Polymorphisms in *MEN1* and *DRD2* genes are associated with the occurrence and characteristics of pituitary adenomas

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Abstract

Objective: Although pituitary adenomas (PAs) affect a significant proportion of the population, only a fraction have the potential to become clinically relevant during an individual's lifetime, causing hormonal imbalance or complications due to mass effect. The overwhelming majority of cases are sporadic and without a clear familial history, and the genotype–phenotype correlation in PA patients is poorly understood. Our aim was to investigate the involvement of genes known for their role in familial cases on drug response and tumor suppression in the development and pathology of PAs in a patient group from Latvia.
Design: The study included 143 cases and 354 controls, we investigated the role of single-nucleotide polymorphisms (SNPs) in seven genes (*SSTR2*, *SSTR5*, *DRD2*, *MEN1*, *AIP*, *GNAS*, and *PRKAR1A*) associated with pituitary tumor occurrence, phenotype, and clinical symptoms.
Methods: Genotyping of 96 tag and nonsynonymous SNPs was performed in the genomic regions of interest.
Results: We discovered a significant association (OR = 17.8, CI 0.95 = 2.18–145.5, $P = 0.0002$) between a rare *MEN1* mutation (rs2959656) and clinically active adenoma in our patients. Additionally, rs7131056 at *DRD2* was associated with a higher occurrence of extrasellar growth in patients with prolactinoma and somatotropinoma (OR = 2.79, CI 0.95 = 1.58–4.95, $P = 0.0004$).
Conclusions: rs2959656, a nonsynonymous variant in *MEN1*, is associated with the development of clinically active PA. Furthermore, rs7131056 in *DRD2* contributes to either faster growth of the adenoma or reduced symptomatic presentation, allowing PAs to become larger before detection.

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Introduction

Up to 15% of clinically active primary intracranial neoplasms are pituitary adenomas (PAs) (1). Clinically significant PAs affect one individual out of approximately 1000–1300 people in the general population (2, 3). Silent adenomas are more common and are found in 1.5–27.0% (mean 10.7%) of people examined (4). Meta-analysis estimation shows a prevalence of 16.7% in the general population (5). PAs are categorized by their size and type of hormone secreted. The most common type of clinically significant PAs is prolactin-secreting (40% of all PAs), followed by nonfunctioning PAs (NFPAs) (37%) and growth hormone (GH)-secreting (somatotroph) adenomas (13%) (6). It was formerly thought that PA-caused acromegaly had a prevalence of 40–60 (7, 8) patients per million people, but more recent findings show that clinically relevant somatotroph adenomas present a rate of 124/1 000 000 (9) people in Europe and 480/1 000 000 (10) in Brazil. More than 70% of GH-secreting adenomas are larger than 10 mm in diameter (macroadenomas) at the time of diagnosis (11), probably because tumor formation can start as many as 10 years before diagnosis (7).

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There is evidence that both genetic and epigenetic factors are important for the development of human neoplasms (12). It has been shown that PAs are tumors of monoclonal origin (13). At least three separate clinical syndromes have been associated with hereditary PAs, including multiple endocrine neoplasia type 1 (MEN1), Carney complex, and familial isolated PA, caused by mutations in *MEN1*, *PRKAR1A*, and *AIP*, respectively (14, 15, 16). About 5% of PAs can be attributed to these familial syndromes, while others are considered as sporadic cases thought to be caused by mutations in various genes including tumor suppressors in the retinoblastoma tumor suppressor and p53 pathways (17). Research shows that a wider range of clinically significant adenomas tend to aggregate in families (18). The genetic mechanisms underlying common forms of adenoma are less clear, with multiple genes potentially involved in the pathogenicity of PA. A recent genome-wide association study identified two markers on chromosome 10 and one on chromosome 13 to the presence of sporadic mutations in the Han Chinese population (19). A novel finding suggests Xq26.3 microduplication responsible for sizeable proportion of the infantile pituitary gigantism cases, and further examination of the genes in this region in sporadic acromegaly cases suggests *GPR101* as a cause (20). A MEN1-like syndrome (MEN4) has been described more recently in rats and humans and relates to mutations in the *CDKN1B* gene, which may be considered as a rare cause of pituitary adenomas (21). *AIP* mutations, normally associated with familial isolated PA, have also been shown to be rarely involved in the occurrence of sporadic cases (22, 23) and lack of response to somatostatin analogs (SA) in acromegaly (18, 23, 24). Somatostatin acting through somatostatin receptors (SSTRs) is an important mediator of GH secretion and regulation of tumor proliferation; therefore, this system has been extensively studied in acromegaly (25, 26, 27), and our previous study identified an association between polymorphisms in *SSTR5* with acromegaly and number of disease parameters (28). Similarly, the involvement of the dopamine system in clinically significant PA has been suggested, since dopamine agonists inhibit production of prolactin from the pituitary gland (29). The targeting of *DRD2* with dopamine agonists has been used successfully in patients with elevated hormone levels in prolactin-secreting and/or mixed GH/prolactin adenomas (30). Somatic mutations in the Gsα gene (*GNAS*) are found in 40% of GH-secreting PAs, resulting in increased sensitivity to the inhibitory action of somatostatin (31).

In this research article, we present results from genotyping of tag single-nucleotide polymorphisms (SNPs) for seven candidate genes (*AIP*, *SSTR2*, *SSTR5*, *DRD2*, *GNAS*, *MEN1*, and *PRKAR1A*) and their neighboring regions (in strong linkage disequilibrium (LD) with markers within the gene) in samples from 143 sporadic PA patients and 354 age- and sex-matched controls (at a 1:2.5 ratio of cases to controls) in order to investigate the potential involvement of these genes in nonfamilial PA cases.

Methods

Study population and diagnosis

This study was carried out using DNA samples from the Latvian Genome Database (LGDB) (briefly described previously) (32). DNA was extracted from the whole-blood leukocytes by phenol–chloroform method. Written informed consent was acquired from all LGDB participants. The study protocol was approved by the Central Medical Ethics Committee of Latvia (protocol no. 01.29.1/28, 14 December 2011). The case group (165 participants) was enrolled for this study from the LGDB between 2004 and November 2011. Two university hospitals involved in the treatment of PA in Latvia participated in the enrollment of study cases. One hundred and fifty-eight patients were recruited from Pauls Stradiņš Clinical University Hospital and seven patients were from Riga Eastern Clinical University Hospital. Hormonal profiles (typically levels of GH, prolactin, insulin-like growth factor 1 (IGF1), adrenocorticotropic hormone (ACTH), and others, depending on the presence of clinical indications) from the blood were determined at an independent commercial laboratory (E. Gulbis Laboratory, Riga, Latvia). Prolactin was detected by electrochemical luminescence (Cobas 6000, Roche), while GH, IGF1, and ACTH levels were measured with Imulite 2000 (Siemens AG, Berlin, Germany). In cases with elevated levels of these hormones, magnetic resonance imaging (MRI) was performed. The presence of a pituitary tumor was determined using high-definition MRI data from a 1 mm slice series of the pituitary gland without contrasting agent with a Siemens Magneton 1.5T (Siemens AG, Berlin, Germany). During the first visit to the endocrinologist, phenotypic features (primary diabetes, primary hypertension, heart dysfunction, carpal bone syndrome, sleep apnea, macrognathia, and morphology of the nose and frontal bone) were also considered in the diagnosis, and further blood serum hormone testing was performed. Anthropometric and social data,

health records, and family histories were obtained when including the patient in the study. Data used in this study include sex, age, age at tumor diagnosis, PA size, extrasellar growth, and hormone-secreting type, treatment type, drug prescription information, and the occurrence of other tumors. PA was designated as a "macroadenoma" if its diameter according to the MRI data was larger than 10 mm. It must be noted that age at diagnosis does not correspond to the age of tumor incidence or age when clinical symptoms attributed to PA occurred. Phenotypic data were collected based on hospital records and interviews for all the patients selected for the study. Samples were excluded from the study due to missing phenotype data (age or sex; $N=9$), misdiagnosed as having PA in the primary examination ($N=5$), inadequate DNA sample quality ($N=2$), or rediagnosis as a pituitary carcinoma ($N=1$). Due to the low number of cases, we also excluded patients with Cushing's disease ($N=4$), leaving 144 cases eligible for genotyping.

Age- and sex-matched controls were selected from LGDB participants without endocrine or metabolic diseases or other chronic disease. Matching of samples was performed by dividing the case sample into groups based on sex, then further dividing subjects into age bins by decade. The sample of suitable controls ($N=738$) was divided in a similar manner. Controls were picked randomly from each subgroup according to a number based on each respective case group. Samples with phenotypic information missing (e.g., sex, age, or BMI) were excluded from random matching. It should be noted that sex and age matching was performed before genotyping, and thus, it was influenced by exclusion of some samples as a result of genotyping quality check. However, the medians of age and interquartile range remained similar for all respective case–control groups, and in case of linear and logistic regression, sex was used as a covariate controlling for this issue. A total of 365 controls and one interplate positive control were used in this study. The case-to-control ratio was 1:2.5.

Candidate gene and SNP selection

Seven candidate genes were selected based on an extensive literature search about known and probable PA genetics in February 2011. The genes chosen were *AIP, MEN1, GNAS, SSTR2, SSTR5, PRKAR1A*, and *DRD2*. Tag SNPs were selected within these genes and in the upstream and downstream regions in strong linkage disequilibrium (LD) ($r^2>0.8$) with markers within each gene using Haploview v4.2 software (33) and HapMap release #28 (NCBI build 36, dbSNP b126, CEU analysis panel) (34) project information available in February 2011. Nonsynonymous SNPs in these genes were also included, and the remaining two slots in the 96-SNP assay were filled with the most informative tag SNPs from *PRKAR1A*. Detailed information about the regions chosen and LD plots are available upon request. The SNP list generated by Haploview was analyzed by the Illumina Assay Design Tool (Illumina, San Diego, CA, USA). SNPs with low predicted genotyping success rates were removed and each gene was reanalyzed in Haploview using the "force exclude" option for the marked polymorphisms. The designated genotyping success rate of 0.5 was deemed acceptable in cases where no other SNP was present to capture the marker or important nonsynonymous variant selected. Otherwise, a "Designability Rank=1" was required. The final number of SNPs corresponded to the requirements of the Custom VeraCode GoldenGate Genotyping platform, offering discrimination of 96 SNPs in a single well of a 96-well microplate as one of the standard options. A full list of all SNPs and respective information about each is available upon request.

Genotyping and quality control

All 96 SNPs were genotyped using the Illumina BeadXpress system (Illumina GoldenGate genotyping assay) (35). Genotyping was carried out according to the manufacturer's instructions. In order to ensure quality control (QC) and a high intrasubject concordance rate, 9.3% or 46 DNA samples were randomly chosen and distributed to other plates for repeated genotyping. All QC steps in the manufacturer's instructions were meticulously implemented. One positive sample was used in all plates. There was >99.98% observed concordance; there was one mismatched allele call in 46 repeat pairs (one out of 5796), excluding uncalled SNPs and alleles. Additionally, only one mismatched allele call was observed among seven positive controls; therefore, no assay plates were removed from the study. Primary genotyping data analysis was performed by Illumina GenomeStudio V2010.3 Genotyping module V1.8.4. software. The Gene Call threshold was set to 0.25. The cluster images of the signal intensity were manually reviewed.

Sanger sequencing was performed to confirm rs2959656 heterozygous state in seven carriers. Polymerase chain reaction primers were 5′-AGCCAGCACTGGACAAGG-3′ and 5′-CCTTCATGCCCTTCATCTTC-3′. Sequencing

primers were 5′-GGAAGCCTCCTGGGACTGT-3′ and 5′-TCTGGAAAGTGAGCACTGGA-3′.

Statistical analysis

PLINK v1.07 (36) was used for statistical analyses. A basic association test was used to test the difference between cases and controls. Logistic regression was used to test for a difference between cases and controls, adjusting the results for smoking status and sex. Linear regression was used to test the association with quantitative variables. *P*-values lower than 0.00081 to account for Bonferroni multiple testing correction for 62 SNPs tested were considered significant. Initial *P*-values lower than 0.05 were deemed noteworthy and such results were examined closer.

Statistical power was calculated using Quanto v1.2.4 for SNP with frequencies of 1, 5, 10, and 50% using the assumptions of gene-only hypothesis, log-additive inheritance, and a population risk of 0.001 in a two-tailed test using our sample (Fig. 1).

Age was not normally distributed in the whole case or control groups due to the fact that PAs are age-related and controls were age-matched to cases. However, the quantitative variable 'age at diagnosis' was normally distributed in PA patients and subgroups of different adenoma types according to a chi-square goodness-of-fit test ($P>0.05$). Normally distributed quantitative variables are presented as mean ± S.D. Quantitative variables that do not follow normal distribution are presented as medians and interquartile range (Table 1).

Mann–Whitney rank sum test was used to compare quantitative variables that are not normally distributed.

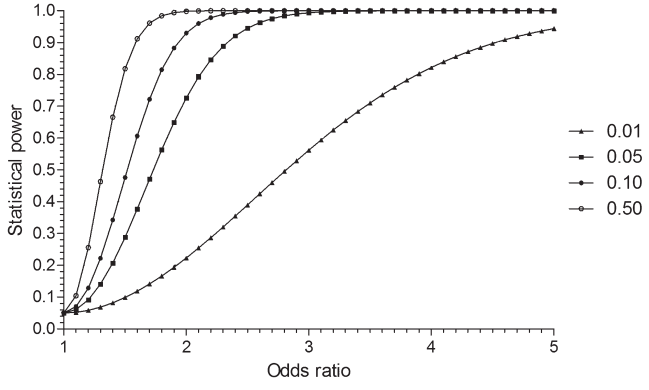

Figure 1
Statistical power calculated for samples in this study (143 cases and 355 controls) at single-nucleotide polymorphism frequencies of 0.01, 0.05, 0.1, and 0.5.

Results

The aim of this study was to genotype a set of 96 SNPs from seven candidate genes, which according to available literature data could be potentially involved in PA in 144 cases and 366 controls. Thirty-three SNPs were excluded from further analysis, with 11 SNPs excluded due to undistinguishable clusters and 22 excluded for being monomorphic (having a single cluster). Twelve samples (11 controls and one case) were excluded due to call rates of less than 90%. One SNP (rs4938025) did not pass the Hardy–Weinberg equilibrium test ($P<0.01$) according to PLINK and was excluded. After QC, 497 individuals genotyped for 62 SNPs remained eligible for further statistical analysis. Detailed characteristics of the study subjects are given in Table 1.

The strongest association signal was observed in *MEN1*. Carriers of the rare nonsynonymous SNP rs2959656 had a significantly increased risk for developing a pituitary tumor (OR = 17.8, $P=0.0002$) (Table 2). Seven heterozygous carriers of this SNP were observed in the case group, while only one 26-year-old individual from the control group had the same variant. The presence of this SNP was not associated with the specific hormonal profile of adenoma. Five of the seven

Table 1 Characteristics of study population.

Variable	Cases	Controls
n	143	354
SNPs analyzed	62	62
PA (overall) male-to-female ratio	1:1.86	–
PA (overall) mean age at diagnosis ± S.D.	44.3 ± 15.9	–
GH-secreting adenomas (*n*)	66	–
GH-secreting adenomas male-to-female ratio	1:2.14	–
GH-secreting adenomas mean age at diagnosis ± S.D.	44.7 ± 14.0	–
Prolactinomas (*n*)	46	–
Prolacinomas male-to-female ratio	1:1.56	–
Prolacinomas mean age at diagnosis ± S.D.	38.9 ± 15.6	–
NFPA (*n*)	31	–
NFPA male-to-female ratio	1:1.82	–
NFPA mean age at diagnosis ± S.D.	51.0 ± 17.6	–
PAs with extrasellar extension (*n*, %)	77 (53.8%)	–
Median age, years (Q1–Q3)	50 (35.5–60.5)	51 (39.75–62.0)
Male sex (*n*, %)	50 (35.0%)	96 (27.1%)
Smoking history (*n*, %)	87 (60.8%)	227 (64.1%)

GH, growth hormone; NFPA, nonfunctioning pituitary adenoma; PA, pituitary adenoma; Q1–Q3, interquartile range; S.D., standard deviation.

Table 2 Association results of SNPs with categorical variables (condition vs control group) (not corrected for multiple testing).

Condition	Gene	SNP	F_PA	F_C	OR (CI 95%)	*P*	*P**
PA	*MEN1*	rs2959656	0.024	0.001	17.8 (2.18–145.5)	0.0002	0.005
GH-secreting PA	*SSTR5*	rs34037914	0.113	0.046	2.63 (1.36–5.08)	0.003	0.006
	SSTR5	rs2076421	0.262	0.363	0.62 (0.41–0.95)	0.03	0.04
	SSTR5	rs169068	0.554	0.456	1.48 (1.01–2.16)	0.04	0.04
Prolactinoma	*MEN1*	rs624975	0.228	0.141	1.80 (1.06–3.06)	0.03	0.05
NFPA	*DRD2*	rs7125415	0.194	0.105	2.06 (1.05–4.04)	0.03	0.05
GH- or prolactin-secreting PA with extrasellar growth	*DRD2*	rs7131056	0.587	0.337	2.79 (1.58–4.95)	0.0004	0.0002
	DRD2	rs4938025	0.585	0.326	2.92 (1.60–5.30)	0.0004	0.002

CI, confidence interval; F_C, SNP frequency in controls; F_PA, SNP frequency in cases; OR, odds ratio; PA, pituitary adenoma; *, adjusted for covariates: sex, BMI, and smoking history.

carriers had clinically significant microadenoma and two others had macroadenomas.

However, several other SNP–phenotype associations with certain types of adenoma or specific disease phenotypes were noted and examined closer. None of these, however, remained statistically significant after Bonferroni correction for multiple testing (correcting for 62 SNPs genotyped). The strongest association trend with acromegaly was rs34037914 at *SSTR5*. A number of disease phenotypes in our study were adenoma-specific or were not equally distributed among different types of tumors. Thus, extrasellar growth of adenomas was more frequently observed in NFPA (77%) compared with both hormone-secreting adenoma groups (46%) ($P=0.004$). Therefore, we assessed the association with this phenotype in these subgroups. While none of the SNPs were associated with extrasellar growth in the NFPA group, two *DRD2* SNPs (rs7131056 and rs4938025) were associated with extrasellar growth in the joint analysis of GH- and prolactin-secreting adenomas. Association results of categorical parameters are given in Table 2.

More closely investigated association results with age at diagnosis are given in Table 3. Four SNPs show association trend with a difference in the age at diagnosis of PA. The minor alleles of three SNPs decreased the age at PA diagnosis by 5–17 years, while the minor allele of *DRD2* variant rs2734849 increased the age at diagnosis by almost 6 years. In the subgroup of cases with acromegaly, two other SNPs show trend of association with decreased age at diagnosis (rs624975 in *MEN1* and rs1800497 in *DRD2*) (Table 3).

Haplotype reconstruction using our genotyping data did not reveal strong LD between the chosen tag SNPs in the Latvian population. Differences in the allele frequencies of SNPs between the Latvian and CEU populations are available upon request.

Discussion

In this study, multiple tag SNPs were genotyped in seven candidate genes in order to investigate their influence on the development of PAs and specific disease phenotypes. Due to the limited sample size, our primary aim was to detect clinically relevant genetic changes with large effects in relation to observed phenotypes ($OR>3$; $-5>\beta>+5$). Only recently, the first genome-wide association study on PA has been published. In that study, two markers on chromosome 10 and one on chromosome 13 were associated with elevated PA risk in the Han Chinese population. With ORs ranging from 1.28 to 1.44, these SNPs have modest influence on increased risk for PA development (19). Even

Table 3 Top association results of SNPs with quantitative variables (not corrected for multiple testing).

Condition	Gene	SNP	Nc	Nwt	Beta	*P*	*P**
Age at PA diagnosis	*DRD2*	rs2734849	103	40	5.84	0.002	0.001
	DRD2	rs2002453	69	72	−5.06	0.02	0.01
	MEN1	rs607969	4	137	−17.6	0.03	0.03
	SSTR5	rs34037914	18	113	−7.07	0.04	0.04
Age at GH-secreting PA diagnosis	*MEN1*	rs607969	4	62	−19.07	0.007	0.006
	SSTR5	rs34037914	12	50	−9.21	0.009	0.02
	MEN1	rs624975	18	48	−9.12	0.009	0.01
	DRD2	rs1800497	19	27	−8.62	0.013	0.007

Beta, regression coefficient; GH, growth hormone; Nc, number of minor allele carriers; Nwt, number of wild-type carriers; PA, pituitary adenoma; *, adjusted for covariates: sex, smoking history.

if this data were available at the design stage of our study, the chance of detecting effects at these ORs in our limited population size would be low (Fig. 1).

Few candidate gene studies have suggested potential contributors to PA development. For example, *LRRC4* has been shown to have a protective effect for the T allele at rs6944446 (OR=0.44, P=0.036) (37). Another study found that two *CYP2D6* alleles increased susceptibility to pituitary tumors (38).

Recently, there is more attention on research of genetic changes in tumor samples that give insights into mutation rate and mechanisms in formation of PA and their clinical phenotypes (20, 39, 40). Main conclusion from these studies is that mutations detectable with current sequencing technologies in the exome of the somatic cells are zero (40) to seven (41) per tumor and that corresponds to generally slow proliferation of benign pituitary adenomas. However, no recurrent somatic mutations were detected in the exomes of seven NFPAs and 24 controls (41) or 12 genomes of prolactin and GH-secreting Pas (40). The heterogeneity of genetical causes of PA seems to be a major hurdle in understanding the PA formation in greater detail. This issue has been overcome in the study of early-onset pituitary gigantism where the impact of microduplication of Xq26.3 encompassing *GPR101* (gene was also found to harbor mutation in 11 sporadic acromegaly patients in the same study) was detected in 14 of 43 study participants demonstrating value of using phenotypic group as uniform as possible (20). Same conclusion can be made to discover that somatic mutations in *USP8* cause corticotroph Pas (42, 43).

The main discovery in our study is the association between the nonsynonymous *MEN1* SNP rs2959656 and the development of PA. Mutations in this tumor-suppressor gene are known to cause sporadic PA and familial MEN1 syndrome (17, 44, 45); however, this particular SNP has not been described previously as a PA marker in the literature. According to the NCBI SNP database, rs2959656 is rare among the CEU population from the HapMap project (34), but the global minor allele frequency is 0.261. It is unlikely that this SNP is the causal variant itself, given the high minor allele frequency in non-European populations. Most likely, this variant is in strong LD with the true causal variant, unique to Europeans or rare in other populations. These assumptions derive from previously published studies, indicating that none of the handful of known *MEN1* mutations is predominantly the cause of neuroendocrine tumors. To date, more than 1300 mutations are known, although there are no mutation hotspots, and functional regions of *MEN1* have not been deduced from these genotype alterations (46, 47).

There were seven rs2959656 in *MEN1* carriers among 143 PA cases and only one carrier in the 354 age- and sex-matched controls. All carriers were heterozygous and all were women. No distinct hormone-secreting profile was observed for carriers. The only minor allele carrier in controls was a 26-year-old female without a pituitary MRI. Her age (26 years) is well below mean age at PA diagnosis (44.3 years) in our sample. Literature data also suggest that pituitary tumors are more likely to affect females and with significantly higher tumor lifetime risk when *MEN1* is mutated (starting from 6.7% at the age of 20–53.4% at the age of 60) (48). This gender discrepancy has been attributed to a difference in the transcriptional regulation of the estrogen and androgen receptors (49). Often, LOH has been observed in patients with MEN1 syndrome (50). LOH in this locus can affect both *MEN1* and *AIP* or either one of them, and such situations have also been associated with Pas (51). Same could be true with inherited large deletion in this region, but not in this case, where all eight carriers were confirmed to be heterozygous for this SNP.

Several associations between SNPs and different PA types, depending on the hormone secretion profile, were observed. The strongest was a previously reported association (28) of rs34037914 in *SSTR5* with acromegaly. A similar association was observed for rs169068 (*SSTR5*), which is in LD with rs34037914. The OR in this study is within the 95% CI range of the previous study (2.63 (1.36–5.08) vs 4.51 (1.76–11.6)) (28). Nevertheless, in this study, we used a larger number of genotype markers resulting in a nonsignificant P-value after correction for 62 SNPs tested. Association of rs34037914 with age at diagnosis of acromegaly in patients with somatotroph adenoma also remained consistent with previous report. Two other markers, rs624975 in *MEN1* and rs1800497 in *DRD2*, showed similar effects as rs34037914 on younger age of the patient at acromegaly diagnosis, while the previously discussed rare rs607969 variant in *MEN1* had a more than two times higher impact on the age at diagnosis. Patients carrying this variant were younger at the time of diagnosis.

Extrasellar growth of adenomas refers to the state when the tumor enlarges to the degree that it extends outside the bony cavity (*sella turcica*) where the pituitary normally resides. Extrasellar growth may be infrasellar (into the bone), suprasellar (toward the optical chiasma and hypothalamus), or into the cavernous sinus. Not surprisingly, most (77% in our sample) of the NFPA cases

exhibited extrasellar growth at the time of diagnosis, because the physical tumor size that usually causes health issues leads to the detection of the disease. Meanwhile, in both hormonally active adenoma groups, where seeking medical help is primarily initiated due to issues linked with hormone oversecretion, the extrasellar growth rate was 46% and was identical in both groups. Due to this heterogeneity, association of extrasellar growth with genotype was carried out only in hormonally active PAs. It is still unclear why several *DRD2* and *GNAS* SNPs might be associated with extrasellar growth of adenomas. First, it is possible that these SNPs increase the aggressiveness of the tumor due to decreased *DRD2* or $G_s\alpha$ content, leading to either less-efficient receptor signaling or signal transduction, resulting in reduced suppression of PA proliferation. In order to test this hypothesis, several consecutive MRI measurements would be needed to assess adenoma dynamics. A second possibility is that these SNPs in *DRD2* and *GNAS* lead to lower hormone secretion; therefore, the adenoma has more time to grow before a person seeks medical help. Third, a completely different mechanism may involve changes in social behavior in the case of *DRD2*. Altered DRD2 signaling may lead to higher anxiety against visiting a medical center and undergoing tests, thus giving more time for tumor growth before its detection. From eight initial associations with the extrasellar growth of PAs, rs7131056, located in the first intron of the *DRD2*, remained significant after correction for covariates and multiple testing for 62 SNPs. According to previous studies, this SNP is weakly associated with migraine (52, 53), nicotine dependence (54), and social phobia (55). The latter association could support the hypothesis that people are more likely to postpone medical examination, allowing their adenoma excess time to grow.

An important limitation of this study is that patient recruitment for the study of acromegaly from two hospitals started earlier than for the rest of the PA cases, leading to overrepresentation of somatotroph adenomas in our sample, which does not reflect the typical proportion of adenoma types in the general population. Other research has shown that the most common type of adenomas is prolactin-secreting (40%), NFPAs (37%), and somatotropinomas (13%) (6). One of the main limitations of this research, and of rare disease genetics in general, is a small sample size that only allows reliable identification of genetic effects with large impacts (OR>3.6 when the allele frequency is 1%). Another limitation of our study is that the control sample had not undergone MRI diagnostics for the pituitary and therefore might contain undiagnosed PAs, especially ones with clinically silent NFPAs. However, cases with silent adenomas that become clinically relevant are generally rare and thus would not affect the analysis significantly. Finally, it should be noted that candidate genes were selected on the basis of their ability to initiate the development of clinically significant tumors rather than tumorigenesis of PA in general.

In conclusion, we have, for the first time, shown an association between rs2959656, a nonsynonymous *MEN1* SNP, with increased risk for PA development. We have also identified several other SNPs in *SSTR5*, *DRD2*, and *GNAS* that show an association trend with the occurrence of PA and their clinical characteristics, but the effects are too small to be reliably proven with our sample set. This study remains one of the few genetic studies using the representative selection of tag SNPs from several candidate genes in order to identify genetic causes of PA.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

1 Vattoth S, DeLappe RS Jr & Chapman PR. Endocranial lesions. *Seminars in Ultrasound, CT and MRI* 2013 **34** 393–411. (doi:10.1053/j.sult.2013.08.002)
2 Daly AF, Rixhon M, Adam C, Dempegioti A, Tichomirowa MA & Beckers A. High prevalence of pituitary adenomas: a cross-sectional study in the province of Liege, Belgium. *Journal of Clinical Endocrinology & Metabolism* 2006 **91** 4769–4775. (doi:10.1210/jc.2006-1668)
3 Fernandez A, Karavitaki N & Wass JA. Prevalence of pituitary adenomas: a community-based, cross-sectional study in Banbury (Oxfordshire, UK). *Clinical Endocrinology* 2010 **72** 377–382. (doi:10.1111/cen.2010.72.issue-3)
4 Molitch ME. Pituitary tumours: pituitary incidentalomas. *Best Practice & Research. Clinical Endocrinology & Metabolism* 2009 **23** 667–675. (doi:10.1016/j.beem.2009.05.001)
5 Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML & McCutcheon IE. The prevalence of pituitary adenomas: a systematic review. *Cancer* 2004 **101** 613–619. (doi:10.1002/(ISSN)1097-0142)

6 Famini P, Maya MM & Melmed S. Pituitary magnetic resonance imaging for sellar and parasellar masses: ten-year experience in 2598 patients. *Journal of Clinical Endocrinology & Metabolism* 2011 **96** 1633–1641. (doi:10.1210/jc.2011-0168)

7 Scacchi M & Cavagnini F. Acromegaly. *Pituitary* 2006 **9** 297–303. (doi:10.1007/s11102-006-0409-4)

8 Holdaway IM & Rajasoorya C. Epidemiology of acromegaly. *Pituitary* 1999 **2** 29–41. (doi:10.1023/A:1009965803750)

9 Daly AF, Tichomirowa MA & Beckers A. The epidemiology and genetics of pituitary adenomas. *Best Practice & Research. Clinical Endocrinology & Metabolism* 2009 **23** 543–554. (doi:10.1016/j.beem.2009.05.008)

10 Rosario PW. Frequency of acromegaly in adults with diabetes or glucose intolerance and estimated prevalence in the general population. *Pituitary* 2011 **14** 217–221. (doi:10.1007/s11102-010-0281-0)

11 Melmed S. Medical progress: acromegaly. *New England Journal of Medicine* 2006 **355** 2558–2573. (doi:10.1056/NEJMra062453)

12 Peltomaki P. Mutations and epimutations in the origin of cancer. *Experimental Cell Research* 2012 **318** 299–310. (doi:10.1016/j.yexcr.2011.12.001)

13 Jacoby LB, Hedley-Whyte ET, Pulaski K, Seizinger BR & Martuza RL. Clonal origin of pituitary adenomas. *Journal of Neurosurgery* 1990 **73** 731–735. (doi:10.3171/jns.1990.73.5.0731)

14 Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, Emmert-Buck MR, Debelenko LV, Zhuang Z, Lubensky IA, Liotta LA *et al*. Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* 1997 **276** 404–407. (doi:10.1126/science.276.5311.404)

15 Kirschner LS, Sandrini F, Monbo J, Lin JP, Carney JA & Stratakis CA. Genetic heterogeneity and spectrum of mutations of the PRKAR1A gene in patients with the carney complex. *Human Molecular Genetics* 2000 **9** 3037–3046. (doi:10.1093/hmg/9.20.3037)

16 Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, Tuppurainen K, Ebeling TM, Salmela PI, Paschke R *et al*. Pituitary adenoma predisposition caused by germline mutations in the AIP gene. *Science* 2006 **312** 1228–1230. (doi:10.1126/science.1126100)

17 Zhou Y, Zhang X & Klibanski A. Genetic and epigenetic mutations of tumor suppressive genes in sporadic pituitary adenoma. *Molecular and Cellular Endocrinology* 2014 **386** 16–33. (doi:10.1016/j.mce.2013.09.006)

18 Couldwell WT & Cannon-Albright L. A heritable predisposition to pituitary tumors. *Pituitary* 2010 **13** 130–137. (doi:10.1007/s11102-009-0212-0)

19 Ye Z, Li Z, Wang Y, Mao Y, Shen M, Zhang Q, Li S, Zhou L, Shou X, Chen J *et al*. Common variants at 10p12.31, 10q21.1 and 13q12.13 are associated with sporadic pituitary adenoma. *Nature Genetics* 2015 **47** 793–797. (doi:10.1038/ng.3322)

20 Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, Schernthaner-Reiter MH, Szarek E, Leal LF, Caberg JH *et al*. Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. *New England Journal of Medicine* 2014 **371** 2363–2374. (doi:10.1056/NEJMoa1408028)

21 Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Hofler H, Fend F, Graw J & Atkinson MJ. Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *PNAS* 2006 **103** 15558–15563. (doi:10.1073/pnas.0603877103)

22 Cazabat L, Guillaud-Bataille M, Bertherat J & Raffin-Sanson ML. Mutations of the gene for the aryl hydrocarbon receptor-interacting protein in pituitary adenomas. *Hormone Research* 2009 **71** 132–141. (doi:10.1159/000197869)

23 Daly AF, Tichomirowa MA, Petrossians P, Heliovaara E, Jaffrain-Rea ML, Barlier A, Naves LA, Ebeling T, Karhu A, Raappana A *et al*. Clinical characteristics and therapeutic responses in patients with germ-line AIP mutations and pituitary adenomas: an international collaborative study. *Journal of Clinical Endocrinology & Metabolism* 2010 **95** E373–E383. (doi:10.1210/jc.2009-2556)

24 Chahal HS, Trivellin G, Leontiou CA, Alband N, Fowkes RC, Tahir A, Igreja SC, Chapple JP, Jordan S, Lupp A *et al*. Somatostatin analogs modulate AIP in somatotroph adenomas: the role of the ZAC1 pathway. *Journal of Clinical Endocrinology & Metabolism* 2012 **97** E1411–E1420. (doi:10.1210/jc.2012-1111)

25 Taboada GF, Luque RM, Neto LV, Machado Ede O, Sbaffi BC, Domingues RC, Marcondes JB, Chimelli LM, Fontes R, Niemeyer P *et al*. Quantitative analysis of somatostatin receptor subtypes (1-5) gene expression levels in somatotropinomas and correlation to in vivo hormonal and tumor volume responses to treatment with octreotide LAR. *European Journal of Endocrinology* 2008 **158** 295–303. (doi:10.1530/EJE-07-0562)

26 Lania A, Mantovani G & Spada A. Genetic abnormalities of somatostatin receptors in pituitary tumors. *Molecular and Cellular Endocrinology* 2008 **286** 180–186. (doi:10.1016/j.mce.2007.08.013)

27 Wildemberg LE, Neto LV, Costa DF, Nasciuti LE, Takiya CM, Alves LM, Rebora A, Minuto F, Ferone D & Gadelha MR. Low somatostatin receptor subtype 2, but not dopamine receptor subtype 2 expression predicts the lack of biochemical response of somatotropinomas to treatment with somatostatin analogs. *Journal of Endocrinological Investigation* 2013 **36** 38–43.

28 Ciganoka D, Balcere I, Kapa I, Peculis R, Valtere A, Nikitina-Zake L, Lase I, Schioth HB, Pirags V & Klovins J. Identification of somatostatin receptor type 5 gene polymorphisms associated with acromegaly. *European Journal of Endocrinology* 2011 **165** 517–525. (doi:10.1530/EJE-11-0416)

29 Stack J & Surprenant A. Dopamine actions on calcium currents, potassium currents and hormone release in rat melanotrophs. *Journal of Physiology* 1991 **439** 37–58. (doi:10.1113/jphysiol.1991.sp018655)

30 Jallad RS & Bronstein MD. The place of medical treatment of acromegaly: current status and perspectives. *Expert Opinion on Pharmacotherapy* 2013 **14** 1001–1015. (doi:10.1517/14656566.2013.784744)

31 Landis CA, Harsh G, Lyons J, Davis RL, McCormick F & Bourne HR. Clinical characteristics of acromegalic patients whose pituitary tumors contain mutant Gs protein. *Journal of Clinical Endocrinology & Metabolism* 1990 **71** 1416–1420. (doi:10.1210/jcem-71-6-1416)

32 Ignatovica V, Latkovskis G, Peculis R, Megnis K, Schioth HB, Vaivade I, Fridmanis D, Pirags V, Erglis A & Klovins J. Single nucleotide polymorphisms of the purinergic 1 receptor are not associated with myocardial infarction in a Latvian population. *Molecular Biology Reports* 2012 **39** 1917–1925. (doi:10.1007/s11033-011-0938-4)

33 Barrett JC, Fry B, Maller J & Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005 **21** 263–265. (doi:10.1093/bioinformatics/bth457)

34 International HapMap C. The International HapMap Project. *Nature* 2003 **426** 789–796. (doi:10.1038/nature02168)

35 Lin CH, Yeakley JM, McDaniel TK & Shen R. Medium- to high-throughput SNP genotyping using VeraCode microbeads. *Methods in Molecular Biology* 2009 **496** 129–142. (doi:10.1007/978-1-59745-553-4)

36 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics* 2007 **81** 559–575. (doi:10.1086/519795)

37 Xiao L, Tu C, Chen S, Yu Z, Lei Q, Wang Z, Xu G, Wu M & Li G. LRRC4 haplotypes are associated with pituitary adenoma in a Chinese population. *Medical Oncology* 2014 **31** 888. (doi:10.1007/s12032-014-0888-5)

38 Gomes L, Lemos MC, Paiva I, Ribeiro C, Carvalheiro M & Regateiro FJ. CYP2D6 genetic polymorphisms are associated with susceptibility to pituitary tumors. *Acta Médica Portuguesa* 2005 **18** 339–343.

39 Luque RM, Sampedro-Nunez M, Gahete MD, Ramos-Levi A, Ibanez-Costa A, Rivero-Cortes E, Serrano-Somavilla A, Adrados M, Culler MD, Castano JP *et al.* In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters. *Oncotarget* 2015 **6** 19619–19633. (doi:10.18632/oncotarget)

40 Valimaki N, Demir H, Pitkanen E, Kaasinen E, Karppinen A, Kivipelto L, Schalin-Jantti C, Aaltonen LA & Karhu A. Whole-genome sequencing of growth hormone (GH)-secreting pituitary adenomas. *Journal of Clinical Endocrinology & Metabolism* 2015 **100** 3918–3927. (doi:10.1210/jc.2015-3129)

41 Newey PJ, Nesbit MA, Rimmer AJ, Head RA, Gorvin CM, Attar M, Gregory L, Wass JA, Buck D, Karavitaki N *et al.* Whole-exome sequencing studies of nonfunctioning pituitary adenomas. *Journal of Clinical Endocrinology & Metabolism* 2013 **98** E796–E800. (doi:10.1210/jc.2012-4028)

42 Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, Meitinger T, Mizuno-Yamasaki E, Kawaguchi K, Saeki Y *et al.* Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nature Genetics* 2015 **47** 31–38. (doi:10.1038/ng.3166)

43 Perez-Rivas LG, Theodoropoulou M, Ferrau F, Nusser C, Kawaguchi K, Stratakis CA, Faucz FR, Wildemberg LE, Assie G, Beschorner R *et al.* The gene of the ubiquitin-specific protease 8 is frequently mutated in adenomas causing Cushing's disease. *Journal of Clinical Endocrinology & Metabolism* 2015 **100** E997–E1004. (doi:10.1210/jc.2015-1453)

44 Cuny T, Pertuit M, Sahnoun-Fathallah M, Daly A, Occhi G, Odou MF, Tabarin A, Nunes ML, Delemer B, Rohmer V *et al.* Genetic analysis in young patients with sporadic pituitary macroadenomas: besides AIP don't forget MEN1 genetic analysis. *European Journal of Endocrinology* 2013 **168** 533–541. (doi:10.1530/EJE-12-0763)

45 Frohman LA & Eguchi K. Familial acromegaly. *Growth Hormone & IGF Research* 2004 **14** (Supplement A) S90–S96. (doi:10.1016/j.ghir.2004.03.021)

46 Wautot V, Vercherat C, Lespinasse J, Chambe B, Lenoir GM, Zhang CX, Porchet N, Cordier M, Beroud C & Calender A. Germline mutation profile of MEN1 in multiple endocrine neoplasia type 1: search for correlation between phenotype and the functional domains of the MEN1 protein. *Human Mutation* 2002 **20** 35–47. (doi:10.1002/humu.10092)

47 Lemos MC & Thakker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. *Human Mutation* 2008 **29** 22–32. (doi:10.1002/humu.v29:1)

48 Goudet P, Bonithon-Kopp C, Murat A, Ruszniewski P, Niccoli P, Menegaux F, Chabrier G, Borson-Chazot F, Tabarin A, Bouchard P *et al.* Gender-related differences in MEN1 lesion occurrence and diagnosis: a cohort study of 734 cases from the Groupe d'etude des Tumeurs Endocrines. *European Journal of Endocrinology* 2011 **165** 97–105. (doi:10.1530/EJE-10-0950)

49 Lips CJ, Dreijerink KM & Hoppener JW. Variable clinical expression in patients with a germline MEN1 disease gene mutation: clues to a genotype-phenotype correlation. *Clinics* 2012 **67** (Supplement 1) 49–56. (doi:10.6061/clinics/2012(Sup01)10)

50 Thakker RV. Multiple endocrine neoplasia type 1 (MEN1). *Best Practice & Research. Clinical Endocrinology & Metabolism* 2010 **24** 355–370. (doi:10.1016/j.beem.2010.07.003)

51 Georgitsi M, Heliovaara E, Paschke R, Kumar AV, Tischkowitz M, Vierimaa O, Salmela P, Sane T, De Menis E, Cannavo S *et al.* Large genomic deletions in AIP in pituitary adenoma predisposition. *Journal of Clinical Endocrinology & Metabolism* 2008 **93** 4146–4151. (doi:10.1210/jc.2008-1003)

52 Todt U, Netzer C, Toliat M, Heinze A, Goebel I, Nurnberg P, Gobel H, Freudenberg J & Kubisch C. New genetic evidence for involvement of the dopamine system in migraine with aura. *Human Genetics* 2009 **125** 265–279. (doi:10.1007/s00439-009-0623-z)

53 Corominas R, Ribases M, Camina M, Cuenca-Leon E, Pardo J, Boronat S, Sobrido MJ, Cormand B & Macaya A. Two-stage case-control association study of dopamine-related genes and migraine. *BMC Medical Genetics* 2009 **10** 95. (doi:10.1186/1471-2350-10-95)

54 Huang W, Payne TJ, Ma JZ, Beuten J, Dupont RT, Inohara N & Li MD. Significant association of ANKK1 and detection of a functional polymorphism with nicotine dependence in an African-American sample. *Neuropsychopharmacology* 2009 **34** 319–330. (doi:10.1038/npp.2008.37)

55 Sipila T, Kananen L, Greco D, Donner J, Silander K, Terwilliger JD, Auvinen P, Peltonen L, Lonnqvist J, Pirkola S *et al.* An association analysis of circadian genes in anxiety disorders. *Biological Psychiatry* 2010 **67** 1163–1170. (doi:10.1016/j.biopsych.2009.12.011)

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