Genomic Profiling of Thyroid Cancer Reveals a Role for Thyroglobulin in Metastasis

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Papillary thyroid carcinoma (PTC) has a wide geographic variation in incidence; it is most common in Saudi Arabia, where it is only second to breast cancer as the most common cancer among females. Genomic profiling of PTC from Saudi Arabia has not been attempted previously. We performed whole-exome sequencing of 101 PTC samples and the corresponding genomic DNA to identify genes with recurrent somatic mutations, then sequenced these genes by using a next-generation gene-panel approach in an additional 785 samples. In addition to *BRAF, N-RAS,* and *H-RAS,* which have previously been shown to be recurrently mutated in PTC, our analysis highlights additional genes, including thyroglobulin (*TG*), which harbored somatic mutations in 3% of the entire cohort. Surprisingly, although *TG* mutations were not exclusive to mutations in the RAS-MAP kinase pathway, their presence was associated with a significantly worse clinical outcome, which suggests a pathogenic role beyond driving initial oncogenesis. Analysis of metastatic PTC tissue revealed significant enrichment for *TG* mutations ($p < 0.001$), including events of apparent clonal expansion. Our results suggest a previously unknown role of *TG* somatic mutations in the pathogenesis of PTC and its malignant evolution.

Introduction

Thyroid cancer is the most common endocrine cancer, and its incidence is increasing worldwide.[1] Among females, it is the second most common malignancy, after breast cancer, in Saudi Arabia, where it accounts for 9% of all cancers and 12% of all malignant cancers in females.[2] Among the several histological subtypes of thyroid cancer, papillary thyroid carcinoma (PTC) is the most common subtype, comprising approximately 80%–90% of all thyroid cancers.[3] Although PTC is usually a well-differentiated thyroid cancer with favorable prognosis, the clinical behavior of PTC varies widely.[4,5] Approximately 20% of PTCs demonstrate tumor recurrence, and some reach advanced stages.[6] Various clinicopathological variables, such as tall-cell variant, advanced stage, vascular invasion, and nodal or distant metastasis, are related to aggressive behavior of PTC.[7,8] However, the factors and mechanisms determining the aggressive behavior of some papillary carcinomas that result in recurrence and metastatic lesions are not fully known. Although predisposition to PTC is known to occur secondary to germline mutations in a few genes, including *PTEN*[9,10](MIM: 601728), *APC*[11](MIM: 611731), *SEC23B*[12](MIM: 610512), and *HABP2*[13](MIM: 603924), the overwhelming majority of PTC cases are sporadic in nature.[14] Available data from genomic analysis of PTC suggest that it is a generally "quiet" cancer in terms of overall mutation burden, a feature that facilitated the identification of driver mutations from the relatively low background of passenger mutations.[15] These recent data are in agreement with previous studies in that a major class of driver mutations in PTC are those in genes encoding various components of the mitogen-activated protein kinase (MAPK) signaling pathway. Such genes include *BRAF* (MIM: 164757); *RAS* (*HRAS* [MIM: 190020]; *NRAS* [MIM: 164790] and *KRAS* [MIM: 190070]), *RET* (MIM: 164761), and *NTRK1*[16–19](MIM: 191315). Recurrent somatic mutations that are likely to drive oncogenesis in PTC have also been identified in *EIF1AX* (MIM: 300186), *PPM1D* (MIM: 605100), and *CHEK2*[15](MIM: 604373). Targeted sequencing of candidate genes has also demonstrated the prominent role of driver mutations in *BRAF* (59%), followed by *PIK3CA* (12%) (MIM: 171834), *TP53* (8%) (MIM: 191170), and *NRAS* (4%).[20]

Despite the recent advances in our understanding of the genomic architecture of PTC, important issues remain to be addressed. For example, the study by the Cancer Genome Atlas Research Network did not target populations such as Italy,[21] Japan,[22] Denmark,[23] and Saudi Arabia,[2] which are all known to have a high incidence of PTC. This is likely to be relevant because the mutation profile of cancer can vary greatly between different populations.[24,25] Specific examples include the demonstration of a higher incidence of *PIK3CA* amplifications (53.1%) in Saudi thyroid cancer,[26] higher incidence of amplification of *ESR1* (20.6%) (MIM: 133430), *HER2* (31%) (MIM: 164870), and *MYC* (16%)(MIM: 190080) in Saudi breast cancer,[27,28] higher rate of *EGFR* (MIM: 131550) amplifications (15.3%) in Saudi lung cancers,[29] lower incidence of *BRAF* mutations (2.5%) in Saudi colorectal cancer,[30] and lower incidence of *PIK3CA* mutations (3.9%) along with

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higher incidence of *PIK3CA* amplification (40%) in Saudi ovarian cancer.[31]

Therefore, genomic profiling of PTC in a country, such as Saudi Arabia, where the disease is especially prevalent is likely to provide new insights. Furthermore, previously published genomic profiling specifically excluded PTC individuals with aggressive disease.[15] Although this might have ensured increased homogeneity of the study samples as intended by those authors, it might have also limited the authors' ability to identify factors whose pathogenic role concerns the advanced stages of malignancy, e.g., metastasis. In an attempt to address these gaps, we present the results of our genomic profiling of >880 PTC samples from Saudi individuals who span the full clinicopathological spectrum. In addition to replicating previously identified genes with recurrent somatic mutations, we identify and validate a few candidates. In particular, we highlight a previously unrecognized role for somatic mutations of the thyroglobulin gene (*TG* [MIM: 188450]) as a marker of poor prognosis and a potential driver of metastasis.

Materials and Methods

Clinical Samples

A total of 909 individuals diagnosed and treated between 1988 and 2015 at King Faisal Specialist Hospital and Research Centre and Prince Sultan Military Medical City, Riyadh, Saudi Arabia were selected. Fresh tumor samples were collected from the operation room (OR) and were subjected to immediate frozen-section examination in the department of pathology. Tumor samples were included in the study if examination both of frozen sections and of formalin-fixed paraffin embedded (FFPE) sections confirmed PTC. None of the individuals from whom fresh cancer tissues were collected received any preoperative treatment in the form of chemotherapy or radiotherapy. Along with fresh cancer tissue, fresh blood in the EDTA tube was also collected for DNA extraction. FFPE blocks were also retrieved from the pathology department, and tumor punches were made available for DNA extraction from the tumor tissue area. Pathological and clinical data were retrieved from medical charts and the integrated clinical information system (ICIS). Clinical risk assessment is given in the supplemental note. All tissue and blood samples were obtained from individuals under approval from the institutional review board of the hospital, and informed consent was obtained from individuals for the study.

Clinicopathological Evaluation of Tumors

Tumor sections from all cases were subjected to histopathological examination and were only included in the study if the tumor area showed an average of 60% of tumor cell nuclei and less than 20% necrosis. The clinical and demographic elements included the following: age at diagnosis, gender, histopathological subtype, regional lymph node status (N), distant metastasis (M), and overall pathologic AJCC stage. The follow-up data elements included the following: vital status (alive/dead), disease status (remission, persistence, or progression), days to last contact or death, and additional radiation therapy given. The median age at diagnosis in the PTC cohort was 37 years, and 64.2% of individuals were younger than 45 years of age. There 24.2% were male, and 75.8% were female; there was a male: female ratio of 1:3. The staging of the submitted tumors was distributed across all four stages: 66.4% were stage I, 4.7% were stage II, 8.6% were stage III, 16.7% were stage IV, and 3.6% of cases had an unknown overall stage. 72.4% were of classical papillary histological subtype, 14.8% were follicular variants, and 7.8% were tall-cell variants. 1.2% were rare types, and 3.8% were without histological annotation. 5.3% of individuals had distant metastatic disease.

Next-Generation Sequencing

We performed whole-exome and capture sequencing on an Illumina HiSeq 2500 DNA Sequencer by using the Nextera Rapid Capture Exome Kit and SureSelect Target Enrichment Kit, respectively, according to the manufacture's instruction.

Clinical Data Analysis

We used the JMP 10.0 (SAS Institute) software package for clinicopathological data analyses. We generated survival curves by using the Kaplan-Meier method and evaluated significance with the Mantel-Cox log-rank test. We calculated risk ratio by using the Cox proportional hazard model. Values of $p < 0.05$ were considered statistically significant.

DNA Isolation

DNA from peripheral blood and from freshly frozen and paraffin-embedded tissues was isolated with the Gentra DNA Isolation Kit according to the manufacturer's recommendations.

PCR and Sanger Sequencing

We used Primer 3 software to design the primers for the particular variants identified by whole-exome or capture sequencing and all coding exons and flanking intronic sequences of *TG* (these are available upon request). PCR was performed in a total volume of 25 μl with 20 ng of genomic DNA, 2.5 μl 10 × Taq buffer, 2.3 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit Taq polymerase, and 0.2 μM each primer and water. We confirmed the efficiency and quality of the amplified PCR products by running them on a 2% agarose gel. The PCR products were subsequently subjected to Sanger sequencing with BigDye terminator V 3.1 cycle sequencing reagents and analyzed on an ABI 3730XL DNA analyzer (Applied Biosystems). Reference sequences were downloaded from GenBank. Sequencing traces were analyzed with the Mutation Surveyor v4.04 (Soft Genetics).

Next-Generation Gene-Panel Sequencing

808 DNA samples were analyzed for target-capture sequencing of 24 genes with the SureSelect Target Enrichment Kit on an Illumina HiSeq 2500 Sequencer. The average coverage of the targeted region was 600×, and 95% of the target was covered at >50×. Sequencing reads were aligned to the human genome (NCBI build 37) with the BWA algorithm on default settings. Finally, 785 cases passed internal quality control and quality matrix and were included in further analyses.

Bioinformatic Analysis

BCL files generated by Illumina for the whole-exome sequencing samples were converted to FASTQ format by CASAVA software (v.1.8.1, Illumina) and aligned to the human reference genome hg19 with the Burrows-Wheeler Aligner[32]. Local realignment, PCR duplicate marking, base-quality recalibration, and calculation of coverage metrics were performed with GATK[33] and Picard tools (v.1.119, http://broadinstitute.github.io/picard/). Putative SNVs

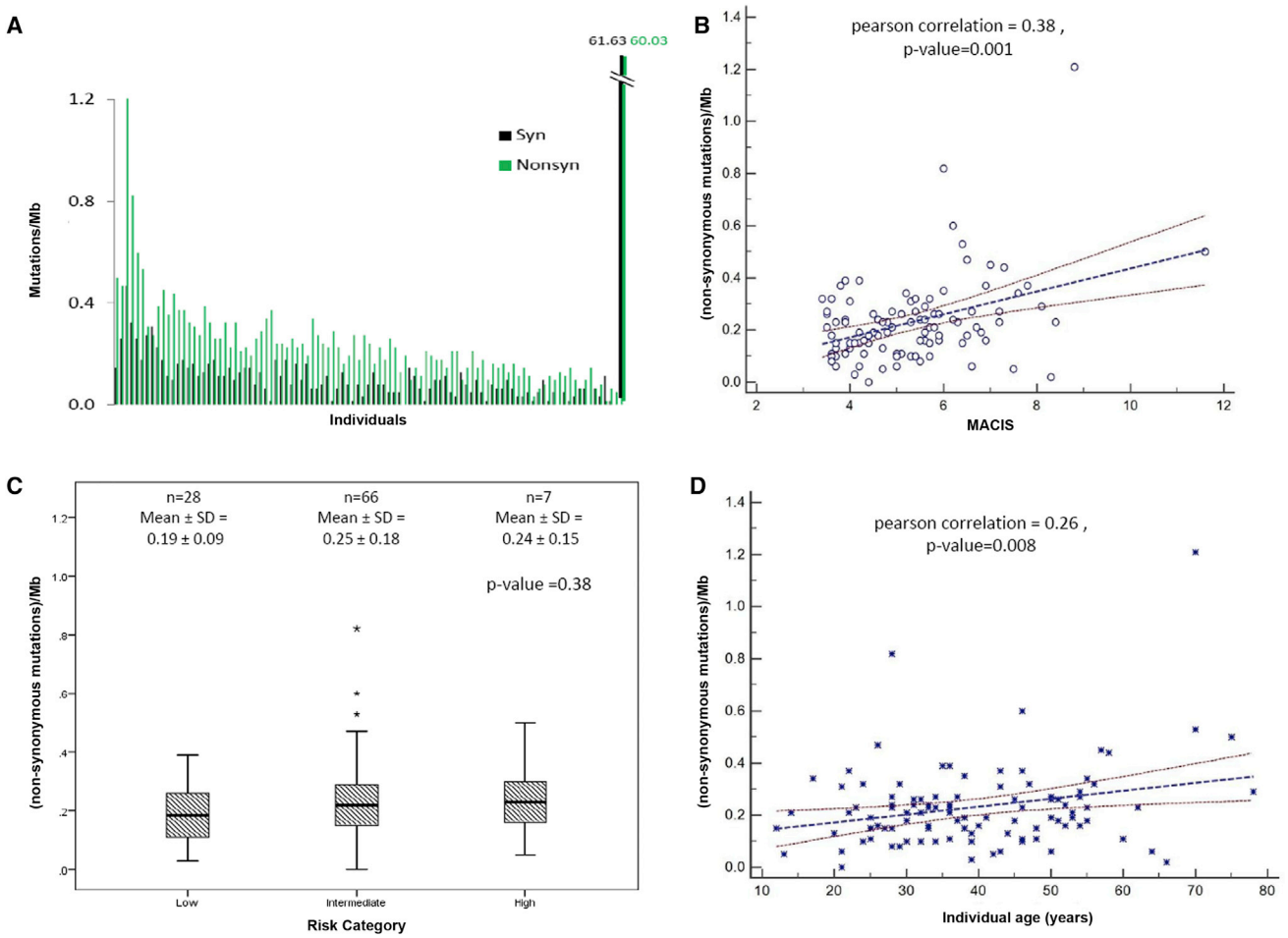

Figure 1. Correlation of Somatic Mutation Density with Clinical Parameters
(A) Somatic mutation density (in MB) in the 101 PTC exome cohort. The maximum mutation density is 1.2 MB, and the median is 0.21 nonsynonymous somatic mutations per MB. Synonymous mutation density is compared with non-synonymous mutation density.
(B) Correlation of non-synonymous mutation density with MACIS score for mortality risk, which is significant ($p = 0.001$).
(C) Correlation of non-synonymous mutation density with risk of recurrence ($p = 0.38$).
(D) Correlation of non-synonymous mutations with individual's age ($p = 0.008$).
One outlier case was excluded for figure presentation in (B), (C) and (D). P values were estimated by Student's t test.

were called with MuTect,[34] and putative somatic indels were called with VarScan2 (http://varscan.sourceforge.net); tumor-normal bam files were used as input. The identified variants were annotated with ANNOVAR.[35] We only considered variants that passed the standard Mutect and VarScan2 filters, and we excluded common SNPs with minor allele frequency of > 0.001 as recorded in dbSNP, the NHLBI exome sequencing project, 1000 Genomes, and our in-house data from exome sequencing of around 700 normal samples. We also excluded variants in non-coding regions, synonymous variants, and variants present in highly repetitive regions. In addition, manually checking the mutations with the Integrated Genomics Viewer filtered out the false positives. Similar filters were also applied to the cohort of 785 capture sequencing samples.

Results

PTC Is a Relatively Quiet Tumor

Compared to other cancers, the thyroid cancer genome has been shown to be relatively quiet and to involve fewer genetic mutations, which might explain why the disease is often slow growing.[15] PTC in our cohort of 101 cases showed on average 0.82 nonsynonymous somatic mutations per Mb. Surprisingly, one of the 101 samples accounted for nearly 83% of all somatic mutations (16,821, compared to a total of 20,118 from all samples combined). When this sample was excluded, the average somatic mutation rate dropped to 0.23 nonsynonymous somatic mutations per Mb, which is comparable to what was reported by the Cancer Genome Atlas Research Network (0.41) and comparatively lower than the rate found in other cancers (bladder = 7.1, breast = 1.2, colorectal = 3.1, head and neck = 3.9, and ovarian = 1.7)[36] (Figure 1A). The minimum density in our cohort was 0.00/MB, the maximum was 1.21/MB, and the median was 0.21/MB). Mutation density was associated with age ($p = 0.008$) and mortality risk (as measured by MACIS score; $p = 0.001$) but not risk of recurrence (ATA guideline 2009) ($p = 0.38$) (Figures 1B–1D).

Mutations in MAPK-Signaling-Pathway Genes Play a Major Role in PTC Pathogenesis

The role of the MAPK pathway in tumorigenesis of differentiated thyroid cancer is well known, and this pathway is dysregulated in most cases of thyroid cancer.[37] This pathway is mainly activated in thyroid cancer by mutations of *BRAF* and *RAS* (*HRAS, NRAS*, and *KRAS*) or rearrangements of *RET-PTC*, *PAX8* (MIM: 167415), and/or *PPARγ* (MIM: 601487).[38] In more than 95% of cases, the activating point mutation of *BRAF* occurs at c.1799T>A, resulting in p.Val600Glu (GenBank: NM_004333, NP_004324) substitution.[38] Recent data have also suggested that mutations in *BRAF* are associated with a more aggressive phenotype.[39] *RAS* genes (*HRAS, KRAS*, and *NRAS*) encode G proteins that signal to both the MAPK and PI3K/AKT pathways. Point mutations in the *RAS* genes typically occur in codons 12, 13, and 61 and are found in 40%–50% of follicular carcinomas and in 10%–20% of PTCs.[40–42]

In our cohort, the MAPK-pathway gene mutations are seen at a relatively high frequency of 66.6% (590/886) (Table 1). This was primarily driven by *BRAF* mutation, the frequency of which was 59.5% (521/875). *BRAF* mutation demonstrated strong association with adverse clinical parameters such as old age ($p < 0.001$), tall-cell variant ($p < 0.001$), extra-thyroidal extension ($p < 0.001$), positive surgical margin ($p < 0.001$), lymph-node metastasis ($p < 0.001$), and stage IV disease ($p < 0.001$). The second most frequently mutated class of genes was *RAS* genes (Table 1). *NRAS* was mutated in 5.4% of all cases (48/876) and showed strong association with follicular variants of PTC ($p < 0.001$). Mutual exclusivity was observed between *NRAS* mutations and both lymph-node metastasis ($p < 0.001$) and extra-thyroidal extension ($p = 0.001$). *HRAS* mutations were seen at a frequency of 2.7% (24/886) and showed an inverse association with lymph-node metastasis ($p = 0.02$), extra-thyroidal extension ($p = 0.02$), and surgical-margin positivity ($p = 0.02$). Both *HRAS* and *NRAS* were mutually exclusive with *BRAF* mutations ($p < 0.001$).

Identification of Candidate Genes in PTC Pathogenesis

We analyzed WES from PTC and matched normal tissue to search for genes with at least two somatic mutations in our cohort of 101 samples. In addition to the MAPK-signaling-pathway genes mentioned above (*BRAF*, *NRAS*, and *HRAS*), we identified 21 genes (Table 1) that are recurrently mutated in PTC tissues. In order to investigate a potential role of these genes in the pathogenesis of PTC, we set out to validate them in a replication cohort of 808 PTC individuals by capturing their exons and flanking intronic sequences and then performing next-generation sequencing. DNA quality was suboptimal for capture sequencing in 23 samples, so we excluded these and proceeded with the analysis of 785 samples. 15 out of the 21 genes had at least one additional somatic mutation in the replication cohort (Table 1 and Figure S1). By combining the two cohorts, we identified recurrent mutations in *TG* (3.05%), *TP53* (0.9%), *DNMT3A* (1.47%) (MIM: 602769), and several candidate genes, including *PKHD1* (1.14%) (MIM: 606702), *SPTBN5* (1.14%) (MIM: 605916), *DNAH1* (0.79%) (MIM: 603332), *CABIN1* (0.68%) (MIM: 604251), and *MACC1* (0.56%) (MIM: 612646) (Table 1 and Figure 2).

TG Somatic Mutations Predict Poor Outcome and Might Drive Metastatic Potential

We were particularly intrigued by the finding that *TG* is recurrently mutated in PTC because of reports of thyroid cancer in individuals with germline mutations in *TG* with associated congenital hypothyroidism.[43,44] First, we set out to investigate the clinical relevance of *TG* somatic mutations. In our cohort, 27 mutant cases were identified; 20 (74.1%) carried frameshift or splicing site mutations. Seven (15.9%) cases harbored missense mutations. The sequencing traces of three representative mutations, c.2241dupT; p.Asn748*, c.1548_1550delAGA; p.Glu516del, and c.4529+1_2del (NM_003235) are presented in Figure S2. Although only 3.05% of the entire cohort harbored somatic mutations in *TG* (Table 1 and Figure S3), these individuals had a significantly poorer clinical outcome ($p < 0.001$) (Table S1) and were significantly associated with aggressive clinical parameters such as old age ($p = 0.01$), recurrence ($p = 0.007$), metastasis ($p < 0.001$), and stage IV ($p = 0.006$) (Table S2). We then asked whether this association was independently driven by *TG* mutations rather than coexisting driver mutations because 77.7% (21/27) of *TG* mutation-positive PTC samples had co-existing mutations in MAPK-pathway genes. Specifically, we compared the clinical outcome of individuals with coexisting mutations in *TG* and MAPK-pathway genes to those with mutations in MAPK-pathway genes only and found the former to be associated with significantly poorer outcome ($p < 0.001$) (Figure 3). The observation that *TG* mutations are mostly observed in the context of mutations in MAPK-pathway genes and that their presence significantly worsens the clinical outcome raises the intriguing possibility that *TG* mutations might not drive the initial oncogenesis of PTC but instead contribute significantly to its malignant evolution. In particular, distant metastasis, an already rare occurrence in PTC, was significantly more common in *TG*-mutation-positive individuals than in those who lacked these mutations ($p < 0.001$). In order to test this hypothesis, we selectively analyzed an independent cohort of 71 PTC-affected individuals with documented distant metastasis and found that the frequency of somatic *TG* mutations increases by more than 4-fold in these individuals as compared to the original unselected cohort (12.7% versus 3.05%, $p < 0.001$). Based on these results that suggest a link between *TG* mutations and distant metastasis, we set out to fully sequence *TG* in distant metastases (brain, kidney, and bone) from 11 PTC-affected individuals for whom distant

Table 1. Recurrent Somatic Mutations from Exome and Gene Panel Sequencing

		Exome 101 Cohort				Gene Panel 785 Cohort				Exome and Gene Panel			
Serial Number	Gene	Number of Mutations	Number of Samples	Total	Frequency	Number of Mutations	Number of Samples	Total	Frequency	Number of Mutations	Number of Samples	Total	Frequency
1	*BRAF*	60	60	101	59.41	464	461	774	59.56	524	521	875	59.54
2	*NRAS*	1	1	101	0.99	47	47	775	6.06	48	48	876	5.48
3	*TG*	2	2	101	1.98	27	25	785	3.18	29	27	886	3.05
4	*HRAS*	2	2	101	1.98	22	22	785	2.8	24	24	886	2.71
5	*DNMT3A*	4	4	101	3.96	9	9	785	1.15	13	13	886	1.47
6	*PKHD1*	3	3	101	2.97	7	7	780	0.9	10	10	881	1.14
7	*SPTBN5*	2	1	101	0.99	10	9	781	1.15	12	10	882	1.13
8	*TP53*	2	2	101	1.98	6	6	785	0.76	8	8	886	0.9
9	*DNAH1*	2	2	101	1.98	5	5	782	0.64	7	7	883	0.79
10	*CABIN1*	2	2	101	1.98	4	4	781	0.51	6	6	882	0.68
11	*MACC1*	2	1	101	0.99	5	4	785	0.51	7	5	886	0.56
12	*GPR98*[a]	2	2	101	1.98	3	3	785	0.38	5	5	886	0.56
13	*SNED1*	2	2	101	1.98	2	2	780	0.26	4	4	881	0.45
14	*TRIOBP*	2	1	101	0.99	3	3	781	0.38	5	4	882	0.45
15	*LTBP2*	3	1	101	0.99	5	3	782	0.38	8	4	883	0.45
16	*CAMKK2*	2	2	101	1.98	2	2	785	0.25	4	4	886	0.45
17	*ATP8B2*	2	1	101	0.99	2	2	785	0.25	4	3	886	0.34
18	*MLXIPL*	2	2	101	1.98	0	0	783	0	2	2	884	0.23
19	*GLRA3*	2	1	101	0.99	1	1	784	0.13	3	2	885	0.23
20	*NEFH*	2	2	101	1.98	0	0	784	0	2	2	885	0.23
21	*ZNF677*	2	1	101	0.99	0	0	783	0	2	1	884	0.11
22	*DENND4B*	2	1	101	0.99	0	0	784	0	2	1	885	0.11
23	*CRTAM*[b]	2	1	101	0.99	0	0	785	0	2	1	886	0.11
24	*SMU1*	2	1	101	0.99	0	0	785	0	2	1	886	0.11

[a]Not validated by Sanger sequencing.
[b]Two hits in the same codon in exome data.

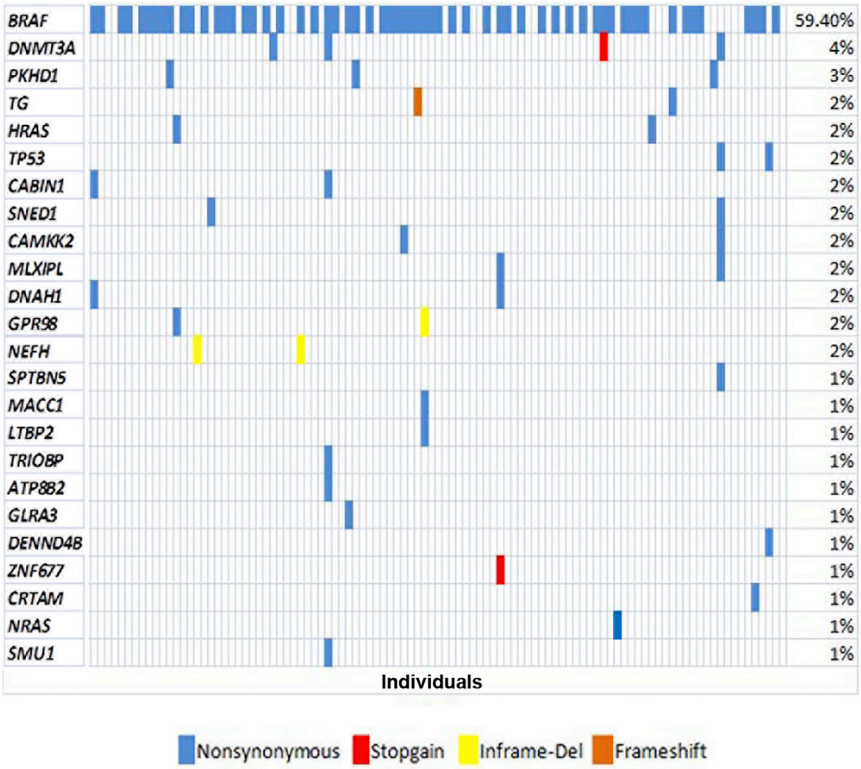

Figure 2. Frequency and Types of Mutation in 24 Genes Detected by Exome Sequencing

Different types of mutations are colored differently. The majority of mutations are missense (blue); there are two stop-gains (red), three in-frame deletions (yellow color), and one frame-shift mutation (brown). The genes with a frequency of 1% share two somatic mutations except that *CRTAM* shows two single nucleotide changes within the same codon.

metastatic tissue samples were available to us. Strikingly, the frequency of *TG* somatic mutations was much higher in this subgroup (36%) than in the overall cohort (3.05%) ($p < 0.001$) (Figure 4). Importantly, our analysis of the four metastatic samples with *TG* somatic mutations revealed prominent heterozygous peaks, indicating that most of the cells in the distant metastasis harbored these mutations (Figure 5). This is particularly important in that the corresponding primary PTC tissue was negative for these *TG* mutations in three cases (Figure 4A). In order to further investigate the possibility that metastatic tissues are clonal expansions of cells with *TG* mutation, we carefully analyzed the next-generation-sequencing reads of the corresponding *TG* segment in the corresponding primary PTC in those three cases and were able to detect the *TG* mutation at an extremely low frequency in one case (Figure S4).

Discussion

Cancer genomics is one of the most promising applications of personalized medicine: the traditional categorization of cancers based on their histopathological appearance is replaced by genomic profiling aimed at identifying the unique molecular signature that characterizes each tumor and ultimately devising personalized targeted therapy. Most previous studies of PTC genetics suffer from the inherent bias of the search for somatic mutations in pre-selected genes. As shown by The Cancer Genome Atlas study, a hypothesis-free genomics approach is needed for the identification of candidate driver mutations in order to better inform the design of targeted therapeutics.[15]

Using a similar genomics approach on a relatively large collection of PTC-affected individuals from a country with one of the world's highest incidence of the disease allowed us to unravel important molecular characteristics. Consistent with our previous experience with gene-level analysis of PTC in Saudi Arabia, we showed that the MAPK signaling pathway is an important player in the pathogenesis of PTC and that somatic mutations in its various components probably drive the oncogenic process. We have shown previously the synergistic effect of *PIK3CA* alteration on the MAPK pathway in tumorigenesis of PTC and the possibility of targeting this pathway for effective anticancer therapy[26]. The role of the MAPK pathway in thyroid carcinogenesis has previously been reported in aggressive histological subtypes such as anaplastic thyroid cancer. Liu et al.[45] have shown that up to 95.8% of anaplastic thyroid carcinomas harbor at least one genetic alteration in genes of the receptor tyrosine kinase (RTK), PI3K/AKT, and MAPK pathways. The same group earlier showed that PTC develops de novo after oncogenic alteration of MAPK pathway genes and, moreover, that its progression to aggressive anaplastic thyroid carcinoma variant is facilitated by activation of the PI3K/AKT pathway.[46]

In addition to the recurrently mutated MAPK pathway genes in our PTC cohort, *TG* was found to be recurrently mutated (3.05%), as were *TP53* (0.9%), *DNMT3A* (1.47%), and a number of candidates, including *PKHD1* (1.14%), *CABIN1* (0.68%), and *MACC1* (0.56%). *TP53* is one of the most frequently mutated genes in human cancers,[47] and a role for *TP53* mutations in aggressive transformation of thyroid cancer has been proposed.[48] A high *TP53* mutation rate of 3.5% (2/57) has been reported in PTC on the basis of targeted next-generation sequencing of a small cohort.[20] The lower frequency observed in our study (0.9%), which is similar that observed in the Cancer

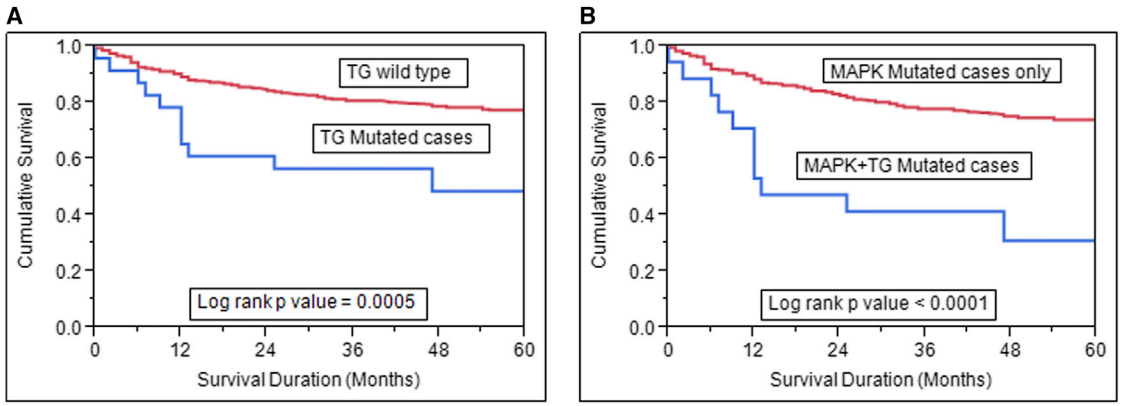

Figure 3. Survival Analysis of Individuals Cohort
Kaplan Meier survival plot showing statistically significant poor survival of (A) *TG*-mutated cases compared to *TG* wild-type cases in the overall cohort ($p = 0.0005$) and (B) significant poor survival of *TG*-mutated cases in the MAPK-positive cohort as compared to MAPK-positive individuals without *TG* mutation ($p < 0.0001$).

Genome Atlas Research Network study (0.7%), is probably more representative in light of the larger sample size. No significant difference was observed between the recurrent genes in our cohort and the TCGA gene list except for *NRAS*, which was less frequently mutated in our cohort, as shown in Table S3.

The role of *DNMT3A* is well documented and studied in hematological malignancies such as acute myeloid leukemia (AML [MIM: 601626]) and myelodysplastic syndromes (MDS [MIM: 614286]).[49,50] Somatic mutation of *DNMT3A* is predicted to have a poor outcome in individuals with MDS.[51] Mutations in this gene have also been reported in other solid tumors, such as lung, breast, prostate, colorectal, and gastric carcinomas.[52] In our cohort, somatic *DNMT3A* mutations were significantly associated with adverse clinicopatholgical parameters such as old age ($p = 0.002$), extra-thyroidal extension ($p = 0.002$), and late-stage tumors ($p = 0.001$). Although germline mutations in *PKHD1* cause autosomal-recessive polycystic kidney disease (ARPKD [MIM: 263200]), it is considered one of the candidate cancer genes for colorectal cancer,[53] and a role in pancreatic ductal adenocarcinoma has been documented.[54] *PKHD1* mutations in our PTC cohort were correlated with adverse clinicopatholgical parameters such as old age ($p = 0.001$) and late-stage tumors ($p = 0.009$). Calcineurin-binding protein 1 (*CABIN1*) has been shown to be a negative regulator of *TP53*.[55] This gene was mutated in 0.7% (6/881) of our PTC cohort and was significantly associated with adverse clinicopatholgical parameters such as old age ($p = 0.01$) and late-stage tumors ($p = 0.003$). *CABIN1* has been identified by gene-expression profiling as a discriminating gene that predicts tumor recurrence in stage III colorectal cancers.[56] It is also one of the cisplatin-resistance genes (CRA) responsible for chemoresistance to cisplatin therapy and is a potential candidate for targeted therapies for counteracting cancer chemotherapy resistance.[57] The gene encoding Metastasis Associated Colon Cancer 1 (*MACC1*) was mutated in 0.7% (5/885) of our PTC cohort. *MACC1* is a candidate gene that was first discovered in 2009 as a prognostic marker and predictor of metastasis in colorectal cancer.[58] It was later shown that this gene plays a significant role in a number of other cancers, such as ovarian, liver, pancreatic, lung, breast, and gastric cancers; esophageal osteosarcoma; and glioma.[59–67] This gene is not known to play a role in thyroid cancer at this time.

One particularly intriguing finding of ours pertains to *TG*. Several reports have highlighted the oncogenic potential of congenital hypothyroidism-associated germline *TG* mutations.[44] In this study, we found that somatic mutations in *TG*, although uncommon (3.05%), appear to exert significant effect on the evolution of PTC. The observation that most *TG* mutation-positive PTC samples also harbored mutations in genes encoding components of the MAPK signaling pathway suggests that *TG* mutations are not driver mutations per se. However, we show that these *TG* mutations were nonetheless associated with a significantly worse clinical outcome than were driver mutations in the absence of *TG* mutations. This is consistent with a role of *TG* mutations in driving the evolution of PTC to a more aggressive phenotype. Consistent with this notion is our finding that *TG* mutations appear to be enriched in metastatic PTC compared to the localized PTC. Metastasis in PTC is a very rare event, as can be observed from our own cohort, where only 47 (5.3%) of the 909 were diagnosed with distant metastasis. The molecular underpinning of distant metastasis is poorly understood in cancers in general but is presumed to involve additional mutational events that endow a subpopulation of the primary cancer cells with new properties that allow them to detach, migrate, invade, and seed in distant locations.[68] Our finding that *TG*-mutation-positive metastasis appear to have the *TG* mutation in the entire population of tumor cells, whereas the corresponding primary tissue can be negative for the same mutation is highly suggestive that the *TG* mutation is one such event that endows a few cells with the metastasis-conducive new properties. Further support for this notion can be gleaned from the

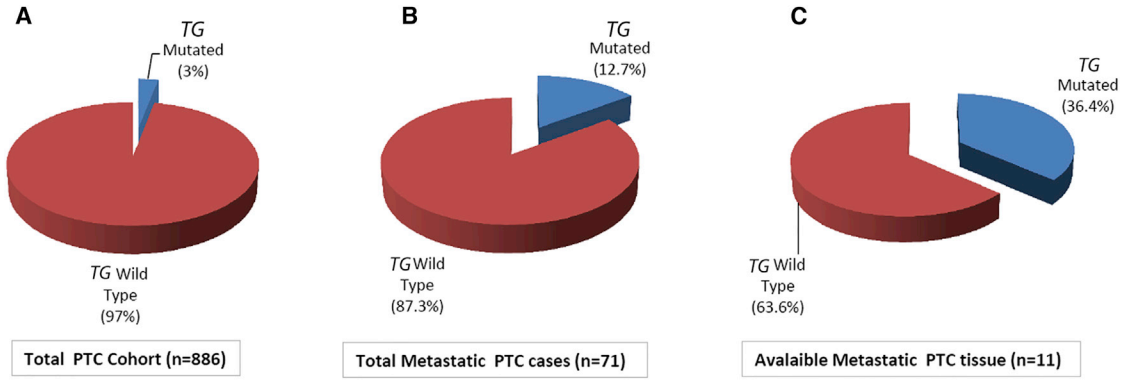

Figure 4. Increasing Proportion of *TG*-Mutated Cases in a Different PTC Cohort
(A) *TG* mutation in overall PTC cohort (3%).
(B) *TG* mutation in an independent metastatic PTC cohort (12.7%).
(C) *TG* mutation in available metastatic tissues (36.4%).

one individual in whom a somatic mutation in *TG* was present at an extremely low level in the primary tumor but was nearly homogeneous in the distant metastasis. These results suggest a model wherein *TG* somatic mutations are rare events in PTC but when present greatly enhance the metastasis potential of the tumor. If confirmed by future studies, this model will have important and clinically relevant consequences, not the least of which is the potential of deep sequencing of *TG* to detect low-level mosaicism in PTC as an independent prognostic indicator of the cancer's aggressiveness, including metastasis potential.

In conclusion, we show that genomic profiling of PTC in Saudi Arabia confirms the previously reported role of mutations in MAPK pathway genes as likely drivers in the majority of cases. A few additional candidate genes were also identified; elucidation of the role of these genes in PTC pathogenesis awaits replication by future studies. Finally, we show that *TG* mutations are strongly correlated with poor clinical outcome, and although they do not appear to drive initial oncogenesis, they are likely to drive the aggressive evolution of PTC, including metastatic potential. To the best of our knowledge, this is the first report of a likely driver of metastasis in PTC.

Accession Numbers

The exome sequencing data of 101 pairs of tumors and matched normal samples have been deposited to the European Genome-phenome Archive (EGA) with accession number EGAS00001001788.

Supplemental Data

Supplemental Data include a supplemental note, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.04.014.

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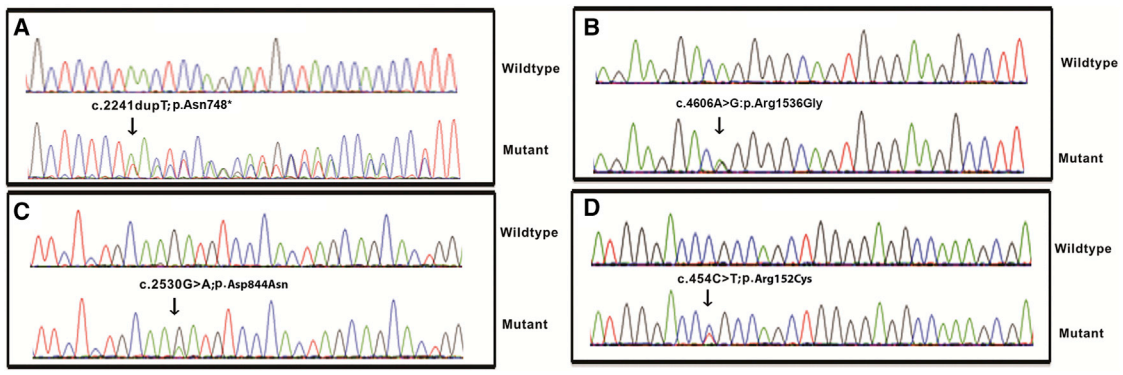

Figure 5. *TG* Mutations Identified in Metastatic Tissue Samples
Sequencing traces of four *TG* mutations identified in metastatic tissue samples. Three mutations, c.2530G>A (B), c.4604A>G (C), and c.454C>T (D), were not detected in corresponding PTC primary tumor tissue by Sanger sequencing. Lower panels show sequencing traces of mutations identified in metastatic tissue samples, and top panels show sequencing traces for corresponding normal examples.

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Web Resources

1000 Genomes, http://www.1000genomes.org/
CADD, http://cadd.gs.washington.edu/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
Ensemble, http://www.ensembl.org/
Exome Variant Server, http://evs.gs.washington.edu/
Integrated Genomics Viewer, https://www.broadinstitute.org/igv/
OMIM, http://www.omim.org/
UCSC, https://genome.ucsc.edu/

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