Whole-Exome Sequencing Studies of Nonfunctioning Pituitary Adenomas

Paul J. Newey, M. Andrew Nesbit, Andrew J. Rimmer, Rosie A. Head, Caroline M. Gorvin, Moustafa Attar, Lorna Gregory, John A. H. Wass, David Buck, Niki Karavitaki, Ashley B. Grossman, Gilean McVean, Olaf Ansorge, and Rajesh V. Thakker

Academic Endocrine Unit (P.J.N., M.A.N., R.A.H., C.M.G., R.V.T.), Nuffield Department of Clinical Medicine, University of Oxford, Oxford OX3 7LJ, United Kingdom; Bioinformatics and Statistical Genetics Group (A.J.R., G.M.) and High-Throughput Genomics Group (M.A., L.G., D.B.), Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, United Kingdom; Department of Endocrinology (J.A.H.W., N.K., A.B.G.), Oxford Centre for Diabetes, Endocrinology, and Metabolism, Oxford University Hospitals Trust, Oxford OX3 7LJ, United Kingdom; and Department of Neuropathology (O.A.), John Radcliffe Hospital, Oxford University Hospitals Trust, Oxford OX3 9DU, United Kingdom

Context: The tumorigenic role of genetic abnormalities in sporadic pituitary nonfunctioning adenomas (NFAs), which usually originate from gonadotroph cells, is unknown.

Objective: The objective of the study was to identify somatic genetic abnormalities in sporadic pituitary NFAs.

Design: Whole-exome sequencing was performed using DNA from 7 pituitary NFAs and leukocyte samples obtained from the same patients. Somatic variants were confirmed by dideoxynucleotide sequencing, and candidate driver genes were assessed in an additional 24 pituitary NFAs.

Results: Whole-exome sequencing achieved a high degree of coverage such that approximately 97% of targeted bases were represented by more than 10 base reads; 24 somatic variants were identified and confirmed in the discovery set of 7 pituitary NFAs (mean 3.5 variants/tumor; range 1–7). Approximately 80% of variants occurred as missense single nucleotide variants and the remainder were synonymous changes or small frameshift deletions. Each of the 24 mutations occurred in independent genes with no recurrent mutations. Mutations were not observed in genes previously associated with pituitary tumorigenesis, although somatic variants in putative driver genes including platelet-derived growth factor D (*PDGFD*), N-myc down-regulated gene family member 4 (*NDRG4*), and Zipper sterile-α-motif kinase (*ZAK*) were identified; however, DNA sequence analysis of these in the validation set of 24 pituitary NFAs did not reveal any mutations indicating that these genes are unlikely to contribute significantly in the etiology of sporadic pituitary NFAs.

Conclusions: Pituitary NFAs harbor few somatic mutations consistent with their low proliferation rates and benign nature, but mechanisms other than somatic mutation are likely involved in the etiology of sporadic pituitary NFAs. **(*J Clin Endocrinol Metab* 98: E796–E800, 2013)**

Anterior pituitary tumors have a prevalence of 70–90 cases per 100 000 of the population and account for 15% of all intracranial neoplasms (1, 2). Pituitary nonfunctioning adenomas (NFAs), which mostly arise from gonadotroph cells, represent the second most frequent pituitary tumor after prolactinomas. Pituitary NFAs are clinically challenging because they present at a late stage with local mass effects (eg, visual impairment) or hypop-

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Abbreviations: *AIP*, aryl hydrocarbon receptor interacting protein; *CDKN1B*, cyclin-dependent kinase 1B; *MEN1*, multiple endocrine neoplasia type 1; mTOR, mammalian target of rapamycin; NDRG4, N-myc down-regulated gene family member 4; NET, neuroendocrine tumor; NFA, nonfunctioning adenoma; *PDGFD*, platelet-derived growth factor D; *PRKAR1A*, protein kinase, cAMP-dependent, regulatory, type I-α; SNV, single-nucleotide variant; *ZAK*, zipper sterile-α-motif kinase.

ituitarism; there are no consistently effective medical therapies available for NFAs; and surgical resection is often incomplete with high rates of tumor recurrence. Furthermore, the absence of reliable hormonal secretion or biomarkers hampers their detection and monitoring of progression (3).

Pituitary NFAs, in common with other pituitary adenomas, are monoclonal in origin (4), although the underlying molecular mechanisms, which likely involve genetic and epigenetic factors, altered signaling pathways, and local paracrine and microenvironmental components, remain incompletely defined (1, 5, 6). For example, pituitary NFAs may demonstrate the down-regulation of cell cycle inhibitors (eg, p15, p16), maternally expressed protein 3A, and zinc-finger protein pleiomorphic adenoma gene-like 1 through promoter hypermethylation; the increased expression of cyclins (eg, cyclin D1), pituitary tumor transforming gene (*PTTG*), pituitary tumor-derived fibroblast-growth factor receptor-4 variant, and specific transcription factors (eg, steroidogenic factor 1 and GATA2); and altered signaling through ERK, mammalian target of rapamycin (mTOR), and canonical Wnt pathways (1, 5, 6). However, the primary initiating events and involvement of somatic mutations of oncogenes or tumor suppressors in pituitary NFA formation are unknown. Indeed, studies investigating somatic mutation involving genes associated with hereditary pituitary adenomas [eg, multiple endocrine neoplasia type 1 (*MEN1*), cyclin-dependent kinase 1B (*CDKN1B*), the aryl hydrocarbon receptor interacting protein (*AIP*), and protein kinase, cAMP-dependent, regulatory, type I-α (*PRKAR1A*) genes] or activating mutations of the α-subunit of the G stimulating protein ($G_s\alpha$), found in sporadic somatotrophinomas and McCune-Albright syndrome have reported that these are rarely observed in pituitary NFAs (1, 5–8).

In addition, such studies have not identified mutations in classical oncogenes or tumor suppressor genes (1). Therefore, to identify such somatic mutations in pituitary NFAs, we have used whole-exome sequencing, which has been successfully used to find mutations in multiple tumor types including sporadic pancreatic neuroendocrine tumors (NETs) and parathyroid tumors (9–12). Thus, these studies have reported that *MEN1* mutations occur in approximately 45% and approximately 35% of sporadic pancreatic NETs and parathyroid tumors, respectively (9–11), and that mutations of death domain-associated protein (*DAXX*) and α-thalassemia/mental retardation syndrome X-linked (*ATRX*), or genes encoding proteins of the mTOR pathway, occur in approximately 40% and 15% of pancreatic NETs, respectively.

Materials and Methods

Patients and tumors

Informed consent was obtained from individuals using protocols approved by local and national research ethics committees (MREC/02/2/93). Pituitary NFAs, obtained from 7 patients [4 males and 3 females, mean age 55 years (range 39–82 years)], who did not have a family history of endocrine neoplasia, were histologically confirmed as gonadotroph adenomas (Table 1 and Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The tumors, which all had a Ki-67 index of 3% or less, did not have features of atypia, and these constituted the discovery set of tumors for exome capture and DNA sequence analysis. Twenty-four additional pituitary NFAs, histologically confirmed as gonadotroph adenomas, were obtained from 9 females and 15 males [mean age 61 years (range 36–83 years)], and these constituted the validation set.

Exome capture, DNA sequence, and bioinformatic analysis

DNA from fresh-frozen pituitary NFAs and leukocytes was extracted using the ArchivePure DNA kit (5 PRIME, Hamburg,

Table 1. Clinical Details of Patients and Somatic Variants in Discovery Set of 7 Pituitary NFAs[a]

Patient			Tumor			
Tumor Number	Age, y	Sex	Tumor Volume, cm^3[b]	Histology[c]	Ki-67 Index	Number of Somatic Variants
1	64	M	15.9	GA (FSH 70%, LH 15%)	2–3%	2
2	59	M	9.1	GA (FSH 5–10%)	2–3%	7
3	59	M	4.2	GA (FSH 15%)	2%	5
4	42	F	0.7	GA (FSH 30%)	2%	5
5	82	F	2.4	GA (FSH 20–30%)	1–2%	1
6	36	F	2.2	GA (LH 5%)	2–3%	2
7	40	M	9.0	GA (FSH 60%, LH 2%)	2–3%	2

Abbreviation: GA, gonadotroph adenoma (immunostaining for LH and/or FSH).

[a] Seven samples were used in the discovery set for exome capture and sequencing studies because power calculations indicated that this number would be able to identify common driver mutations with a greater than 90% probability of identifying 1 or more mutations in genes involved in 30% of tumors (Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, et al. *Nature* 2011;469:539–542).

[b] Estimated tumor volume using the Di Chiro-Nelson method of $V = 1/2(h \times w \times l)$ as documented on preoperative magnetic resonance imaging.

[c] None of the pituitary NFAs had features of atypia.

Germany), as described (10). Exome sequencing was performed using the SureSelect human all exon 50-Mb kit (Agilent Technologies, Santa Clara, California) and HiSeq2000 (Illumina, San Diego, California), as previously described (10). All data were aligned to the hg19/GRCh37 reference genome build (Stampy, http://www.well.ox.ac.uk/project-stampy), stored in BAM format, and variants identified using an in-house variant-caller, Platypus, (http://well.ox.ac.uk/platypus), which has been successfully validated in other studies (10). Variants were examined using the Human hg19 assembly on the Integrative Genomics Viewer (www.broadinstitute.org/igv/) to confirm their tumor-specific nature. Tumor-specific (somatic) variants were confirmed by dideoxynucleotide sequencing using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies, Grand Island, New York) and an automated detection system (ABI 3730 automated capillary sequencer; Applied Biosystems, Foster City, California). Primer sequences are available on request. Only those variants validated by confirmatory sequencing are reported.

Identification of candidate driver mutations

The functional significance of each variant was assessed using the programs Polyphen-2 (genetics.bwh.harvard.edu/pph2/), SIFT (sift.jcvi.org/) and MutationTaster (www.mutationtaster.org/), which scored variants by their predicted effect on protein function. Mutational analysis of the genes *PDGFD*, *ZAK*, and *NDRG4* were undertaken in the validation set of 24 pituitary NFAs (primer sequences available on request).

Results

Identification of tumor-specific somatic variants

Whole-exome capture using DNA from the discovery set of 7 pituitary NFAs and matched leukocyte samples yielded excellent target region coverage with approximately 97% and approximately 94% of bases covered with more than 10 and more than 20 reads in the tumor samples, respectively (Supplemental Table 1). Germline mutations were not observed in genes associated with familial pituitary tumor syndromes (eg, *MEN1*, *CDKN1B*, *AIP*, or *PRKAR1A*) in any of the leukocyte samples (Supplemental Table 2), consistent with the absence of a family history of endocrine neoplasia. Using stringent variant calling and filtering parameters, 28 somatic mutations were identified in the 7 tumors, and 24 of these were confirmed by dideoxynucleotide sequencing (Table 2 and Supplemental Figure 2), indicating approximately 86% concordance between the somatic variant calling algorithm and confirmatory sequencing. Thus, the tumors in the discovery set revealed a mean somatic variant rate of 3.5 variants/tumor (range 1–7). Of the 24 confirmed variants, 21 occurred as single-nucleotide variants (SNVs), and the remaining three comprised 2 small deletions and 1 deletion-insertion (indels) (Table 2). Approximately 90% of SNVs resulted in missense amino-acid changes, whereas the remaining approximately 10% were synonymous changes. More than 70% of the SNVs occurred as C:G>T:A transitions, and less than 30% were transversions. No correlation between tumor size and the number of somatic mutations was observed.

Identification of candidate driver mutations

Somatic mutations in genes previously associated with pituitary tumorigenesis including *MEN1*, *AIP*, *CDKN1B*, *PRKAR1A*, *GNAS*, *HRAS*, *RB1*, *PTTG*, *PIK3CA*, and *ZAC1* were not identified (Supplemental Table 2), and each of the 24 confirmed somatic variants occurred in independent genes, thereby indicating that mutations of a single gene are unlikely to be responsible for a high proportion (eg, >30%) of sporadic pituitary NFAs. To identify putative driver mutations, further analysis of each of the somatic variants was performed. We hypothesized that putative driver mutations would typically: 1) have a deleterious effect on protein function [as predicted by protein prediction software (ie, Polyphen2, MutationTaster, and SIFT]; 2) be present at sufficient allele frequency to represent likely heterozygous or homozygous changes (ie, present from early in the tumorigenic process), although deviation from the expected heterozygous or homozygous allele frequencies may represent either contamination with normal tissue or the preference of the sequence and alignment process for the wild-type allele as previously reported (9, 10); and 3) be involved in biological processes relevant to tumorigenesis. Using these criteria, 3 of the 24 genes were selected for further evaluation, and these comprised platelet-derived growth factor-D (*PDGFD*), N-myc down-regulated gene family member 4 (*NDRG4*), and Zipper sterile-α-motif kinase (*ZAK*) (Supplemental Figure 2). *PDGFD* is reported to be involved in the development and progression of several human tumors (eg, prostate, brain, renal, lung) and regulates multiple cellular processes including proliferation, transformation, apoptosis, angiogenesis, and cellular migration and acts via signaling pathways that include phosphatidylinositol 3-kinase/Akt, mTOR, MAPK, and Notch (13). *NDRG4* is required for cell cycle progression and survival and has been implicated in glioblastoma and meningioma development (14, 15), whereas *ZAK* is a member of the MAPK kinase kinase family of signal transduction molecules implicated in cell cycle checkpoint regulation and is reported to act as a tumor suppressor through regulation of ERK and c-Jun N-terminal kinase signaling pathways (16). Each of the variants identified in these 3 genes were predicted to have a significantly deleterious effect on protein function (Supplemental Figure 2). However, DNA sequence analysis of the coding region and splice sites of *PDGFD*, *NDRG4*, and *ZAK* in the validation set of 24 pituitary NFAs did not

Table 2. Somatic Variants Identified in Sporadic Pituitary NFAs[a]

Gene Symbol	Gene Name	Transcript	Nucleotide Change	Protein Change	Mutation Type	Predicted Zygosity (Variant Allele %)[b]	Variant Type	Tumor Number
ABCA10	ATP-binding cassette, subfamily A (ABC1), member 10	NM_080282	c.A841G	p.T281A	Missense	het (33%)	SNV	6
ASS1	Argininosuccinate synthase 1	NM_054012	c.A1223G	p.K408R	Missense	het (40%)	SNV	3
CRTAC1	Cartilage acidic protein 1	NM_001206528	c.G89A	p.R30Q	Missense	het (32%)	SNV	4
DOCK9	Dedicator of cytokinesis 9	NM_001130048	c.A4006G	p.I1336V	Missense	het (12%)	SNV	5
GRM7	Glutamate receptor, metabotropic 7	NM_000844	c.G1140A	p.G380G	Synonomous	het (33%)	SNV	2
KEL	Kell blood group, metallo-endopeptidase	NM_000420	c.G937A	p.A313T	Missense	het (42%)	SNV	3
KLHL4	Kelch-like 4	NM_019117	c.G1441A	p.V481M	Missense	het (25%)	SNV	2
MYBPH	Myosin binding protein H	NM_004997	c.A1G	p.M1V	Missense	het (41%)	SNV	3
NDRG4	**N-myc down-regulated gene family member 4**	**NM_001130487**	**c.167_168del**	**p.T56RfsX6**	**Frameshift**	**het (30%)**	**indel**	**4**
NFXL1	Nuclear transcription factor, X-box binding-like 1	NM_152995	c.C1300T	p.H434Y	Missense	het (39%)	SNV	6
PDGFD	**Platelet derived growth factor D**	**NM_025208**	**c.A791G**	**p.N264S**	**Missense**	**het (30%)**	**SNV**	**2**
POMT2	Protein-O-mannosyltransferase 2	NM_013382	c.C1317T	p.V439V	Synonomous	het (40%)	SNV	2
PPP3R2	Protein phosphatase 3, regulatory subunit B, β	NM_147180	c.C167T	p.P56L	Missense	het (42%)	SNV	4
RNF135	Ring finger protein 135	NM_032322	c.854_855delinsCA	p.H285P	Missense	het (30%)	indel	7
ROPN1L	Rhophilin associated tail protein 1-like	NM_031916	c.T116A	p.L39Q	Missense	het (39%)	SNV	2
SETBP1	SET binding protein 1	NM_015559	c.C3718A	p.Q1240K	Missense	het (41%)	SNV	4
SLC35E3	Solute carrier family 35, member E3	NM_018656	c.A95T	p.N32I	Missense	het (43%)	SNV	1
SLC5A10	Solute carrier family 5 (sodium/glucose cotransporter), member 10	NM_001042450	c.T404A	p.M135K	Missense	het (29%)	SNV	4
SORCS1	Sortilin-related VPS10 domain containing receptor 1	NM_001013031	c.G940T	p.V314L	Missense	het (34%)	SNV	1
SPHKAP	SPHK1 interactor, AKAP domain containing	NM_001142644	c.A3773G	p.N1258S	Missense	het (33%)	SNV	2
SPTBN5	Spectrin, β, non-erythrocytic 5	NM_016642	c.G913A	p.A305T	Missense	het (60%)	SNV	2
TCF7L2	Transcription factor 7-like 2	NM_001198530	c.A407G:	p.H136R	Missense	het (23%)	SNV	3
TOMM70A	Translocase of outer mitochondrial membrane 70 homolog A	NM_014820	c.613delG	p.E205NfsX3	Frameshift	het (40%)	indel	7
ZAK	**Zipper sterile-α-motif kinase**	**NM_016653**	**c.G592C**	**p.E198Q**	**Missense**	**het (39%)**	**SNV**	**3**

Abbreviations: het, heterozygous; homo, homozygous; indel, insertion or deletion. Genes in boldface were identified as putative candidate driver mutations and sequenced in an additional 24 pituitary NFAs.

[a] Only somatic variants that were confirmed by a second method of capillary sequencing are shown. The concordance between the variant calling algorithm and confirmatory capillary sequencing for somatic variants was 86%, which is lower than that observed for germline variants. This lower concordance, which has been reported by other studies (9, 10, 17), may be due to the occurrence of variants with low-allele frequencies in tumor samples, which may be genetically heterogeneous, and the limitations of capillary sequencing that may not be able to detect variants with an allele percentage of less than 15% (10). The Platypus variant calling algorithm was used to identify the somatic variants because it has been successfully validated by other studies including the 1000 Genomes Project (1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, *et al. Nature.* 2012;491:56–65; Nesbit MA, Hannan FM, Howles SA, Reed AA, Cranston T, Thakker CE, et al. *Nat Genet.* 2013; 45:93–97). However, it remains possible that the use of alternate variant calling algorithms may identify some different somatic variants and reduce the false-positive rate.

[b] The variant allele percentage was calculated by dividing the total number of variant allele calls by the total number of calls for that nucleotide. Deviation from predicted heterozygote (50%) and homozygote (100%) allele frequencies may reflect contamination with normal tissue, genetic heterogeneity of tumor tissue, and/or a preference for the wild-type allele in the sequencing and alignment process.

identify any additional mutations, thereby indicating that these genes are unlikely to represent common driver mutations in pituitary NFAs. The possibility that mutations in the remaining 21 genes may make a significant contribution to the etiology of pituitary NFAs remains to be investigated.

Discussion

Our whole-exome sequencing studies of sporadic pituitary NFAs have identified that these tumors typically harbor few somatic variants. The low mutation rate (~3.5 mutations/tumor) is significantly lower than that reported for a variety of other malignant tumors [eg, non-small cell lung cancer, 104 variants/tumor (17); gastric cancer, 62 variants/tumor (18)] but is similar to that reported for greater than 95% of sporadic parathyroid tumors [3.6–7 variants/tumor (10, 11)]. The low mutation rate observed in pituitary NFAs is consistent with their typically low proliferation rates and benign phenotype. In addition, our studies did not identify somatic mutations in: established oncogenes; tumor suppressor genes; genes associated with familial pituitary syndromes; or genes previously implicated in pituitary tumorigenesis, consistent with previous reports (1). Furthermore, the absence of recurrent mutations within specific genes suggests that there is no common driver gene responsible for a high proportion (eg, >30%) of NFAs. Instead, pituitary NFAs may occur as a

result of either low frequency driver mutations [ie, mutations in genes affecting low proportions of tumors as reported for chronic lymphocytic leukemia in which multiple genes are mutated in ≤15% of tumors (19)] or mechanisms other than somatic mutation. This may include copy number variations (eg, deletions or amplifications), chromosomal rearrangements, or epigenetic changes that would not be detected by exome sequencing. For example, methylation of several genes including cell-cycle regulators and putative tumor suppressor genes has been reported in pituitary tumors (1, 5, 6), whereas a recent study using methylome analysis has reported that pituitary NFAs demonstrate higher levels of methylation than other pituitary tumor types (20). In addition, the current methodology does not exclude the possibility of missing causative somatic variants in the approximately 3% of exonic regions that were either uncaptured or represented by less than 10 base reads or in most noncoding regions. However, our study did identify potential pathogenic variants in several genes that are involved in tumorigenic pathways which have not previously been associated with pituitary tumorigenesis (eg, *PDGFD*, *ZAK*, and *NDRG4*), although the absence of recurrent mutations in the validation cohort of an additional 24 tumors suggest that such genes are not responsible for a significant proportion of pituitary NFAs.

In summary, our studies demonstrate that the somatic mutation rate in pituitary NFAs is low when compared with other tumor types and that events other than a recurring somatic mutation (eg, epigenetic changes, copy number variations) are likely to be responsible for the development of most pituitary NFAs.

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Address all correspondence and requests for reprints to: Rajesh V. Thakker, MD, FRCP, FRCPath, FMedSci, Academic Endocrine Unit, Nuffield Department of Clinical Medicine, University of Oxford, Oxford Centre for Diabetes, Endocrinology, and Metabolism, Churchill Hospital, Headington, Oxford OX3 7LJ, United Kingdom. E-mail: rajesh.thakker@ndm.ox.ac.uk.

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P.J.N. is a National Institute for Health Research Academic Clinical Lecturer in Endocrinology.

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