Molecular Screening of PROKR2 Gene in Girls with very early Idiopathic Central Precocious Puberty.

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Research

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Abstract

Background Prokineticin receptor 2 (PROKR2) loss of function mutations have been described as cause of hypogonadotropic hypogonadism. In 2017 a first case of central precocious puberty (CPP) caused by heterozygous gain of function mutation in PROKR2 was described in a 3.5-year-old girl. No other cases have been reported yet. This study performs a molecular screening in girls with “early” onset CPP (breast budding before 6 years of age) in order to identify possible alterations in PROKR2.

Methods We analyzed DNA of 31 girls with idiopathic CPP diagnosed via basal LH levels > 0.3 IU/L or peak-LH > 5UI/L after stimulation, negative for MKRN3 mutations. The Fisher exact test was used to compare allele frequency of polymorphism found to Exome Aggregation Consortium (ExAC) dataset.

Results No rare variants were identified. Five polymorphisms were found (rs6076809, rs8116897, rs3746684, rs3746682, rs3746683). All except one (i.e. rs3746682) had a minor allele frequency similar to that reported in literature. rs3746682 presented a minor allele frequency higher than described in The Exome Aggregation Consortium (ExAC) (0.84 in our population vs 0.25 from ExAC).

Conclusions As for other G-protein-coupled receptors (i.e. GPR54), mutations in PROKR2 do not seem to be a frequent cause of CPP in girls.

Background

Idiopathic central precocious puberty (CPP) results from premature activation of hypothalamic GnRH secretion in absence of congenital or acquired organic lesions in Central Nervous System. The prevalence of CPP is higher in girls than in boys (1). Although around 70% of variation in pubertal timing seems due to genes, genetic mechanisms leading to CPP are still a field of scientific investigation (2). So far, MKRN3 loss of function mutations are the most frequently identified monogenic cause of CPP (3, 4). Heterozygous activating mutations in KISS1 and KISS1R genes have been reported as cause of few cases of CPP (5, 6). DLK1 loss-of-function mutations determine a more complex, yet infrequent phenotype characterized by CPP, overweight, early Type 2 Diabetes, hyperlipidemia and Polycistic Ovary Syndrome (7). Besides that, rare cases of patients with CPP primarily related to clinical syndromes or chromosomal abnormalities have been identified (8).

In 2017 Fukami et al. performed an extended molecular analysis by next generation sequencing and identified a new heterozygous frameshift mutation in PROKR2 gene in a 3.5 years old girl affected by CPP (9). PROKR2 is a G-protein coupled receptor (GPCR) expressed on the membrane of GnRH neurons, whose activation promotes GnRH secretion. Loss-of-function mutations in this gene accounts for about 5% congenital hypogonadotropic hypogonadism with or without anosmia (10). In contrast with other mRNA with frameshift mutations producing a stop-codon, the mRNA with the mutation identified by Fukami and colleagues, namely p.C242fsX305, showed to escape nonsense-mediated mRNA decay mechanism. In-vitro assay demonstrated that this variant receptor did not exert activity itself whereas boosted the activity of wild-type receptor in heterozygous state. The precise mechanism of action still
remains unclear. Perhaps, hyperactive mutant-wild-type heterodimers have a greater ligand affinity or a reduced mechanism of receptor internalization due to the trunked in C-tail domain polypeptide.

The aim of this study was to perform a molecular screening of PROKR2 in a cohort of girls with CPP. Considering the exceptionally young age of the patient described by Fukami et al., we limited the molecular screening to girls with “very early” onset CPP, namely before the age of 6 years.

To date, no other data of mutations in PROKR2 in CPP are available.

**Methods**

**Setting**

In order to investigate the possible role of PROKR2 in the etiopathology of CPP, a prospective, observational multicenter study was set up and carried out over a 2-year period.

4 Italian centres were involved in this study: Department of Woman, Child, General and Specialized Surgery of Università degli Studi della Campania “Luigi Vanvitelli”, Naples, Pediatric Section-Department of Translational Medical Sciences, University of Naples Federico II, Naples, Institute for Maternal and Child Health IRCCS, “Burlo Garofolo”, Trieste, and the Department of Paediatrics, Bologna University, Bologna. Ethics committee of the Università degli Studi della Campania “Luigi Vanvitelli” approved the protocol then subscribed by ethics committee of the other centres. According to the World Medical Association Declaration of Helsinki, written informed consent from parents and oral consent from all participants was collected.

**Subjects**

We recruited 31 females with diagnosis of CPP that met these following criteria: thelarche occurred before age of 6 years, defined as Tanner stage B $\geq$ 2 at physical examination, a diagnosis of central HPG activation identified by pubertal basal luteinizing hormone (LH) levels ($>0.3$ UI/l) or a positive Gn-RH stimulating test and normal brain MRI.

Exclusion criteria included: congenital defects or abnormalities possibly related to syndromic features, central nervous system pathology (i.e., tumours or nonspecific cerebral anomalies associated with CPP). All the patients involved in this study were Italian.

**Protocol**

All girls underwent a clinical examination with special regard to auxological parameters and staging of breast development according to Tanner’s classification. Right hand and wrist X-ray was performed for bone age evaluation by TW2 method. Peripheral blood samples were collected for all patients for hormonal dosage and genetic analysis.
Chemiluminescence assay (LIAISON, Diasorin) was used to measure FSH and LH concentrations, with detection limits of 0.06 and 0.05 U/L, respectively, and intra- and inter-assay CV less than 5%. Radioimmunoassay was used to measure serum estradiol (CisBiO International). The analytical and functional detection limits for plasma estradiol were 4 and 8 pg/mL, respectively.

GnRH stimulating test was provided in patients in which basal hormone level did not meet diagnostic criteria for CPP. Peak-LH > 5UI/L after administration of 0.1 mg of Relefact LH-RH (Sanofi-Aventis, Frankfurt am Main, Germany) was considered positive.

Thin-section, contrast-enhanced MRI examination of sellar region with T1-weighted and T2-weighted sagittal, coronal sequences, 3DT2 thin section axial sequence and FLAIR and EPI DWI on axial sequence was acquired for all patients.

**Genetic analysis**

Genomic DNA was extracted from peripheral whole blood using a DNA extraction kit (Promega, Madison WI, USA) following the manufacturer's instructions. Each of the two coding exons and the intron-exon boundaries of the PROKR2 gene was amplified by polymerase chain reaction (PCR) using two couples of primers each and subsequently analysed by direct sequencing (ABI PRISM 3100, Perkin Elmer, USA) under standard conditions. In the attempt to reduce possible bias of selection in the sample due to other mutations, an analogous procedure was used to screen MKRN3 gene sequence to rule out the most frequent genetic cause of CPP nowadays individuated. All genetic analysis was performed at Department of Woman, Child, General and Specialize Surgery of Università degli Studi della Campania “L. Vanvitelli”, Naples, Italy.

**Data Analysis**

Epidemiological data were expressed as medians (interquartile ranges). The Fisher exact test was used to compare allele frequency of polymorphism found in our screening to Exome Aggregation Consortium (ExAC) dataset. Results reach statistical significance at a p value less than 0.05. All statistical analyses were performed using Stat-Graph Centurion XVII software for Windows.

**Results**

Clinical and laboratory characteristics of our cohort are shown in Table 1. Median age at first occurrence of thelarche was 5.6 (min-max: 1.1–5.9 years). Family history of precocious sexual development was identified in 26.9% of them. No mutation was found in MKRN3 locus in the whole sample.
All patients were treated by GnRH analogues (triptorelin) with a good response. In particular, two patients with a breast budding occurred before 3 years of age, included in our cohort, had a very rapidly progressive form of precocious puberty, with accelerated growth, thelarche progression and in one case uterine bleeding, both requiring blocking treatment. All these clinical features excluded the alternative diagnosis of minipuberty in these patients.

No rare variants in the coding region of PROKR2 were identified. Five polymorphisms were found as listed in Table 2. All except one had a minor allele frequency (MAF) similar to that reported in literature. SNP rs3746682 presented a MAF higher than described in the ExAC (0.84 in our population vs 0.25 from ExAC).

Table 1
Clinical and laboratoristic features of CPP girls. All continuous variables are expressed as median (IQR25-75)

| Clinical Feature                          | Median (IQR)     |
|-------------------------------------------|------------------|
| Age at diagnosis years                    | 6.0 (5.0-6.5)    |
| Age at thelarche occurrence years         | 5.6 (4.2–5.8)    |
| PH ≥ 2 N (%)                              | 17 (54.8%)       |
| B > 2 N (%)                               | 12 (38.7%)       |
| Height SDS                                | 0.89 (-0.18 to 1.43) |
| BMI SDS                                   | 0.47 (-0.24 to 0.82) |
| Δ bone age – chronological age years      | 2.66 (1.75–3.45) |
| Basal LH UI/L                             | 0.8 (0.4–2.4)    |
| Basal FSH UI/L                            | 3.9 (3.4–5.1)    |
| Basal E2 pg/ml                            | 16 (11-27.7)     |
| Peak LH UI/L                              | 11.3 (8.2–26.7)  |

Table 2
MAF of SNP identified in our cohort and their corresponding in The Exome Aggregation

| SNP Position | MAF in our cohort | MAF in ExAc |
|--------------|-------------------|-------------|
| rs6076809    | c.-8-40C > T      | 0.06 0.03   |
| rs8116897    | c.458 + 62G > A   | 0.47 0.49   |
| rs3746684    | c.465C > T        | 0.37 0.37   |
| rs3746682    | c.585G > C        | 0.84 0.28   |
| rs3746683    | c.525C > G        | 0.18 0.10   |
Consortium.

Discussion

*PROKR2* plays a critical role to regulate olfactory bulb morphogenesis and sexual maturation (11). A hyperactive prokineticin system appears to be an obvious pathogenetic mechanism of sexual precocity. Nevertheless, no sequence rare variation was detected in the coding region of *PROKR2* in our cohort. Although its relatively small size, our sample is.

We do realize that our sample size is relatively small, however it is exceptionally homogenous due to stricter requirements of selection: all patients had a very early onset CPP and genetic analysis negative for *MKRN3*. This recruitment method allows to exclude the “gray zone” between 7–8 years where normal variants of “accelerated puberty/rapid progressive thelarche” can occur without medical intervention needed and set up a cohort with the most similar clinical features to the index case of *PROKR2* mutant precocious puberty reported by Fukama et al. So, although negative, the finding from our study remains interesting. Indeed, we can speculate that if mutation in coding region of *PROKR2* could explain 10% of CPP, we would have had identified at least one mutation within our sample size with a 95% level of confidence and a precision of 90%. Therefore, we can suggest that *PROK2R* is not at least a common cause for CPP, even in very early onset CPP.

Those findings suggest that as for other GPCR (e.g. *KISS1R*) (6), gain of function variants are a very rare cause of CPP probably because hypersignalling in the pathway are barely tolerated due to the critical role exerted by those genes.

Since we evaluated only coding region and intron-exon joints, we can not rule out presence of possible mutations outside those regions. Promoter regions and miRNAs regulatory elements should be the next target of further investigations as they may also play an important role in transcriptional or post-transcriptional control of gene expression as the experience with *MKRN3* gene in CPP has taught us (12). We do believe the difference observed in MAF of rs3746682 polymorphism individuated in our cohort is probably due to the small sample size and need to be investigated in larger cohorts.

Besides, it is important to remember that murine studies on the prokineticin system demonstrated that *PROK2*-PROKR2 signalling seems to be implicated in many other hypothalamic functions such as the regulation of SCN circadian clock (13). Therefore, it is possible that mutations of this gene might cause not isolated CPP in the context of more complex phenotypes. The role of prokineticins system in the regulation of pubertal timing and its disruption in humans still warrants further investigation.

Conclusions
This study states *PROKR2* gene variants are not a common cause of CPP, also in very young girls. At the best of our knowledge, this is the first study providing a molecular screening of *PROKR2* in a selected group of patients with idiopathic CPP. Genetic aetiology of central precocious puberty remains an interesting field of research, the finding of our screening supports the idea that gain of function mutations of genes involved in hypogonadotropic hypogonadism seem to be a very rare cause of CPP.

**List Of Abbreviations**

CPP: Central Precocious Puberty

MAF: Minor Allel Frequency

GPCR: G-Protein Coupled Receptor

LH: Luteinizating Hormone

PCR: Polymerase Chain Reaction

ExAC: Exome Aggregation Consortium

**Declarations**

**Ethics approval and consent to participate:** All research followed Ethical Standards stated in World Medical Association Declaration of Helsinki. The study was approved by the “Comitato Etico” AOU Università degli Studi della Campania “L. Vanvitelli”, Naples, Italy. All patients’parents provided parental written informed consent to partecipate.

**Consent for publication:** Parental written informed consent for publication was collected.

**Availability of data and material:** the datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interest:** the authors declare that they have no financial or non-financial competing interest.

**Funding:** Nothing to declare.

**Authors’ contribution:** FA wrote the manuscript, GC and GU performed the laboratory analysis, AC, RD, GT and EM enrolled the patients and performed all clinical examination, performed the data analysis, contributed to data interpretation, AG designed the study, corrected the draft and analyzed the data.

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