Whole-exome sequencing identifies variants in invasive pituitary adenomas

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Abstract. Pituitary adenomas exhibit a wide range of behaviors. The prediction of invasion or malignant behavior in pituitary adenomas remains challenging. The objective of the present study was to identify the genetic abnormalities associated with invasion in sporadic pituitary adenomas. In the present study, the exomes of six invasive pituitary adenomas (IPA) and six non-invasive pituitary adenomas (nIPA) were sequenced by whole-exome sequencing. Variants were confirmed by dideoxynucleotide sequencing, and candidate driver genes were assessed in an additional 28 pituitary adenomas. A total of 15 identified variants were mainly associated with angiogenesis, metabolism, cell cycle phase, cellular component organization, cytoskeleton and biogenesis immune at a cellular level, including 13 variants that occurred as single nucleotide variants and 2 that comprised of insertions. The messenger RNA (mRNA) levels of diffuse panbronchiolitis critical region 1 (DPCR1), KIAA0226, myxovirus (influenza virus) resistance, proline-rich protein BstNI subfamily 3, PR domain containing 2, with ZNF domain, RIZ1 (PRDM2), PR domain containing 8 (PRDM8), SPANX family member N2 (SPANXN2), TRIO and F-actin binding protein and zinc finger protein 717 in IPA specimens were 50% decreased compared with nIPA specimens. In particular, DPCR1, PRDM2, PRDM8 and SPANXN2 mRNA levels in IPA specimens were approximately four-fold lower compared with nIPA specimens (P=0.003, 0.007, 0.009 and 0.004, respectively). By contrast, the mRNA levels of dentin sialophospho protein, EGF like domain, multiple 7 (EGFL7), low density lipoprotein receptor-related protein 1B and dynein, axonemal, assembly factor 1 (LRRC50) were increased in IPA compared with nIPA specimens (P=0.041, 0.037, 0.022 and 0.013, respectively). Furthermore, decreased PRDM2 expression was associated with tumor recurrence. The findings of the present study indicate that DPCR1, EGFL7, the PRDM family and LRRC50 in pituitary adenomas are modifiers of tumorigenesis, and most likely contribute to the development of oncocytic change and to the invasive tumor phenotype.

Introduction

Pituitary adenomas account for 10-15% of all intracranial neoplasms and are incidentally identified in <27% of non-selected autopsies (1). The clinical presentation of pituitary adenomas depends on the structural and functional characteristics of the tumor (2). The World Health Organization (WHO) categorizes pituitary tumors into typical adenomas, atypical adenomas and pituitary carcinomas; of which, typical adenomas constitute the major class. However, the WHO classification does not offer an accurate association between the histopathological findings and the clinical behavior of the tumor (3). An estimated 35-55% of pituitary adenomas demonstrate invasion into bones, dura or adjacent structures, including the cavernous or sphenoid sinuses (4). Clinically defined invasive pituitary adenomas (IPAs) demonstrate earlier and more frequent recurrences, and may be resistant to conventional treatments, such as surgery and radiotherapy (5). Specific biomarkers that distinguish between aggressive and nonaggressive pituitary adenomas have not yet been identified, although certain studies suggest that the Ki-67 proliferation index may be of diagnostic value (3). The WHO classification of endocrine tumors indicates that invasion of the surrounding structures, size at presentation, an elevated mitotic index, a Ki-67 labeling index of >3% and extensive tumor protein p53 (p53) expression are indicators of aggressive behavior (6,7). However, Ki-67 and p53 labeling index evaluations demonstrate subjective variability, and the cutoff values...
are controversial (8). Clinically, endocrine tumors present a challenging management problem, with a high frequency of incomplete resections, tendency for recurrence and notable morbidity (9).

Previously, several studies attempted to identify novel molecular markers [fibroblast growth factor receptor 4, matrix metalloproteinases, Ki-67, p53, cyclooxygenase-2, galectin-3, angiogenesis molecules and pituitary tumor-transforming 1 (PTTG)] that require additional validation (10-13). In a previous study, multivariate Cox regression analysis assessed galectin-3 immunohistochemical expression in ≥30% of neoplastic cells; galectin-3 messenger RNA expression was indicated to be a strong predictive factor of recurrence or tumor progression (P<0.001); and a Ki-67 labeling index of >3% (P=0.019) was indicated in the 81 cases with available follow-up data (12). PTTG expression may be associated with tumor invasiveness and microvessel density of pituitary adenomas (13). Apoptosis and mitoses represent two adverse and asynchronous events that maintain the optimal cell numbers; cyogenetic analysis may, therefore, be useful in defining the biological invasion of pituitary tumors (14). In addition, predicting the subsequent risk of disease invasion or drug sensitivity is challenging. However, mutations in classic oncogenes and tumor-suppressor genes are rarely associated with these tumors (3-6,8-15). Nonfunctioning pituitary adenomas (NFPAs) result in few somatic mutations, which is consistent with the associated low proliferation rates and benign nature; however, mechanisms other than somatic mutation are likely to be involved in the etiology of sporadic NFPAs (16). The majority mechanisms of endocrine tumorigenesis differ significantly from those associated with haematological malignancies and non-endocrine tumors (17). In addition, the genetic events underpinning the development of invasion or refractory pituitary adenomas are not yet understood (18).

In order to identify the genetic events that may be contributing to the invasion of pituitary adenomas, whole-exome sequencing, which has been successfully used to find variants in multiple tumor types, was applied (16,19-21). Through stringent variant calling and filtering parameters, 15 identified variants were mainly associated with cell cycle phase, cellular component organization and biogenesis at cellular level by whole-exome sequencing in combination with homozygosity mapping between IPAs and non-invasive pituitary adenomas (nIPA). The present study supports the role of somatic variants of the PR domain (PRDM) gene family, which is known to control cell proliferation in cancer and in normal development, in IPAs.

Materials and methods

Patients and specimens. Specimens from six IPAs and six nIPAs were obtained from patients that underwent endoscopic transsphenoidal surgery between December 2009 and January 2010 at Beijing Tiantan Hospital, Beijing, China. Informed consent was obtained from all individuals and ethical approval was obtained form the Institutional Review Board of Beijing Tiantan Hospital Affiliated to Capital Medical University. Pituitary adenomas, obtained from 12 patients (5 men and 7 women; mean age, 40.7 years; range, 16.0-63.0 years) that did not have a family history of endocrine neoplasia, were characterized based on presurgical clinical and biochemical findings, including a pituitary hormone test. This tested for 12 types of pituitary hormone: Growth hormone, adrenocorticotropic hormone, follicle-stimulating hormone, luteinizing hormone, estradiol, progesterone, human growth hormone, cortisol, total triiodothyronine, total thyroxine, thyroid-stimulating hormone and prolactin (PRL) levels (4 patients in normal range, 8 patients with increased PRL levels; normal range, 2.5-17 ng/ml). Pituitary adenomas were also characterized based on morphological and immunohistochemical analysis of removed tissue samples (Table I). Cases with multiple hormonal changes according to the clinical and pathological data were excluded. The following IPA diagnostic criteria were adopted: i) Knosp classification grade III-IV tumors and Hardy classification invasive adenomas; ii) tumor cells confirmed via pathology as invading the sphenoid bone or adjacent dura mater; iii) tumor cells invading the sphenoid sinus cavity or peripheral vascular and nerve; iv) Ki-67 labeling index of >3% (22). The tumors did not have atypical features, and constituted the ‘discovery’ set of tumors for exome capture and DNA sequence analysis. An additional 28 pituitary adenomas, histologically confirmed, were obtained from 13 women and 15 men (mean age, 61 years; range, 17-71 years), and these constituted the ‘validation’ set. For histological analysis, the tumor specimens were divided into two sections. One was stored in liquid nitrogen and the other was fixed in 4% paraformaldehyde for 24 h (Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China) within 0.5 h of surgery. After washing for 6 h in flowing water, the specimens underwent gradient dehydration in alcohol, were embedded in paraffin wax (Leica Biosystems Richmond Inc., Richmond, IL, USA) and sectioned at a thickness of 5 µm. Sections were incubated with primary mouse anti-human monoclonal PRDM2 antibody (catalog no., ab3791; dilution, 1:200; Abcam, Cambridge, MA, USA) at 4°C overnight. Next, sections were washed three times with phosphate-buffered saline (PBS; ZSBG-BIO), then incubated with DyLight-conjugated AffiniPure secondary antibody (goat anti-mouse IgG H+L; catalog no., ZF-0313; ZSBG-BIO, Beijing, China) with fluorescence was added at room temperature for 1 h followed by 3 washes with PBS (5 min each). Streptavidin-Biotin Complex (ZSBG-BIO) was added for 20 min and then the sections were washed with PBS. Next, sections were mounted with ProLong Gold Antifade reagent (ZSBG-BIO) with DAPI (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Staining was visualized using a LEICA-TCS-SP5II microscope (Leica, Wetzlar, Germany). The percentage of DAPI-stained cells exhibiting PRDM2 immunoreactivity was analyzed in 5 randomly selected high power fields.

Specimen preparation, exome capture, DNA sequencing and bioinformatics analysis. Total DNA was extracted from pituitary adenomas using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). An aliquot containing 5 µg of genomic DNA was purified and quantified from each specimen. Exome enrichment was performed by using an ABI SOLiD optimized SureSelect Human All Exon kit (Agilent Technologies, Inc., Santa Clara, CA, USA), which included the exonic sequences of ~18,000 genes, covering a total of 42 Mb of genomic sequences. The enriched exome libraries were then
amplified by emulsion polymerase chain reaction (ePCR; Ion PI™ Hi-Q™ OT2 200 kit; cat no. a26434), according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.), and based on a library concentration of 0.5 pM. The PCR products were then sequenced on a SOLiD5500 sequencer (Thermo Fisher Scientific, Inc.), and one quadrant of a SOLiD sequencing slide was required for each sample.

Color-space reads were mapped to the hg19 reference human genome using SOLiDBioScope software (5500 W Series Genetic Analyzer V2.0; Thermo Fisher Scientific, Inc.), which is suitable for a repetitive mapping approach. Single-nucleotide polymorphisms (SNPs) were then called using the diBayes algorithm with conservative default call stringency. Known SNPs available from the Single Nucleotide Polymorphism Database Table II. Methylation validation primers.

| Gene     | Forward primer | Reverse primer |
|----------|----------------|----------------|
| PRDM8    | 5'-ATTCCCTTTCAACGACAGA-3' | 5'-AAGAGTTGGAATACGGTACAA-3' |
| PRDM2    | 5'-GGCCAAGAAGGGAGAAGGC-3' | 5'-AAGTCCAGGACTCAATGTGAC-3' |
| MGAM     | 5'-GGCGGAGTCTGGCTTATT-3' | 5'-GTATGACAGTGCAGTTCAGGA-3' |
| SPANXN2  | 5'-GAGGGAGGACGAGGGCCCTTACA-3' | 5'-CTCAGGTACCAATGGCCATGTA-3' |
| TRIOBP   | 5'-CCAGGCTCCCTCATGACAC-3' | 5'-TGTTGCGAGCAAGACCT-3' |
| ZNF717   | 5'-CCTTCCCTGGTATGCTTACCT-3' | 5'-TCAGAGAATCTCATGGTTCAGCA-3' |
| PRB3     | 5'-CCCCACAAAGGGAGAAGGAAACCAt-3A-3' | 5'-CCCAAGGAGGACAAGGAGT-3' |
| DPCR1    | 5'-TTCTGATTGGACTCCCTCTC-3' | 5'-TAGTGGATCTCCTGACCT-3' |
| DSPP     | 5'-ATCTCTTGTATTTAGCTACCCC-3' | 5'-AATATAATTTGATACATCAAC-3' |
| MX2      | 5'-AAGAGTCGGAGCCAGGCACCC-3' | 5'-GGGTTGACAGGACCC-3' |
| EGFL7    | 5'-TCCTGGGTGGGTGGCTCAGACGTCG-3' | 5'-AATIGGATGATGGCCTATG-3' |
| LRR5C50  | 5'-CGAGACCATCTACTGCACCACACA-3' | 5'-TGTTCCCTTGTTGAGGAC-3' |
| LRPIB    | 5'-ACGCAATTTGGAATCTTGCTTA-3' | 5'-ACGCGTACATTACATACCC-3' |
| MAST4    | 5'-CTTGAATCTCTGTCTCAGATGTG-3' | 5'-ACAGGAATCTGTTGGATC-3' |
| RP1L1    | 5'-GCTTCCTTTGATATCTTCTCTTTT-3' | 5'-TTCACTCTGGAAAATCTTACCC-3' |
| GAPDH    | 5'-CAGCTGAGGGACCCTATGAA-3' | 5'-AGGATGTCATCTGAGGTG-3' |

PRDM8, PR domain containing 8; PRDM2, PR domain containing 2, with ZNF domain, RIZ1; MGAM, maltase-glucoamylase (α-glucosidase); SPANXN2, SPANX family member N2; TRIOBP, TRIO and F-actin binding protein; ZNF717, zinc finger protein 717; PRB3, proline-rich protein BstNI subfamily 3; DPCR1, diffuse panbronchiolitis critical region 1; DSPP, dentin sialophospho protein; MX2, myxovirus (influenza virus) resistance; EGFL7, EGF like domain, multiple 7; LRRC50, dynein, axonemal, assembly factor 7; LRPIB, low density lipoprotein receptor-related protein 1B; MAST4, microtubule associated serine/threonine kinase; RP1L1, retinitis pigmentosa 1-like 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Color-space reads were mapped to the hg19 reference human genome using SOLiDBioScope software (5500 W Series Genetic Analyzer V2.0; Thermo Fisher Scientific, Inc.), which is suitable for a repetitive mapping approach. Single-nucleotide polymorphisms (SNPs) were then called using the diBayes algorithm with conservative default call stringency. Known SNPs available from the Single Nucleotide Polymorphism Database

Table I. Clinical data of patients.

| Specimen | Tumor sub-type | PRL, ng/ml | Age, years | Gender | Tumor volume, cm³ | Histology | Ki-67 index, % | Invasive variants | No. of variants |
|----------|----------------|------------|------------|--------|-------------------|-----------|----------------|------------------|-----------------|
| 1        | NFPA           | 5.8        | 63         | F      | 16.4              | (-)       | >3             | Yes              | 23              |
| 2        | NFPA           | 13.2       | 53         | F      | 4.8               | (-)       | 1-2            | No               | 12              |
| 3        | NFPA           | 9.4        | 42         | M      | 13.2              | (-)       | >3             | Yes              | 28              |
| 4        | NFPA           | 11.7       | 53         | F      | 7.4               | (-)       | 1-2            | No               | 14              |
| 5        | PRL            | 182        | 25         | F      | 10.7              | PRL (+)   | >3             | Yes              | 27              |
| 6        | PRL            | 1,625      | 16         | F      | 12.2              | PRL (+)   | >3             | Yes              | 37              |
| 7        | PRL            | 3,117      | 54         | M      | 14.3              | PRL (+)   | >3             | Yes              | 26              |
| 8        | PRL            | 268        | 29         | M      | 9.2               | PRL (+)   | >3             | Yes              | 32              |
| 9        | PRL            | 123        | 32         | F      | 2.4               | PRL (+)   | 1-2            | No               | 21              |
| 10       | PRL            | 233        | 43         | F      | 3.1               | PRL (+)   | 1-2            | No               | 20              |
| 11       | PRL            | 2,899      | 34         | M      | 1.9               | PRL (+)   | 1-2            | No               | 25              |
| 12       | PRL            | 2,830      | 44         | M      | 4.1               | PRL (+)   | 1-2            | No               | 19              |

PRL normal range, 2.5-17 ng/ml. NFPA, nonfunctioning pituitary adenomas; PRL, prolactin; F, female; M, male.
Table III. Reverse transcription-quantitative polymerase chain reaction primers.

| Gene     | Forward primer | Reverse primer |
|----------|----------------|----------------|
| DPCR1    | 5'-AGTGTCTGCTCCCTCTCCTTCTCTCTCTTA-3' | 5'-GGGAGCTCTGGAGGTCTTTTGC-3' |
| DSPP     | 5'-GCAATTTGGCGATAGCTACGG-3' | 5'-CTGACACATTGTATCCTGGCAGGAG-3' |
| MGAM     | 5'-GGCGGAGTGCTCCTTGTCTTAT-3' | 5'-GTATGACAGTGCGTTCTCACAGA-3' |
| EGFL7    | 5'-ATGTGGATGAATGCACTGCT-3' | 5'-TGGCCACCTCTGGTGGTT-3' |
| MX2      | 5'-GCCAGCTGGAGTAAAAGTACACAA-3' | 5'-AGGTCAGTATGTCAGTTGG-3' |
| LRRC50   | 5'-CGAGACACTCTGACCAACAC-3' | 5'-TGGTTCCTTGTAGTTCTTGAGATA-3' |
| PRDM2    | 5'-AGCAGCTGGATTTAGGAGA-3' | 5'-CAGAGGTAAAATCTCGGCT-3' |
| PRDM8    | 5'-ATTCCTCCCTTAACACGACCAAGA-3' | 5'-AGAAGTTGGATACGTCGTAAA-3' |
| LRP1B    | 5'-AGCCCAATTCGAATCCCTGTA-3' | 5'-TTGCAATGACTAATCCATGTTT-3' |
| RP1L1    | 5'-AGAAGCGAGGCAGTTAACCTTCTG-3' | 5'-TCACACTCCTGGCTTGTT-3' |
| PRB3     | 5'-CCTCCCAGCAAGATGCTAGTT-3' | 5'-GGGAGATCTTCTCCGGCTA-3' |
| ZNF717   | 5'-CCTTTTGCTGTAGCTATCTTCT-3' | 5'-TCAGAAGATCTGCTGGA-3' |
| MAST4    | 5'-CTTTGACATCCGCTCACAAGGACTTC-3' | 5'-ACAAGAATGTTGTTGAC-3' |
| SPANXN2  | 5'-GAGAGGAGCAAGGCTCCTAGA-3' | 5'-CTCATCTCAATGGCCATGTA-3' |
| TRIOBP   | 5'-CCAGGCTTTCTCTATGACAC-3' | 5'-TGTTGACAGCAGGCAGC-3' |
| GAPDH    | 5'-TGAGGGCGGATTCTCCTTCTTA-3' | 5'-TGTTGACACCAACTCTGTTAGA-3' |

DPCR1, diffuse panbronchiolitis critical region 1; DSPP, dentin sialophospho protein; MGAM, maltase-glucoamylase (α-glucosidase); EGFL7, EGF like domain, multiple 7; MX2 myxovirus (influenza virus) resistance; LRRC50, dynein, axonemal, assembly factor 1; PRDM2, PR domain containing 8; RP1L1, retinitis pigmentosa 1-like 1; PRB3, proline-rich protein BstN1 subfamily 3; ZNF717, zinc finger protein 717; MAST4, microtubule associated serine/threonine kinase; SPANXN2, SPANX family member N2; TRIOBP, TRIO and F-actin binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(dbSNP) version 130, which is maintained by the National Center for Biotechnology Information, were excluded.

Mutation validation. Primer3 software (version 0.4.0; http://frodo.wi.mit.edu/primer3/) was used to generate primers for the PCR amplification of variants identified via exome sequencing or exons covered in additional screening using a SOLiD5500xl sequencer (Thermo Fisher Scientific, Inc.; Table II). The DNA ladder (DL1000; Takara Bio, Inc., Otsu, Japan) and ethidium bromide were purchased from Takara Bio, Inc. Amplification products of an appropriate size were identified using agarose gel electrophoresis (100 V, 30 min). Amplicons from 3 normal pituitary and 28 pituitary tumor DNA molecules coupled with leukocyte were sequenced using SOLiD sequencing or exons covered in additional screening using SOLiD5500xl. Sequencing products were subjected to gradient dewatering, removed of water, treated with fresh 3% hydrogen peroxide (ZSGB-BIO) at room temperature for 10 min and washed with phosphate-buffered saline (pH 7.2; ZSGB-BIO) 3 times for 5 min each. For microwave repair, the specimens were placed in 0.01% citric acid (pH 6.0; ZSGB-BIO), kept warm in a microwave oven (600 W; LG Electronics Appliances Co., Ltd., Tianjin, China) for 10 min, allowed to cool to room temperature and washed once with PBS for 10 min. Antibody repair solution was added at room temperature for 10 min, and then washed 3 times with PBS for 5 min each time. PRDM2 antibody (monoclonal; Abcam Inc., Eugene, OR, USA) was added at a 1:200 dilution and incubated at 4˚C overnight. The DyLight™-conjugated AffiniPure secondary antibody with fluorescence (ZSGB-BIO) was added at room temperature for 1 h, followed by 3 washes with PBS for 5 min each time. Streptavidin-biotin complex was added for 20 min and then washed with PBS for 5 min. Sections were mounted with ProLong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen; Thermo Fisher Scientific, Inc.). Sections were analyzed with a LEICA-TCS-SP5U (Leica Microsystems GmbH, Wetzlar, Germany) to estimate the percentage of DAPI-stained cells displaying PRDM2 immunoreactivity.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from frozen normal pituitary and pituitary adenomas (~50 mg) using the TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed as described previously (23), using the Applied Biosystems 7500 Fast System (Thermo Fisher Scientific, Inc.) and the primers indicated in Table III. The fold-change in differential expression for each gene was calculated using the comparative Cq method (also known as the 2-ΔΔCq method), as previously described (24).

Immunohistochemical analysis. Pituitary adenomas and pituitary gland specimens were sectioned to a thickness of 5 µm in paraffin wax (Leica Biosystems Richmond Inc.). The sections were subjected to gradient dewatering, removed of water, treated with fresh 3% hydrogen peroxide (ZSGB-BIO) at room temperature for 10 min and washed with phosphate-buffered saline (pH 7.2; ZSGB-BIO) 3 times for 5 min each. For microwave repair, the specimens were placed in 0.01% citric acid (pH 6.0; ZSGB-BIO), kept warm in a microwave oven (600 W; LG Electronics Appliances Co., Ltd., Tianjin, China) for 10 min, allowed to cool to room temperature and washed once with PBS for 10 min. Antibody repair solution was added at room temperature for 10 min, and then washed 3 times with PBS for 5 min each time. PRDM2 antibody (monoclonal; Abcam Inc., Eugene, OR, USA) was added at a 1:200 dilution and incubated at 4˚C overnight. The DyLight™-conjugated AffiniPure secondary antibody with fluorescence (ZSGB-BIO) was added at room temperature for 1 h, followed by 3 washes with PBS for 5 min each time. Streptavidin-biotin complex was added for 20 min and then washed with PBS for 5 min. Sections were mounted with Prolong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen; Thermo Fisher Scientific, Inc.). Sections were analyzed with a LEICA-TCS-SP5U (Leica Microsystems GmbH, Wetzlar, Germany) to estimate the percentage of DAPI-stained cells displaying PRDM2 immunoreactivity.

Statistical analysis. All statistical analyses were performed using SPSS version 20.0 (IBM SPSS, Armonk, NY, USA). For comparisons, one-way analyses of variance, χ² tests, Wilcoxon rank-sum tests and two-tailed Student’s t-tests were performed as appropriate. Binary logistic regression was performed
Table IV. Variants identified in sporadic pituitary adenomas.

| Gene        | Gene name                                      | Exon-ID | Coverage | Variant | Start   | End     | Reference | Gene type | Protein change | Catalog (case ≥4) | Mutation type |
|-------------|------------------------------------------------|---------|----------|---------|---------|---------|-----------|-----------|-----------------|------------------|---------------|
| DPCR1       | Diffuse panbronchiolitis critical region 1     | DPCR1-2 | 104      | SNV     | 30919188| 30919188| A         | C         | N983H           | IPA              | Missense       |
| DSPP        | Dentin sialophospho protein                    | DSPP-5  | 109      | SNV     | 88536900| 88536900| A         | G         | N1029S          | IPA              | Missense       |
| EGFL7       | EGF-like-domain, multiple 7                    | EGFL7-10| 38       | SNV     | 139565452| 139565452| G         | A         | D208N           | nIPA             | Missense       |
| KIAA0226    | KIAA0226                                       | KIAA0226-16| 52      | SNV     | 197409451| 197409451| C         | T         | -               | IPA              | Synonymous     |
| LRRC50      | Dynein, axonemal, assembly factor 1            | LRRC50-8| 33       | SN      | 84203660| 84203660| G         | A         | -               | IPA              | Synonymous     |
| LRP1B       | Low density lipoprotein receptor-related protein 1B | LRP1B-7  | 46       | SNV     | 141946094| 141946094| G         | A         | V303I           | nIPA             | Missense       |
| MAST4       | Microtubule associated serine/threonine kinase family member 4 | MAST4-32 | 57       | Insert  | 65892764| 65892764| -         | GCT       | -               | nIPA             | Frame shift    |
| MGAM        | Maltase-glucoamylase (α-glucosidase)            | MGAM-3  | 37       | SNV     | 141708332| 141708332| C         | A         | P52T            | nIPA             | Missense       |
| MX2         | Myxovirus (influenza virus) resistance         | MX2-10  | 33       | SNV     | 42771182| 42771182| G         | A         | -               | IPA              | Synonymous     |
| PRB3        | Proline-rich protein BstNI subfamily 3         | PRB3-3  | 37       | SNV     | 11420621| 11420621| G         | A         | P188W           | nIPA             | Missense       |
| PRDM2       | PR domain containing 2, with ZNF domain, RIZ1   | PRDM2-10| 53       | Insert  | 14106398| 14106398| -         | TCC       | -               | IPA              | Frame shift    |
| PRDM8       | PR domain containing 8                         | PRDM8-10| 32       | SNV     | 81122528| 81122528| A         | G         | N102D           | nIPA             | Missense       |
| SPANXN2     | SPANX family, member N2                        | SPANXN2-2| 50      | SNV     | 142796177| 142796177| T         | C         | L167Q           | nIPA             | Missense       |
| TRIOBP      | TRIO and F-actin binding protein                | TRIOBP-7| 36       | SNV     | 38119487| 38119487| A         | C         | E308D           | nIPA             | Missense       |
| ZNF717      | Zinc finger protein 717                        | ZNF717-5| 70       | SNV     | 75786450| 75784650| G         | A         | T775H           | nIPA             | Missense       |

SNV, single nucleotide variant; Insert, insertion; IPA, invasive pituitary adenoma; nIPA, non-invasive pituitary adenoma.
to identify the independent factors associated with pituitary adenoma recurrence. *P*<0.05 was used to indicate a statistically significant difference.

**Results**

Identification of variant genes by whole-exome sequencing.
For the identification of tumor-specific somatic variants, whole-exome capture using DNA from the discovery set of six IPAs and six nIPAs yielded excellent target region coverage, with ~72% of the exome covered to a depth of at least 30-fold between the somatic variant calling algorithm and confirmatory sequencing. Several prioritization steps were taken to decrease the number of genetic variants and to find the potentially pathogenic variants, as follows: i) Variants should have a deleterious effect on protein function (as predicted by protein prediction software, such as PolyPhen-2, MutationTaster and SIFT); ii) variants should be present at sufficient allele frequency to represent likely heterozygous or homozygous changes (i.e., 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 (25,26); and iii) variants should be involved in biological processes relevant to tumorigenesis (27). Approximately 90% of single-nucleotide variants (SNVs) resulted in missense amino-acid changes, whereas the remaining (~10%) were synonymous changes. Over 70% of the SNVs occurred as CG-T-A transitions, and <30% were transversions. Using stringent variant calling and filtering parameters (16), 233 variants were identified in the specimens.

In addition, five variants (C8orf79, chr8:12879694; FSHD region gene 1 family member B, pseudogene, chr20:29632674; mucin 2, oligomeric mucus/gel-forming, chr1:1092715; mucin 6, oligomeric mucus/gel-forming, chr11:1018092; and solute carrier family 5 member 3, chr21:35467473) were present in all specimens, and 47 were detected in either the IPA or nIPA. Of these, 15 were somatic variants confirmed by dideoxynucleotide sequencing. Of the 15 confirmed variants, 13 occurred as SNVs, including three synonymous SNVs, and two comprised insertions (Table IV). The genes with variants were generally associated with angiogenesis, metabolism, cell cycle phase, cellular component organization, cytoskeleton and biological immunity at a cellular level. The genes include: EGF like domain, multiple 7 (EGFL7), associated with angiogenesis; low density lipoprotein receptor-related protein 1B (LRP1B) and maltase-glucoamylase (α-glucosidase) associated with cell metabolism; dentin sialophospho protein (DSPP), PR domain containing 2, with ZNF domain, RIZ1; mRNA, values at or above 50th percentile were classified as high levels. The genes with variants were most likely pathogenic variants, as follows:

| Parameter       | Higha | Low    | χ²   | P-value |
|-----------------|-------|--------|------|---------|
| All cases       | 12    | 12     |      |         |
| Age, years      |       |        |      |         |
| ≥50             | 4     | 6      | 0.689| 0.406   |
| <50             | 8     | 6      |      |         |
| Gender          |       |        |      |         |
| Male            | 6     | 7      | 0.168| 0.682   |
| Female          | 6     | 5      |      |         |
| Tumor size, cm  |       |        |      |         |
| ≥2              | 5     | 4      | 0.178| 0.673   |
| <2              | 7     | 7      |      |         |
| Recurrenceb     |       |        |      |         |
| Yes             | 4     | 10     | 6.511| 0.011   |
| No              | 8     | 2      |      |         |

*Median expression level was used as the cutoff between the high and low mRNA levels. Recurrence was defined as the discovery of an elevated PRL level at any time in the postoperative surveillance period after an initial remission. Low PRDM2 mRNA levels were defined as values below the 50th percentile of the 12 patients; values at or above 50th percentile were classified as high levels. PRDM2, PR domain containing 2, with ZNF domain, RIZ1; mRNA, messenger RNA.

Analysis of the expression of variant genes by RT-qPCR.

RT-qPCR was used to test whether the invasion of pituitary adenomas was associated with differences in the expression levels of 15 genes. Expression of DPCR1, KIAA0226, MX2,
and age, gender or tumor size. However, decreased PRDM2 protein levels were more frequently observed in recurrent tumors (Fig. 3). Furthermore, binary multivariate regression revealed that decreased levels of PRDM2 were independently associated with tumor recurrence (odds ratio, 0.065; 95% confidence interval, 0.050-0.832; P=0.036).

Discussion

Tumor invasion may be based on clinical, radiological and pathological features (2-6,8-21,23,27-30). However, no standard or comparable score on IPA is generally accepted, except for radiological classification. IPAs are associated with a poor prognosis, as therapeutic options are limited. In addition, IPAs tend to recur quickly following initial treatment, are generally unresponsive to therapy and are a challenge to manage (5). In the present study, 15 somatic variants that are mainly associated with metabolism, cell cycle phase, cellular component organization, cytoskeleton and biological immunity at a cellular level, but not with genes previously implicated in pituitary adenomas, were identified by whole-exome sequencing.

A growing body of evidence suggests a coevolutionary model of cancer, wherein the cross-talk between tumor cells and the host determine the malignant potential of individual tumors (31). Endogenous T cells respond to and infiltrate tumors, significantly delaying malignant progression in mouse models (32). Low expression levels of interleukin-6 and signal transducer and activator of transcription 3 were indicated to be significant in the dysimmunity of pituitary adenoma (33). DPCR1, located between major histocompatibility complex (MHC), class I (HLA)-B and HLA-A on chromosome 6p21.33, is classified as one of the HLA molecules. The DPCR1 gene may contain markers for diagnosis of diffuse pan-bronchiolitis, a bronchiolar disease that affects human airways (34). To fully escape the immune system, cancer cells typically mutate to decrease the expression of antigens, lose expression of HLA proteins or employ an aberrant antigen processing pathway (35). However, to the best of our knowledge, the association between DPCR1 variations and the risk of IPA has not yet been investigated. The mRNA level of DPCR1 is approximately four-fold lower in IPA compared with in nIPA specimens; therefore, the invasion of pituitary adenomas is hypothesized to be associated with the induction of immune-escape via the downregulation of DPCR1.

The expansion of solid tumors depends on the continuous growth of novel blood vessels from pre-existing capillaries. The role of angiogenesis and tumor blood vessels in the pathogenesis of pituitary tumors remains a mystery. A previous study indicated the involvement of prolactin during vasculature remodelling by acting on the endothelial and perivascular cells in pituitary adenomas (36). The vascular-specific secreted factor EGFL7 is a component of the interstitial extracellular matrix (ECM) and regulates the proper spatial organization of endothelial cells within each filopodia, affecting the collective movement of the cells (37). A previous study indicated that the expression of EGFL7 in neural stem cells (NSCs) in vitro decreased NOTCH-specific signaling and resulted in the decreased proliferation and self-renewal of NSCs (38). EGFL7 acts as a soluble NOTCH inhibitor, which is in contrast to the typical NOTCH

Figure 3. Confocal images show the number of PRDM2-positive puncta in recurrent pituitary adenomas. (A) Normal pituitary; (B) non-invasive pituitary adenoma; (C) invasive pituitary adenoma. Green, PRDM2; dilution, 1:200. Blue, 4',6-diamidino-2-phenylindole; dilution, 1:3,000. Scale bar, 50 μm. PRDM2, PR domain containing 2, with ZNF domain, RIZ1.

PRDM2 levels are associated with recurrence in pituitary adenomas. The usual morphological signs of tumor aggression are poorly associated with the invasive potential of pituitary tumors, proliferation capacity, tendency of post-surgical recurrence and global biological behavior (28). PRDM2 contains a PR domain that demonstrates histone H3 lysine 9 methylation activity (29). Therefore, whether decreased levels of PRDM family mRNA in pituitary adenomas was associated with certain clinical parameters was assessed (Table V). No significant association was indicated between PRDM2 mRNA levels
heterogeneity and uncertainty of the boundaries of diagnostic classification. Whole-exome sequencing may be useful in the future for a wide range of applications to medicine.

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