Whole-Exome Sequencing Study of Thyrotropin-Secreting Pituitary Adenomas

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Context: Thyrotropin (TSH)-secreting pituitary adenomas (TSHomas) are a rare cause of hyperthyroidism, and the genetic aberrations responsible remain unknown.

Objective: To identify somatic genetic abnormalities in TSHomas.

Design and Setting: A single-nucleotide polymorphism (SNP) array analysis was performed on 8 TSHomas. Four tumors with no allelic losses or limited loss of heterozygosity were selected, and whole-exome sequencing was performed, including their corresponding blood samples. Somatic variants were confirmed by Sanger sequencing. A set of 8 tumors was also assessed to validate candidate genes.

Patients: Twelve patients with sporadic TSHomas were examined.

Results: The overall performance of whole-exome sequencing was good, with an average coverage of each base in the targeted region of 97.6%. Six DNA variants were confirmed as candidate driver mutations, with an average of 1.5 somatic mutations per tumor. No mutations were recurrent. Two of these mutations were found in genes with an established role in malignant tumorigenesis (SMOX and SYTL3), and 4 had unknown roles (ZSCAN23, ASTN2, R3HDM2, and CWH43). Similarly, an SNP array analysis revealed frequent chromosomal regions of copy number gains, including recurrent gains at loci harboring 4 of these 6 genes.

Conclusions: Several candidate somatic mutations and changes in copy numbers for TSHomas were identified. The results showed no recurrence of mutations in the tumors studied but a low number of mutations, thereby highlighting their benign nature. Further studies on a larger cohort of TSHomas, along with the use of epigenetic and transcriptomic approaches, may reveal the underlying genetic lesions.

*These authors contributed equally to this work.

Abbreviations: cnLOH, copy number-neutral loss of heterozygosity; LOH, loss of heterozygosity; SNP, single-nucleotide polymorphism; SNV, single-nucleotide variant; TSG, tumor suppressor gene; TSH, thyrotropin; TSHoma, thyrotropin-secreting pituitary adenoma; WES, whole-exome sequencing.

Thyrotropin (TSH)-secreting pituitary adenomas (TSHomas) account for 0.5% to 2.8% of all pituitary adenomas, and an increasing number of these tumors have been reported during the last decade (1, 2). Although goiter and hyperthyroidism are hallmark features in patients with these tumors, many plurihormonal tumors also present with the features of excess growth hormone and prolactin (3, 4). Similarly, because most of these tumors are macroadenomas and are invasive, neurologic features, such as ocular symptoms and headaches due to mass effects, also cause considerable morbidity (4). Surgery remains the treatment of choice; however, surgical resection is often incomplete for macroadenomas, and, consequently, most research recently performed has focused on medical management (5–7). Therefore, elucidating the genetic events that underlie TSHomas could lead to advances in their management.
The molecular mechanisms underlying tumorigenesis in TSHomas have not yet been clarified. Although these tumors are considered to be monoclonal in origin (8), no intrinsic genetic defects in common proto-oncogenes or tumor suppressor genes (TSGs) leading to tumor initiation or promotion have been identified. Germ-line mutations in the AIP and MEN1 genes have been reported (9, 10); however, such familial occurrence constitutes only a small number of cases, with most of these tumors being sporadic. Similarly, although candidate gene screening approach using polymorphic markers have detected loss of heterozygosity (LOH) at the MEN1 gene locus (11q13) in a small number of TSHomas, a concurrent mutation in the MEN1 gene was not found in the same samples (11). Furthermore, mutant isoforms of thyroid hormone receptors were searched as possible candidate oncogenes to explain the refractoriness of these tumors to the inhibitory effects of triiodothyronine; however, mechanisms other than mutations were more likely to account for the phenomenon of inappropriate TSH secretion in these tumors (12, 13). Moreover, in contrast to somatotropinomas, in which somatic mutations in the α-subunit of the stimulatory G (GNAS) gene have been detected in up to 40% of tumors (14–16), screening for the G protein subunits Gaα, Ga11, and Gαs, as well as the thyrotropin (TSH)-releasing hormone receptor among TSHomas, did not reveal the activating mutation (17). More recently, mutations in an orphan G protein–coupled receptor, GPR101, and the deubiquitination gene, USP8, have been reported in somatotropinomas and Cushing disease, respectively (18, 19). However, USP8 and GPR101 mutations in TSHomas have not yet been reported. Besides mutations, several chromosomal aberrations have been described in various pituitary tumors using comparative genomic hybridization as well as whole-genome sequencing and single-nucleotide polymorphism (SNP) array technology (15, 20). However, genetic studies involving TSHomas are limited or have been conducted by using only a small number of isolated markers.

The use of an array-based SNP analysis is an efficient method for a genome-wide copy number analysis as well as for the detection of cryptic chromosomal changes, such as LOH; it has also been successfully used for a wide range of tumors (21–23). Similarly, whole-exome sequencing (WES) is a well-validated approach to identify mutations and has been used in several tumor types, including pituitary adenomas (24). In the current study, we conducted a combinatorial approach using an SNP array analysis and WES to identify the genetic landscape of TSHomas. Using WES, we identified 6 candidate mutations. Although these mutations were not recurrent, considering the rarity of TSHomas, we attempted to highlight the somatic landscape of these very rare tumors, and our results provide important information for future studies directed toward detecting tumorigenesis in these tumors. Furthermore, focal as well as chromosomal arm-length copy number gains were frequent and were a more recurrent finding than losses. In addition, we found several broad regions of copy number–neutral loss of heterozygosity (cnLOH).

Materials and Methods

Patient material

Twelve patients with TSHomas were included in the current study (Table 1). Written informed consent was obtained from all participants, and the Ethics Committee of Gunma University and Toranomon Hospital approved the study. Hormonal studies on all patients showed elevated free triiodothyronine and free thyroxine levels; 7 patients had elevated TSH levels. Seven of the resected tumors were microadenomas, and 5 were macroadenomas. All tumors were histologically confirmed as TSHomas and preserved in RNAlater (ThermoFisher Scientific, Waltham, MA) immediately after surgery. The first set of 8 tumor samples was randomly selected for the SNP array. Four of these samples, which had no or a limited region of LOH, constituted the discovery set for WES. The remaining 4 tumor samples and 4 additional samples (8 tumor samples total) constituted the validation set.

Isolation of genomic DNA

DNA was isolated from tumor and blood samples by using the commercially available Qiagen tissue kit (Qiagen, Hilden, Germany) and a Genomix kit (Austin, TX), respectively, according to the manufacturer’s guidelines. All specimens were quantified by using PicoGreen® (ThermoFisher Scientific).

SNP array analysis

The HumancytoSNP-12v2.1 BeadChip kit (Illumina Inc., San Diego, CA) was used to perform a genome-wide SNP array. Genomic DNA from tumors was hybridized to the BeadChip by using the Illumina protocol, and arrays were imaged by using the Illumina HiScan system. Manufacturer-provided cluster files were used to make genotype calls, and data were analyzed by examining the allelic composition and signal intensity.

Whole-exome capture, sequencing, and bioinformatics analysis

Exome enrichment was performed by using the SureSelect Human All Exon V5 target enrichment technique (Agilent Technologies, Santa Clara, CA), and libraries were prepared according to the standard Illumina protocol for paired-end sequencing. Sequencing was performed on the Illumina HiSeq2000 platform outputting 100–base pair reads. Sequencing data were aligned to hg19/GRCh37 using the Burrows-Wheeler aligner (version 0.7.8). Single-nucleotide variants (SNVs) and small insertions and deletions (indels) were identified by using SomaticSniper, version 1.0.3 (The Genome Institute at Washington University School of Medicine, St. Louis, MO), and GATK SomaticIndel Detector, version 1.6 (Broad Institute, Cambridge, MA), respectively.
Sequence validation

Tumor-specific variants were confirmed by Sanger sequencing using the BigDye Terminator, version 3.1, cycle sequencing kit (ThermoFisher Scientific) and ABI 3730 automated capillary sequencer (ThermoFisher Scientific). The confirmed DNA variants were further assessed in a validation set of 8 tumors.

The method used in this study is described in detail in the Supplemental Materials and Methods.

Results

Analysis of chromosomal copy number alterations

The overall pattern of copy number alterations found by using the SNP array analysis among TSHomas is shown in Fig. 1. Copy number changes involving the whole chromosome (the entire p and q arm) signifying an aneuploidy event were common, with 62.5% of tumors showing at least 1 whole chromosome copy number gain or loss (Supplemental Table 1). Similarly, a long single stretch of copy number alterations involving the gain of an entire arm of the chromosome (the entire p or q arm), referred to here as a chromosomal arm-length gain, was most frequent on 4p, 5p, 7, and 19q (50%, 4 of 8 samples), followed by 4q, 15q, 16p, 19p, and 21q (37.5%, 3 of 8 samples) (Supplemental Table 2). Copy number gain at 15q is of particular interest because it harbors the locus for the USP8 gene (19). In contrast to gains, chromosomal arm-length losses were infrequent and noted only on the chromosomal arms 18p and 18q (tumor sample 1).

Regional analysis of copy number changes

In a regional analysis of copy number alterations, 106 regions (range, 0 to 41 per sample) of focal (less than the chromosomal arm-length) gains were found. All focal gains are presented in Supplemental Table 3. Tumor sample 5 showed the highest number of focal gains because of the high number of these events, particularly on chromosome 1p and chromosome 2. Chromosome 1p on this sample harbored 9 regions, and chromosome 2 had 22 regions of focal gains mainly clustered in its p arm; all these closely spaced focal gains were the gain of a single copy number (Supplemental Tables 3 and 4). This pattern resembles the SNP array-based finding of the chromothripsis-like pattern (23, 25). In addition, multiple loci of recurrent gains were detected. Although most of these gains were a single copy gain, the recurrent gain of 2 copies was observed across 7 loci (1q31.1-32.1, 1q41, 1q43, 7p21.3-21.2, 7p12.1-11.2, 7q21.11-21.13, and 13q31.1-31.3) (Supplemental Table 4). Several of these loci harbor genes for micro-RNA; importantly, recurrently targeted loci 1q31.1-32.1, involved in copy number gains in 50% of our tumors, harbored the BRINP3 gene, which was previously reported in relation to gonadotrope-cell pituitary adenoma (26). Similarly, the focal gain at chromosome 20q (position: 31733956-58602753) on

Table 1. Clinical Details of All Patients With TSHomas

<table>
<thead>
<tr>
<th>Tumor Sample No.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Tumor Size</th>
<th>Histology (Immunostaining)</th>
<th>MIB-1 (%)</th>
<th>TSH&lt;sup&gt;a&lt;/sup&gt; (pg/mL)</th>
<th>FT&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (pg/mL)</th>
<th>FT&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (ng/dL)</th>
<th>GH&lt;sup&gt;d&lt;/sup&gt; (ng/mL)</th>
<th>PRL&lt;sup&gt;e&lt;/sup&gt; (ng/mL)</th>
<th>Preoperative Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>M</td>
<td>Micro</td>
<td>TSH, PRL</td>
<td>2</td>
<td>3.584</td>
<td>10.46</td>
<td>2.24</td>
<td>&lt;0.1</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>M</td>
<td>Micro</td>
<td>TSH, GH</td>
<td>&lt;1.0</td>
<td>4.564</td>
<td>5.76</td>
<td>2.01</td>
<td>&lt;0.1</td>
<td>10.8</td>
<td>SSA</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>F</td>
<td>Micro</td>
<td>TSH, GH, PRL</td>
<td>0.3</td>
<td>1.892</td>
<td>4.83</td>
<td>1.75</td>
<td>3.9</td>
<td>17.9</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>M</td>
<td>Macro</td>
<td>TSH, PRL</td>
<td>1</td>
<td>10.565</td>
<td>7.88</td>
<td>1.84</td>
<td>1</td>
<td>51.4</td>
<td>SSA</td>
</tr>
<tr>
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<td>Micro</td>
<td>TSH</td>
<td>1.5</td>
<td>4.976</td>
<td>5.1</td>
<td>1.77</td>
<td>&lt;0.1</td>
<td>19.6</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
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<td>F</td>
<td>Micro</td>
<td>TSH</td>
<td>&lt;1.0</td>
<td>2.28</td>
<td>7.3</td>
<td>3.4</td>
<td>0.6</td>
<td>14.9</td>
<td>SSA</td>
</tr>
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<td>7</td>
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<td>F</td>
<td>Micro</td>
<td>TSH, GH</td>
<td>0.2</td>
<td>5.22</td>
<td>4.93</td>
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<td>0.3</td>
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<tr>
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<td>2.349</td>
<td>5.72</td>
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<td>0.2</td>
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</tr>
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<td>9</td>
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<td>Macro</td>
<td>TSH, PRL</td>
<td>*</td>
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<td>Macro</td>
<td>TSH, PRL</td>
<td>*</td>
<td>1.52</td>
<td>4.22</td>
<td>1.94</td>
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<td>17.1</td>
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</tr>
<tr>
<td>11</td>
<td>28</td>
<td>F</td>
<td>Macro</td>
<td>TSH</td>
<td>*</td>
<td>5.45</td>
<td>28.33</td>
<td>7.77</td>
<td>0.87</td>
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<td>SSA</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>F</td>
<td>Micro</td>
<td>TSH</td>
<td></td>
<td>8.88</td>
<td>5.56</td>
<td>2.49</td>
<td>0.56</td>
<td>17</td>
<td>SSA</td>
</tr>
</tbody>
</table>

—Patients 1, 3, and 5 did not receive SSA or DA before surgery. *MIB-1% data not available for tumor samples 9–12.

Abbreviations: DA, dopamine agonist; F, female; GH, growth hormone; M, male; MIB-1%, cell proliferation marker-labeling index; PRL, prolactin; SSA, somatostatin analog.

<sup>a</sup>Reference range: 0.54–4.26 μIU/mL.

<sup>b</sup>Reference range: 2.29–4.17 pg/mL.

<sup>c</sup>Reference range: 0.72–1.52 ng/dL.

<sup>d</sup>Reference range: <2.1 ng/mL.

<sup>e</sup>Reference range: 4.4–31.2 ng/mL.
tumor sample 7 harbored the GNAS gene, which together with the 20q chromosomal arm-length gain on tumor samples 1 and 8 represent important loci involved in the copy number gain in 37.5% of tumor samples.

In contrast to focal gains, only 2 tumors showed focal losses. Hemizygous deletion leading to focal loss was identified in tumor sample 4 (chromosome 15) and tumor sample 8 (chromosome 22) (Figs. 1 and 2 and Supplemental Table 5).

Copy number-neutral LOH
Several broad regions of LOH without any change in the copy number were found in our SNP array analysis. These areas of copy number-neutral LOH (cnLOH) were common, with 62.5% of TSHomas harboring at least 1 cnLOH event. Our results showed that 66 regions across 11 chromosomes ranging in size from 1.29 Mb to 67 Mb were involved in cnLOH (Figs. 1 and 2; Supplemental Table 6). As shown in Figs. 1 and 2, tumor samples 3, 6, and 7 harbored no cnLOH events, whereas tumor sample 1 had cnLOH involving 4 chromosomes and tumor samples 2, 4, and 8 had cnLOH involving 2 chromosomes. Chromosomes 5, 7, and X (tumor sample 5) and chromosome 12 (tumor sample 2) had limited cnLOH regions, and chromosomes 1, 6, 8, 10, 11, 18, and 22 had larger regions involved in cnLOH. The most common region involved in cnLOH was in chromosomes 1p and 8 (25% each, 2 of 8 samples). Recurrent cnLOH at 1p on tumor sample 1 (position: 48723161-54018483, 5.29 Mb) and tumor sample 4 (position: 38201371-54633847, 16 Mb) contained loci for the CDKN2C gene, which codes for proteins involved in cell-cycle regulation. Similarly, cnLOH at chromosome 11 (position: 55091268-73356872, 18 Mb) on tumor sample 1 harbored loci for MEN1 and AIP. The largest cnLOH event (position: 44936177-112494578, 67 Mb) on chromosome 10 in tumor sample 1 harbored 10q21.1 loci, 1 of the 3 significant susceptibility loci for sporadic pituitary adenoma reported by Zhou et al. in a genome-wide association study (27). All cnLOH events were located in regions of segmental duplication, and almost half of them were on previously identified fragile sites (28).

Because mutant allele frequencies in tumor DNA are consistent with mutations being heterozygous in nature, we included TSHoma samples with no LOH (tumor samples 3, 6, and 7; Fig. 2) or minimal LOH (tumor sample 5; Fig. 2) for the WES study. A similar approach to WES has been successfully used to study aldosterone-producing adenomas (29).

Identification of tumor-specific somatic variants in a discovery cohort
WES was performed on 4 tumors (tumor samples 3, 5, 6, and 7; Table 1) and their matched leukocyte samples.

Figure 1. Overview of the pattern of copy number alterations in TSH-secreting adenomas across autosomal chromosomes. The genomic location and size of the copy number variant and LOH events are depicted on the left side of the ideograms for tumor samples 1 to 8 (position based on GRCh37). Green bars, orange bars, and gray bars represent copy number gains, copy number losses, and copy neutral LOH events, respectively. The red mark within the ideogram represents the centromere. Copy number gains and copy number-neutral LOH were more common than copy number losses. (Note: closely spaced focal gains at chromosome 2 on tumor 5, as well as closely spaced cnLOH in tumor samples, appear in a single small bar.) The graph was plotted by using KaryoStudio software (Illumina Inc.). Chromosomal gains found in sex chromosomes are shown in Supplemental Fig. 1.
On average, 4.7 Gb of high-quality sequence data were generated per sample. Of sequence reads, 99.98% were aligned to the human reference genome (hg19/GRCh37). The average distinct coverage of each base in the targeted region was 97.63%, with 66.71% of the targets covered to a depth of 50X. The summary statistics of WES are presented in Supplemental Table 7.

Consistent with the absence of a family history, none of the matched leukocyte samples showed germline mutations in the following genes: MEN1, CDKN1B, AIP, and PRKAR1A, which are known to be associated with familial pituitary adenomas. As shown in Table 2, WES identified a total of 1003 (250.75/tumor) high-quality SNVs and tumor-specific indels, among which 108 variants were predicted to be functionally damaging variants, including 67 missense, 20 simple insertion and deletion, 11 frame-shift, 7 splice-site, and 3 nonsense variants. Seventeen of these variants were previously unreported somatic DNA variants. Because 3 of these unknown variants (HLA-DRB1, HLA-DQA1 [in tumor sample 3], and CYP21A2 [in tumor sample 5]) occurred in the highly polymorphic human leukocyte antigen region, we selected the remaining 14 genes as bearing probable candidate driver mutations. Sanger sequencing performed on these 14 variants confirmed 6 of them (Fig. 3; Supplemental Table 9), with an average of 1.5 confirmed putative somatic mutations per tumor (range, 0 to 4 per sample). All 6 somatic DNA variants were heterozygous SNVs. Five of the 6 variants (83.3%) were missense changes, whereas 1 was a nonsense variant (16.6%). Two of these variants, involving the genes ZSCAN23 and SYTL3, occurred across chromosome 6, and 1 variant each
Identification of candidate driver mutations in the validation cohort

The 6 SNVs confirmed in the discovery cohort were subjected to a validation screen in a set of 8 tumors (tumor samples 1, 2, 4, 8, and 9 to 12; Table 1) to identify recurrent mutations. Recurrent mutations were not found in the validation set of 8 TSHomas, thereby indicating that the detected mutations represent rare drivers for tumorigenesis or passenger mutations and are unlikely to represent common driver mutations in TSHomas. No mutations were found in known oncogenes, TSGs, or genes previously implicated in other pituitary adenomas. However, 2 genes mutated in tumor sample 5, namely SMOX and SYTL3, have previously been implicated in tumorigenesis and acted via oxidative DNA damage and the Rab pathway, respectively (30–32).

Integration of SNP array and WES results

As shown in Supplemental Table 10, the integration of our results from WES to the SNP array analysis revealed copy number gains on chromosome 4p (position: 48283-49553810) harboring the CWH43 gene in 50% of our genotyped samples. Similarly, the copy number gains on chromosome 9 (position: 71034203-141044489) in 37.5% of tumor samples harbored the ASTN2 gene loci. Furthermore, copy number gains at chromosomes 12 and 20, each of which was observed in 25% of our genotyped samples, harbored the loci for the R3HDM2 and SMOX genes, respectively. Although concomitant gain in copy number and mutation were observed together in the case of CWH43 in tumor sample 3, in all other tumor samples, gain in copy numbers was not observed with concomitant mutation in genes. In addition, cnLOH at chromosome 6 (position: 27601587-32435044) on tumor sample 4 harbored loci for the ZSCAN23 gene, which was involved in the mutation observed in tumor sample 7.

Table 2. Overview of Somatic DNA Variants Found in the Discovery Set of 4 TSHoma Samples

<table>
<thead>
<tr>
<th>Tumor Sample No.</th>
<th>Variant Type</th>
<th>Quality* → On Targetb → Missense</th>
<th>Nonsense</th>
<th>Frame-shift</th>
<th>Splice-Site</th>
<th>Insertions</th>
<th>Deletions</th>
<th>Total</th>
<th>Unknownd</th>
<th>Variants Confirmed</th>
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<tr>
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<td>SNV</td>
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<td>41</td>
<td>14</td>
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<tr>
<td></td>
<td>Indel</td>
<td>111</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
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<td>6</td>
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<td>Indel</td>
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<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>7</td>
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<tr>
<td>6</td>
<td>SNV</td>
<td>141</td>
<td>36</td>
<td>16</td>
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<td>0</td>
<td>0</td>
<td>16</td>
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<tr>
<td></td>
<td>Indel</td>
<td>102</td>
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<td>5</td>
<td>2</td>
<td>0</td>
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<td>9</td>
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<tr>
<td>7</td>
<td>SNV</td>
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<td>1</td>
<td>0</td>
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<td>3</td>
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<td>3</td>
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<tr>
<td>Total</td>
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<td>67</td>
<td>3</td>
<td>11</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>108</td>
<td>17</td>
</tr>
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</table>

Only the number of somatic variants at each filtering step is shown in the table, along with the variant types and functional classes. SomaticSniper was used to detect somatic SNVs, and somatic indels were called using the GATK SomaticIndel detector. Details of all 108 protein-altering variants with amino acid change further indicating the unknown variants are presented in Supplemental Table 8.

*aQuality refers to total number of high-quality somatic SNVs and tumor-specific indels.

bOn target refers to SNVs identified in the targeted region covered by the SureSelect Human All Exon V5 capture system used in this study (which captures known coding DNA sequences from the National Center for Biotechnology Information Consensus CDS Database).

*cAmino acid change refers to DNA variants with moderate to high predicted functional impacts on the encoded protein, as predicted by effect prediction tool snpEFF.

*dUnknown refers to variants not previously reported in the public databases, including dbSNP, 1000 Genome Project, and HapMap-JPT.

Involving the genes CWH43, ASTN2, R3HDM2, and SMOX occurred over chromosomes 4, 9, 12, and 20, respectively. No same mutation was confirmed with Sanger sequencing in more than 1 tumor, such that a recurrent alteration was not identified in the discovery cohort.

Although an average of 1.5 putative somatic mutations were identified per tumor, the number of mutations varied across tumor samples. As shown in Table 3, tumor sample 5 harbored 4 somatic DNA variants (SYTL3; c.158A>G, ASTN2; c.1508C>T, R3HDM2; c.1276G>A, SMOX; c.944T>C). Similarly, although tumor samples 3 (CWH43; c.1240G>A) and 7 (ZSCAN23; c.1069C>A) harbored 1 somatic DNA variant each, tumor sample 6 harbored none. Tumor sample 5, with the highest number of mutations, was the only sample who had the highest cell proliferation marker-labeling index among the samples included for the WES study.

No same mutation was confirmed with Sanger sequencing in more than 1 tumor, such that a recurrent alteration was not identified in the discovery cohort.

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Identification of candidate driver mutations in the validation cohort

The 6 SNVs confirmed in the discovery cohort were subjected to a validation screen in a set of 8 tumors (tumor samples 1, 2, 4, 8, and 9 to 12; Table 1) to identify recurrent mutations. Recurrent mutations were not found in the validation set of 8 TSHomas, thereby indicating that the detected mutations represent rare drivers for tumorigenesis or passenger mutations and are unlikely to represent common driver mutations in TSHomas. No mutations were found in known oncogenes, TSGs, or genes previously implicated in other pituitary adenomas. However, 2 genes mutated in tumor sample 5, namely SMOX and SYTL3, have previously been implicated in tumorigenesis and acted via oxidative DNA damage and the Rab pathway, respectively (30–32).
Discussion

The current study characterized the comprehensive genetic landscape of TSHomas using the combined approach of SNP array and WES. Our results suggest a lower number of somatic SNVs in TSHomas (1.5 per tumor) compared with that reported for surface-derived malignant tumors (33). However, our results are in line with previous findings among somatotropinomas (15) and nonfunctioning pituitary adenomas (24), wherein similar number of mutations per tumor were observed. This finding highlights the low mitotic activity and benign nature of TSHomas.

The results of our WES study are consistent with previous findings on pituitary adenomas with regard to the absence of mutations in established classical oncogenes and TSGs (15, 16, 24). However, in contrast to previous studies of other pituitary adenomas, we found no mutation involving the GNAS, GPR101, or USP8 genes (14–16, 18, 19). Nevertheless, the loci harboring the genes USP8 and GNAS were involved in recurrent amplification in our copy number analysis, thereby highlighting the importance of investigating these genes in future studies involving a larger cohort of TSHomas.

Although we did not find the same mutation in more than 1 tumor, 6 SNVs were identified in the genes CWH43, ZSCAN23, SYTL3, ASTN2, R3DHM2, and SMOX. Two of these genes, SMOX and SYTL3, have previously been implicated in the tumorigenesis of several cancers. SMOX is involved in polyamine metabolism,
and Cag A of *Helicobacter pylori* has been shown to induce its expression; this, in turn, leads to oxidative DNA damage and rendering cells resistant to apoptosis, and, hence, confers a high risk of gastric cancer (30). Similarly, oxidative DNA damage resulting from an increase in the expression of spermine oxidase has been described in prostate cancer (31). SYTL3 has been shown to encode synaptotagmin-like proteins, which play a role in vesicular transport with their interaction with RAB27. The deregulation of this Rab pathway along with Rab effector genes has been implicated in bladder cancer (32). Oxidative DNA damage and the Rab effector pathway both contribute to the expression of spermine oxidase, which has been implicated in prostate cancer (31).

Table 3. Details of Somatic Variants Confirmed in the Discovery Set of 4 TSHomas

<table>
<thead>
<tr>
<th>Position</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Nucleotide Change</th>
<th>Protein Change</th>
<th>Transcript</th>
<th>Mutation Type</th>
<th>Predicted Zygosity</th>
<th>Variant Type</th>
<th>Tumor Sample No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>49019319</td>
<td>CWH43</td>
<td>Cell wall biogenesis-43</td>
<td>c. G1240A</td>
<td>A414T</td>
<td>NM_025087.2</td>
<td>Missense</td>
<td>Heterozygous</td>
<td>SNV</td>
<td>3</td>
</tr>
<tr>
<td>28402343</td>
<td>ZSCAN23</td>
<td>Zinc finger and SCAN</td>
<td>c. G1069T</td>
<td>G357R</td>
<td>NM_001012455.1</td>
<td>Missense</td>
<td>Heterozygous</td>
<td>SNV</td>
<td>7</td>
</tr>
<tr>
<td>159086474</td>
<td>SYTL3</td>
<td>Synaptotagmin-like 3</td>
<td>c. A158G</td>
<td>N53S</td>
<td>NM_001009991.3</td>
<td>Missense</td>
<td>Heterozygous</td>
<td>SNV</td>
<td>5</td>
</tr>
<tr>
<td>119738995</td>
<td>ASTN2</td>
<td>Astrotactin 2</td>
<td>c.C1508T</td>
<td>W503STOP</td>
<td>NM_014010.4</td>
<td>Nonsense</td>
<td>Heterozygous</td>
<td>SNV</td>
<td>5</td>
</tr>
<tr>
<td>57674167</td>
<td>R3HDM2</td>
<td>R3H domain containing 2</td>
<td>c.G1276A</td>
<td>P426S</td>
<td>NM_014925.3</td>
<td>Missense</td>
<td>Heterozygous</td>
<td>SNV</td>
<td>5</td>
</tr>
<tr>
<td>4163070</td>
<td>SMOX</td>
<td>Spermine oxidase</td>
<td>c.T944C</td>
<td>V315A</td>
<td>NM_175839.2</td>
<td>Missense</td>
<td>Heterozygous</td>
<td>SNV</td>
<td>5</td>
</tr>
</tbody>
</table>

Another important result of our SNP array analysis was the chromothripsis-like pattern of copy number alterations observed for chromosomes 1 and 2 in tumor sample 5. However, the accurate inference of breakpoint rearrangements observed in chromothripsis is not possible from SNP array data alone (25). Furthermore, because chromothripsis has been reported in growth hormone–secreting adenomas (15) and their potential for tumorigenesis in benign tumors has been described for uterine leiomyomas (35), our results in TSHomas need to be confirmed further by using more reliable approaches, such as massively parallel sequencing (25).

Our study provides evidence for cnLOH in TSHomas. These genetic lesions have previously been described in relation to various tumors (36–38). We did not examine matched constitutive samples; however, we noted several broader regions of cnLOH in contrast to the generally lower size of germline-cnLOH reported in other study (36). This suggests that some of these observed cnLOH represent somatic uniparental disomy events. Furthermore, because these events were identified at regions of segmental duplication and fragile sites, they may have resulted from a recombination event or gene conversion during the repair of double-strand breaks at these sites, as described in various other tumors (36, 37, 39). However, recurrent cnLOH events were observed only on chromosome arms 1p and 8p. cnLOH at 1p encompasses loci to be elucidated; however, the genetic lesions identified serve as a reference point for future studies aimed at identifying candidate driver genes. One exceptional finding during our SNP array analysis was the absence of any detected copy number alteration in tumor sample 6; surprisingly, in WES, no mutations were confirmed in the same sample. These findings may reflect tumor sample 6 possibly representing the early stage of the disease; however, analysis of the duration of symptoms as well as the age of patients with the observed number of copy-number aberrations among all samples did not establish a pattern or trend with regard to genetic evolution of the tumor.
harboring the CDKN2C gene, the inactivation of which by promoter methylation has been reported in up to 20% of pituitary tumors (40). Although cnLOH may result in the duplication of a methylated gene and cause the effective knockout of TSG, causing clonal selection, further epigenetic approaches need to be used to verify the involvement of such a mechanism (36).

The current study examined somatic events in TSHomas; however, some caveats are worth noting. The sample size used for the study was small. To investigate this rare tumor, we used a set of 12 samples, 4 of which were used in the discovery cohort for WES. Multicenter studies with more TSHomas may enhance the landscape of somatic genetic events further. Furthermore, in our SNP array analysis, we relied on manufacturer-provided cluster files to obtain genotype calls. Studies involving a higher-resolution SNP array from constitutive matched pair samples along with the inclusion of mixing studies may lead to the more accurate identification of somatic cnLOH events.

In conclusion, we identified several candidate somatic mutations and changes in copy numbers in TSHomas. Our results showed no recurrence of mutations in the tumors studied but a low number of mutations per tumor, thereby highlighting their benign nature. Further studies on a larger cohort of TSHomas, in combination with epigenetic and transcriptomic approaches, may reveal the underlying genetic lesions.

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