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The Genomic Landscape of Sporadic Prolactinomas

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Abstract

Somatic *GNAS* and *USP8* mutations have been implicated in sporadic somatotrophinomas and corticotrophinomas, respectively. However, no genes are known to be recurrently mutated in sporadic prolactinomas. The prevalence of copy number variants (CNV), which is emerging as a mechanism of tumorigenesis in sporadic pituitary adenomas in general, is also unclear in prolactinomas. To characterize the genetic events underpinning sporadic prolactinomas, we performed whole exome sequencing of paired tumor and germline DNA from 12 prolactinoma patients. We observed recurrent large-scale CNV, most commonly in the form of copy number gains. We also identified sequence variants of interest in 15 genes. This included the *DRD2*, *PRL*, *TMEM67*, and *MLH3* genes with plausible links to prolactinoma formation. Of the 15 genes of interest, CNV was seen at the gene locus in the corresponding tumor in 10 cases, and pituitary expression of eight genes was in the top 10% of tissues. However, none of our shortlisted somatic variants appeared to be classical driver mutations as no variant was found in more than one tumor. Future directions of research include mechanistic studies to investigate how CNV may contribute to prolactinoma formation, larger studies of relevant prolactinoma subsets according to clinical characteristics, and additional genetic investigations for aberrations not captured by whole exome sequencing.

Keywords Prolactinoma · Pituitary adenoma · Whole exome sequencing · Copy number variation · Loss of heterozygosity · Driver mutation

Introduction

The genetic basis of sporadic prolactinomas is currently unknown. This is in contrast to well-described somatic events in other pituitary tumors, namely: *GNAS* mutations in somatotrophinomas and occasional non-functioning pituitary adenomas [1–3]; *USP8* [3, 4] and rarely *NR3C1* [3, 6] mutations in corticotrophinomas; and *CTNNB1* (encoding β-catenin) and *BRAF* mutations in the vast majority of adamantinomatous and papillary craniopharyngiomas, respectively [5]. Some of these somatic events recapitulate multisystem disorders, including McCune-Albright syndrome due to

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GNAS somatic mosaicism [6], and a newly described syndromic disorder including pediatric Cushing's disease due to a germline heterozygous *USP8* mutation [7].

Gene-specific assessment of sporadic prolactinomas has shown nil or only rare somatic variants in biologically plausible genes. This includes genes where germline variants cause familial pituitary tumor syndromes (FPTS), such as *MEN1* [8] and *AIP* [9], and genes implicated in sporadic pituitary adenomas, including *GNAS* [1], *USP8* [4], and *TP53* [10, 11]. A recurrent germline gain-of-function *PRLR* mutation has been identified in prolactinoma patients, but no such variants have been found in the somatic setting [12]. A somatic *HRAS* variant has been identified in an aggressive prolactinoma, but this association was not borne out in an extension study including 72 prolactinomas [13].

A limitation of single gene studies is the reliance on existing knowledge to select candidate genes. "Orphan" genes of hitherto unknown function could be contributory to prolactinomas akin to other genetic discoveries, such as the roles of *GPR101* in X-linked acrogigantism [14] and *ARMC5* in bilateral macronodular adrenal hyperplasia [15]. Whole exome or genome sequencing offers an unbiased approach to novel gene discovery. Only three pangenomic studies of prolactinomas have been performed to date, all employing whole exome sequencing (WES). Wang et al. [16] focused on point variants conferring bromocriptine resistance in a cohort of 12 prolactinomas and identified 11 candidate genes between initial and follow-up [17] studies. Bi et al. [18] investigated 41 pituitary macroadenomas, including three prolactinomas. Six genes were mutated in more than one tumor, but none of these were prolactinomas. Song et al. [3] examined 125 pituitary adenomas, including 20 prolactinomas. Two genes were considered to be potential tumorigenesis genes but only one prolactinoma harbored a variant in these genes. Overall, these studies did not find recurrent sequence variants amongst the prolactinomas that could constitute driver mutations. There is, however, emerging evidence of recurrent copy number variants (CNVs) in sporadic pituitary adenomas, including the small number of prolactinomas thus far studied [2, 3, 18, 19].

The aim of the present study was to perform WES in a pure prolactinoma cohort to identify recurrent somatic genetic events. We hypothesized that, like other pituitary tumors, somatic driver mutations and/or CNVs might also underlie the development of prolactinomas.

Materials and Methods

Patients

Twelve patients with clinically evident prolactinomas that had been surgically resected were recruited from two tertiary referral pituitary centers in Australia. Clinical data were collated using medical records.

DNA Extraction

Patients provided fresh blood samples for germline DNA extraction. Operative tumor specimens were retrieved for somatic DNA extraction. Tumor specimens had either been stored as fresh frozen ($n = 6$) or formalin-fixed paraffin-embedded (FFPE; $n = 6$) tissue. Duration of tumor storage ranged from 7 months to 8 years. DNA was extracted using commercially available kits (Qiagen and Bioline) according to manufacturer protocols. FFPE samples were deparaffinized and additional DNA repair steps were performed using uracil-N-glycosylase to enzymatically remove formalin-induced cytosine deamination artifacts.

Whole Exome Sequencing

WES of germline and tumor DNA samples was performed using the Roche NimbleGen SeqCap EZ MedExome v3.0 target enrichment kit, and the Illumina NextSeq 500 sequencing platform. The average of mean depth of coverage amongst all samples was 129x, and 97% of target bases were covered ≥ 20x.

Filtration of Sequence Variants

Bioinformatic analysis was performed in the ACRF Cancer Genomics Facility of the Centre for Cancer Biology, SA Pathology (Adelaide, Australia). BWA-MEM was used to align short reads to GRCh37/hg19 (version b37+decoy). Small variants (typically < 50 bp) were called using Genome Analysis Toolkit (GATK) HaplotypeCaller package version 3.4. Raw WES data were initially filtered for variants that were: high quality (by GATK internal filters); very rare (< 0.2% population); potentially functional (by snpEFF impact, branching/binding predictions, GERP, or CADD); and not in regions of segmental duplication.

Germline variants were considered further if they had a GATK genotype quality (GQ) score > 50 and depth of coverage > 30x, and were not situated in a low complexity region. Drawing on existing literature, we searched for germline variants in known FPTS genes: *AIP*, *CDH23*, *CDKN1B*, *DICER1*, *GPR101*, *MAX*, *MEN1*, *PRKAR1A*, *SDHA*, *SDHB*, *SDHC*, and *SDHD* [14, 20–23].

Artifact was observed in tumor DNA results due to reasons including presumed normal tissue admixture and DNA degradation in FFPE specimens. Raw data from tumor DNA were thus re-analyzed by a dedicated in-house somatic variant calling pipeline to identify variants present in tumor DNA and absent in germline DNA. To increase the reliability of somatic variant calls, this pipeline integrates four variant callers that

detect insertions/deletions ("indels") and single-nucleotide variants (SNVs): Shimmer (v e5bafb4), Seurat (v 2.6), Strelka2 (v 2.9.0), and VarScan2 (v 2.4.0); and three callers that detect SNVs only: MuTect (v 1.1.4), SomaticSniper (v 1.0.5), and Virmid (v 1.1.1). Only somatic indels and SNVs that were detected by at least two or five variant callers, respectively, were considered to be candidate somatic sequence variants.

These candidate somatic variants were shortlisted to a final list of somatic variants of interest that were absent in population genomic databases (dbSNP, 1KG, UK10K, gnomAD, ExAC, and ESP) with evidence of being highly damaging (high snpEFF impact). Pituitary expression of these final genes of interest was determined using the Genotype-Tissue Expression (GTEx) project database (https://gtexportal.org), comprising 53 non-diseased tissue sites including 183 pituitary samples.

All germline variants in known FPTS genes and somatic variants of interest were verified by inspection of raw sequencing data in Integrated Genomics Viewer (IGV).

Identification of Somatic Copy Number Variants

Raw WES data were interrogated for copy number variation (CNV) via in-house scripts, with calculation of copy number using a normalized read depth of coverage against control samples and correlation with minor allele frequency. Coverage plots of sequence read depth and minor allele frequency was manually inspected to identify chromosomal and arm level copy number gains and losses as well as copy number neutral loss of heterozygosity (LOH).

Statistical Analysis

IBM SPSS Statistics 25.0 was used for statistical analysis. The Mann-Whitney *U* test was used to assess differences in the median numbers of candidate somatic variants and chromosomes affected by CNV or copy neutral LOH per tumor according to relevant categorical clinical characteristics. *P* values < 0.05 were considered statistically significant.

Results

Clinical Characteristics

The study cohort consisted of six women and six men aged 16–65 years at prolactinoma diagnosis. The tumors hypersecreted prolactin alone. Apart from one patient who presented with pituitary apoplexy, all patients were treated with dopamine agonists (DA) preoperatively. In all cases, surgical resection was by the trans-sphenoidal route and histopathology confirmed pituitary adenomas with positive immunostaining for prolactin. Postoperative tumor remnants or recurrences were observed in ten patients, all of whom had macroadenomas or giant adenomas at the baseline scan. The remaining two patients had microadenomas that were resected because of DA intolerance, with gross total resection achieved and no evidence of tumor recurrence to date. No patients had received other medical therapies or radiotherapy at study enrollment. Other clinical characteristics of the patient cohort are given in Table 1.

Pathological Characteristics

Pathological characteristics are described in Table 2. Most tumors were densely granulated lactotroph adenomas. Ki-67 index was only available in a minority of tumors. Few or no mitoses were observed in the remaining tumors, arguing against a significant degree of proliferation [24]. Histological invasion was found in only three tumors. Despite DA pretreatment in all but one patient, fibrosis was only observed in 4/11 DA-treated tumors (Fig. 1).

Germline Sequence Variants

The only known FPTS gene with germline sequence variants after filtration was *CDH23*, with missense variants observed in Patient 6 (c.1103G>A (p.Arg368His), population prevalence in gnomAD 0.06%, CADD 26.2, GERP 4.5) and Patient 11 (c.4510G>T (p.Ala1504Ser), gnomAD 0.01%, CADD 25, GERP 5.06; and c.4907C>T (p.Ala1636Val), gnomAD 0.07%, CADD 22.5, GERP 5.75).

Somatic Sequence Variants

Filtration of WES data revealed 138 candidate somatic variants, none of which were found in more than one tumor. Only one gene (*PHTF1*) was mutated in more than one tumor. Another two genes (*NBEAL2*, *TMEM67*) were each mutated twice in the same tumor from Patient 1. Of the 135 different genes containing candidate somatic variants, there was no overlap with genes implicated in FPTS or sporadic pituitary adenomas (i.e., *AIP*, *CDH23*, *CDKN1B*, *DICER1*, *GNAS*, *GPR101*, *MAX*, *MEN1*, *NR3C1*, *PRKAR1A*, *PRLR*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *TP53*, *USP8*).

Each tumor harbored multiple candidate somatic variants (median 9.5 per tumor, range 3–23). There was no significant difference in the median number of variants according to gender (male 7.5 vs female 15.0, $P = 0.107$), indication for surgery (DA intolerance 9.5 vs other indications 14.0, $P = 0.624$), tumor consistency (no cystic component 9.0 vs cystic component 15.0, $P = 0.114$), or extent of resection (no remnant 9.5 vs remnant 14.0, $P = 0.780$).

The 138 candidate somatic variants were shortlisted to 15 variants of interest (Table 3; Fig. 2) that were absent in

Table 1 Clinical characteristics at time of diagnosis

Patient	Age (yr), gender	Tumor maximum diameter (mm)	Hardy's score	Tumor consistency	PRL (xULN)	Surgical indication	Postoperative remnant[#]	Tumor recurrence[*]
1	40 F	16^	3^	Solid^	33^	DA resistance	Yes	N/A
2	16 F	8	1	Cystic	9	DA intolerance	No	N
3	56 M	8	3	Solid	10	DA intolerance	No	N
4	42 F	11	3	Solid	5	DA intolerance	Yes	N/A
5	28 F	60	3	Solid	278	DA resistance	Yes	N/A
6	53 M	18	3	Solid	145	DA intolerance	Yes	N/A
7	32 M	26	3	Solid	20	DA resistance	Yes	N/A
8	64 M	52	3	Mixed	576	Apoplexy at Dx	Yes	N/A
9	65 M	37	3	Solid	67	DA resistance	Yes	N/A
10	32 F	16^	2^	Solid^	28^	DA intolerance	No	Y
11	40 M	41	3	Solid	215	DA resistance	Yes	N/A
12	61 F	46	3	Mixed	72	DA resistance	Yes	N/A

DA, dopamine agonist; *Dx*, diagnosis; *F*, female; *M*, male; *N/A*, not applicable; *N/S*, not stated in report and images not available for review; *PRL*, prolactin; *xULN*, absolute level divided by upper limit of normal; *yr*, year

[#] Based on postoperative imaging and serum prolactin results

[*] Only applies to tumors that were completely resected

^ Preoperative results used as results at initial diagnosis unavailable

population genomic databases and highly damaging ($n = 14$), or situated in a gene with another candidate somatic variant in another tumor ($n = 1$, *PHTF1*). The shortlist included nonsense or frameshift variants in three genes (*DRD2*, *PRL*, *TMEM67*) with known associations with the pituitary gland and the *MLH3* gene which is a tumorigenesis gene in other tissues. Using the GTEx database, we observed that the pituitary was in the top 10% of expressing tissues for 8/15 shortlisted genes of interest. We next used the STRING database (https://string-db.org) of known and predicted protein-protein interactions to look for interactions between the 15 genes of interest. The only interaction was the known link between *PRL* encoding prolactin and *DRD2* encoding the D2 dopamine receptor (D2R), which are co-expressed in multiple species and co-mentioned in medical literature.

No patient had a germline variant in the same gene containing a somatic variant of interest in their corresponding tumor. Conversely, no candidate somatic variants were found in *CDH23* in the two patients with germline *CDH23* variants.

Somatic Copy Number Variants

All but one tumor contained chromosomal or arm level CNVs and/or copy neutral LOH (median 10.5 chromosomes affected per tumor, range 0–21). There was no significant difference in the median number of chromosomes affected according to gender (male 10.0 vs female 12.0, $P = 0.377$), surgical indication (DA intolerance 8.0 vs other indications 12.0, $P = 0.514$), tumor consistency (no cystic component 10.0 vs cystic component 17.0, $P = 0.266$), or extent of resection (no remnant 10.0 vs remnant 10.0, $P = 0.926$). CNV most commonly manifested as whole or partial chromosomal gain (median 10 chromosomes per tumor, range 0–20), and occasionally as whole or partial chromosomal loss (median 0 chromosomes per tumor, range 0–2). Copy neutral LOH was also seen (median 0.5 chromosomes per tumor, range 0–6).

Recurrent and single cases of chromosomal gain, loss, and copy neutral LOH are shown in Table 4. Examples of CNV calling are depicted in Fig. 3. The most frequent chromosomes affected were Chr 8, 9, and 14 followed by Chr 3, 7, 12 and 20 for gains, and 1 and 15 for copy neutral LOH. No chromosomes showed recurrent losses.

Each tumor was assessed for regional overlap between its CNV results and any observed variant of interest. Copy number gain was present in the corresponding tumor at the locus of 10/15 genes of interest and corresponding monosomy was observed for 1/15 genes.

Discussion

The major somatic event in our cohort of 12 patients with prolactinomas was large-scale CNV, most commonly in the form of copy number gains. We also observed sequence variants of interest in 15 genes, including genes of putative interest in prolactinoma tumorigenesis. Although we found that pituitary expression is in the top 10% of tissues for over half of our genes of interest, these somatic variants do not appear to

Table 2 Pathological characteristics of patient cohort at time of diagnosis

Patient	Positive hormone IHC	Granulation pattern	Mitoses	Ki-67 index	Histological invasion	Fibrosis
1	PRL	Undetermined	Scant	U/A	No	No
2	PRL	Densely granulated	< 1/10hpf	U/A	No	No
3	PRL	Densely granulated	Nil	U/A	No	Yes
4	PRL	Undetermined	Scant	U/A	No	No
5	PRL, LH	Densely granulated	Scant	U/A	Yes—sphenoid, nasopharynx	Yes
6	PRL	Densely granulated	< 1/10hpf	U/A	Yes—dura	No
7	PRL, TSH, LH, FSH	Undetermined	Scant	U/A	No	No
8	PRL	Sparsely granulated	Nil	U/A	Yes—sphenoid	No
9	PRL	Densely granulated	Nil	<1%	N	Yes
10	PRL	Densely granulated	< 1/10hpf	3%	No	No
11	PRL	Densely granulated	Nil	< 1%	No	Yes
12	PRL	Sparsely granulated	< 1/10hpf	U/A*	No	No

FSH, follicle-stimulating hormone; *IHC*, immunohistochemistry; *LH*, luteinizing hormone; *PRL*, prolactin; *TSH*, thyroid-stimulating hormone; *U/A*, unavailable

*Topoisomerase index 5%

be classical driver mutations as none were found in more than one tumor. We also found rare missense germline variants in the recently recognized FPTS gene, *CDH23*. However, somatic second-hits were not found in the corresponding tumors and *CDH23* is a notably large gene with 69 exons, which may increase the propensity for variants of uncertain significance. Our results recapitulate the findings of the few systematic genomic studies of prolactinomas that have been performed to date (Table 5), whereby CNV is common and recurrently mutated genes are rare.

We observed several recurrent copy number gains and copy neutral LOH. This is in keeping with the pituitary macroadenoma WES study by Bi et al. [18], and their follow-up study of 114 pituitary adenomas including 14 prolactinomas [2], that indicated two patterns of CNV: a highly disrupted group mostly consisting of functional adenomas (including prolactinomas) or atypical null cell adenomas with CNV involving a mean of 39% of the genome; and a less disrupted group mostly consisting of non-functioning adenomas with CNV involving a mean of 0.5% of the genome. Song et al. [3] also found a high degree of CNV in their mixed cohort of pituitary adenomas, with almost one-third of pituitary adenomas showing CNV involving > 80% of the genome. Our patients' tumors demonstrated recurrent gains in Chr 7, 9, and 14 similar to Bi et al. [18] and in Chr 1, 3, 7, 16, and 20 similar to Song et al. [3]. In contradistinction to these former studies, we observed additional recurrent gains in Chr 5–7, 10, 12, 17–19, 21, 22, and X. We also found no recurrent chromosomal losses, whereas Bi et al. [18] found losses to be particularly common in Chr 1p and 11 in hormonally active adenomas. While we found recurrent copy neutral LOH in Chr 1, 4, and 15, Bi et al. [18] only found Chr 11q LOH. Discrepancies between the different cohorts are at least partly explained by the heterogenous mixes of different pituitary adenoma subtypes in previous studies. Our pure prolactinoma cohort should be considered separately to these previous studies because of the potential for specific DA treatment effects in 11 of our 12 patients who were treated with a DA preoperatively. DA resistance may have led to the observed high rate of CNV or vice versa.

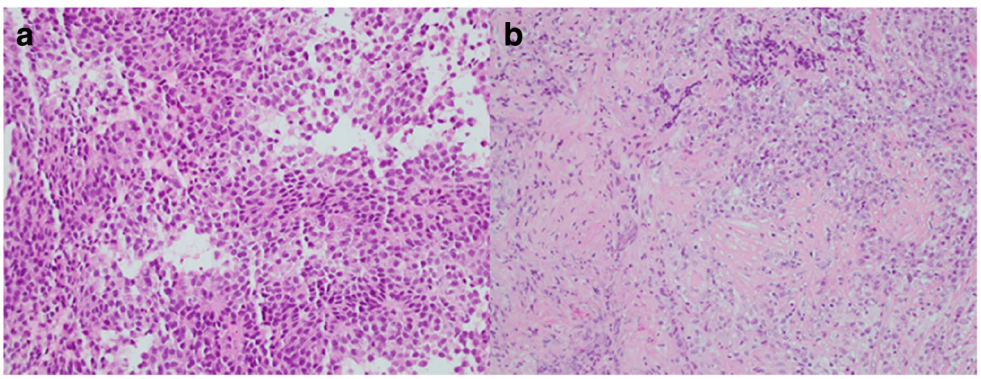

Fig. 1 Hematoxylin & eosin appearance of prolactinomas at medium power in a case demonstrating no fibrosis (**a** Patient 12) and another demonstrating marked fibrosis (**b** Patient 11)

Table 3 Somatic sequence variants of interest

Gene, ID[a]	Chr locus	Pituitary rank amongst all tissue expression[b]	CNV at locus[c]	Pt: variant	VAF in tumor DNA
ANKS3, ENSG00000168096	16: 4780135	3	Trisomy	Pt 1: c.15delC (p.Ser5fs)	33%
C19orf25, ENSG00000119559	19: 1475427	3	Trisomy	Pt 7: c.216dupG (p.Ile73fs)	12%
C9orf163, ENSG00000196366	9: 139379389	2	Trisomy	Pt 1: c.491delC (p.Pro164fs)	15%
CAST, ENSG00000153113	5: 96077063	35	Tetrasomy (2:2)	Pt 5: c.888+1G>T	30%
DCAF10, ENSG00000122741	9: 37860080	31	Tetrasomy (2:2)	Pt 12: c.1202_1203delCT (p.Pro401fs)	26%
DRD2, ENSG00000149295	11: 113283323	1	Monosomy	Pt 1: c.1093C>T (p.Gln365*)	19%
KLRD1, ENSG00000134539	12: 10460684	4	Trisomy	Pt 1: c.7+1G>C	21%
LDB2, ENSG00000169744	4: 16597359	34	Nil	Pt 9: c.3G>A (p.Met1?)	37%
MLH3, ENSG00000119684	14: 75514552	12	Trisomy	Pt 8: c.1806delA (p.Lys602fs)	27%
NBEAL2, ENSG00000160796	3: 47036629	25	Tetrasomy (2:2)	Pt 1: p.Phe470fs, c.1407_1408delCT	41%
PHTF1, ENSG00000116793	1: 114242872	5	Nil	Pt 4: n.468-7insA Pt 7: n.468-7delA	Pt 4: 33% Pt 7: 16%
PRL, ENSG00000172179	6: 22290411	1	Trisomy	Pt 4: c.483dupA (p.Val162fs)	13%
SKIDA1, ENSG00000180592	10: 21805663	10	Nil	Pt 5: c.1088delC (p.Pro363fs)	16%
SPTBN2, ENSG00000173898	11: 66453356	13	Nil	Pt 4: c.7159A>T (p.Lys2387*)	35%
TMEM67, ENSG00000164953	8: 94797512	2	Trisomy	Pt 1: c.1194C>A (p.Tyr398*)	40%

Chr, chromosomal; *CNV*, copy number variation; *Pt*, Patient number corresponding to tumor in which variant detected; *VAF*, variant allele frequency

[a] HGNC gene symbol, gene ID by snpEFF

[b] Pituitary rank after sorting all 53 non diseased tissue types by median TPM(transcripts per million) in GTEx

[c] Corresponding CNV at the gene locus in the same tumor

By contrast to the high burden of CNV, we found relatively few sequence variants per tumor and a lack of recurrent sequence variants between tumors. This argues against a major role of driver mutations in the pathogenesis of prolactinomas. This differs from the experience of studying somatotrophinomas, corticotrophinomas, and craniopharyngiomas, but mimics findings in other pituitary tumor subtypes. Paired tumor-normal WES studies of seven patients with non-functioning pituitary adenomas in 2013 [25] and of four patients with TSHomas in 2016 [26] also found no recurrent variants that could be considered driver mutations. A low number of somatic mutations have been observed in previous studies of pituitary adenomas compared with other neoplasms [3, 18]. However, direct comparison of absolute mutation numbers between studies is limited by differing methods of variant filtration. Within studies, we and others [3] have found no association between prolactinoma clinical characteristics and number of sequence variants.

Some variants within individual tumors were of interest due to their location in genes with a plausible connection to prolactinoma formation. This includes the truncating *DRD2* variant found in a patient with a 40-year history of prolactinoma that showed DA escape over the last 3 years despite increasing doses of cabergoline, necessitating surgery and radiotherapy. We speculate that this variant, found in 19% of tumor DNA, may reflect a subclone that is driving the patient's recent DA resistance. Indeed, D2R expression is typically high in prolactinomas, and downregulation has been hypothesized as a mechanism of DA resistance [27]. Compared with DA-responsive prolactinomas, resistant tumors demonstrate decreased D2R density, overall reduction in D2R mRNA production, and altered expression of D2R mRNA isoforms with lower expression of the more efficient short isoform [28]. In female mouse models, D2R deficiency induces lactotroph hyperplasia [29]. However, we did not find *DRD2* variants of interest in our other five patients with DA

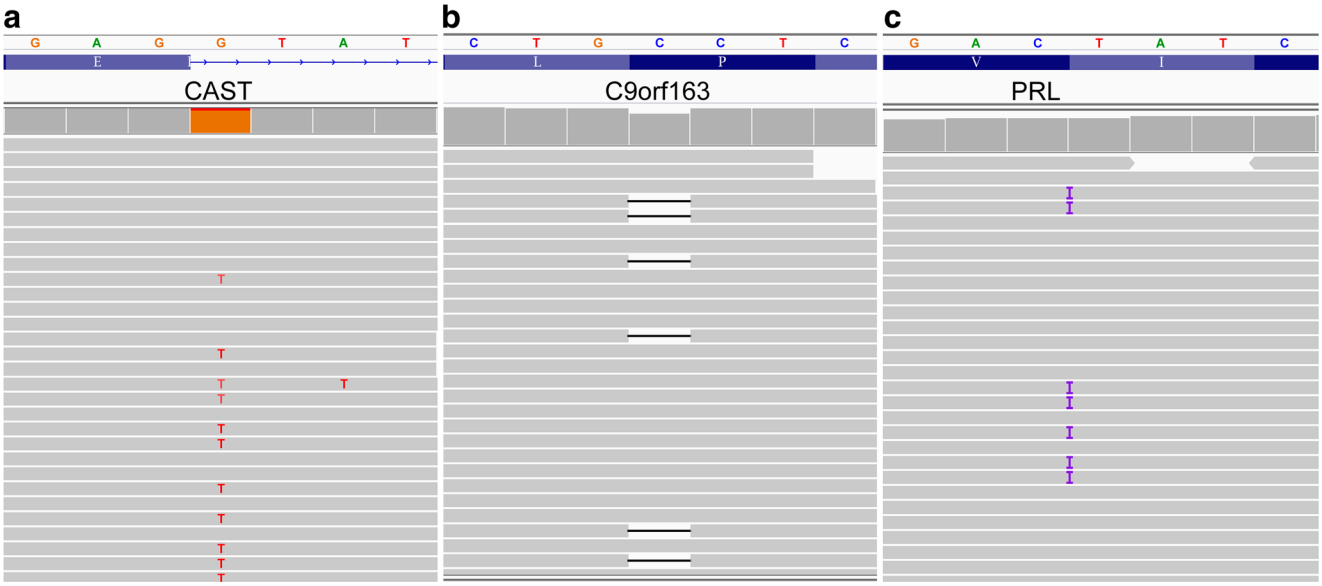

Fig. 2 Examples of somatic point variants as visualized in Integrated Genomics Viewer. **a** *CAST* missense variant in Patient 5: c.888+1G>T. **b** *C9orf163* deletion in Patient 1: c.491delC (p.Pro164fs). **c** *PRL* insertion in Patient 4: c.483dupA (p.Val162fs)

resistance. Wang et al. [16] also reported an absence of *DRD2* sequence variants, although the sensitivity of this study was reduced by its overall low depth of coverage with only 10 x coverage in 80% of the exome. Another tumor in our cohort harbored a frameshift variant in *PRL*, which is well known to be highly expressed in lactotrophs [27]. Autocrine signaling between prolactin and the abundant prolactin receptors on lactotrophs has been postulated as the explanation for the sexual dimorphism in lactotroph hyperplasia in D2R knockout mice [29]. By this theory, male mice lacking the D2R do not reach the prolactin threshold required for the feed-forward loop to activate and trigger lactotroph hyperplasia [29]. We also found isolated somatic variants in *TMEM67* where biallelic inactivating variants have been implicated in hypopituitarism [30], and in *MLH3* which is a mismatch repair gene with a possible role in Lynch syndrome [31]. Although a Lynch syndrome registry study found an increased risk of pituitary adenomas [32], there is currently no evidence of a specific role for *MLH3* in pituitary tumorigenesis.

The remaining variants of interest were located in genes with no currently known associations with the pituitary gland. Comparison with the previously published genomic studies including prolactinomas showed little overlap: Song et al. [3] found one *ANKS3* frameshift variant and two *SKIDA1* variants; and Bi et al. [18] found a *KLRD1* missense variant. None of these variants were seen in the prolactinoma subsets of these studies. In addition, none of our cases fulfilled Knudson's two-hit model of tumor suppressor genes as no patients had germline variants in the 15 genes harboring somatic variants of interest and the two patients with germline *CDH23* variants had no candidate somatic variants in *CDH23*. CNV might have arguably been the second-hit in some of these tumors as 11/15 (73%) of variants of interest were in regions of CNV in a given tumor. Trisomy and tetrasomy could be especially relevant as increased mutant dosage can amplify a dominant negative effect by a sequence variant, thereby contributing to tumorigenesis. On the other hand, the maximum VAF was 41% among the 15 variants of interest despite the frequent coexistence of CNV. Furthermore, most prolactinomas in our study harbored multiple CNV and the CNVs were large; thus, it is unlikely that any single gene in these regions of CNV can explain the pathogenesis of prolactinomas.

Table 4 Somatic CNV analysis summary showing chromosomes with whole or partial gains, losses and copy neutral LOH in either multiple or single tumors

	Copy neutral LOH	Gain	Loss	Mixed CNV and copy neutral LOH
Recurrent	Chr 1, 4, 15	Chr 1, 3, 5–10, 12, 14, 16–22, X	nil	nil
Single	Chr 5, 6, 10, 11, 16, 20	Chr 2, 4, 11, 13, 15	Chr 11, 13, 15, 18, X	Chr 1, 4, 11

Chr, chromosome; *CNV*, copy number variants; *LOH*, loss of heterozygosity

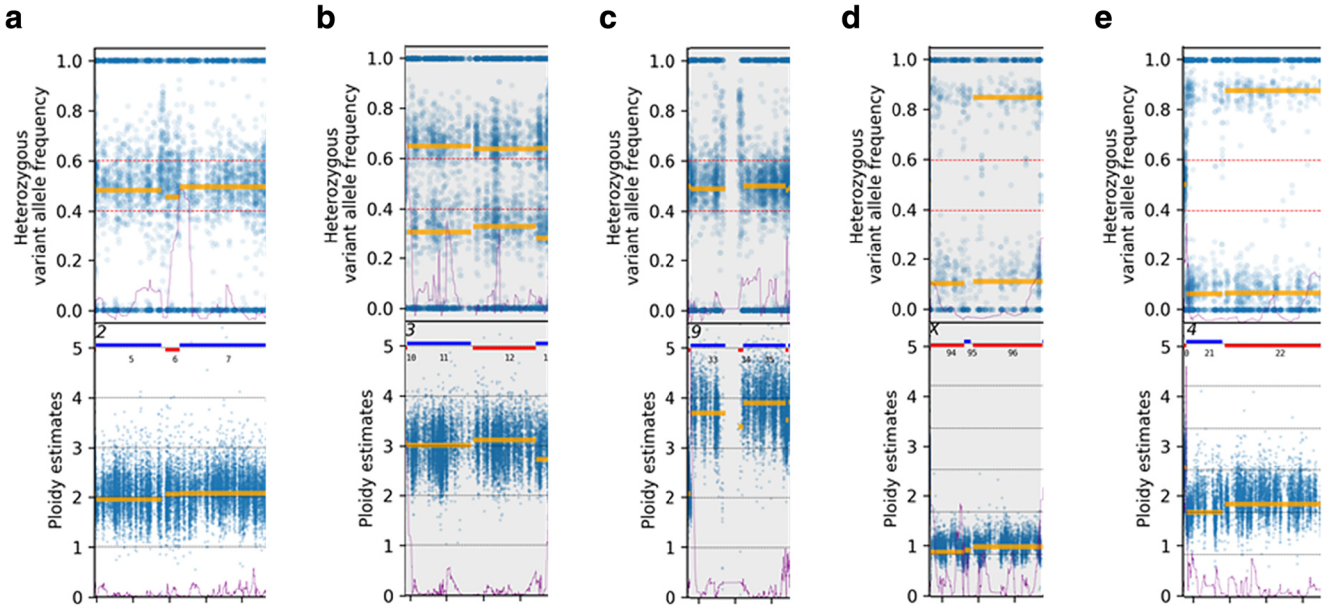

Fig. 3 Examples of tumor DNA calls of normal diploid status, chromosomal gain, chromosomal loss, and copy neutral loss of heterozygosity (LOH) based on somatic heterozygous variant allele frequency (VAF) (top panels) and ploidy estimates using depth of coverage (bottom panels). **a** Disomic baseline in Chr 2 in Patient 6 represented by the usual 0.5 heterozygous VAF and ploidy count of 2. **b** Chr 3 trisomy (2:1) in Patient 10 represented by separation of heterozygous VAF into VAFs of approximately 0.4 and 0.6 and increased ploidy count at 3. **c** Chr 9 tetrasomy (2:2) in Patient 10 represented by usual 0.5 heterozygous VAF but increased ploidy count at 4. **d** Chr X monosomy in Patient 1 (female) represented by separation of heterozygous VAF and decreased ploidy count at 1. **e** Chr 4 copy neutral LOH in Patient 1 represented by separation of heterozygous VAF but normal ploidy count of 2

A key limitation of our study is that WES does not detect deep intronic and intergenic variants, balanced translocations, fusion genes, or epigenetic changes. Integrative genomic analyses of both DNA and RNA [33] as well as the emerging technology of long-read sequencing with real-time analysis of nucleotide binding [34] may elucidate some of these possibilities. Furthermore, half of our tumor samples were FFPE, although we employed an optimized DNA extraction protocol to limit artifactual results because of this. Another limitation of our study is that the identification of CNV and copy neutral LOH was based on broad patterns in VAF and ploidy based on depth of coverage. We were thus only able to categorize CNV and copy neutral LOH at the arm or chromosomal level. Smaller CNVs may have been missed, although other data support the predominance of large-scale CNV, as observed in our tumors, over smaller CNVs [18]. The relatively low allele frequencies of our variants of interest are also noteworthy. This may seemingly contradict the known monoclonal origin of pituitary adenomas [27]. However, the < 50% VAFs seen in our tumor DNA results may reflect CNV, which was a common finding in our tumors, and/or normal tissue admixture, particularly as pituitary adenomas are rarely resected en bloc and interspersed normal pituitary tissue is a common microscopic finding. VAFs < 50% may alternatively represent the presence of multiple tumor clones. The possibility of this may be assessed in future studies through spatial transcriptomics whereby sequencing results are overlaid with tissue sections to compare the transcriptomes of different tumor regions [35]. Finally, the small size of this pilot study limited our ability to identify clinical predictors of the number of somatic variants and the number of chromosomes affected by CNV or copy neutral LOH. An independent validation set of another group of prolactinomas using the same platforms employed in this study would have been ideal to further explore these putative clinicopathological correlations and our identified genes of interest; however, surgery is rarely performed for prolactinomas and thus, further tumors were not available for investigation.

Larger studies involving sufficient numbers of different prolactinoma subsets (e.g., cystic prolactinomas or young onset prolactinomas in males) with use of fresh frozen tumor samples may better elucidate the genetic drivers of tumorigenesis. Our findings of suspicious albeit isolated somatic variants in strong candidate genes such as *DRD2* may be a function of the heterogenous patient and tumor case mix in the prolactinoma studies to date. We kept our inclusion criteria at a minimum in order to capture sufficient numbers of prolactinomas, which otherwise tend to be medically managed with DAs. Routine biobanking of pituitary adenomas will help facilitate future studies, although resected tumor tissue is often

Table 5 Pangenomic studies of paired tumor and germline DNA from prolactinoma patients. All studies employed whole exome sequencing

Study	Cohort	Filter for GOI	GOI	Recurrent CNV
Wang 2014 [16], Gao 2015 [17]	DA responsive vs resistant PRLoma ($n = 12$)	Variants differing between responsive vs resistant PRLoma	*C1orf170* *DPCR1* *DSPP* *KRTAP10–3* *MUC4* *MX2* *POTEF* *PRB3** *PRDM2** *PRG4* *RP1L1*	N/T
Song 2016 [3]	Pituitary adenoma ($n = 120$, incl 20 PRLoma)	Recurrently mutated in multiple PA	*GRB10** *IARS* *KIF5A** *SP100* *TRIP12*	Gains: Chr 1p13.2, 1q31.3, 3p22.3, 7q21.11, 16q12.2, 20p13, 20q13.33 Losses: Chr 1p36.31, 3p21.31, 9q34.11, 11q13.2, 11p15.5, 16p13.3
Bi 2017 [18]	Pituitary macroadenomas ($n = 42$, incl 3 PRLoma)	Recurrently mutated in multiple PA	*ATAD3B* *BHLHE22* *KDM2B* *OR5M1* *TTN** *VPS13B*	Gains: Chr 7, 9q, 14q Losses: Chr 1, 2, 4, 10, 11, 15q, 18 Copy neutral LOH: Chr 11q
Present study	PRLoma ($n = 12$)	Absent in population databases and strong in silico prediction for pathogenicity, or recurrently mutated in multiple PRLoma	*ANKS3* *C19orf25* *C9orf163* *CAST* *DCAF10* *DRD2** *KLRD1* *LDB2* *MLH3* *NBEAL2* *PHTF1* *PRL** *SKIDA1* *SPTBN2* *TMEM67**	Gains: Chr 1, 3, 5–10, 12, 14, 16–22, X Losses: nil Copy neutral LOH: Chr 1, 4, 15

Chr, chromosome; *CNV*, copy number variants; *DA*, dopamine agonist; *GOI*, genes of interest; *incl*, including; *LOH*, loss of heterozygosity; *n*, number of cases that underwent whole exome sequencing; *N/T*, not tested; *PA*, pituitary adenoma; *PRLoma*, prolactinoma

*particular genes of interest

piecemeal, in small quantity and potentially damaged by intraoperative cauterisation.

In conclusion, this systematic genomic study of all coding genes in a pure prolactinoma cohort demonstrated variants in genes of biologically plausible interest within individual tumors, without overlap between prolactinomas in this study or with the few other published pangenomic studies [2, 3, 16, 17]. We instead found a high degree of CNV, corroborating other preliminary studies of sporadic prolactinomas [19] and larger studies of mixed pituitary adenoma subtypes [2, 3, 18]. Further research is required to determine how CNV may contribute to prolactinoma formation and ways in which this could be therapeutically targeted.

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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 institutional research committees (Melbourne Health: HREC/16/MH/132; Royal

Adelaide Hospital: SSA/18/CALHN/445) and with the National Health and Medical Research Council guidelines.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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