Characterization of Two Constitutively Active Prolactin Receptor Variants in a Cohort of 95 Women with Multiple Breast Fibroadenomas

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Background: The role of prolactin (PRL) and its receptor (hPRLR) in promoting breast tumors is debated. We recently identified a gain-of-function hPRLR variant (I146L) in four women with multiple breast fibroadenomas (MFA) and no control subject.

Objectives: The specific aims were to describe this cohort of women presenting with MFA to identify and functionally characterize germline variants of hPRL/hPRLR genes and compare phenotypes of all patients.

Design: Ninety-five patients prospectively underwent clinical examination, breast ultrasonography, magnetic resonance imaging, and hormonal evaluation of gonadal and lactotrope functions. We analyzed hPRL/hPRLR coding sequences and made comparisons with a control population of 194 women. Functional characterization of hPRLR variants was performed. Pathology and immunohistochemistry were systematically carried out after surgical removal of tumors.

Results: One third of patients had a family history of breast disease. No hormonal imbalance was observed, except 30.7% of explosive stimulated PRL. Prolactin receptor variants were identified in exon 5 (I76V: 10 patients, eight controls) and exon 10 (one patient, no control). Both I146L and I76V variants exhibited constitutive activity. Pathology showed common fibroadenomas and identified six benign phyllodes tumors. Estrogen and progesterone receptors were detected in 85 and 98% of samples, respectively. Ki-67 median staining was less than 5%. No phenotypic difference was observed between carriers and noncarriers of either hPRLR variant.

Conclusion: We present the largest population with MFA ever described, 15% of which had a hPRLR exhibiting basal activity in vitro. This questions the involvement of the hPRLR in MFA etiology and the potential relevance of therapeutic inhibition of PRLR signaling in patients. (J Clin Endocrinol Metab 95: 271–279, 2010)
Prolactin (PRL) is a polypeptide hormone mainly secreted by lactotrope cells of anterior pituitary. More than 300 separate functions or molecules activated by its receptor (PRLR) have been reported (1). PRL is notably involved in mammary gland development and has a prominent role in lactation (2). Implication of the PRL axis in breast diseases is a matter of debate. The role of PRL in breast tumorigenesis remains difficult to affirm, despite wide epidemiological studies showing that high-normal PRL levels are associated with an increased risk of breast cancer (3, 4). Unfortunately, data from large cohorts of hyperprolactinemic patients are lacking to further support this association. In vitro studies established that human (h) PRL has a mitotic action on mammary tumor cells and that hPRLR is overexpressed in samples of breast tumors compared with adjacent normal breast tissue (5). In contrast to humans, the role of PRL in rodent mammary tumor development is unanimously admitted. Whereas transgenic mice overexpressing PRL develop both benign and malignant mammary tumors (6, 7), mammary development and tumor rate are concomitantly impaired in mice deficient for genes encoding PRL, its receptor, or signal transducer and activator of transcription (Stat)-5A, the major signaling molecule of the hPRLR in the mammary gland (8–11). A human genetic model has yet to be identified to strengthen the role of hPRL/hPRLR in breast diseases. Some authors reported associations between PRL or hPRLR single-nucleotide polymorphisms (SNPs) and risk of breast cancer (12–14), but no functional analysis was undertaken to provide any mechanistic insight to these associations.

We recently identified in a population of women with multiple breast fibroadenomas (MFA), a germline heterozygous variant in exon 6 of the hPRLR gene, encoding Ile146 to Leu (I146L) substitution in the extracellular domain of the receptor (15). This sole substitution was sufficient to confer constitutive activity to the receptor variant, as reflected by PRL-independent activation of the PRLR/Stat5 cascade and proliferative/antiapoptotic effects in various cell lines. In such context, this study was aimed at phenotypically describing the largest cohort ever of women with MFA, including the identification and functional characterization of additional hPRLR variants, with particular focus on patients harboring hPRLR variants.

**Patients and Methods**

**Patients and design**

This cohort study was carried out in the Department of Endocrinology and Reproductive Medicine at the Pitie´-Salpeˆtrie`re Hospital in Paris, France, from September 2004 to March 2009. It was approved by the local ethical committee, and all patients and controls provided written informed consent before the initiation of the study.

We included 95 premenopausal women (of which 72 were Caucasian) having at least three fibroadenomas (FAs) in one breast (16) and being free of any hormonal treatment influencing the gonadal axis for at least 1 month. The evaluation took place during a 1-d hospitalization and consisted of a careful recording of personal and family history, with a particular attention paid to mammary and gynecological histories, and a mammary gland examination. Hormonal evaluation of gonadal axis and lactotrope function was performed. A blood sample was drawn for sequencing of PRL and hPRLR exons. On the same day, patients underwent breast ultrasound and magnetic resonance imaging (MRI). After evaluation, surgical removal of one FA was suggested to obtain a precise pathological analysis, perform immunohistochemistry, and constitute a bank of frozen tissues for functional studies.

A control cohort of 120 Caucasian women was established for the PRLR genotyping part of this study. Inclusion criteria were no history of breast disease, no pituitary disorder, and normal PRL levels. To minimize the risk of including subjects who could later develop MFA, we included women over age 35 yr. Another cohort of 74 control women for whom we had no clinical information was also studied, raising the number of controls to 194.

**Hormone assays**

Hormonal assays were performed in the 95 MFA premenopausal women meeting the inclusion criteria. All basal blood samples were drawn fasting at 0800 h. LH and FSH plasma levels were measured by chemiluminescent immunometric assay (Siemens, Surrey, UK). RIA was used to measure estradiol (DiaSorin, Stillwater, MN), progesterone (PROG-CTRIA; CIS Bio International, Gif-sur-Yvette, France), testosterone (Orion Diagnostica, Espoo, Finland), androstenedione (Immunotech, Marseille, France). PRL, dehydroepiandrosterone-sulfate, and SHBG were measured by Immulite 2000 chemiluminescence assay equipment (Diagnostica Products Corp., Deerfield, MA). PRL was measured before and after a stimulation test (either with 250 µg TSH or 10 mg metoclopramide).

**Radiology**

Breast ultrasonography was performed for 78 women, with a Xario XG (Toshiba, Tokyo, Japan). Patients were in dorsal decubitus and mammary glands were scanned with a 7- to 14-MHz high frequency probe.

Breast MRI was performed for 87 women with a 1.5 Tesla (Philips, Best, The Netherlands). It included both breasts in the field of view and patients were in ventral decubitus. Transverse planes in T2-weighted, and short inversion time inversion-recovery sequences were used. Then transverse planes of 4 mm section thickness in T1-weighted (Gradient Recalled Echo) sequence were performed with one precontrast and four postcontrast acquisitions. A subtraction of precontrast to postcontrast images was done to better visualize the enhancing foci. We ended with sagittal planes of 0.8 mm section thickness in late three dimensional T1-weighted sequences. T1-shortening contrast agent used was 0.1 mmol/kg of iv gadolinium chelate.
Pathological analysis, construction of tissue macroarrays (TMAs), and immunohistochemistry

Seventy patients (73.7%) underwent surgical removal of a FA. Surgically isolated tissues were fixed in neutral formalin and embedded in paraffin. Whole hematoxylin-eosin-safran sections of tumoral and adjacent tissues were reviewed by one pathologist. TMA were done for 46 of the 70 samples (37 with both tumor and adjacent tissues). Five areas were marked for each section of each block of FA and adjacent tissues. The array blocks were incubated overnight at 37°C to improve adhesion between cores and paraffin of recipient blocks. They were cut (3 μm sections) at room temperature with a standard microtome. Using automated immunohistochemical technique (Ventana, Benchmark, VT), we applied primary antibodies directed against estrogen receptor (ER; 1:50, clone 1D5, Dako, Trappes, France) or progesterone receptor (PR; 1:50, clone PgR 636, Dako). ER and PR were considered positive when 10% nuclear staining was observed (17). TMAs were also used for Ki-67 (1:50, clone MIB-1; Dako, Trappes, France) immunohistochemical staining, which was analyzed by counting the fraction of positively stained nuclei. For each spot, 5 to 10 vision fields were analyzed.

DNA sequencing

Genomic DNA was extracted and analyzed as previously described (15). The 11 exons of the hPRLR gene and five exons of the hPRL gene were entirely sequenced in both directions using primers matching intronic boundaries (primer sequences available on request).

Functional analysis of hPRLR coding gene variants

Functional characterization of hPRLR variants was performed using HEK293 human fibroblasts (binding) or Ba/F3 mouse pro-B lymphoid cells (proliferation), transfected using expression vector for either hPRLR (wild type or variants). Experimental procedures were extensively described in two recent publications (15, 18). Briefly, stable clones (HEK) or populations (Ba/F3) were selected by addition of 0.7 mg/ml neomycin analog (G418; PAA, Cölbe, Germany) and, for Ba/F3 cells only, hPRL (Ba/F3) were selected by addition of $4 nM$. Binding affinities of PRLRs were determined using cell homogenates prepared from stable HEK clones, using displacement of $^{125}$I-hPRL by unlabeled hPRL. Ba/F cell proliferation/survival assays were performed by incubating each stable population with or without hPRL over 3 d; living cells were quantified daily using WST-1 reagent (Roche Diagnostics, Mannheim, Germany). Activation of Stat5 and ERK1/2 was assessed by Western blot analysis of Ba/F cell lysates (50 μg), using antiphospho-Stat5 (AX1; Advantex Bioreagents, El Paso, TX) and antiphospho-ERK1/2 (E10; Cell Signaling, Beverly, MA) monoclonal antibodies. Anti-Stat5 (C17; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-ERK (06-182; Millipore, Bed ford, MA) were used to assess equal loading.

Statistical analysis

Analyses were processed with StatView version 5 (Abacus Concepts, Berkeley, CA). Descriptive statistics were performed for each variable; quantitative results are presented as mean ± SD and qualitative results are presented as a distribution of a number of patients. To compare two groups, two-tailed Fisher test was used for qualitative variables and t test for quantitative variables. For functional assays, we used nonparametric ANOVA for comparing parameters of three PRLR variants and Mann Whitney for two group comparison. In all cases, a $P < 0.05$ was considered significant.

Results

Clinical parameters

Patients were 28.1 ± 8.7 yr old (14–51 yr) and had a mean body mass index of 21.5 ± 3.0 kg/m². Their personal medical history did not show any recurrent event, except for thyroidopathy, found in 6.3% of the cohort. Personal genital history showed a normal mean age for menarche (12.7 ± 1.3 yr), with regular menses for 76.8% of patients. In this young cohort, 74.7% of the women were nulliparous. Before evaluation, only 10.5% of the women were free of having ever taken any hormonal treatment. Thirty-four percent of patients reported one or more female relative with breast cancer. Family history of benign breast diseases (BBD) were reported for 38.9% of patients and 14% of the women mentioned family history of both breast cancer and BBD. In most cases (63.2%), FAs were first discovered by the women themselves, otherwise by a clinical breast examination. The first FA appeared at age 21.1 ± 6.6 yr and progression to MFA was usually short, with a mean age of 25.5 ± 8.8 yr at diagnosis. Forty percent of women had already undergone surgical removal of at least one FA before the study. The clinical examination did not reveal any abnormality, besides palpable lumps in breasts (Table 1). Half of patients reported mastalgia. Galactorrhea was a very uncommon feature, observed in only six patients, all but one with normal PRL levels.

Variants of hPRLR and PRL genes

The PRL exons were sequenced for 85 MFA patients. The hPRLR exons were sequenced for the entire 95 MFA cohort as well as for 11 additional patients who had not disrupted hormonal treatment in due time to be included in the global study and for the 194 controls.

No nonsynonymous coding SNP was identified in the PRL gene. We found three germline coding variants in hPRLR gene in MFA patients (Table 2). The A95G SNP in exon 5 (encoding amino acid substitution I76V) was previously published in National Center for Biotechnology Information database (rs 16871473; Bethesda, MD). We found a new SNP in exon 10 (G877A encoding amino acid substitution E554Q). We previously published the A105C variant in exon 6 (encoding amino acid substitution I46L) found in four unrelated Caucasian patients (3.7%) and none of the controls ($P < 0.01$). We recently reported that hPRLR$^{I146L}$ variant was constitutively active (15). This variant was further characterized in comparison with
The first number of patients corresponds to the MFA women meeting the inclusion criteria and the added number corresponds to MFA women who did not stop hormonal treatment in due time for inclusion in the global study, but for whom PRLR was sequenced; \( a \) and \( b \). PRLR variants were sequenced in 70% of gastric and in 95% of breast tumors. For each exon, the number of variants found is given for the MFA cohort and for the control cohort (Caucasian and non-Caucasian).

### Hormonal characteristics

Hormonal characteristics are described in Table 3. PRL was slightly elevated in seven cases (23–36.2 ng/ml) without an obvious cause, except in one case in which the patient was under neuroleptic medication. Clinically, two of them had irregular menses and one had galactorrhea. The only unusual and recurrent hormonal finding was an explosive response (>100 ng/ml) of PRL to stimulation test in 31.2% of patients. For both clinical and biological parameters, there was no statistical difference between Caucasian and non-Caucasian women nor between women with and without a PRLR variant.

### Radiological evaluation

Breast ultrasound revealed multiple round, oval, or bilobate well-demarcated masses of low echogenicity. MRI displayed multiple well-circumscribed encapsulated masses of isointensity in both T1- and T2-weighted sequences (Fig. 2). Internal septas of low signal intensity were pathognomonic of FAs. Dynamic contrast-enhanced imaging demonstrated a low and moderate enhancement of the masses. In the whole cohort, mean number of FAs per breast found on ultrasound and MRI were 4.4 ± 4.0 and 3.9 ± 3.2, respectively; with a mean size of 12.6 ± 6.8 and 12.9 ± 5.6 mm. Results of ultrasound and MRI were not statistically different for FA number and size (Table 1). There was no difference between Caucasian and non-Caucasian patients nor between women with and without a hPRLR variant.

### Pathological results and immunohistochemistry

We report mostly FAs with typical pathological features: benign biphasic tumor, with epithelial and meso-
enchymal components. The epithelial component was typical, describing duct-like spaces surrounded by fibroblastic stroma. Depending on the amount and relationship between these two components, there were two main histological features. Intracanalicular FAs showed a stromal component predominating and compressing the ducts, which were irregular, reduced to slits. Pericanalicular FAs displayed a fibrous stroma spread around the ductal spaces, which remained round or oval, on cross-section. Most surprisingly, benign phyllodes tumors and/or FAs with phyllodes features were found in six cases (6.3%). We also found an intraductal carcinoma adjacent to an FA. In very few cases, FA contiguous breast tissue was not normal, harboring fibrocystic mastopathy, adenosis, or papilloma.

Immunohistochemical analysis was performed in 53 women. ER and PR were detected in 85 and 98%, respectively. All ER-positive patients were also PR positive. Ki-67 like immunoreactivity was observed in nuclei of epithelial cells in most cases (Fig. 3, arrows). Positive staining ranged from 0 to 25% for both adjacent and FA tissues, and the median of staining was lower than 5% for both groups. Ki-67-positive staining of mesenchymal component was generally less than 1% of the nuclei, and it was observed in adjacent tissue of only eight patients and in FA of 18 patients.

For the pathological and immunohistochemical findings, there was no difference between Caucasian and non-Caucasian patients or between women with and without a PRLR variant.

Discussion

FAs are benign tumors of the breast that represent 75% of all breast lesions in young adult females (19). Simple FAs are very common, unlike MFA, which is a rare disease with uncertain implications (16). Our study shows that MFA also occurs in young women and that the progression from simple to multiple FAs, when it could be assessed, was fast. But whether MFAs arise from the continued progression of FAs or is a separate de novo condition remains unknown (16). Evolution of FA to MFA may be associated with predisposition factors, which are currently difficult to predict because the mechanisms controlling FA development and growth are poorly understood (20). The high rate of relatives with breast diseases in our cohort may reflect a predisposition state; however, this could also represent a bias because our patients are probably more likely to consult in breast clinics because of their family history. Findings in our cohort did not show any histological difference between the FAs harvested from MFA patients and the common isolated FAs; in addition, breast tissue adjacent to the tumor was most often normal. The questions of specific breast sensitivity in women who develop MFA rather than having one FA and of a higher risk in developing malignancy remain unanswered. Studies of the potential link between FA and subsequent breast cancer are controversial (21, 22) and never include MFA, but common risk factors may exist. Past studies suggested a possible role for some hormones in the pathogenesis of FAs (23–25). Our study failed to highlight any hormonal imbalance, which could explain MFA development. Half of the cohort reported mastalgia, although the majority had regular menses and a balanced hormonal profile. Almost all patients had normal PRL levels, but nearly one third had an explosive response to PRL stimulation. Some case-control

FIG. 1. A, Displacement of $^{125}$I-PRL by unlabeled hPRL using stable HEK293 clones expressing either receptor (as indicated in the top right corner). Data are shown as means ± SD (n = 3–4, in duplicates). B, Basal (no PRL) and PRL-induced growth/survival of BaF-PRLR<sub>WT</sub> (diamonds), BaF-PRL<sub>146L</sub> (squares), and BaF-PRL<sub>176V</sub> (circles) populations were monitored using WST-1 reagent (OD 450 nm). Error bars, SD, from one representative experiment (of three) performed in triplicate. a, P < 0.05 vs. BaF-PRLR<sub>WT</sub>; b, P < 0.05 for each population vs. its nonstimulated counterpart. C, Stat5 and ERK1/2 phosphorylation in serum-starved (6 h) BaF-PRLR<sub>WT</sub>, BaF-PRL<sub>146L</sub>, and BaF-PRL<sub>176V</sub> cells stimulated (+) or not (−) with PRL (15 min).
studies reported higher basal and stimulated PRL levels in women with BBD compared with controls (26, 27), with an improvement of mastalgia and BBD after 3 months of bromocriptine (28).

The implication of PRL in breast diseases in humans remains a matter of debate. The role of PRL on breast cell proliferation/differentiation and the tumor growth-promoting effect of PRL signaling on the mammary gland are well documented in animal models (2, 8). This is less clear in humans. Some studies have reported associations between SNPs in PRL/hPRLR genes and breast cancer risk, but functional analysis was never carried out (12–14). In addition, no study has examined SNPs in relation to BBD. We therefore decided to investigate anomalies of these genes in our cohort of MFA patients. We identified three missense germline variants in the hPRLR. Functional analyses were focused on the two variants identified in more than one patient. As already reported, PRLR<sub>146L</sub> is a constitutively active receptor (15). This variant was further characterized in this study with respect to intracellular signaling. We also identified PRLR<sub>76V</sub> as another gain-of-function variant because it also displayed enhanced...

### TABLE 3. Hormonal characteristics in the 95 premenopausal MFA patients

<table>
<thead>
<tr>
<th></th>
<th>Follicular phase (n = 63)</th>
<th>Preovulatory phase (n = 10)</th>
<th>Luteal phase (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (mUI/ml)</td>
<td>Mean ± SD</td>
<td>5.6 ± 3.7</td>
<td>22.8 ± 14.8</td>
</tr>
<tr>
<td></td>
<td>Range (Normal values)</td>
<td>0.5–18.6</td>
<td>9.6–52</td>
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<tr>
<td>FSH (mIU/ml)</td>
<td>Mean ± SD</td>
<td>8.3 ± 7.2</td>
<td>11.2 ± 5.7</td>
</tr>
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<td></td>
<td>Range (Normal values)</td>
<td>1.9–34.5</td>
<td>4.8–19.4</td>
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<tr>
<td>E2 (pg/ml)</td>
<td>Mean ± SD</td>
<td>41.7 ± 25.0</td>
<td>228.57 ± 118.8</td>
</tr>
<tr>
<td></td>
<td>Range (Normal values)</td>
<td>10.6–162.0</td>
<td>77.0–438.0</td>
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<tr>
<td>E1 (pg/ml)</td>
<td>Mean ± SD</td>
<td>57.3 ± 30.2</td>
<td>149.0 ± 88.4</td>
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<td></td>
<td>Range (Normal values)</td>
<td>19.0–153.0</td>
<td>25.0–259.0</td>
</tr>
<tr>
<td>P (ng/ml)</td>
<td>Mean ± SD</td>
<td>0.3 ± 0.3</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Range (Normal values)</td>
<td>0.1–1.4</td>
<td>0.1–0.9</td>
</tr>
<tr>
<td>PRL (ng/ml)</td>
<td>Mean ± SD</td>
<td>11.0 ± 6.0</td>
<td>13.0 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>Range (Normal values)</td>
<td>1.9–36.2</td>
<td>6.2–23.0</td>
</tr>
<tr>
<td>SHBG (ng/ml)</td>
<td>Mean ± SD</td>
<td>53.7 ± 23.0</td>
<td>70.1 ± 22.2</td>
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<td></td>
<td>Range (Normal values)</td>
<td>0.3–126.0</td>
<td>38.0–104.0</td>
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<tr>
<td>T (ng/ml)</td>
<td>Mean ± SD</td>
<td>0.3 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Range (Normal values)</td>
<td>0.1–0.9</td>
<td>0.2–0.6</td>
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<tr>
<td>A (μg/ml)</td>
<td>Mean ± SD</td>
<td>1.6 ± 0.7</td>
<td>1.9 ± 0.4</td>
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<td>Range (Normal values)</td>
<td>0.6–4.1</td>
<td>1.3–2.3</td>
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<tr>
<td>DHEA–S (ng/ml)</td>
<td>Mean ± SD</td>
<td>1506 ± 808</td>
<td>1517 ± 907</td>
</tr>
<tr>
<td></td>
<td>Range (Normal values)</td>
<td>195–3810</td>
<td>408–3203</td>
</tr>
</tbody>
</table>

Results expressed in mean ± SD and range. ND, Not detectable; E2, estradiol; E1, estrone; DHEA-S, dehydroepiandrosterone-sulfate; P, progesterone; T, testosterone; A, androstenedione.
basal activity in Ba/F3 cells, albeit less marked than PRLRI146L.

Despite their remarkable functional features, these two modified receptors did not discriminate MFA patients, with respect to clinical, hormonal, radiological, and pathological parameters. We did not observe hyperprolactinemia, which was rather unexpected for patients presenting with a genotype encoding a constitutively active hPRLR. The latter patients did not have more or larger FAs than the rest of the cohort. The intrinsic functionality of these two variants suggests that an association between PRLR genotype and MFA may exist, but they are clearly not the sole parameter involved. The role of PRLRI76V in MFA physiopathology remains difficult to apprehend. Indeed, it is a known polymorphism, observed in our study in the same proportion of patients and controls; however, it was clearly found to have constitutive activity in vitro.

Another argument for its potential role in breast pathology is the high rate of family history of benign and malignant breast tumors in MFA patients bearing the I76V variant. Otherwise, there was no particular family history of breast disease in patients with the I146L variant. Nevertheless, further studies are needed because we investigated only breast phenotypes, whereas others should probably be examined because PRL has many other functions (1). In cases in which familial study was possible, both gain-of-function variants did not appear de novo but were transmitted by either parent. Keeping in mind the small number of subjects involved, the interpretation of both their roles in vivo is rendered even more difficult by the fact that among the carrier mothers, some were free of breast pathology whereas the others presented benign or malignant breast tumors. Even though it is a polymorphism, our results encourage us to remain cautious in the follow-up of MFA patients bearing the PRLRI76V and even more for those with PRLRI146L. One of the latter, indeed, developed a rapidly evolving grade III invasive ductal carcinoma at age 38.

Another reason to advocate careful follow-up is the unexpectedly high rate of phyllodes tumors observed in our study. Six percent of MFA patients exhibited benign phyllodes tumors, sometimes associated with FAs, whereas they usually constitute 0.3–0.9% of all breast neoplasms and occur in an older age group than FAs (29). Although most

![FIG. 3. A, Representative images of Ki 67-like immunoreactivity in adjacent and fibroadenoma tissues of the MFA patients of three genotypes: PRLR_{WT}/PRLR_{WT}, PRLR_{WT}/PRLR_{I146L}, and PRLR_{WT}/PRLR_{I76V}. Arrows highlight positively stained nuclei. Bar, 50 μm. B, Quantification of Ki 67 immunoreactivity: red line, PRLR_{WT}/PRLR_{I146L} patient; green lines, PRLR_{WT}/PRLR_{I76V} patients.](image-url)

![Ki67 positive cells, %](image-url)
phyllodes tumors behave in a benign fashion, they can undergo malignant progression (30). Simple FAs, on the other hand, are always benign. To address the question of whether they displayed an abnormal rate of cell proliferation in MFA, which could possibly be associated with a higher risk of malignant progression, we studied Ki-67 expression. Ki-67 is a classical proliferation marker used as a prognostic factor in breast cancer and has also been suggested as a useful parameter in distinguishing benign from malignant breast tumors (30–32). We found the same range of positive staining in MFA as in usual BBD, namely a low proliferative activity irrespective of FAs number and size. The results were the same for FAs and benign phyllodes tumors.

In conclusion, this study is the first one to date describing a large cohort of MFA patients and characterizing hPRLR gene variants exhibiting constitutive activity in vitro. However, long-term follow-up is needed because many questions remain to be answered. The mechanisms by which some women develop multiple breast tumors have to be unraveled, but several predisposition factors are most likely involved. We are particularly concerned about the potential risk for subsequent breast cancer, especially in patients with the I146L variant, and in regard to the unexpectedly high rate of phyllodes tumors in our cohort. In addition, according to the poorly understood etiology of all BBD, current treatments are mostly empirical and not evaluated. Development of new therapeutic approaches, such as specific hPRLR antagonists (33), should be relevant, especially in patients harboring a PRLR allele encoding a constitutively active receptor, which can obviously not be targeted by classical drugs down-regulating pituitary PRL secretion.

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