Identification of a gain-of-function mutation of the prolactin receptor in women with benign breast tumors

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There is currently no known genetic disease linked to prolactin (Prl) or its receptor (PrlR) in humans. Given the essential role of this hormonal system in breast physiology, we reasoned that genetic anomalies of Prl/PrlR genes may be related to the occurrence of breast diseases with high proliferative potential. Multiple fibroadenomas (MFA) are benign breast tumors which appear most frequently in young women, including at puberty, when Prl has well-recognized proliferative actions on the breast. In a prospective study involving 74 MFA patients and 170 control subjects, we identified four patients harboring a heterozygous single nucleotide polymorphism in exon 6 of the PrlR gene, encoding Ile146→Leu substitution in its extracellular domain. This sole substitution was sufficient to confer constitutive activity to the receptor variant (PrlRI146L), as assessed in three reconstituted cell models (Ba/F3, HEK293 and MCF-7 cells) by Prl-independent (i) PrlR tyrosine phosphorylation, (ii) activation of signal transducer and activator of transcription 5 (STATS) signaling, (iii) transcriptional activity toward a Prl-responsive reporter gene, and (iv) cell proliferation and protection from cell death. Constitutive activity of PrlRI146L in the breast sample from a patient was supported by increased STATS signaling. This is a unique description of a functional mutation of the PrlR associated with a human disease. Hallmarks of constitutive activity were all reversed by a specific Prl antagonist, which opens potential therapeutic approaches for MFA, or any other disease that could be associated with this mutation in future.
mutation in the PrlR gene, leading to a constitutively active receptor.

**Results**

**Patients and Controls.** Seventy-four Caucasian women with MFA were consecutively recruited by the BBDS study group, from 9 different centers, and referred to our outpatient clinic. Inclusion was possible based on the existence of at least 3 FAs in one breast in patients receiving no treatment influencing gonadal axis for at least 1 month. All underwent basic clinical investigations. For those who underwent surgery, FA and adjacent tissue were obtained whenever possible.

A cohort of 96 control Caucasian subjects was constituted based on stringent inclusion criteria including no history of benign or malignant breast disease, no pituitary disorder, normal Prl levels. To minimize the risk of including young subjects who could later develop an MFA, we fixed the cut-off age at 35. A random population of 74 unrelated control women (no inclusion criteria) was also analyzed.

**PrlR Genotype Associated with MFAs.** No missense single nucleotide polymorphism (SNP) was identified in the PrlR gene of any patient. With respect to the PrlR, we found in both MFA patients and control subjects (with no difference) the sole coding SNP reported in the NCBI database (rs16871473, C/T in exon 5, encoding I76V substitution at protein level), as well as many known SNPs in intronic regions bordering exons [supporting information (SI) Text]. In four unrelated patients (5.6%), we found a coding SNP that was not reported in the NCBI database (deposit procedure in progress). In four unrelated patients (5.6%), we found a coding SNP that was not reported in the NCBI database (deposit procedure in progress). In four unrelated patients (5.6%), we found a coding SNP that was not reported in the NCBI database (deposit procedure in progress). In four unrelated patients (5.6%), we found a coding SNP that was not reported in the NCBI database (deposit procedure in progress).

**Establishment of Cell Models to Study I146L SNP.** The impact of I146L substitution on PrlR properties (mutant is referred to as PrlR_{I146L}) was characterized by using transfected cell models and multiple well-established readouts for structure-function studies of the PrlR (Ba/F3 mouse lymphoid cells, HEK293 human embryonic kidney fibroblasts). To avoid any bias, stable clones (HEK) or populations (Ba/F3) to be compared were generated and selected based on similar expression levels of WT and mutated PrlRs as determined by semiquantitative PrlR immuno blotting (Fig. S2) and/or radioligand receptor assay. HEK-PrlR_{WT} and HEK-PrlR_{I146L} clones expressed approximately 5,000 surface receptors/cell, whereas Ba/F-PrlR_{WT} and Ba/F-

**Mutation I146L Encodes a Constitutively Active PrlR.** In stable HEK-PrlR_{WT} and MCF-7-PrlR_{WT}, Prl stimulation induced tyrosine phosphorylation of the PrlR (Fig. 3 A and B), which is known to be mediated by the receptor-associated tyrosine kinase, JAK2 (14). Otherwise, strong receptor phosphorylation was observed in non Prl-stimulated HEK-PrlR_{I146L} cells, but not in HEK-PrlR_{WT} cells (Fig. 3A). This was also observed in MCF7-PrlR_{WT}, stable clones were noted MCF7-PrlR_{WT,WT} versus MCF7-PrlR_{WT,I146L}.

![Fig. 1. MRI of a MFA patient showing several fibroadenomas (arrows) mainly located in the left breast.](image-url)
The interleukin 3-dependence of Ba/F3 cells for proliferation/survival can be shifted to any other cytokine providing they express the cognate receptor. Accordingly, Ba/F-PrtlRWT cells grew in the presence of Prol but underwent massive apoptosis in the absence thereof within 24 h (Fig. 3F). Otherwise, Ba/F-PrtlR146L survived (Fig. 3F) and even proliferated over several days (Fig. 3G) irrespective of the addition of Prol. The ability of PrlR146L to shift cells to the S/M phase was further increased by Prol stimulation, and, as observed in the luciferase assay, it attained higher levels than in stimulated Ba/F-PrtlRWT cells (Fig. 3F and Fig. S5).

MCF7-PrtlRWT,WT cells were not dependent on Prol for survival but failed to proliferate in Prol-serum-deprived medium (Fig. 3H).

**Table 1.**

<table>
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<tr>
<th>Constructs</th>
<th>Luciferase activity (normalized)</th>
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<tr>
<td>PrlRWT</td>
<td>0.05</td>
</tr>
<tr>
<td>PrlR146L</td>
<td>0.10</td>
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shown), indicating that I146L mutation leads to constitutive receptor activation. Signal transducer and activator of transcription 5 (STAT5) is the main signaling target triggered by the PrlRWT (15). Accordingly, Prol induced STAT5 phosphorylation in the three cell models expressing either receptor (Fig. 3A–C). In agreement with the constitutive activation of PrlR146L, STAT5 was phosphorylated in the absence of Prol only in cells expressing the mutant receptor, although to different extent depending on cell lines (Fig. 3A–C).

The relevance of Prol-independent activation of PrlR146L/STAT5 cascade was assessed in functional assays. Despite similar levels of PrlR expression in stable clones, HEK-PrlR146L cells exhibited significantly higher basal activity of the Prol-responsive lactogenic hormone response element (LHRE)-luciferase reporter gene compared to HEK-PrlRWT cells (Fig. 3D). The same effect was observed in other stable clones or populations (not shown), and in transient transfections (Fig. 3E). The C-terminal tyrosine of the PrlR (Tyr-587) is critical for receptor phosphorylation, activation of STAT5 and transcription of downstream target genes (16). This residue was mutated into Phe in both PrlRWT and PrlR146L, and the cognate receptor mutants were transiently expressed in HEK cells (Fig. 3E). As expected, replacement of Tyr-587 drastically reduced the ability of Prol to activate the reporter gene via both PrlRWT and PrlR146L (15 min).

The Y587F mutation also reversed the higher basal activity of PrlR146L back to the level observed for the other constructs, indicating that the constitutive activity of PrlR146L involves phosphorylation of the C-terminal tyrosine. Besides constitutive activity, we also noticed that the activity of PrlR146L in the presence of any concentration of Prol was systematically higher than that displayed by PrlRWT (Fig. 3D and Fig. S5).

Cell cycle distribution of Ba/F-PrtlRWT and Ba/F-PrtlR146L cells was monitored by FACS analysis. Cells were starved by Prol depletion and stimulated with 8 nM Prol (Right) or not (Left) for 24 h. DNA content analysis of propidium iodide-stained cells is represented. Numbers indicate the average proportion of cells ± SD (seven to nine independent series) exhibiting <2n (subG1), 2n (G0/G1), and >2n (S/M). In the absence of Prol, expression of PrlR146L protects cells from death and stimulates division (Lower Left). (G and H) Proliferation of MCF7-PrtlRWT,WT and MCF7-PrtlRWT,146L cells was monitored by using WST-1 reagent. The data presented that expression of PrlR146L is autonomous for growth. Error bars indicate SD (slopes differences; three independent experiments performed in triplicate), * P < 0.05.
In the same conditions, MCF7-PrlRWT,I146L cells proliferated to a submaximal level, as Prl could further enhance cell division (not shown). This demonstrates that the growth-promoting effect of PrlRWT,I146L as observed for receptor and STAT5 phosphorylation, occurs irrespectively of co-expression of the WT receptor, which is particularly important regarding the fact that the mutation is heterozygous in our MFA patients.

**Increased Stat5 Signaling in Breast Tissue from Mutated Patients.** Biopsies of MFA and adjacent tissue from one of these four heterozygous patients (PrlRWT,Wt/I146L) was available for histological studies and was compared to samples from two homozygous patients (PrlRWT,Wt). Activation of the PrlR-STAT5 pathway was investigated by immunohistochemical analysis of the receptor, STAT5, phospho-STAT5, and fatty-acid synthase (FAS), a downstream target gene of STAT5 in mammary cells (17). In MFA samples, irrespective of the PrlR genotype, the nuclear location of phospho-STAT5 labeling (arrows on Fig. 4) and the intense FAS labeling supported activation of that pathway in tumors. Remarkably, phospho-STAT5 and FAS labeling was also observed in adjacent tissue from the mutated patient but not in those from non mutated patients, suggesting increased activation of PrlR-triggered cascades also occurs in healthy tissue expressing the mutated receptor. PrlR and STAT5 labeling were similar in all samples analyzed (not shown), indicating that activation of STAT5 cascade was not caused by over-expression of these proteins.

**Inhibition of PrlRWT,I146L Constitutive Activity by PrlR Signaling Inhibitors.** We investigated whether strategies known to inhibit PrlR-induced activation of PrlRWT,I146L could down-regulate the constitutive activity of PrlRWT,I146L. Tyrophostin B42 (AG490) is a pharmacological inhibitor of JAK2 activity and Del1–9-G129R-hPrl is a specific, competitive PrlR antagonist (18). Both inhibited constitutive phosphorylation of PrlRWT,I146L stably expressed in HEK cells (Fig. 5A). A single treatment with the PrlR antagonist markedly reduced STAT5 activation in Ba/F-PrlRWT,I146L cells over 24 h (Fig. 5B) as well as the number of spontaneously dividing (S/M) cells (from 41 ± 3% to 22 ± 4% in favor of G0/G1 cells; 3 independent experiments, data not shown). In agreement, three-day proliferation of Ba/F-PrlRWT,I146L cells was also significantly reduced by a single treatment with the PrlR antagonist (Fig. 5C). Similar growth inhibition by the antagonist was observed on MCF7-PrlRWT,I146L cells (Fig. 5D).

**Discussion**

Our data represent a unique functional characterization of a genetic anomaly of the PrlR gene associated with a human disease. The multiple functional assays used in this study unambiguously converge to the evidence that PrlRWT,I146L exhibits constitutive activity, highlighting the remarkable effect of this single substitution on the biological properties of the PrlR. Importantly, these conclusions were confirmed by using mammary epithelial cells (MCF-7) co-expressing both PrlRWT (endogenous) and PrlRWT,I146L (exogenous), which is presumably the most representative model of the situation found in the breast tissue of the heterozygous patients.

The molecular mechanism by which I146L mutation confers constitutive activity to the PrlR is currently unknown. This is not the first example of a membrane receptor on which such a conservative substitution has functional consequences (19). Based on the three-dimensional structure of the dimerized rat PrlR (20), Ile146 is located just under the surface of interaction of both receptor molecules (Fig. S7). It is reasonable to postulate that I146L mutation could force the PrlR to fold in a confor-
mimicking that normally induced upon Prl binding. This is in good agreement with the ability of the PrlR antagonist to reduce significantly the constitutive activity of PrlR_{I146L}. An engineered mutation of the topologically equivalent Ile residue in the common beta chain of the human IL-3 receptor (E74N) also led to a receptor exhibiting constitutive activity in some cell lines, suggesting that this position represents a hot spot in cytokine receptors to achieve constitutive activity (21).

Engineered PrlR variants exhibiting ligand-independent activity have also been generated by deleting half up to almost the entire extracellular domain (22, 23). The cellular effects induced by these artificial variants were very similar to those reported in this study for the natural PrlR_{I146L} mutant. Mammary-specific by these artificial variants were generated (24). Phenotypes included premature over-development of the gland in virgin and pregnant animals, and, after parturition, impaired terminal differentiation, functional failure, and delayed involution. Unfortunately, this study failed to reveal whether these morphological anomalies developed into mammary tumors because only young animals were used (24). We recently showed that transgenic mice over-expressing Prl in the differentiating/factating mammary gland developed various benign lesions from the age of approximately 1 year (25). Using a permanently active promoter, others showed that autocrine Prl induced mammary carcinomas in virgin females from the age of approximately 15 months (26). Although the mechanisms leading to the development of benign versus malignant tumors are not yet fully understood, they may involve various parameters such as genetic background or the state of differentiation of the gland (25), these studies highlighted the ability of PrlR-triggered pathways to promote mammary tumorigenesis in rodents. In humans, the link between Prl levels and breast carcinogenesis has recently emerged from the Nurse Health study (27, 28). The description of a gain-of-function mutation of the PrlR associated with a BBD adds a new facet to the involvement of this hormonal system in the pathogenesis of breast tumors. Interestingly, this substitution has recently been reported as an uncharacterized SNP in three breast cancer patients (4, 5). Our findings strengthen the need to perform large epidemiological studies to determine whether PrlR_{I146L} may also participate in breast carcinogenesis. The mechanisms involved downstream constitutive PrlR activation in MFAs remain to be elucidated. The role of STAT5 transcription factor in breast tumorigenesis has been underlined in recent studies (29, 30). STAT5 appeared to be constitutively phosphorylated in the three cell models expressing PrlR_{I146L}. Moreover, it was also activated in the mammary tissue of the mutated patient, and this was true in both the tumor and the adjacent tissue (Fig. 4). Active STAT5 was also observed in MFA from patients expressing only the WT receptor. More than being a marker of PrlR_{I146L}, constitutive activity, phosphorylated STAT5 is a thus good candidate contributing to the pathogenesis of MFAs. The molecular pathways that could mediate its effects may involve FAS, as the latter was proposed to stimulate survival and proliferation of breast cancer cells via complex mechanisms interfering with ER actions (31). Similar mechanisms could also occur in MFA, in which ER expression was also assessed (not shown). It is clear however that MFA pathogenesis remains a complex process presumably involving several other mechanisms yet to be identified (8).

Clinically, none of our mutated patients displayed any obvious sign of hyperprolactinaemia, although full clinical phenotyping was not performed. Also, because serum samples for endocrine evaluation were only performed during the early follicular phase, potential luteal phase defects cannot be ruled out. In fact, germline activating mutations of receptors can lead to phenotypes that are different from those expected. For example, gain-of-function mutations of the LH receptor result in precocious puberty only in boys (19), whereas a physiological role of this receptor on the ovary is widely recognized. Also, the pathological consequences of FSH receptor activating mutations are only apparent during pregnancy in women with no apparent fertility or ovarian troubles (32). In our patients, the association of the PrlR mutation with a breast phenotype highlights this tissue as the main Prl target. In agreement, the increased risk of developing a breast cancer among women with high-normal versus low-normal Prl levels (27, 28) reinforces the idea that the first consequence of a slightly increased Prl stimulus is related to a breast phenotype. The higher sensitivity of the breast is likely related to the expression level of the PrlR, which is one of the highest of all Prl target organs, actually several fold more than seen in the ovary (33). Future investigations will be directed to identifying to what extent the increased signaling of PrlR_{I146L}, demonstrated in various in vitro assays, participates in triggering quantitatively and/or qualitatively different downstream events that could be correlated to breast pathogenesis.

Because of the Prl-independent activity of PrlR_{I146L}, no beneficial effect of dopamine agonists would be expected in these mutated patients. The field of BBDs therefore constitutes an opportunity to study the antiproliferative activity of alternative molecules. Competitive PrlR antagonists are a new class of potential drugs which act directly at the level of PrlR activation (34). Our study clearly shows that these molecules are able to exert inhibitory effect on PrlR_{I146L}, constitutive activity in vitro, including on STAT5 signaling. These findings encourage consideration in the future of adapting therapeutic management of patients harboring this mutated receptor.

Methods

Patients. The study was approved by the local ethical committee and written informed consent was obtained from all patients and controls. DNA was extracted from whole blood cells, and the 11 exons of the PrlR gene were sequenced in both directions. Reference sequences were obtained from Ensembl (OTTHUMG000000090789) and National Center for Biotechnology Information online databases (NM.000949).

Cell Cultures and Transfections. HEK293 and MCF-7 were cultured in DMEM and Ba/F3 in RPMI as detailed in SI Text. Cells were cotransfected (Fugene 6, Roche) by using plasmids encoding the human PrlR of interest and, for HEK cells only, the vector encoding firefly luciferase under control of STAT5 response elements (LHRE) (35). Stable clones were selected in growth medium containing 500 μg/ml active G-418 (gentamicin).

Binding Assays. Expression level and affinity parameters of receptors were determined by routine radioligand binding assay as described in SI Text.

Cell-Based Assays. Luciferase assays (HEK cells) were performed as earlier described by using a luciferase kit (Promega) and a luminometer (Lumat LB 9501, Berthold) as detailed in SI Text. Cell proliferation/survival (Ba/F3, MCF-7) was measured over 3 days by daily measurement of tetrazolium salt conversion (WST-1 assay). Cell cycle distribution was checked by FACS analysis using propidium iodide labeling (35).

Signaling Studies. Intracellular signaling was analyzed by routine immunoprecipitation/immunoblot procedures as detailed in SI Text. Immunoprecipitations were performed by using anti-human PrlR antibody (1A2B1, 2ymed) or polyclonal anti-STAT5 (C-17, Santa Cruz Biotechnology). Immunoblotting involved either anti-phosphotyrosine antibody (4G10, Upstate), anti-phosphorylated STAT5 (AX1, Advantex BioReagents), and, after stripping, the anti-PrlR or anti-STAT5 antibodies referenced above.

Immunohistochemical Analysis. The analyzes of breast biopsies were performed by using 3-μm sections from paraffin-embedded, formalin-fixed tissues, stained by using the antibodies described above, or anti-fatty acid synthase (H-300, Santa Cruz Biotechnology). Protocol is detailed in SI Text.

Statistical Analyses. The frequencies of the mutation in both cohorts were compared by the two-tailed Fisher test. Results of bioassays are expressed as mean ± SD, and multiple groups were analyzed by the Kruskall-Wallis test.
with the Mann–Whitney U test as a post hoc comparison. Cell proliferation assay was analyzed by linear regression analysis, with comparison of slopes by ANCOVA analysis. Statistical analysis was performed by using PRISM software (GraphPad Software). P < 0.05 was regarded as statistically significant.

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