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Germline Prolactin Receptor mutation is not a major cause of sporadic prolactinoma in humans

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Abstract

Background/Aims. No genetic anomalies specifically predisposing humans to prolactinomas have so far been identified. The prolactin receptor (PRLR) is a good candidate, however, as *Prlr* knockout mice develop prolactinomas, and a case of familial hyperprolactinemia has been linked to *PRLR* mutation. The main objective of this study was to detect germline *PRLR* mutations in patients with sporadic prolactinomas unrelated to *AIP* or *MEN1* mutation.

Methods. We sequenced all *PRLR* exons and intron-exon junctions on genomic DNA from 88 patients with a median age of 24 years.

Results. We identified four *PRLR* variations (p.Ile76Val, p.Ile146Leu, p.Glu108Lys and p.Glu554Gln) in 16 patients. One patient had the rare variant p. Glu554Gln in the heterozygous state. Another patient had the extremely rare p.Glu108Lys variant described here for the first time. The other two variants (p.Ile76Val and p.Ile146Leu) are relatively common in the general population. All these four variants have been functionally tested *in vitro* and have no effect on PRLR expression, localization and signalling after prolactin stimulation.

Conclusion. Inactivating germline variations of *PRLR* are not associated with sporadic prolactinoma in this series. Nevertheless, somatic disruption of *PRLR* has not been excluded in this subset of pituitary tumors.

Introduction

Prolactin (PRL) is a hormone mainly secreted by lactotroph cells of the anterior pituitary gland. As its name implies, its main role is in lactation. PRL acts via its membrane receptor PRLR, which belongs to the hematopoietic cytokine superfamily [1,2]. The human *PRLR* gene is located on chromosome 5 and contains 11 exons [3]. Non physiologic hyperprolactinemia occurs in about 0.1% to 0.3% of the general population and can result in hypogonadism, infertility and galactorrhea [4,5]. About 50% of cases of hyperprolactinemia unrelated to pregnancy are due to benign PRL-secreting pituitary tumors called prolactinomas. The vast majority of these tumors are sporadic, and little is known of the mechanisms underlying their development.

Genes known to be involved in pituitary tumorigenesis include *aryl hydrocarbon receptor interacting protein (AIP)* and *multiple endocrine neoplasia type 1 (MEN1)* [6,7], mutations of which are associated with various subtypes of pituitary adenoma. Recently, two new genes were implicated in tumorigenesis arising from specific pituitary cell lineages: *GPR101* and the *encoding ubiquitin-specific protease 8 (USP8)* gene were reported to be specifically involved in the development of somatotroph and corticotroph adenomas, respectively [8,9]. To date, no gene mutations have been shown to specifically predispose humans to pituitary lactotroph hyperplasia or pituitary adenomas. However, many years ago, our team developed a model of *Prlr*-deficient mice [10], which exhibited hyperprolactinemia in both sexes. Female *Prlr*-null mice developed pituitary hyperplasia by 6 months of age and large tumors by late adulthood (14 months). Male *Prlr*-null mice exhibited a less severe phenotype, with only moderate pituitary enlargement at 18 to 21 months of age [11]. The proposed mechanism of prolactinoma's development in this model is through a loss of negative dopaminergic growth control, which results from a lack of PRL action on the hypothalamus. We therefore suspected that germline *PRLR* mutation might also be a specific genetic cause of prolactinoma in humans. This suspicion was reinforced in late 2013 by the identification of the first inactivating germline mutation of the human *PRLR* gene, in three sisters with familial idiopathic hyperprolactinemia [12]. The diagnosis was based on high plasma PRL levels in the absence of medications or medical conditions known to provoke hyperprolactinemia. No adenoma was found on magnetic resonance imaging

(MRI), but the presence of a microadenoma too small to be detected by MRI could not be ruled out [12].

The aim of this single-center study was to examine the possible involvement of *PRLR* mutations in a series of patients with prolactinoma.

Patients and Methods

Patients

We studied 88 unrelated patients with sporadic prolactinomas referred to Bicêtre hospital between January 2010 and January 2015. Clinical characteristics of the patients are summarized in Table 1. Median age at diagnosis was 24 years. Most of the patients had macroprolactinomas (diameter >10 mm). All 88 patients had a genetic analysis: the entire *AIP* and *MEN1* coding region and intron-exon junctions were amplified and sequenced [13], they were also screened for large deletions or duplication of the *AIP* and *MEN1* genes by multiplex ligation-dependent probe amplification [14]. Patients with *MEN1* and *AIP* mutations were excluded. Informed consent was obtained from all the patients prior to blood sampling and molecular studies.

DNA sequencing

Genomic DNA was isolated from human blood leukocytes by standard methods. Reference sequences were obtained from NCBI (Gene ID: 5618). The entire coding sequence and intron-exon junctions of the *PRLR* gene (NG 029042.1) were amplified from gDNA with 10 sets of primers (Supplemental Table 1). Following a 5-minute hot start, DNA was amplified for 35 cycles (95°C for 45 s, 56°C for 1 min, and 72°C for 45 s) in 1X buffer containing 2 mM MgCl₂, 0.5 mM dNTPs, 0.5 μM each primer, 100 ng of gDNA and 1.5 U of *DreamTaq* (Fermentas) in a total volume of 50 μL. The amplified samples were subjected to agarose gel electrophoresis to check for the presence of single bands. The PCR products were purified with the QIAquick kit (Qiagen) and then directly sequenced on an automated 3100 Applied Biosystems device, using the same primers as for PCR. The reported nucleotide numbering corresponds to cDNA numbering (www.hgvs.org/mutnomen), with +1 corresponding to the A of the ATG translation initiation codon (codon 1) in the reference sequence.

In silico prediction of PRLR variants

We used PolyPhen-2 v.2.2.5 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://sift.jcvi.org/>), SNPs&GO (<http://snps-and-go.biocomp.unibo.it/>), UMDpredictor (<http://umd-predictor.eu/>), and Mutation Taster (<http://www.mutationtaster.org/>) software to predict the possible functional effect of PRL amino acid variations.

Structural location of the mutations

The structure of the human PRL/PRLR complex (code 3D48 [15]) was loaded from the Protein Data Bank (PDB: <http://www.rcsb.org/pdb/home/home.do>).

Amino acid replacements and figures were performed with Pymol (DeLano, <http://pymol.sourceforge.net/>).

Antibodies

Antibodies directed against HA (sc-805, Santacruz, 1:1000 dilution), STAT5 (C-17, Santa Cruz Biotechnology, 1:1000 dilution), phosphorylated STAT5 (C11C5, Cell Signaling 1:500 dilution), α -tubulin (T6199, Sigma, 1:5000 dilution) and horseradish peroxidase-linked secondary antibodies (Thermoscientific, 1:10 000 dilution) were used for western blot analyses and/or immunoprecipitation assays. Antibody directed against HA (Roche diagnostics, 1:500 dilution) was used for indirect immunofluorescence. The secondary antibody used for immunofluorescence analysis was donkey anti rat Alexa fluor (Invitrogen, 1:2000 dilution).

Plasmids and mutagenesis

PRLR mutations were introduced by means of site-directed mutagenesis using the QuickChange XLII Kit (Stratagene) into a *HA-PRLR* construct. The LHRE (lactogenic hormone response element)-firefly luciferase reporter plasmid contains six repeats of the STAT5 DNA binding sequence from the beta casein gene promoter [16]

Cell culture

Human embryonic kidney 293 (HEK293) and monkey kidney (COS-7) cell lines were grown in 10% fetal bovine serum in DMEM-F12, supplemented with L-glutamine and penicillin-streptomycin antibiotics at 37°C in a humidified atmosphere of 5% CO₂. Cells were transiently transfected with wild-type or mutant *HA-PRLR* constructs using FuGENE 6 (Promega), according to the manufacturer's protocol.

Immunofluorescence and confocal microscopy

COS-7 cells were prepared on glass coverslips, which were then placed in 24-well tissue culture plates. They were transiently transfected with wild-type or mutant HA-PRLR-expression constructs. After 48 hours, cells were incubated for 1 h at 4°C with an anti-HA-antibody diluted in PBS, 1% BSA to observe membrane expression. The cells were washed and fixed for 15 min in 4% formaldehyde. After saturation with PBS, 1% BSA for 1 h the cells were incubated for 1 h with a fluorescent secondary antibody. Cells were fixed in 4% paraformaldehyde (SigmaAldrich) and permeabilized using 0.2% Triton X-100 in PBS, before being incubated with anti-HA-antibody diluted in PBS, 1% BSA. The cells were washed three times and then incubated with appropriate secondary antibodies diluted in the same buffer. After washing, the samples were mounted in Vectashield plus 4',6-diamidino-2-phenylindole (DAPI Fluoromont G, Southern Biotech) that stains nuclei. Imaging was carried out on a SP8 Leica confocal microscope.

Western blot analysis

Six-well plates of HEK293 cells were transiently transfected with wild-type or mutant HA-PRLR-expression constructs. Thirty hours following transfection, cells were incubated in serum-free media for 6 hours, followed by 15 minutes of 1000 ng/mL PRL treatment. Cells were then lysed in 65 mM Tris, pH 6.8, 4% SDS, 1.5% β -mercaptoethanol and held at 100°C for 5 min. Proteins were separated on 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene difluoride membrane. For immunoblotting, the membranes

were probed overnight with relevant antibodies and then incubated with appropriated fluorescent secondary antibodies. Bound immunoglobulins were revealed by fluorescent detection (Odyssey, Li-Cor).

Immunoprecipitation assays

For immunoprecipitation study, cells were lysed at 4 °C for 2 hours in lysis buffer (20 mM Tris HCl, 50 mM NaCl, 1% Triton, proteases and phosphatases inhibitors) followed by centrifugation at 16,000 g for 30 min at 4 °C to remove cell debris. Supernatants were incubated overnight at 4 °C with polyclonal anti-STAT5 antibody and Protein G-Sepharose beads (Sigma). Protein G-Sepharose beads were washed three times with lysis buffer and three times with 20 mM Tris, pH 7.5, 50 mM NaCl. The immune complexes were eluted at 95 °C in 65 mM Tris pH 6.8, 4% SDS and 1.5% β -mercaptoethanol and analyzed by SDS-PAGE using anti-phosphorylated-STAT5 antibody, and after stripping, the anti-STAT5 antibody mentioned above.

LHRE-luciferase reporter assay

HEK293 cells were transiently co-transfected in 96-well plates using FuGENE 6 (Promega), according to the manufacturer's protocol with 40 ng of either wild-type or mutant PRLR-expression constructs, 100 ng of LHRE-firefly luciferase reporter plasmid, and 20 ng of the control vector containing Renilla luciferase (Promega). Following transfection, cells were incubated in serum-free media overnight, treated with 0-1000ng/mL PRL for 24 hours in serum-free media and then lysed with passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured consecutively by using dual-luciferase assays (Promega) and a TriStar reader (Berthold) and are expressed as relative light units. The experiment was performed three times in sixplicates.

Statistical analyses

Data are expressed as a mean \pm S.E.M. of at least three experiments. Two-way anova with Bonferroni post-test was performed using PRISM software (GraphPad Software).

Results

Prevalence of PRLR variants in the cohort

Analysis of the *PRLR* gene revealed 4 missense variants in 16 of the 88 prolactinoma patients. These substitutions were found in the heterozygous and homozygous states in respectively 14 and 2 patients. Three variants, two recurrent (p.Ile76Val and p.Ile146Leu [17,18]) and one novel (p.Glu108Lys), were located in the extracellular domain of the protein. The fourth, p.Glu554Gln, lays the intracellular domain (Figure 1). The p.Ile76Val and p.Ile146Leu variants were referenced with allelic frequencies of 4.3 and 1.9%, respectively, in the new Exome Aggregation Consortium (ExAC) Browser Database <http://exac.broadinstitute.org>, which includes more than 120 000 alleles, whereas p.Glu108Lys and p.Glu554Gln were referenced with very minor frequencies. The allele frequency of each variant was compared with the one reported in ExAC database (Table 2), suggesting that only p.Glu554Gln variant was overrepresented in our series.

In silico analysis

We then conducted *in silico* analyses (Table 3) using 5 different tools to predict the possible impact of the amino acid substitutions on the structure and function of PRLR. Among the four identified variants, p.Glu554Gln was predicted to be "probably detrimental" by 2 of the 5 tools. The p.Glu108Lys and p.Ile146Leu variants were predicted to be disease-causing by only 1 of the 5 tools. The p.Ile76Val variant was predicted to be benign by all the tools. In addition, we performed multiple protein sequence alignment of PRLR residues in orthologs for the new variant p.Glu108Lys, which indicated a moderate evolutionary conservation of this novel variant (Figure 2.A).

Modeling of the novel p.Glu108Lys missense variant

The crystal structure of the PRLR extracellular domain is available to analyze the consequence of the p.Glu108Lys homologous human mutation in the complex formed between the extracellular domain of PRLR and the PRL ligand (Figure 2. B and C). This revealed that amino acid 108 faced

outwards. Then, the mutation in lysine does not apparently modify the general conformation of the protein, indicating that this amino acid substitution is probably a polymorphic variant rather than a mutation.

Functional characterization of the four missense variants

To investigate the functional activity of these four missense variants, we used HEK293 and COS-7 cells, which express components of the JAK2–STAT5 pathway. COS-7 and HEK293 cells that were transfected with *wild-type* and mutated *PRLR* constructs had similar levels of expression and cellular localization of the respective PRLR (Figure 3.A and B). Furthermore, wild-type, Ile76Val, Glu108Lys, Ile146Leu and Glu554Gln PRLR showed a similar increase in phosphorylated STAT5 when treated with PRL (Figure 3.C). In accordance to the effect on STAT5 phosphorylation, the variants induced similar activation on LHRE-luciferase reporter gene expression (Figure 3.D), whereas transfection of the *PRLR* loss of function mutant His188Arg reported by Newey et al. [12], used as a negative control, failed to activate the reporter gene expression.

Discussion

Prl or *Prlr* knockout causes hyperprolactinemia and promotes the formation of lactotroph tumors in mice [10,19], suggesting that the PRL pathway might also be involved in the development of prolactinomas in humans. No specific genetic abnormality has yet been described in prolactinoma patients. However, very recently, Newey and colleagues reported the first loss-of-function mutation in the human *PRLR* gene, in three patients with familial hyperprolactinemia [12], further supporting *PRLR* as a candidate gene for lactotroph cell tumorigenesis. To test this possibility, we screened for *PRLR* germline mutations in a large cohort of patients with sporadic prolactinomas. To enhance the probability of finding a relevant genetic event, we focused mainly on young patients with macroprolactinomas [13,20].

We detected four variants affecting 16 of the 88 patients with sporadic prolactinomas. Two of these variants are very rare (p.Glu554Gln and p.Glu108Lys) in the general population, and thus could

have been involved in prolactinoma development. The p.Glu554Gln *PRLR* variant was found in one patient, in the heterozygous state. This variant is very rare, as it is referenced with very low allelic frequencies in ExAC Browser database. Two of five *in silico* analysis tools suggested it was "likely deleterious" but the current study showed a JAK2-STAT5 signaling *in vitro* and a transcriptional activity not different to those of the wild-type *PRLR*. Thus, the p.Glu554Gln substitution, which affected only one patient in our cohort, does not seem to predispose humans to prolactinoma. The other original variant, p.Glu108Lys, found in one patient in the heterozygous state, is not fully conserved across species and was predicted to be benign by 4 of the 5 *in silico* tools. It is extremely rare in the general population, found on only 2 of 121 250 alleles in the ExAC server. Modelling suggests that this missense variation does not modify the general protein conformation. According to this prediction, the functional characterization of this variant demonstrated that it could not be considered as pathogen.

Several considerations argue against a role of the other two recurrent variants in human prolactinoma development. The most frequent variant in our cohort was p.Ile76Val, which was identified in the heterozygous and homozygous state in respectively 9 and 2 of our 88 patients. This variant is already known to be frequent in the general population, with allelic frequency of 4.3% in ExAC Browser database, suggesting it is a simple polymorphism. Moreover, all the *in silico* analysis tools used here predicted it to be benign, and our functional analyses showed no loss of function *in vitro*. It may therefore be considered as a simple polymorphism with no discernable effect.

The p.Ile146Leu variant was found in three of our patients, always in the heterozygous state. Given its frequency of about 2% in the general population, it is also likely to be a simple polymorphism [12]. Moreover, its structure was predicted to be benign by 4 of the 5 *in silico* analysis tools used here. Functional results reported for this variant are conflicting, even within the same team, some describing it as a gain-of-function mutation [14] and others finding no effect [21]. Here, we demonstrated no effect of this variant on *PRLR* expression, localization, ability to be phosphorylated after PRL stimulation and transcriptional activity. So, we think it is very unlikely that this variant is associated with prolactinoma development.

This is the first screening study of *PRLR* germline mutations in a significant number of patients with prolactinomas. No variants were found in 72 patients (82%), showing that most prolactinomas occur independently of germline genetic events affecting the coding regions of the *PRLR* gene. Four different variants were found in the remaining 16 patients, including two variants (p.Glu108Lys and p.Glu554Gln) functionally untested so far. Our *in vitro* studies suggest that the four variants are non-pathogenic. Germline loss-of-function *PRLR* mutations thus do not appear to be a common occurrence in patients with sporadic prolactinoma. It is important to underline that somatic disruptions of *PRLR* in the prolactinomas have not been excluded and could be the object of future investigations.

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Figure Legends

Figure 1. Schematic representation of the four *PRLR* variants found in the cohort.

The 11 exons of the human *PRLR* gene are symbolized by boxes and the corresponding protein domains are represented. Black arrows indicate the four *PRLR* variants found in the cohort. White arrows indicate alternative splicing sites generating the long receptor (with exon 10) or the short receptor (with exon 11). TM = transmembrane domain.

Figure 2. Structural modeling and multiple sequence alignment of the Glu108Lys mutation.

Panel A shows multiple protein sequence alignments of prolactin receptor residues in orthologs, indicating a moderate evolutionary conservation of Glu108 (arrow). Panels B represents the three-dimensional homologous human structure of the complex formed between the extracellular domain of the wildtype prolactin receptor (blue) and a modified prolactin (green) (PDB code 3D48, (13)). Panel C corresponds to the p.Glu108Lys model presented in the same orientation. The residue at position 108 is drawn in a ball-and-stick representation and colored yellow.

Figure 3.

Panel A shows cell surface expression of wild-type, Glu108Lys and Glu554Gln *PRLR* receptors in permeabilized or non-permeabilized COS-7 cells as example. The expression of other 2 variants was similar. Confocal microscopy was used to study the cellular distribution of receptors. Bar scale = 10 μ m. Panel B shows a representative immunoblot analysis to detect *PRLR* and α -tubulin. Panel C shows a representative immunoblot of phosphorylated STAT5 (pSTAT5) after immunoprecipitation of total STAT5 without or with 1000ng/mL prolactin stimulation of HEK293 cells. Panel C represents *PRL* dose-response of LHRE-luciferase gene induction after transient transfection of HEK293 cells with wild-type *PRLR* and with all mutated *PRLR* found in 88 patients with prolactinoma. As a negative control, was also evaluated the loss of function His188Arg *PRLR* mutant reported by Newel et al [12].

Figure 1

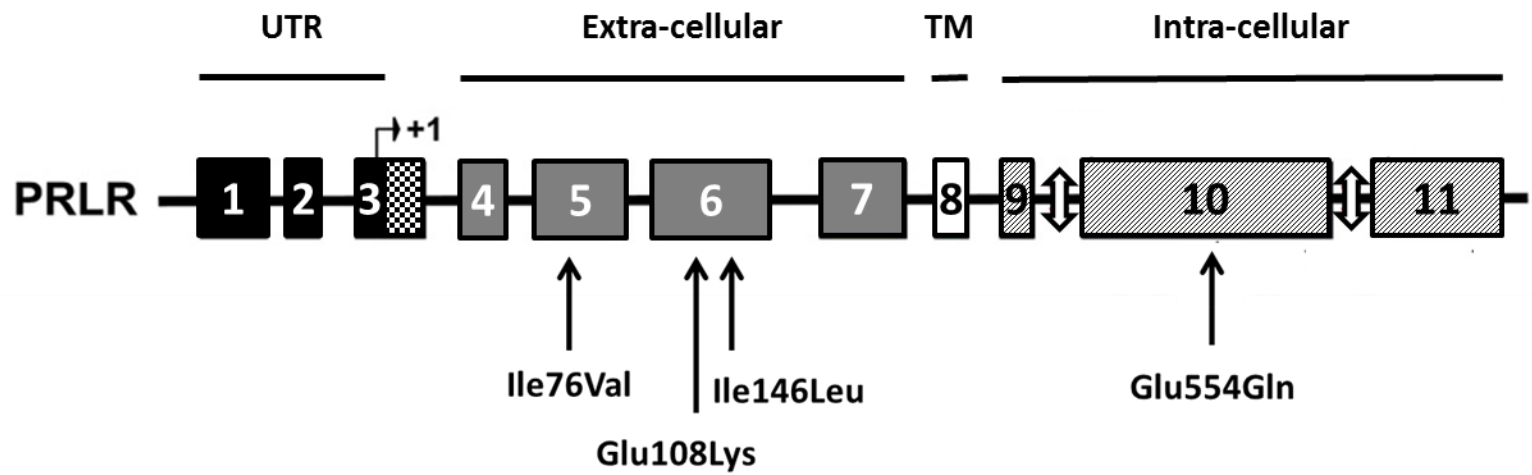
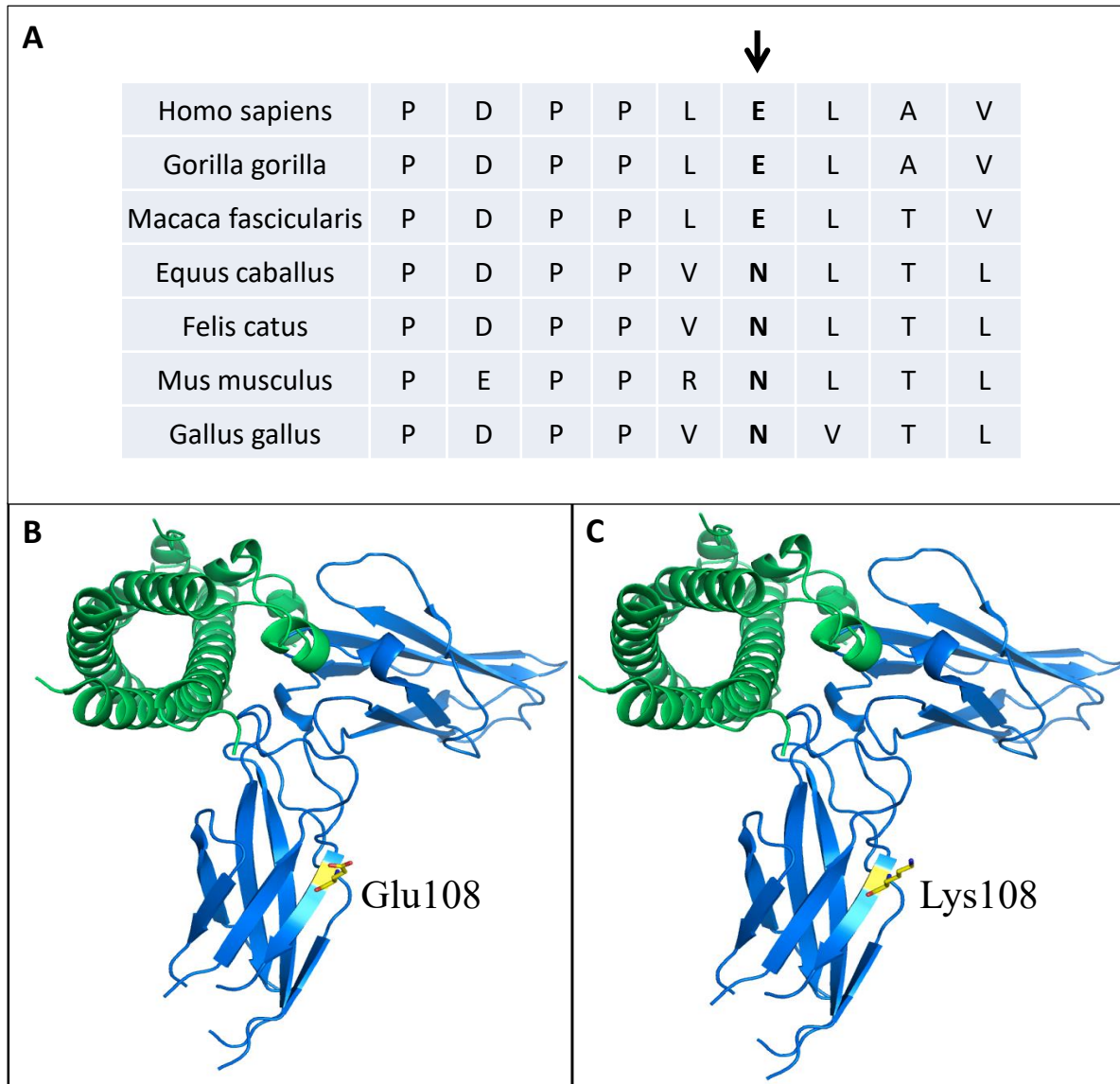
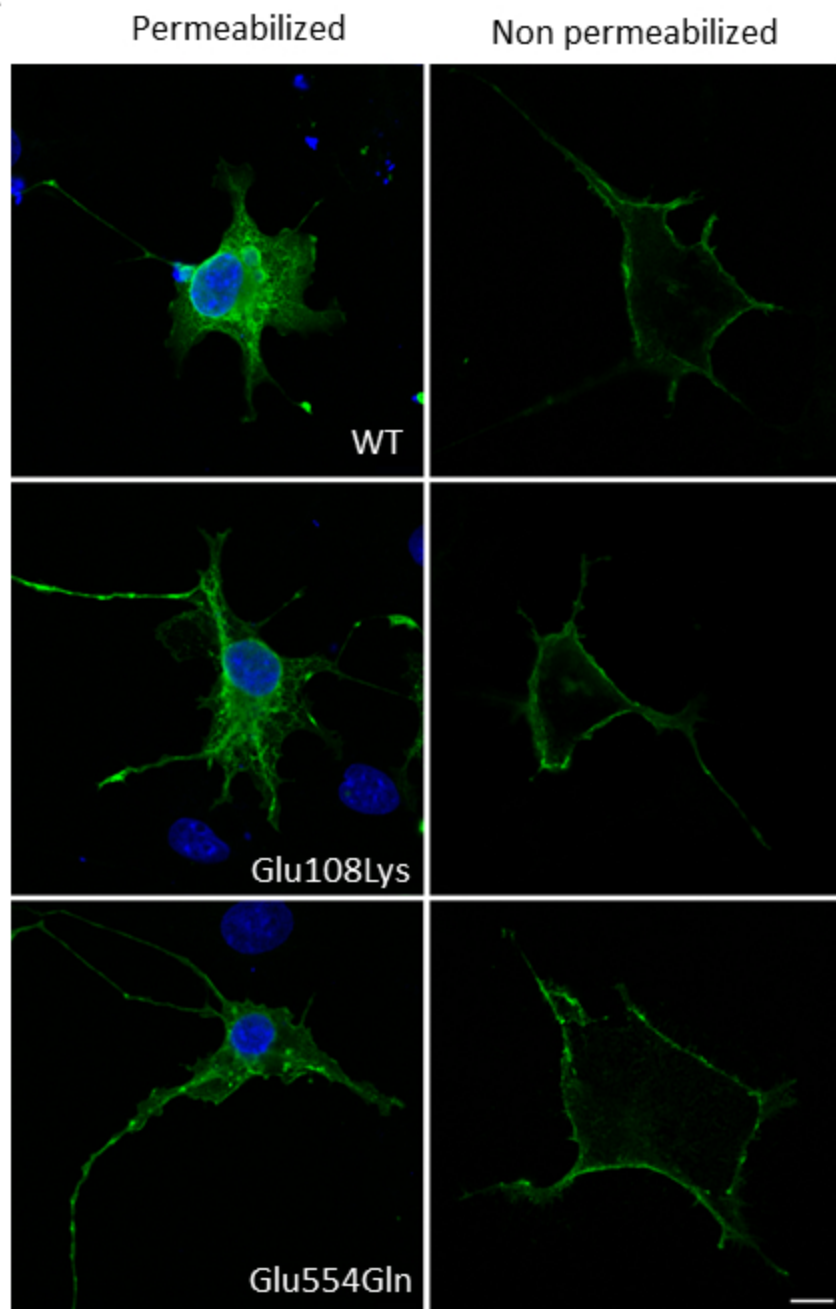


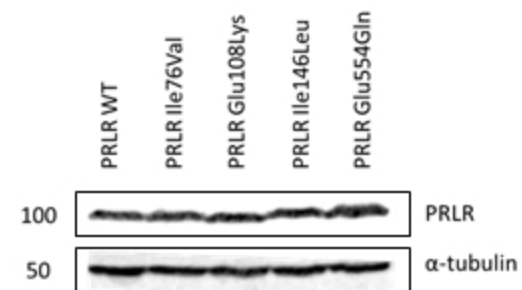
Figure 2



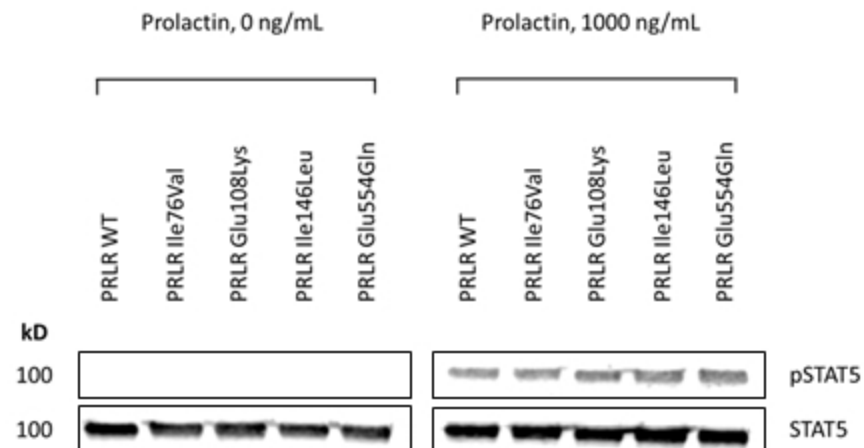
A



B



C



D

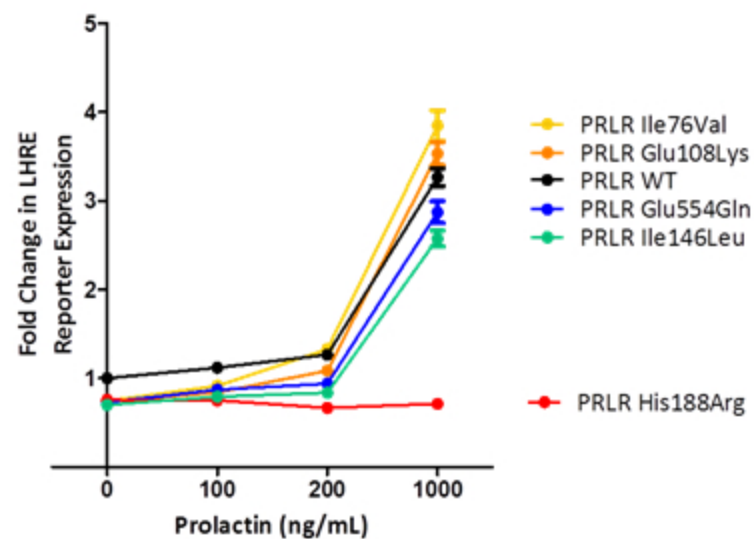


Table 1. Clinical characteristics of the 88 patients with prolactinoma

Gender	F: 54 (61%) M: 34 (39%)
Age at diagnosis, years (median, range)	24 (11-55)
Type of adenoma	
Microadenoma (<10mm)	6
Macroadenoma (>10mm)	82
Tumor diameter at diagnosis, mm (median, range)	20 (4-110)
PRL plasma level at diagnosis, ng/mL (median, range)*	564 (50-34900)
Main clinical symptom at diagnosis in women	
Primary amenorrhea	9 (16%)
Secondary amenorrhea/infertility	21 (39%)
Galactorrhea	17 (32%)
Tumor syndrome (headache, vision problems)	2 (4%)
Other/Unknown	5 (9%)
Main clinical symptoms at diagnosis in men	
Delayed puberty	3 (9%)
Gynecomastia	3 (9%)
Erectile dysfunction	6 (18%)
Tumor syndrome (headache, vision problems)	16 (47%)
Other/Unknown	6 (17%)

* Plasma with elevated total PRL concentrations are sub-fractionated using polyethylene glycol precipitation to provide a more meaningful clinical measurement of the bioactive monomeric PRL content. Normal range = 12-24 ng/mL.

Table 2. *PRLR* sequencing results in 88 patients with prolactinomas

Sequence variation	Location	Amino acid variation	dbSNP ID	Allele frequency		
				Patients with prolactinoma	ExaC Browser	p value*
c.298A>G	Exon 5	p.Ile76Val	rs78705921	0.07386	0.04345	0.06
c.394G>A	Exon 6	p.Glu108Lys	ND	0.00568	0.0000165	0.004
c.508A>C	Exon 6	p.Ile146Leu	rs72478580	0.01705	0.01899	1.00
c.1732G>C	Exon 10	p.Glu554Gln	rs146459777	0.00568	0.0009477	0.16

ND: non determined

* p value was calculated using Fisher's exact test.

Table 3. *In silico* analysis of missense variants

Variants	Polyphen ^a	UMD Predictor ^b	Mut taster ^c	SNPs&GO ^d	SIFT/Provean ^e
p.Ile76Val	Benign	Polymorphism	Polymorphism	Neutral	neutral
p.Glu108Lys	Benign	Polymorphism	Disease Causing	Neutral	neutral
p.Ile146Leu	Benign	Probable polymorphism	Disease Causing	Neutral	neutral
p.Glu554Gln	Probably damaging	Polymorphism	Polymorphism	Neutral	deleterious

a) PolyPhen-2 v.2.2.5 (<http://genetics.bwh.harvard.edu/pph2/>).

b) UMD Predictor (<http://umd-predictor.eu/>)

c) Mutation Taster default version (<http://www.mutationtaster.org/>).

d) SNPs&GO default version (<http://snps-and-go.biocomp.unibo.it/>).

e) SIFT default version (<http://sift.jcvi.org/>). Analyses were carried out using the SIFT Human Protein model.