Regulation of steroidogenesis in a primary pigmented nodular adrenocortical disease-associated adenoma leading to virilization and subclinical Cushing's syndrome

Johannes Hofland 1, Wouter W de Herder 1, Lieke Derks 1, Leo J Hofland 1, Peter M van Koetsveld 1, Ronald R de Krijger 2, Francien H van Nederveen 2, Anelia Horvath 3, Constantine A Stratakis 3, Frank H de Jong 1 and Richard A Feelders 1

1 Section of Endocrinology, Department of Internal Medicine, Room Ee-532 and 2 Department of Pathology, Erasmus Medical Center, PO Box 2040, 3000 CA Rotterdam, The Netherlands and 3 Section on Endocrinology and Genetics, Program on Developmental Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA

(Correspondence should be addressed to J Hofland; Email: j.hofland@erasmusmc.nl)

Abstract

Context: Primary pigmented nodular adrenocortical disease (PPNAD) can lead to steroid hormone overproduction. Mutations in the cAMP protein kinase A regulatory subunit type 1A (PRKAR1A) are causative of PPNAD. Steroidogenesis in PPNAD can be modified through a local glucocorticoid feed-forward loop.

Objective: Investigation of regulation of steroidogenesis in a case of PPNAD with virilization.

Materials and methods: A 33-year-old woman presented with primary infertility due to hyperandrogenism. Elevated levels of testosterone and subclinical ACTH-independent Cushing's syndrome led to the discovery of an adrenal tumor, which was diagnosed as PPNAD. In vivo evaluation of aberrantly expressed hormone receptors showed no steroid response to known stimuli. Genetic analysis revealed a PRKAR1A protein-truncating Q28X mutation. After adrenalectomy, steroid levels normalized. Tumor cells were cultured and steroidogenic responses to ACTH and dexamethasone were measured and compared with those in normal adrenal and adrenocortical carcinoma cells. Expression levels of 17β-hydroxysteroid dehydrogenase (17β-HSD) types 3 and 5 and steroid receptors were quantified in PPNAD, normal adrenal, and adrenal adenoma tissues.

Results: Isolated PPNAD cells, analogous to normal adrenal cells, showed both increased steroidogenic enzyme expression and steroid secretion in response to ACTH. Dexamethasone did not affect steroid production in the investigated types of adrenal cells. 17β-HSD type 5 was expressed at a higher level in the PPNAD-associated adenoma compared with control adrenal tissue.

Conclusion: PPNAD-associated adenomas can cause virilization and infertility by adrenal androgen overproduction. This may be due to steroidogenic control mechanisms that differ from those described for PPNAD without large adenomas.

European Journal of Endocrinology 168 67–74

Introduction

Primary pigmented nodular adrenocortical disease (PPNAD) constitutes a rare cause of adrenocortical hyperplasia and ACTH-independent Cushing's syndrome. PPNAD can occur sporadically or in conjunction with other tumors in Carney complex (1). Known genetic causes of PPNAD and Carney complex are mutations in components of the cAMP protein kinase A (PKA) pathway: PRKAR1A (2), PDE11A (3), and PDE8B (4). The net effect of these mutations is increased activity of the PKA catalytic subunits (2). Aberrant cAMP–PKA signaling in the adrenal cortex leads to hyperplasia, the formation of multiple pigmented nodules, and the sporadic formation of a large tumor. The latter has been linked to mutations in CTNNB1, which lead to constitutive activation and nuclear translocation of its product β-catenin (5). Other manifestations of Carney complex include lentiginosis, myxomas, and pituitary, thyroid, and testicular tumors (6).

PPNAD is the most common endocrine manifestation of Carney complex (7) and can lead to ACTH-independent Cushing's syndrome (8). Testosterone hypersecretion from PPNAD has also been described in two female patients (9). In addition, the autonomous cortisol production by the adrenal nodules reacts paradoxically to dexamethasone administration in 69% of patients during the course of Liddle's test (8), due to increased glucocorticoid receptor (GR (NR3C1))
expression (10) and possibly specific interactions between the GR and the PKA catalytic subunits (11).

Macronodular adrenal hyperplasia and adrenocortical adenomas with autonomous cortisol production can be associated with aberrant expression of one of several eutopic and ectopic G-protein-coupled receptors (GPCRs) that are functionally coupled to steroidogenesis (12, 13). Eutopic or ectopic GPCR expression does not appear to play a major role in controlling steroidogenesis in PPNAD, unlike in macronodular hyperplasia or solitary adrenocortical adenomas (14), although clinical testing for aberrant responses has only been reported for two PPNAD patients (10).

Here, we describe a patient presenting with virilization and subclinical Cushing’s syndrome due to PPNAD and a single adenoma that developed in her right adrenal. We performed several in vivo stimulation tests to screen for eutopic or ectopic stimuli that could possibly regulate the peculiar hypersecretion of cortisol and androgens. To obtain further insight in the regulation of steroidogenesis in this single tumor, in vitro studies were performed in which we examined the effects of ACTH and dexamethasone on steroidogenic enzyme expression and steroid production. In addition, expression levels of the testosterone-producing enzymes 17β-hydroxysteroid dehydrogenase (17β-HSD) types 3 and 5 and of the glucocorticoid and androgen receptors were measured in PPNAD as well as in other adrenal tissues.

Materials and methods

Clinical case

A 33-year-old Caucasian woman was referred to our department because of primary infertility and hyperandrogenism. The patient had been investigated for infertility for several years. Two years before referral, fertility screening showed no abnormalities in the patient or her partner. Six intra-uterine insemination sessions and an IVF attempt did not result in pregnancy. The patient was then referred to the Department of Gynecology of our center for a second opinion; here, laboratory analysis showed an increased serum level of testosterone.

The patient had menarche at the age of 13 years. Soon thereafter, she started using oral contraceptives because of facial acne and hirsutism. Seven years before presentation, the patient stopped oral contraceptive use and regained regular menstrual cycles. She noticed increased and coarse hair on her face, abdomen, and upper legs with concomitant frontotemporal hair loss. During the past years, libido had increased and her clitoris grew larger. Her past medical history and family history were unremarkable nor did she take any medication or hormonal preparations.

Upon physical examination, the patient displayed a female phenotype with overt hirsutism and a male pattern baldness. Her extremities and torso were covered with multiple lentigines; clitoromegaly was confirmed upon pelvic examination. Endocrinological evaluation showed increased levels of testosterone and 17-hydroxyprogesterone (17-OHP) and a suppressed ACTH level (Table 1). Morning and midnight cortisol levels were 263 and 246 nmol/l respectively. Cortisol and androgen levels were not adequately suppressed after the overnight 1 mg dexamethasone test. Abdominal CT scan subsequently showed a nodular enlargement in the right adrenal (19×14 mm). Hounsfield units measured 45 at basal, rising to 135 after i.v. administration of contrast. Magnetic resonance imaging (MRI) confirmed the right adrenal nodule (Fig. 1A) with increased signal on the T2-weighted image, which enhanced after i.v. gadolinium administration. No signal loss was observed during the washout phase.

The patient was tested for ectopic hormone receptor expression by measuring cortisol, 17-OHP,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Serum hormone levels.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preoperative</td>
</tr>
<tr>
<td></td>
<td>Reference values</td>
</tr>
<tr>
<td>Cortisol (nmol/l)</td>
<td>200–800</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>0.5–3</td>
</tr>
<tr>
<td>17-OHP (nmol/l)</td>
<td>0.5–10</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>2–15</td>
</tr>
<tr>
<td>11-Deoxycorticisol (nmol/l)</td>
<td>0–50</td>
</tr>
<tr>
<td>DHEA (nmol/l)</td>
<td>1.4–25</td>
</tr>
<tr>
<td>DHEAS (μmol/l)</td>
<td>0.8–10</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>100–1000</td>
</tr>
<tr>
<td>ACTH (pmol/l)</td>
<td>0–11</td>
</tr>
<tr>
<td>LH (U/l)</td>
<td>2–8</td>
</tr>
<tr>
<td>FSH (U/l)</td>
<td>1–8</td>
</tr>
<tr>
<td>Urine cortisol (nmol/day)</td>
<td>0–850</td>
</tr>
</tbody>
</table>

www.eje-online.org
and an upright posture test (15). The patient failed to meal (116 g carbohydrates, 27 g proteins, and 14 g fat); pressin (10 IU i.m.) administration; a standard mixed i.v.). metoclopramide (10 mg i.v.), and arginine–vaso-tropin-releasing hormone (200 mg i.v. following LH-releasing hormone (100 μg i.v.). thyrotrpin-releasing hormone (200 μg i.v.), glucagon (1 mg i.v.), metoclopramide (10 mg i.v.), and arginine–vaso-pressin (10 IU i.m.) administration: a standard mixed meal (116 g carbohydrates, 27 g proteins, and 14 g fat); and an upright posture test (15). The patient failed to show an increase in steroid levels of >50% after stimulation by the above-mentioned procedures.

The patient underwent an open right-sided adrenalectomy because of the suspicion of adrenocortical cancer. Postoperative testing showed nondetectable testosterone, DHEAS, and ACTH levels, whereas 17-OHP and androstenedione concentrations were markedly diminished (Table 1). Pathological examination of the right adrenal showed a ‘black’, round adenoma that measured ~2 cm in diameter. Microscopically, the tumor consisted of eosinophilic cells with extensive granular pigmentation (Fig. 1B). The tumor was embedded within an atrophic adrenal cortex peppered with multiple other pigmented nodules (Fig. 1C). The histopathological picture of the remaining cortex was consistent with that of PPNAD (1). Immunohistochemistry of β-catenin, using a mouse MAB (#610154, diluted 1:200, BD Biosciences, San Jose, CA, USA (16)), displayed a membranous and cytoplasmic staining in both the adenoma and the surrounding cortex (Fig. 1D).

Leukocyte DNA sequencing, performed as published elsewhere (17), revealed a previously reported PRKAR1A protein-truncating Q28X mutation (18), confirming the diagnosis of PPNAD. Separate sequencing of microdissected adenoma and PPNAD tissues, using methods (19) and primers (5) previously reported, subsequently showed no mutations in exon 3 of CTNNB1.

Screening of the patient for pituitary tumors and for cardiac myxoma was negative, whereas the patient did suffer from several nonfunctional benign thyroid nodules and fibro-adenomas in both breasts. No family members showed signs of Carney complex-associated disease. Six months after the operation, the patient reported to be 7 weeks pregnant. At 39 weeks of pregnancy, she successfully gave birth to a healthy boy. Endocrinological evaluation 2 years after operation showed normal HPA axis and normal levels of androgens (Table 1).

**Control samples**

Patient samples for in vitro examination were also obtained from one normal adrenal cortex obtained at radical nephrectomy due to renal cell carcinoma, from two patients with adrenocortical hyperplasia due to metastasized ACTH-producing neuroendocrine tumors and from four patients with adrenocortical carcinoma. Analysis of the normal and hyperplastic adrenal samples were combined in the group designated as ‘controls’.

Three other patients with histologically proven PPNAD and clinical Cushing’s syndrome were identified and adrenal samples were subsequently used for mRNA analysis. These included a 33-year-old female (PPNAD2), a 24-year female (PPNAD3), and a 24-year-old male (PPNAD4). PPNAD2 also showed no significant increases in cortisol production following in vivo testing for aberrantly expressed GPCRs. None of the PPNAD patients underwent the full Liddle’s test, but PPNAD3 and PPNAD4 subjects did undergo a 7 mg i.v. dexamethasone test, which increased cortisol levels by 19 and 13% respectively. Other samples for mRNA analysis of the 17β-HSD types 3 (HSD17B3) and 5 (AKR1C3) and steroid receptors included normal adrenals (n = 7). ACTH-dependent hyperplasia (n = 10), and clinically nonfunctional (n = 3), cortisol- (n = 6) and aldosterone-secreting (n = 6) adrenocortical adenomas.

Informed consent was obtained from all patients before operation. The study was approved by the Medical Ethics Committee of the Erasmus MC and the DNA studies were completed under an approved Eunice Kennedy Shriver National Institute of Child Health and Human Development clinical protocol.

**Tissue processing**

Shortly after resection, adrenal tissue samples were snap-frozen and kept at −80 °C for mRNA analysis or put in DMEM-F12 (Invitrogen) containing 5% FCS, penicillin, and streptomycin (Invitrogen) for purposes of primary culture. A monolayer culture was obtained by treating the tissue with collagenase (Sigma–Aldrich) as described previously (20). After allowing the cells to

![Figure 1](https://example.com/figure1.png)
attach overnight, the medium was changed to serum-free medium. The next day, cells were incubated with vehicle, 10 ng/ml ACTH1-24 (Novartis) or 1 μM dexamethasone (Sigma–Aldrich). After 48 h of incubation, the supernatants were removed and stored at −20°C; cells were simultaneously snap-frozen and stored at −80°C. Hormone measurements were performed as previously reported (19).

**mRNA and protein analysis**

RNA was isolated from plated cells and homogenized frozen adrenal tissues with TRIzol reagent (Invitrogen). RNA measurement, reverse transcriptase reactions, and quantitative PCR (qPCR) were performed as described previously (21). The qPCR was performed in a 12.5 μl volume for HPRT1, STAR, CYP11A1, HSD3B2, CYP17A1, CYP21A2, CYP11B1 (21), AKR1C3, HSD17B3, and steroid receptors GR and AR (assay on demand, Hs00366267_m1, Hs00970002_m1, Hs00230818_m1, and Hs00907242_m1, Applied Biosystems). Expression levels were calculated relative to that of the housekeeping gene HPRT1, expression of which was stable between incubations and the different groups of tissues, using the ΔΔCt method.

Immunohistochemistry of HSD17B3 and AKR1C3 protein was performed with methods equal to that of β-catenin staining, using MABs purchased from Sigma–Aldrich (HP A015307, 1:30) and Abcam (ab49680, 1:3000) respectively.

**Statistical analysis**

All data on steroid hormone levels and mRNAs were analyzed using paired t-test or one-way ANOVA with post hoc Newman–Keuls multiple comparison test. Logarithmic conversion was applied to obtain normality if necessary. Statistical significance was assumed at P<0.05.

**Results**

**Primary cultures**

Primary cultures were obtained from the patient’s adrenal tissue, one normal adrenal, two hyperplastic adrenals, and four adrenocortical carcinomas. Forty-eight-hour incubation with ACTH of the patients’ tumor cells led to significant increases in cortisol (5.3-fold), androstenedione (4.9-fold), and testosterone (3.6-fold) levels (Fig. 2A). The increase in cortisol secretion following ACTH stimulation in the control group was 6.8-fold and that of androstenedione 3.7-fold, whereas ACTH did not stimulate testosterone production by normal adrenal cells. Dexamethasone incubation did not alter steroid levels compared with vehicle. All steroidogenic enzymes studied were present in cells from the PPNAD. Of the predominant human 17β-HSD isoforms that are known to be involved in testosterone production, AKRTC3 mRNA expression in the cultured PPNAD cells was 68-fold higher than that of HSD17B3.

**Figure 2** (A) Cortisol, androstenedione (A’dione), and testosterone levels in the supernatant of primary cultures following 48 h of ACTH or dexamethasone incubation. mRNA levels of steroidogenic enzymes in primary cultures of the PPNAD, normal and hyperplastic adrenals (control, n=3), and adrenocortical carcinomas (n=4) following 48 h of ACTH (B) or dexamethasone (C) incubation. *P<0.05, **P<0.01, ***P<0.001 compared with control. Data are expressed as mean±S.E.M.
mRNA. ACTH increased the expression of STAR, CYP17A1, CYP21A2, and CYP11B1 in the PPNAD (Fig. 2B), whereas dexamethasone had no significant effect on steroidogenic enzyme mRNAs (Fig. 2C). Also, in control and carcinomatous adrenals, ACTH stimulated steroidogenic enzyme expression whereas dexamethasone did not affect these mRNAs.

17β-HSds and steroid receptors

The mRNA levels of HSD17B3 and AKR1C3 were measured in the fresh frozen tissue samples of the patient’s tumor, three other PPNAD samples, normal adrenals, and adrenocortical adenomas. Overall, adrenal AKR1C3 levels were higher than those of HSD17B3. Within the PPNAD group, our patient had the highest levels of AKR1C3 and HSD17B3 (Fig. 3, depicted as a filled square). Compared with all other adrenal samples, the virilizing PPNAD had the highest expression of HSD17B3. Of the different adrenal tissues, nonfunctional adenomas had higher levels of AKR1C3 mRNA expression compared with cortisol-producing adenomas (P < 0.05, Fig. 3). Immunohistochemistry of both types of 17β-HSD revealed specific, cytoplasmic staining in the control adrenals. AKR1C3 was expressed in the zona reticularis and clustered to a lesser extent in the zona glomerulosa, whereas HSD17B3 was expressed in the zona reticularis and adrenal medulla (Fig. 3). In the PPNAD tissue, both proteins showed a heterogeneous staining pattern among the large adenoma, the small nodules, and the remaining cortex. Staining of AKR1C3 was more intense in the PPNAD-associated adenoma than in the surrounding cortex or normal adrenals and more prevalent than that of HSD17B3 (Fig. 3).

Because of the paradoxical rise in cortisol after dexamethasone described in cases of PPNAD and the hyperandrogenism of the current PPNAD case, we also measured GR and AR mRNAs in these samples in order to investigate potential feed-forward loops of steroid receptor levels and glucocorticoid or androgen overproduction. The steroid receptor mRNAs were expressed in all samples, but there were no significant differences between GR or AR expression in any of the tissue types that were studied (Fig. 3).

Discussion

As the first description of Carney complex in 1985 (1), the genetic basis of the disease has been elucidated for the majority of the patients (22). However, the factors controlling the development of clinically significant PPNAD in individual patients remain largely unknown.

A patient presenting with PPNAD with primary infertility has not been described previously. The high serum levels of androgens and androgen precursors, combined with the absence of a cortisol diurnal rhythm and a suppressed ACTH, led to the discovery of an adrenal tumor with atypical radiological features. As such high androgen levels have not been previously reported for PPNAD and the patient’s left adrenal showed no abnormalities, an adrenocortical carcinoma was suspected and an open adrenalectomy was subsequently performed. Removal of the tumor led to normalization of serum androgen levels and a successful pregnancy within a few months. The steroid-secreting PPNAD-associated adenoma was the cause of the patient’s primary infertility, as the serum steroid profile normalized after unilateral adrenalectomy despite the remaining left adrenal presumably also being affected by PPNAD. Unlike a previous report (23), this adenoma was not associated with CTNNB1 mutations or constitutive β-catenin activation. This could reflect that only a subset of PPNAD-associated adenomas is.

Figure 3 AKR1C3, HSD17B3 (top panels), GR, and AR (bottom panels) mRNA expression in normal adrenal glands, adrenocortical hyperplasia (Hyp), PPNAD, and nonfunctional (NFA), cortisol-producing (CPA), and aldosterone-producing (Conn) adrenocortical adenomas. The virilizing PPNAD is depicted as a filled square. Expression is calculated relative to that of the housekeeping gene HPRT1. Bar denotes mean. *P < 0.05. In the centre: immunohistochemistry of AKR1C3 and HSD17B3 showed a cytoplasmic but heterogeneous staining pattern in the PPNAD-associated adenoma. The remaining cortex and smaller nodules showed a similar pattern, with AKR1C3 as the predominant 17β-HSD (magnification, 50×).
characterized by these mutations, as is the case in sporadic adrenocortical adenomas (24, 25).

Steroid overproduction in PPNAD usually results in a mild, ACTH-independent form of Cushing’s syndrome (8). As is evident from our case, other steroids can also be produced in PPNAD, leading to a very different clinical presentation. The Q28X mutation (18) has been detected in two previous patients. These female relatives displayed lentigines, breast myxomas, and PPNAD, but without hyperandrogenism. Recent studies in adrenal cortex-specific Prkar1a knockout mice showed that PKA R1α loss is sufficient to drive PPNAD development and autonomous steroidogenesis. ACTH responsiveness remained intact in these mice (26). Human adrenal cells with mutated PRKAR1A remain cAMP responsive and increase PKA activity even more compared with nonmutated cells as PRKAR1A mutations lead to constitutive PKA activity (2, 9). In vivo and in vitro studies of our patient showed that the PPNAD cells still possessed the ability to respond to ACTH; production of cortisol, androstenedione, and testosterone increased after exposure to ACTH. The ACTH responsiveness in terms of cortisol and androstenedione was comparable between normal adrenocortical and the patient’s PPNAD cells. However, results for testosterone differed: in the control cells, no increase of testosterone production was detected, whereas testosterone production by the PPNAD cells was significantly stimulated by ACTH. The ACTH effect as measured by steroidogenic enzyme mRNA expression levels showed an augmented and diminished response for CYP11B1 and HSD3B2 respectively making it possible that there was an aberrant regulation of steroidogenesis in the patient’s PPNAD cells.

Testosterone is mainly formed from androstenedione by 17β-HSD types 3 and 5, encoded by HSD17B3 and AKR1C3 respectively (27). Whereas HSD17B3 is the predominant testosterone-forming enzyme in the testis, AKR1C3 mainly ensures testosterone formation in nontesticular tissues, such as the adrenal cortex (28) and prostate gland (29). Our patient showed particularly high mRNA levels of HSD17B3 compared with all other adrenal tissues, but this could not be confirmed by immunohistochemistry. AKR1C3 mRNA expression in this PPNAD was augmented compared with the other PPNAD samples and staining of the enzyme revealed positive cell clusters in the large adenoma. Given the higher levels compared with HSD17B3, AKR1C3 forms the principal candidate for the cause of hyperandrogenism in our patient, presumably due to mass effect in the dominant nodule. The origin of the augmented 17β-HSD type 5 levels in this PPNAD-associated adenoma remains elusive as the factors that control adrenocortical AKR1C3 expression are unknown.

In vivo studies in our original patient and PPNAD2 did not show evidence for ectopic expression of hormone receptors known to aberrantly control adrenal function in macronodular hyperplasia. This finding is consistent with the two other cases described previously (10). It therefore appears unlikely that aberrant GPCR-signaling stimuli regulate increased adrenocortical steroidogenesis in human PPNAD cells.

The paradoxical rise in serum cortisol levels following Liddle’s test implied that glucocorticoids can locally regulate adrenocortical steroidogenesis in the majority of PPNAD. Dexamethasone incubation in primary cells from our patient’s PPNAD did not stimulate steroid secretion or steroidogenic enzyme mRNA levels, comparable to the effects in normal and malignant adrenal cells. This implies that overproduction of cortisol and androgens was present without stimulatory effects of the GR. Although the constitutively activated cAMP–PKA pathway could be sufficient cause for the hormonal syndrome in the patient, it could also be speculated that mechanisms different from glucocorticoid action are involved in steroidogenic control within PPNAD-associated adenomas. In addition, using qPCR, we could not show an increased expression of GR mRNA in PPNAD samples from four patients, two of whom showed slightly augmented cortisol levels after dexamethasone in vivo. This finding suggests that in at least some PPNAD tissues, aberrant coupling of the GR to the cAMP–PKA pathway (11) instead of GR overexpression (10) may be the culprit for the dexamethasone-induced rise in cortisol production. Other steroid receptors, such as the progesterone and estrogen receptors, have also been detected in PPNAD tissue (30). The finding that AR mRNA is present in PPNAD is novel, although the AR appears to be expressed similarly in the various adrenal tissues that were examined in this study.

In conclusion, we described a novel clinical presentation for PPNAD, a female with primary infertility due to a virilizing adenoma formed in the context of PPNAD. Successful pregnancy ensued upon adrenalectomy. This tumor also had a unique steroid secretion profile in vitro, suggesting that defects of the PKA pathway may also affect secretion of additional steroids, apart from glucocorticoids. The steroidogenic regulation in this PPNAD-associated adenoma may be different from that in PPNAD without adenomas.

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.

Funding
This work was in part supported by the Intramural Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA.

Acknowledgements
The authors gratefully acknowledge Cobie Steenbergen for sequencing of CTNNB1 and Lisette de Vogel for performing the immunohistochemistry.
References


resurgence in adrenal cortex-specific Prkar1a knockout mice. 
PLoS Genetics 2010 6 e1000980. (doi:10.1371/journal.pgen. 1000980)


Received 11 July 2012
Revised version received 18 September 2012
Accepted 12 October 2012