Table 1) for 30 s and 72 °C for 45 s with an initial denaturation at 94 °C for 2 min and a final extension at 72 °C for 7 min. The amplicons were resolved on 2% agarose gel. Successfully amplified exons were directly sequenced in forward and reverse directions using Big Dye terminator v3.1 cycle-sequencing reaction kit and an ABI PRISM 3730Xl genetic analyzer (Applied Biosystems). The GeneBank accession numbers of *CHEK2*, *PPM1D*, and *EIF1AX* are NM_007194.3, NM_003620.3, and NM_001412, respectively.

Table 1 Primers used for PCR amplification of the *EIF1AX*, *PPDM1*, and *CHEK2*

Gene	Exon	Primers (5′–3′)	T_m (°C)
EIF1AX	1	1F-GGAAAAGCGACGCAAAGAGTC	55
		1R-CTGGGTGACCTGCAATCTACG	
	2	2F-GGTGATAATGTGTTAATGTTG	50
		2R-GTGCCACCACACTTCACCCT	
	6	6F-TCCCATCTTGACTTAGCAGTGA	57
		6R-AGGGGAAAGTTGGTTTCTCCAT	
PPM1D	2	2F-ATTTTATTTCTTATTACAG	50
		2R-GAAAGAGAAAACGACAGAA	
	5	5F-CTTCTCCTTGTTCTTTTGAA	50
		5R-ACTTAAAAACTATGGAACT	
	6	6F-TCACTGGAGGAGGATCCATG	55
		6R-AGGGGTTCTTTCAATTTCTT	
CHEK2	10	10F-TATTCCCTTTTGTACTGAAT	50
		10R-GTGCAGCAATGAAAATATTT	
	12	12F-GTGATTTGCCCAATTGTTG	55
		12R-CTAGCAGGCACTGTCCCAC	
	14	14F-CTCCATTTTCCTTTATTTTCA	50
		14R-AAAAGAAAGATGACAGAGTG	

Results

Patients and tumor characteristics

We studied a total of 301 patients with TC. These included 119 males (39.5%) and 182 females (60.5%) with a median age of 34 years (range, 9–75). Ninety-seven patients (32.2%) were in the pediatric and adolescent age group (≤18 years, median age 16 years, range 9–18) and 204 (67.8%) were adults (median age 39 years, range 19–78). The tumors were classic PTC in 197 cases (65.5%), follicular variant PTC in 58 cases (19.3%), tall cell variant PTC in 20 cases (6.65%), FTC in 6 cases (2%), oncocytic variant in 2 cases (0.7%), diffuse sclerosing variant PTC in 2 cases (0.7%), columnar variant PTC in 2 cases (0.7%), poorly

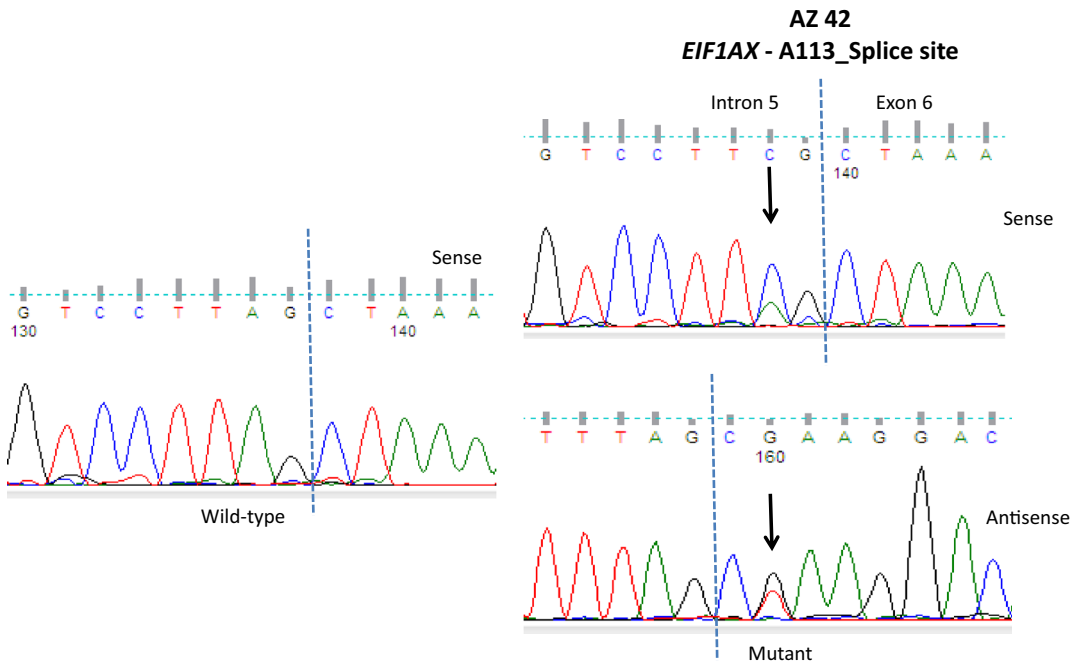

Fig. 1 Sequence chromatogram showing *EIF1AX*–A113_Splice site mutation at the junction between intron 5 and exon 6 in forward and reverse directions. The wild-type sequence is shown on the left panel

differentiated thyroid cancer in 13 cases (4.3%), and anaplastic thyroid cancer in 1 case (0.3%). The median tumor size was 3.0 (0.5–13 cm). There was tumor multifocality in 147 cases (48.8%), extrathyroidal extension/invasion in 120 cases (39.9%), vascular invasion in 79 cases (26%), lymph node metastasis in 144 cases (47.8%), and distant metastasis in 30 cases (10%).

Mutations in *EIF1AX*, *PPM1D*, and *CHEK2*

In this cohort of 301 unselected cases of TC, only one case harbored A113_splice site mutation at the intron 5/exon 6 splice site of *EIF1AX* gene (Fig. 1). None of the three genes studied carried any of the other mutations reported in the TCGA database. A number of previously reported single nucleotide polymorphisms (SNPs) were found in *CHEK2* and *PPM1D* (Table 2). All of these SNPs were in heterozygous forms. *EIF1AX* did not carry any SNP in any of the three exons studied.

Analysis of other frequently mutated genes ($BRAF^{V600E}$, *TERT* promoter, *RAS* genes)

Mutations in more conventional genes were also assessed in the same cohort of patients (Table 3). The $BRAF^{V600E}$ mutation was present in 117 cases (38.87%). *TERT* promoter mutations occurred in 36 cases (11.96%); C250T in 6 cases (1.99%) and C228T mutation in 30 cases (9.96%). *HRAS* T81C (H27H) polymorphism was present in 116 cases (38.2%) but no mutations. *KRAS* Q61R mutation occurred in only 2 cases (0.66%) and *NRAS* Q61R in 18 cases (5.98%) and Q61K in 3 cases (0.99%).

Discussion

In this study, we attempted to ascertain the rates of genetic alterations in three genes that were found to be significantly mutated in DTC in the TCGA data [18]. We used the conventional Sanger sequencing that is considered to be the standard method for detection of mutations and is frequently used for the confirmation of NGS findings. We screened the exons that carried the previously reported mutations in each of the three genes in a sample of TC. Apart from a single tumor that harbored a well-known A113_splice site mutation at the intron 5/exon 6 splice site of *EIF1AX* gene, we found none of the previously reported mutations in this large cohort from the Middle East.

The TCGA included 496 well-differentiated TC [18]. Although the total number of cases was 496 tumors, due to technical reasons, only 390 tumors were analyzed on all platforms and only 402 tumor/normal tissue pairs were whole exome sequenced [18]. The analysis showed a low somatic mutation density score relative to other cancers. Mutation density significantly correlated with the age of the patients and the tall cell variant PTC had the highest mutation densities [18]. The relatively large number of tumors included in the TCGA study and the relatively low

Table 2 Identification of *EIF1AX*, *CHEK2*, and *PPM1D* variants in 301 (97 pediatric and 204 adults) thyroid cancers

Gene	Exon	Nucleotide	Codon	Amino acid	SNP ID	Pediatric TC[a] Mutated/total (%)	Adult TC[a]	All TCs[a]	Zygosity
EIF1AX	06	A>C	A113_splice site			0/89 (0%)	1/185 (0.54%)	1/274 (0.36%)	All heterozygous
CHEK2	10	A1141G	ATG-GTG	M381V	rs375130261	97/97 (100%)	198/198 (100%)	295/295 (100%)	All heterozygous
	10	C1169T	TAC-TGC	Y390C	rs200928781	97/97 (100%)	198/198 (100%)	295/295 (100%)	All heterozygous
	12	C1420T	CGT-TGT	R474C	rs540635787	95/95 (100%)	194/194 (100%)	289/289 (100%)	All heterozygous
	12	C1427T	ACG-ATG	T476M	rs142763740	95/95 (100%)	194/194 (100%)	289/289 (100%)	All heterozygous
	14	C1566T	CCC-CCT	P522P	rs202104749	95/95 (100%)	175/175 (100%)	270/270 (100%)	All heterozygous
PPM1D	05	C1177G	CTG-GTG	L393V	rs771831676	2/77 (2.59%)	2/190 (1.05%)	4/267 (1.49%)	All heterozygous
	06	A1501G	ACT-GCT	T501A	rs61757742	0/82 (0%)	2/186 (1.07%)	2/268 (0.74%)	All heterozygous

[a]Successfully PCR-amplified and sequenced tumor samples

mutation density allowed the detection of significantly mutated genes in as low as 3% of the cases [18]. The TCGA confirmed the previously known data of the high frequency of mutations in the MAPK-related genes such as *BRAF*, *NRAS*, *HRAS*, and *KRAS* genes. These mutations occurred in a total of 300 out of the 402 tumors (74.6%) that were successfully sequenced. *BRAF* mutations (mostly $BRAF^{V600E}$) occurred in 248 cases (61.7%) and *RAS* mutations in 52 cases (13%). *BRAF* and *RAS* mutations were mutually exclusive. Known and novel fusion genes have been identified in 74 (15.3%) tumors of 484 informative tumor sequences [18]. Three novel genes were found to be significantly mutated. These genes were the *EIF1AX*, *PPM1D*, and *CHEK2*.

EIF1AX encodes a protein that mediates a transfer of Met-tRNAF to 40S ribosomal RNA to form the 40S ribosomal preinitiation complex that starts the protein translation. Six mutations (1.5%) were found in the TCGA study and all of them except one were mutually exclusive with other MAPK-related genes [18]. In our study, we found only one tumor [1/274 (0.36%)] harboring A113_splice site mutation at the intron 5/exon 6 splice site of *EIF1AX* gene. We did not find any other genetic alterations in the tested exons in any of the 301 cases that we studied. Other studies have found variable rates of *EIF1AX* mutations ranging from 1.5 to 2.5% in PTC [18, 19], 5.1% in FTC [20], 11% in poorly differentiated TC [21], and 9% in anaplastic TC [21]. *EIF1AX* mutations were also occasionally described in benign adenomas [22] and in combination with other mutations in benign and malignant tumors [21, 23].

CHEK2 encodes a DNA repair enzyme called checkpoint kinase 2, which is activated when there is a double-stranded DNA break [24]. It is a tumor suppressor gene that is activated by the ataxia telangiectasia mutated gene and activates several other proteins including TP53 to induce repair of DNA [24, 25]. Four missense and one nonsense mutations (1.2%) were found in the TCGA study [18]. These mutations were not reported before in DTC and were not mutually exclusive with other MAPK-related genes [18]. In our study, we found none of these mutations. Interestingly, we found five known SNPs; each was present in 100% of our patients (Table 2). In a study from Poland, *CHEK2* rs17879961 germline polymorphism was found to be associated with increased risk of PTC [26]. Another study from the same population showed the c.470C (I157T) homozygous variant increases the risk of PTC by about 13-fold [27].

PPM1D encodes a wild-type TP53-induced phosphatase (Wip1). Wip1 is a serine/threonine phosphatase that is involved in the DNA damage response [28]. It dephosphorylates a number of important checkpoint pathway proteins including TP53 [29], ATM [30], CHEK1 [28], and CHEK2 [28]. Five mutations were also found in the TCGA

Table 3 Identification of *BRAF*, *TERT*, and *RAS* mutations in 301 (97 pediatrics and 204 adults) thyroid cancers

Gene	Exon	Nucleotide	Amino acid	Pediatric TC Mutated/total (%)	Adult TC	All TCs
BRAF	15	T1799A	V600E	23/97 (23.71%)	94/204 (46.07%)	117/301 (38.87%)
TERT		C228T		1/97 (1.03%)	29/204 (14.21%)	30/301 (9.96%)
		C250T		0/97 (0%)	6/204 (2.94%)	6/301 (1.99%)
KRAS	02	A182G	Q61R	0/97 (0%)	2/204 (0.98%)	2/301 (0.66%)
NRAS	02	C181A	Q61K	1/97 (1.03%)	2/204 (0.98%)	3/301 (0.99%)
	02	A182G	Q61R	1/97 (1.03%)	17/204 (8.33%)	18/301 (5.98)

study (one missense and four frameshift mutations) [18]. These also occurred in the presence of other MAPK-related gene mutations. In our study, we did not find these mutations in any of the tumors that were tested. However, two previously reported SNPs (rs771831676 and rs61757742) were found in 4 of 267 (1.49%) and 2 of 268 cases (0.74%) tested, respectively (Table 2).

Excluding the single A113_splice site mutation in *EIFA1X*, why didn't we find any of the previously described mutations [18] in this relatively large cohort of patients? One possibility is the sample size. However, the total number of tumors we studied was 301, not quite different from the 402 cases that were successfully sequenced in the TCGA data. With a frequency of these mutations ranging between 1.2 and 2.7% in the TCGA, it is expected that some tumors in our study would harbor at least a few of the mutations reported in the TCGA. In the TCGA, tumors with aggressive histologies were excluded to ensure a homogenous cohort of tumors. Our patients were mostly also of well-differentiated subtypes of TC and were mostly not different from those included in the TCGA project. Indeed, 250 tumors were similar to those included in TCGA with classic PTC in 172 cases (57.1%), follicular variant PTC in 58 cases (19.3%), and tall cell variant PTC in 20 cases (6.6%). Another possibility for the lack of mutations in our study is the different techniques used. In TCGA, NGS was used while we used the conventional Sanger sequencing. It is known that detection of mutations in Sanger sequencing depends partially on allele frequency. It is plausible that the lack of these mutations in our study is due to low mutant allele frequency and low sensitivity of Sanger sequencing for detection of mutations with low allele frequency. However, in the TCGA study, mutations found in NGS data were confirmed by Sanger sequencing and in some situations, where clonality was assessed, their clonal fraction was high (70–100%) [18]. Therefore, a low clonal fraction of cells carrying these mutations is an unlikely explanation for the lack of mutations in these genes in our patients. Unlike the TCGA project, our study included patients in the pediatric age, the inclusion of pediatric cases also could be a reason for this low frequency as common somatic mutations found in the adult DTC occur at significantly lower frequencies in pediatric DTC [31]. Finally, the absence of the mutations in the three genes studied in our patients compared to the TCGA data may reflect racial differences between the North American population in the TCGA study and the Middle Eastern Arab population in this study. This is not supported by the fact that the rates of the other well-known genes such as $BRAF^{V600E}$, *TERT* promoter, and *NRAS* mutations in our study are similar to those reported in data from the US and other regions [3, 5, 7, 10, 32].

Our study has a number of shortcomings. First, the sample size remains relatively small for the detection of generally rare genetic mutations. However, as mentioned above, the absence of all of the 18 mutations in the three genes is unlikely to be explained by the sample size, especially that it is not much different from the sample size in the TCGA study. It would have been of interest to do NGS in our study at least on a subsample of our patients looking specifically for these mutations and to compare the NGS and Sanger sequencing data but this was not possible due to economic and technical reasons related to the need to sequence a large number of tumors. Although the study did not reveal the mutations reported in the TCGA, it expands the literature on this subject and draws the attention to the fact that racial or technical factors might lead to different detection rates of these mutations. Additional studies from other populations are needed to further assess the rates and importance of mutations in these three genes in TC.

Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent This study was performed only on archived pathology samples and did not obtain any new samples from the patients. With that and according to the Institutional Review Board rules and guidelines, informed consents were waived by the

Institutional Review Board of the King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia.

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