

## Mutation Analysis of the LH Receptor Gene in Leydig Cell Adenoma and Hyperplasia and Functional and Biochemical Studies of Activating Mutations of the LH Receptor Gene

Annemieke M. Boot, corresponding author Serge Lumbroso, Miriam Verhoef-Post, Annette Richter-Unruh, Leendert H. J. Looijenga, Ada Funaro, Auke Beishuizen, André van Marle, Stenvert L. S. Drop, and Axel P. N. Themmen

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### Abstract

#### Context:

Germline and somatic activating mutations in the LH receptor (LHR) gene have been reported.

#### Objective:

Our objective was to perform mutation analysis of the LHR gene of patients with Leydig cell adenoma or hyperplasia. Functional studies were conducted to compare the D578H-LHR mutant with the wild-type (WT)-LHR and the D578G-LHR mutant, a classic cause of testotoxicosis. The three main signal transduction pathways in which LHR is involved were studied.

#### Patients:

We describe eight male patients with gonadotropin-independent precocious puberty due to Leydig cell adenoma or hyperplasia.

#### Results:

The D578H-LHR mutation was found in the adenoma or nodule with hyperplasia in all but two patients. D578H-LHR displayed a constitutively increased but noninducible production of cAMP, led to a very high production of inositol phosphates, and induced a slight phosphorylation of p44/42 MAPK in the absence of human chorionic gonadotropin. The D578G-LHR showed a response intermediate between WT-LHR and the D578H-LHR. Subcellular localization studies showed that the WT-LHR was almost exclusively located at the cell membrane, whereas the D578H-LHR showed signs of internalization. D578H-LHR was the only receptor to colocalize with early endosomes in the absence of human chorionic gonadotropin.

#### Conclusions:

Although several LHR mutations have been reported in testotoxicosis, the D578H-LHR mutation, which has been found only as a somatic mutation, appears up until now to be specifically responsible for Leydig cell

adenomas. This is reflected by the different activation of the signal transduction pathways, when compared with the WT-LHR or D578G-LHR, which may explain the tumorigenesis in the D578H mutant.

The LH receptor (LHR) belongs to the large family of G protein-coupled receptors that share a common structure of seven transmembrane domains and mediate signal transduction by activating heterotrimeric G proteins (1). During male embryogenesis, the action of human chorionic gonadotropin (hCG) through the LHR is responsible for testosterone production and hence virilization of the genital tract. At puberty, the LHR mediates the action of LH on testosterone synthesis by Leydig cells.

Inactivating mutations of the LHR gene are responsible for Leydig cell hypoplasia, a rare form of 46XY disorder of sex development, whereas activating mutations are the cause of familial male limited gonadotropin-independent precocious puberty (FMPP) or testotoxicosis (1, 2). It is an autosomal dominant disorder where in time improper Leydig cell hyperplasia occurs as a result of constitutive active LHR. The sixth transmembrane domain of the LHR represents a hot spot for activating mutations; in particular, various substitutions of the aspartic acid residue at position 578 have been reported: D578G-LHR, D578Y-LHR, and D578H-LHR (1–5). The D578H-LHR mutation has so far been reported only as a somatic mutation, limited to the tumor tissue, in a subset of patients with testosterone-producing Leydig cell adenoma (6–9).

In the Supplemental Data (published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>) and Table 1, we present eight male patients presenting with GnRH-independent precocious puberty not only due to a well circumscribed Leydig cell adenoma but also due to nodular Leydig cell hyperplasia. The aim of the present study was to perform LHR gene mutation analysis after microdissection procedures and to define the functional and biochemical properties of this specific D578H-LHR mutant by comparing with the wild-type (WT) variant (WT-LHR) and with the D578G-LHR mutant frequently reported in testotoxicosis.

## Patients and Methods

### Patients and samples

In total, eight cases were included, of which of one, only frozen testicular tissue was available; of four, only formalin-fixed material; and of three, both frozen as well as formalin-fixed, paraffin-embedded tissue. Research on these samples has been performed according to the Code for Proper Secondary Use of Human Tissue in The Netherlands, as developed by the Dutch Federation of Medical Scientific Societies (FMWV), version 2002, and has been approved by an institutional review board (MEC 02.981).

### DNA isolation

DNA isolation was performed as described before (10, 11). This was done both from the total tissue block as well as after microdissection from 5- $\mu$ m-thick sections, specifically of the LHR-positive areas, as

determined by immunohistochemistry, to allow comparison of the total biopsy and cells of interest (Leydig cells) (see below).

### Immunohistochemistry

Immunohistochemistry for the LHR was detected using a mouse monoclonal antibody (20C3) (12). No antigen retrieval was applied. For frozen tissue sections, the slides (4  $\mu\text{m}$  thick) were fixed for 1 h at room temperature using 4% buffered formaldehyde and subsequently washed with tap water. For the formalin-fixed, paraffin-embedded sections, after deparaffination, endogenous peroxidase activity and biotin were blocked, and sections were incubated with PBS/BSA (1%) at room temperature. The primary antibody was incubated overnight at 4 C at a dilution of 1:2000. Detection of the primary antibody was done with a biotin-labeled rabbit antimouse Ig and a biotinylated horseradish peroxidase-streptavidin complex (Vector Elite kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as chromogen. The staining was scored as absent, weak, or strong.

### LHR mutation analysis

To analyze the presence of the LHR mutation 203C $\rightarrow$ G (D578H-LHR), a highly sensitive locked nucleic acid-mediated PCR clamping and melting curve analysis was done as described before (13). Confirmatory Applied Biosystems (Foster City, CA) sequencing was done on the generated amplification products. The following primers were used: LHCGR, ATGTGATAAGAGGTACTTTGAAGG; LHCGR-R, CCAGTAAACTTTAGAGTTGGTTA; iLC LHCGR, TAAGAAAATGGCAATCC XTCA; Sensor mut, GCAGGTGAAATGGGTGAAGA-FL; and LNA wt, AGGTGAAATCGGTGAA p (developed and generated by TIB MOLBIOL, Berlin, Germany).

### Functional studies

Stable transfected cell lines (derived from 293 cells) were developed in which the expression of the hemagglutinin-tagged LHR cDNA, fused to the green fluorescent protein (GFP), is inducible by tetracycline (Tet-On system; Clontech Laboratories, Palo Alto, CA). This system allows 1) control of the timing and the level of LHR expression and 2) analysis in living cells of the LHR spatiotemporal expression pattern. Construction of the mutant LHR cDNA expression vectors was performed as described before (14). Overnight induction with different tetracycline concentrations was performed. Analysis of three main signal transduction pathways was carried out as described before for cAMP (14) and inositol phosphate induction (15), and for p44/42 MAPK phosphorylation, analysis was carried out according to the manufacturer's instructions (Cell Signaling, Bioké, Leiden, The Netherlands).

To study colocalization of LHR proteins in caveolae and other cell membranes, we used the method previously described by Song et al. (16) based on carbonate lysis and ultracentrifugation in sucrose gradient, which allows a clear separation of the membranes associated with lipid rafts and the other cell membranes. After sucrose gradient centrifugation, 12 fractions of 1 ml were taken. First we used different antibodies to various cell membranes to characterize these fractions obtained. The fractions 1–3 do not

contain any protein or LHR. Fractionation experiments were performed after overnight tetracycline induction, followed by a 20-h rest in Tet-free and serum-free medium. These conditions allow a complete maturation of the LHR and avoid interferences with the neosynthesized receptor. Colocalization of the LHR with caveolin-1 and early endosome antigen 1 (EEA-1) were performed using fluorescently labeled antibodies (BD Transduction Laboratories, Erembodegem, Belgium).

## Results

### LHR mutation analysis

In the tumor of cases 1, 3, 4, 6, and 8 and in the nodules of case 2, a heterozygous mutation of the LHR gene was found in exon 11 encoding a replacement of aspartic acid at position 578 with histidine (Aps578His, the D578H-LHR mutation, Table 1). In none of the cases was the mutation present in blood leukocytes, and in case 2, it was not present in the normal testicular tissue outside the nodules. In case 3, in addition to the D578H mutation found in the tumor only, we retrieved in the peripheral blood as well as in the tumor a heterozygous DNA change Y133N in exon 4 of the LHR gene. In cases 5 and 7, DNA was extracted from a Leydig cell nodule, but sequencing of the LHR gene showed no variants or mutations. In addition, Leydig cells from a nodule were picked by microdissection and pooled, but after DNA extraction and sequencing of exon 11 of the LHR gene, no mutations were found. No known Gs $\alpha$  mutation was detected in case 5.

### Functional studies

#### Control of LHR expression level with inducible system

The inducible expression of LHR under the control of tetracycline allowed us to precisely control the expression level of the three LHR and, hence, to perform functional studies with similar quantities of the various receptors (Fig. 1A). Treating stably transfected HEK293 cells for 16 h with different concentrations of tetracycline induced LHR protein expression levels paralleling the concentrations of tetracycline until a maximal expression is reached at 15, 15, and 30 ng/ml for WT-LHR, D578G-LHR, and D578H LHR, respectively (Fig. 1A). In the following experiments, these concentrations were used to induce LHR protein.

#### The cAMP pathway

Cotransfection of the three expression plasmids with a cAMP-responsive luciferase reporter gene revealed that at all expression levels, as induced by the three concentrations of tetracycline used, the tumorigenic mutant LHR D578H-LHR did not respond to hCG but showed a clear constitutive activity without hCG (Fig. 1B). The WT-LHR did not show any appreciable constitutive activity in the absence of ligand but could be stimulated to a much higher level than the D578H-LHR mutant receptor. The D578G-LHR showed an intermediary response, with a moderately high basal activity, which is slightly increased by hCG.

At lower expression levels after induction with 2.5 ng/ml tetracycline when the protein bands are hardly visible on Western blots, the D578H-LHR remained stable, whereas the WT-LHR and D578G-LHR revealed a lower maximal activity in an otherwise unchanged pattern.

At even very low induction doses (no LHR protein detectable on Western blots), the specific noninducible pattern of the D578H-LHR mutant remained although at a slightly lower maximal activity (data not shown).

#### The inositol trisphosphate pathway

The accumulation of inositol phosphates was determined in the stably transfected HEK293 cell lines before or after induction with tetracycline (Fig. 1C). Similar to the results obtained with the cAMP-response system, the D578H-LHR mutant showed a very high basal activity that is not inducible by hCG. However, both the WT-LHR and the D578G-LHR mutant did not show inositol phosphate accumulation in the absence of ligand, whereas both receptors were able to transduce the stimulatory effect of hCG. The maximal inositol phosphate production is similar for all three LHR variants.

#### The MAPK pathway

To investigate the possible induction of the MAPK pathways, preliminary experiments investigating the phosphorylation of MAPK p38, ERK1/2, and MAPK p44/42 were performed (data not shown). In the stable cell lines, only MAPK p44/42 showed a clear phosphorylation response, and therefore, additional experiments were performed using MAPK p44/42 phosphorylation as readout. In the absence of hCG, a slight phosphorylation of p44/42 MAPK was found with the D578H-LHR mutant but not with the other LHR variants (Fig. 1D). The p44/42 phosphorylation was clearly increased by the addition of hCG, but with a delay when compared with both WT-LHR and D578G-LHR, which showed a clear response at 2 min after addition. The phosphorylation induced by hCG through the D578H-LHR remained high after 60 min, similar to the WT-LHR results. Conversely, the D578G-LHR did not show basal activity, and after a clear induction after 2 min, the phosphorylation decreased more rapidly than with both WT- and D578H-LHR. When the absolute levels of phosphorylation were compared, the activity of the WT-LHR is clearly much higher than the induction of phosphorylation by the two mutant receptors.

### **Biochemical and morphological studies**

#### Localization of LHR in living cells

Enhanced GFP fused to LHR allowed us to follow the LHR protein subcellular localization before and after addition of hCG on the same living cell, using confocal microscopy.

Before addition of hCG, the D578H-LHR already displayed a different pattern compared with the WT-LHR (Fig. 2). Although the WT-LHR staining is almost exclusively located at the cell membrane, the D578H-LHR showed signs of internalization, with small intracellular aggregates near the plasma membrane. Fifteen minutes after addition of hCG, the difference between the two receptors is more pronounced. Some intracellular aggregates begin to appear in the WT-LHR cells, whereas they are much more abundant in the D578H-LHR cells. The pattern displayed by D578G-LHR mutant is different from both the WT-LHR and the D578H-LHR. Although the D578G-LHR mutant also shows intracellular expression in the absence of ligand, there do not appear to be aggregates, suggesting that the protein is present in a different compartment than the D578H-LHR protein. After induction with hCG, the formation of aggregates is similar to the pattern found with WT-LHR, against a background fluorescence, that does not change.

#### Fractionation studies

Study on the localization of the three LHR showed clear differences in the spatial expression pattern among WT-LHR, D578H-LHR, and D578G-LHR. Because the functional properties, depending on the pathway considered, are also different, we postulated that the internalization process used by the different LHR variants might involve different pathways as well. It has been shown for several membrane receptors and for G protein-coupled receptors that their localization in subdomains of the plasma membrane, i.e. in caveolae, are associated with different functional properties of signal transduction and internalization (17).

In addition, caveolin-1, the main component of caveolae, is involved in tumorigenesis processes (18). Therefore, sucrose gradient separation of membrane subdomains was employed to study the association of LHR protein with caveolae. Gradient fractions were subjected to Western blot analysis to determine colocalization different membrane proteins with the WT and mutant LHR protein (Fig. 3A). Fractions 4 and 5 are caveolae fractions (CAV) as determined by the cofractionation of the caveolin-1 protein, whereas fractions 9–12 are noncaveolae membrane fractions (NCM) as indicated by the presence of the early endosome marker EEA-1, the endoplasmic reticulum marker BIP78 (binding immunoglobulin protein 78), and clathrin, one of the proteins of the clathrin-coated pits. Fractions 6–8 are relatively devoid of protein.

Notwithstanding the higher total protein content in the NCM, WT and mutant LHR protein staining was quite intense in CAV, suggesting that LHR protein was concentrated in these fractions. The effect of the induction of internalization of LHR by hCG was also studied. Cells were treated with vehicle or 100 ng/ml hCG for 30 min, and the ratio of CAV or NCM localization after fractionation was determined for each of the LHR variants (Fig. 3B). hCG treatment clearly induces the transfer of the WT-LHR protein from the CAV to the NCM, whereas the D578G and D578H LHR mutants do not show that behavior, probably because of their constitutive activity they are under continuous internalization associated with a transfer from the CAV to the NCM fractions.

#### Colocalization experiments

To further substantiate the membrane subdomain localization of the LHR proteins, we used confocal microscopy to determine the localization of the enhanced GFP-tagged LHR protein with fluorescently labeled antibodies to EEA-1, an early endosome marker, and caveolin-1. Early endosomes are the organelles where receptors are found after internalization through clathrin-coated pits.

Surprisingly, we were not able to find any colocalization of WT or mutant LHR protein with caveolin-1 before or after hCG treatment (data not shown), contrasting with the presence of LHR in the lipid-raft fractions.

Studies of colocalization with EEA-1 revealed a clear difference between D578H-LHR and both WT- and D578G-LHR (Fig. 4). In the absence of hCG, D578H-LHR partly but clearly colocalized with EEA-1, which is not the case for the two other receptors. Quantification of colocalization using the confocal microscopy software showed that 15–20% of D578H-LHR localized in early endosomes, much higher than the 5% found for WT-LHR and D578G-LHR. After a 30-min treatment with hCG, there was a drastic increase in the localization of all three receptors within the early endosomes. About 30% of WT-LHR and D578G-LHR and 45–50% of the D578H-LHR colocalized with EEA-1 (Fig. 4).

#### Discussion

D578H-LHR is found only as a somatic mutation in the testis and seems to be specifically associated with Leydig cell adenomas and nodular hyperplasia of Leydig cells. In some patients, microdissection of the testis material with pooling of the DNA was necessary to identify the LHR mutation. Nevertheless, in two patients, no mutation could be identified. Although the technique is very sensitive, one cannot exclude that the mutation is missed. Other techniques, like pyrosequencing, may be helpful in the future. Another explanation could be that a gene functioning downstream of the LHR might be involved, but until now, no other mutation has been shown to be associated with Leydig cell adenomas. One can conclude that in the majority of the patients, the somatic D578H-LHR mutation is responsible for Leydig cell adenoma.

Nodular Leydig cell hyperplasia in one testis has previously been described in a male with FMPP with a germline Asp564Gly (D564G-LHR) mutation (13). Patient 2 is the first patient with nodular Leydig cell hyperplasia due to a somatic activating mutation of the LHR gene, the Asp578His mutation (D578H-LHR), with nodules in both testes. We hypothesize that in this patient, the somatic mutation occurred in an earlier phase during embryogenesis. Remarkably, the father of patient 2 developed a seminoma similar to an earlier reported patient with FMPP (19), but no mutation was observed in the seminoma of the father. In patients 5 and 7, in whom no mutation was found, the gonadotropins were less depressed, and in patient 5, lower testosterone levels were observed at presentation than in the other patients. Central activation may have played a role; however, histology examination showed Leydig cell hyperplasia and adenoma. The Leydig cell hyperplasia resolved at an older age in patient 5.

The D578H mutation is found only as a somatic mutation, similar to  $G\alpha$  mutations observed in McCune-Albright syndrome. A germline  $G\alpha$  gene mutation is considered to be lethal. Germline lethality is less likely for the D578H LHR mutation because only Leydig cells are potentially affected by a constitutively active LHR. Another explanation for the absence of germline D578H mutations may be the absence of transmission by affected males. There are two possibilities: less likely infertility, because men with FMPP are fertile, or absence of the mutation within germ cells, which is a reasonable explanation because of the different embryological origin of Leydig and Sertoli cells.

Functional experiments on the three main signal transduction pathways in which LHR is involved showed that D578H-LHR and D578G-LHR mutants are not functionally similar. D578H-LHR displayed a constitutive and noninducible production of cAMP, as observed before (4, 20). The activity of D578H-LHR is lower than the maximal activity of the WT-LHR, confirming previous results (4, 20). Angelova et al. (21) showed that this can be due to an improved complementarity for the G protein in the D578H-LHR mutant compared with the WT. D578G-LHR showed an in-between response with a moderate high basal activity, which was also described in other studies (22, 23). The stable cell lines used allowed us to induce comparable LHR protein levels of the various LHR mutants and shows that the D578G can be induced to the constitutive level obtained with the D578H.

The constant high production of cAMP may be causative for the tumorigenesis comparable with the activating mutation of the G $\alpha$  gene in McCune-Albright syndrome, which may cause thyroid and adrenal hyperplasia and pituitary tumors (24). However, although pituitary adenomas are relatively frequent in McCune-Albright syndrome, Leydig cell adenomas are rare. Most activating mutations in other G protein-coupled receptors, like TSH receptor (TSHR) gene and FSH receptor gene, cause a change in amino acid in the sixth transmembrane segment of the receptor, a highly conserved part of the G protein-coupled receptors, similar to the D578H- and D578G-LHR mutants (25, 26). This transmembrane domain has been shown to be involved in Gs coupling (21). Activating mutations in the TSHR gene are associated with nonautoimmune hyperthyroidism and thyroid adenomas (26). In the patient with FMPP with nodular hyperplasia with the Asp564Gly mutation, the cAMP was lower than in the Asp578Gly mutation, which is not associated with nodular hyperplasia (13). Therefore, it seems unlikely that the higher cAMP production alone is responsible for the tumorigenesis.

Compared with previous studies, only minor differences in the inositol phosphate production responses could be observed (4, 20, 27); e.g. we did not observe a further induction of in the D578H-LHR by hCG. Such slight inconsistencies could be explained by the use of different transfection methods used, type of receptor-tagging cell, and/or species type examined. Nevertheless, in particular, the D578H-LHR mutation, associated with Leydig cell adenoma or hyperplasia, leads to a very high production of inositol phosphates. This may be more likely involved in the proliferation of Leydig cells because the basal production was much higher than in the other types, and this pathway is known to control processes like proliferation and cell metabolism.

D578H-LHR, not the WT-LHR or D578G-LHR, induced a slight phosphorylation of p44/42 MAPK in the absence of hCG. The MAPK signaling pathway may play a role in several steps of tumor genesis. However, the activity reacted on the addition of hCG and the activity of phosphorylation of the WT-LHR was about 2-fold larger than of the two mutants, which is not in favor of the MAPK pathway playing an important role in Leydig cell adenomas.

Furthermore, we showed for the first time that the LHR is present at high concentration in lipid rafts components.

No colocalization of WT or mutant LHR protein with caveolin-1 before or after hCG treatment was observed, contrasting with the presence of LHR in the lipid raft fractions. However, the detergent-resistant fractions contain both caveolae, which contain caveolin-1, and noncaveolae lipid rafts, which lack caveolin-1. It is also noteworthy that some cells, although expressing caveolin-1, do not form caveolae structures. It is possibly the case for the Trex 293 cells we used. It would thus mean that the LHR present in the detergent-resistant fraction is localized only in noncaveolae lipid rafts.

Because both early endosomes and caveolae contain scaffold proteins and participate in regulation of several signal transduction pathways in the cells, it is tempting to relate the specific location of the D578H-LHR mutant within the cell and the functional characteristics. More experiments are necessary to prove this link and to determine whether these functional, biochemical, and morphological features of the D578H-LHR mutant are responsible for the development of adenoma in the patients.



Preliminary results have shown that D578H-LHR mutant exhibits a specific spatial pattern within the cells, i.e. spontaneous internalization in the absence of ligand and colocalization with early endosomes. Several lines of evidence show that caveolae could be involved in tumorigenesis (28). In addition, caveolae are involved in the endocytosis process and can regulate the activity of various signaling molecules, like the cAMP, inositol trisphosphate, and MAPK pathway. However, the caveolae of D578H mutation behaved the same as those of the D578G mutation.

In conclusion, the somatic D578H-LHR mutation in the LHR gene was observed in the majority of the patients with Leydig cell adenoma and in a patient with nodular Leydig cell hyperplasia in both testes. The D578H-LHR mutant was compared with the D578G-LHR mutant and the WT-LHR in functional and biochemical studies. The D578H-LHR and D578G-LHR mutants activate the signal transduction pathways in a different manner, which may explain the tumorigenesis in the D578H mutant.

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