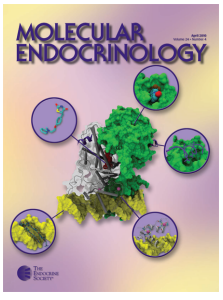
JCEM THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM

Influence of the Fibroblast Growth Factor Receptor 4 Expression and the G388R Functional Polymorphism on Cushing's Disease Outcome

Luciana Pinto Brito, Antonio Marcondes Lerário, Marcello Delano Bronstein, Iberê Cauduro Soares, Berenice Bilharinho Mendonca and Maria Candida Barisson Villares Fragoso

J. Clin. Endocrinol. Metab. 2010 95:E271-E279 originally published online Jul 21, 2010; , doi: 10.1210/jc.2010-0047



Influence of the Fibroblast Growth Factor Receptor 4 Expression and the G388R Functional Polymorphism on Cushing's Disease Outcome

Luciana Pinto Brito, Antonio Marcondes Lerário, Marcello Delano Bronstein, Iberê Cauduro Soares, Berenice Bilharinho Mendonca, and Maria Candida Barisson Villares Fragoso

Unidade de Endocrinologia do Desenvolvimento, Laboratorio de Hormonios e Genetica Molecular LIM/42 (L.P.B., A.M.L., B.B.M., M.C.B.V.F.), Unidade de Neuroendocrinologia (M.D.B.), and Divisão de Anatomia Patológica do Hospital das Clínicas LIM/14 (I.C.S.), Faculdade de Medicina da Universidade de Sao Paulo, 05403-900

Context: Abnormal *FGFR4* expression has been detected in pituitary tumors, especially in larger and invasive adenomas. In addition, the *FGFR4* functional polymorphism G388R has been associated with poor outcome in several human malignancies. Then, we hypothesized that *FGFR4* expression and genotype could be markers of adverse outcome of Cushing's disease after transsphenoidal surgery.

Objectives: The objective was to investigate whether there is an association between the postoperative outcome of Cushing's disease (remission/recurrence) and the *FGFR4* G388R genotype or the *FGFR4* expression in corticotrophinomas.

Design and Patients: Clinical, hormonal, and pathological data of 76 patients who underwent the first transsphenoidal surgery were retrospectively reviewed. All patients were genotyped for G388R polymorphism. *FGFR4* expression was assessed by real-time PCR in 18 corticotrophinomas.

Main Outcome Measures: The outcome measures included the *FGFR4* G388R genotype and *FGFR4* expression in postoperative remission and recurrence of Cushing's disease.

Results: Homozygosis for *FGFR4* glycine (Gly^{388}) allele was associated with reduced disease-free survival, in the univariate analysis (hazard ratio of 6.91; 95% confidence interval of 1.14–11.26; $P = 0.028$). Male gender ($P = 0.036$), lack of pathology confirmation ($P = 0.009$), and cortisol levels more than 2 μg/dl in the early postoperative period ($P < 0.001$) were also significant predictors of Cushing's disease recurrence in the univariate analysis. *FGFR4* overexpression was found in 44% of the corticotrophinomas, and it was associated with lower postoperative remission rate ($P = 0.009$).

Conclusions: Our data suggest that homozygosis for *FGFR4* Gly^{388} allele and *FGFR4* overexpression are associated with higher frequency of postoperative recurrence and persistence of Cushing's disease, respectively. **(*J Clin Endocrinol Metab* 95: E271–E279, 2010)**

Cushing's disease is characterized by an overproduction of glucocorticoids by the adrenal cortex as a result of excessive corticotrophin (ACTH) secretion from a pituitary tumor. The disease occurs more commonly in women than in men, and the corticotroph tumors are usually less than 10 mm and limited to the sellar region (1, 2). Currently, the treatment of choice for patients with Cushing's disease is selective adenomectomy through a transsphenoidal approach (3). The postoperative remission rates vary widely among centers, from 60 to 90% (1, 3–5).

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2010-0047 Received January 7, 2010. Accepted June 10, 2010.
First Published Online July 21, 2010

Abbreviations: FGF, Fibroblast growth factor; FGFR, fibroblast growth factor receptor; MRI, magnetic resonance imaging.

Recent long-term follow-up studies have suggested that ACTH-secreting tumors have a high probability of recurrence, with overall recurrence rates of 5–25% (1, 3, 6). Previous studies have identified factors associated with higher remission rates, such as microadenoma visualized by magnetic resonance imaging (MRI), low postoperative serum cortisol levels, and histological confirmation of an ACTH-staining adenoma. Conversely, macroadenomas, invasive tumors, young age, and male gender were predictors of persistent hypercortisolism after transsphenoidal surgery or higher rates of recurrence during the follow up (1, 4, 7, 8).

Although pituitary corticotroph tumorigenesis has been extensively studied, no molecular marker was found to be associated with Cushing's disease prognosis. Corticotroph pituitary adenomas are monoclonal, suggesting that somatic defects in a pituitary cell are likely the origin of the tumor. However, cumulative evidence has emphasized the involvement of microenvironment permissive factors, such as hypothalamic peptides, hormones, growth factors, and cytokines in the pituitary tumor development and progression (9). Bone morphogenic protein-4, a member of the TGF-β family, is down-regulated in corticotrophinomas, and it was shown to inhibit corticotroph tumor cell growth (10). IL-6 expression, a gp130-related cytokine, affects cell proliferation and ACTH secretion (11). Additionally, members of the fibroblast growth factor (FGF) family have been implicated in pituitary tumors pathogenesis (12–14). FGF-2 and FGF-4 expression have been identified in human pituitary adenomas (13, 14). FGF-4/hst strong immunoreactivity, in particular, correlates with prolactinomas invasiveness (14). The actions of FGFs are mediated by FGF receptors (FGFRs). Normal human adenohypophysis expresses *FGFR1*, *FGFR2*, and *FGFR3* but not *FGFR4* (15, 16). Interestingly, an N-terminally truncated isoform of FGFR4 (pdt-FGFR4) has been detected in pituitary tumors of various subtypes (16, 17). In corticotroph pituitary adenomas, it has been identified in approximately 50% of the tumors (16). FGFR4 belongs to the tyrosine kinase receptors family, and its activation mediates cell proliferation, survival, migration, and resistance to apoptosis (18). Its overexpression has been demonstrated in several human cancers (19, 20). In melanomas, FGFR4 expression was significantly associated with shorter overall survival and disease-free survival (19). FGFR4 staining was also a poor outcome predictor in patients with astrocytoma grade III (20). In a large cohort of pituitary neoplasms, a strong FGFR4 protein expression was more frequently observed in larger adenomas (16). A positive association between pdt-FGFR4 mRNA expression and tumor invasiveness was demonstrated in GH-secreting pituitary tumors (21). Recently, a common *FGFR4* germ-line polymorphism at codon 388 (G388R) was described (22). The presence of the arginine (Arg^{388}) allele has been associated with poor outcome in several cancers, such as prostate, colon, breast, and lung (22–24).

In an effort to identify genetic factors associated with prognosis in Cushing's disease, the aim of the current study was to determine whether the *FGFR4*-G388R polymorphism and *FGFR4* expression levels were predictors of postoperative outcome (remission *vs.* persistent disease and long-term remission *vs.* recurrence) after the first transsphenoidal surgery. In addition, clinical, hormonal, and pathological findings associated with postoperative outcome were evaluated in this series of Cushing's disease patients.

Patients and Methods

Subjects

The study was approved by the Ethics Committee of the Hospital das Clínicas (Sao Paulo, Brazil), and informed written consent was obtained from all patients and/or parents. Seventy-six consecutive patients (60 females and 16 males) who underwent the first-time transsphenoidal surgery for treatment of Cushing's disease between 1989 and 2008 in our institution (Hospital das Clínicas da Faculdade de Medicina da Universidade de Sao Paulo, Endocrinology Department, Sao Paulo, Brazil) and had genomic DNA or tumoral cDNA available for molecular studies were included in this study. All patients had, at least, 1 yr of clinical and laboratorial follow up after the surgical approach. None of patients had undergone previous treatment (surgery or radiotherapy to the pituitary or adrenal). Data regarding the preoperative diagnosis (clinical features, laboratory data, and imaging studies), surgical and histopathological findings and outcome, postoperative follow up, and long-term evolution were retrospectively recorded. To analyze the frequency of FGFR4 G388R genotype in the Brazilian population, a DNA bank of 103 healthy subjects was used as a control group.

Cushing's disease diagnosis

Cushing's disease was diagnosed using currently accepted standard criteria (5, 25). At least two elevated 24-h urine cortisol excretion levels and/or lack of suppression of serum cortisol during a low-dose overnight dexamethasone suppression test were performed to establish endogenous hypercortisolemia in all patients. Nonsuppressed ACTH level, MRI evidence of a pituitary adenoma, high-dose dexamethasone suppression, and/or inferior petrosal sinus sampling tests were used to establish pituitary origin.

Tumor grade

Tumor size and invasiveness were classified on the basis of preoperative radiologic images (MRI/computed tomography) and intraoperative findings using the Hardy criteria modified by Bates *et al.* (26): grade 1, microadenomas (tumors $\leq$ 10 mm in the maximal diameter); grade 2, macroadenomas (tumors $>$ 10 mm); grade 3, microadenomas or macroadenomas with invasive local signals (evidence of bony destruction and/or tumor within

the sphenoid and/or cavernous sinuses); and grade 4, central nervous system or extracranial spread.

FGFR4 genotyping

Genomic DNA was isolated from peripheral blood leukocytes or oral swabs by standard methods. An *FGFR4* exon 9 fragment was PCR amplified using the following primers: 5′-GACCGCAGCAGCGCCCGAGGCCAG-3′ and 5′-AGAGGGAAGAGGGAGAGCTTCTG-3′ as described previously (22). To determine the distribution of FGFR4 Arg^{388} and Gly^{388} allele, the 168-bp amplicon was digested overnight with *Bst*NI (New England Biolabs, Ipswich, MA) according to the instructions of the manufacturer. Restriction fragments were resolved on a 3% agarose gel, and DNA was visualized under UV transillumination after staining with ethidium bromide. The digestion of the 168-bp *FGFR4* region by *Bst*N1 results in 109-, 37-, and 22-bp fragments in the case of the Gly^{388} allele. The Arg^{388} allele digestion results in 82-, 37-, 29-, and 22-bp fragments. Although the 37, 29, and 22 bp are not well visible in the gel system used, homozygosis for either the Gly^{388} or Arg^{388} allele can be characterized by one band of 109 or 82 bp, respectively. Heterozygous cases can be characterized by two bands of 109 and 82 bp (Fig. 1).

Tumors samples and quantitative real-time PCR

The fragments of the tumors were collected from 44 patients who underwent transsphenoidal surgery for Cushing's disease and snap frozen and stored in liquid nitrogen. Serial sections of the fragment were immunostained for ACTH, prolactin, GH, FSH, LH, TSH, and reticulin. Twelve samples were excluded from mRNA expression study because of a lack of pathology confirmation of an ACTH-secreting tumor.

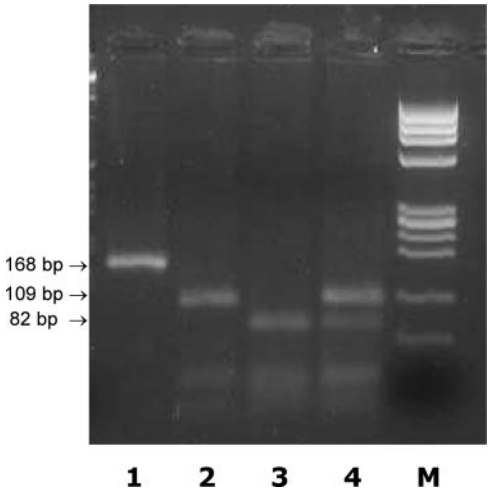


FIG. 1. Representative 3% agarose gel showing *Bst*N1 digestion of a 168-bp *FGFR4* region. The digestion results in 109-, 37-, and 22-bp fragments in the case of the Gly^{388} allele. The Arg^{388} allele digestion results in 82-, 37-, 29-, and 22-bp fragments. Although the 37, 29, and 22 bp are not well visible in the gel system used, homozygosis for either the Gly^{388} or Arg^{388} allele can be characterized by one band of 109 or 82 bp, respectively. Heterozygous samples can be characterized by two bands of 109 and 82 bp. The size marker ϕX 174 DNA/Hinf-1 digest (M) and the undigested PCR sample (*lane 1*) were used as standards. *Lanes 2* and *3* are homozygous samples for the Gly^{388} and Arg^{388} allele, respectively. *Lane 4* is a heterozygous sample.

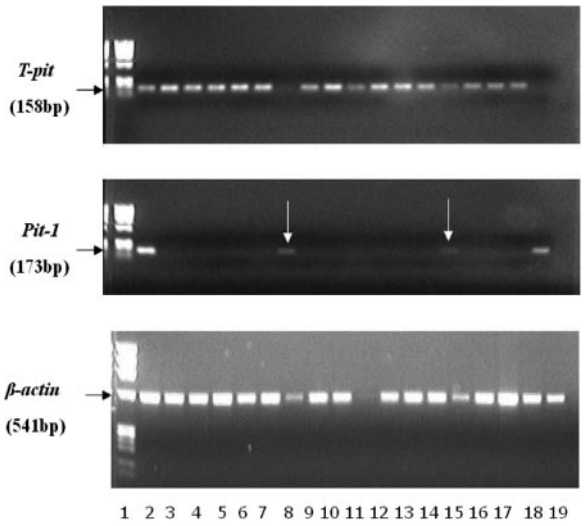


FIG. 2. Representative 1% agarose gels showing *T-pit, Pit-1*, and *β-actin* expression. *Lane 1* shows size marker ϕX 174 DNA/Hinf-1 digest. *Lane 2* shows a pool of normal pituitary gland with positive expression of *T-pit* and *Pit-1*. *Lanes 3–18* are samples of corticotroph tumors. *Lanes 8* and *15* show corticotroph tumors *Pit-1* positive and therefore excluded from additional analysis. *Lane 19* shows an example of a tumor-producing GH (*Pit-1* positive and negative *T-pit*). The expression of *β-actin* is shown as control of amplification.

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA) reagent method from the 32 remaining samples. The RNA quality and concentration were determined by Agilent 2100 bioanalyzer (RNA 6000 nano kit; Agilent Technologies, Santa Clara, CA). Four samples were excluded from additional analysis as a result of RNA degradation signals (RNA integrity ≤ 5). The cDNA was generated from 1.25 μg of total RNA using the High Capacity cDNA Archive kit for RT (Applied Biosystems, Foster City, CA).

To evaluate ACTH-secreting tumors, contamination by nonadenomatous pituitary tissue RT-PCR for *Pit-1* gene was performed. *T-pit* expression detected by RT-PCR was also used to confirm the presence of corticotroph cells in the tumor samples (Fig. 2). *Pit-1* expression was detected in 10 of 28 samples, which were excluded from additional analysis.

Quantitative real-time RT-PCR was performed in the ABI PRISM 7000 Sequence Detector using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The FGFR4 assay used amplifies the eight to nine exon boundaries and detects both intact and pdt-FGFR4 isoform (assay identification number Hs00242558_m1). Three endogenous control genes were used for each sample, and the reactions were performed in triplicate. The relative expression levels of FGFR4 was calculated using the $2^{-\Delta\Delta ct}$ method as described previously (27). A commercial pool of pituitary glands was used as the reference sample (Clontech, Palo Alto, CA). The criteria used for underexpression and overexpression was a 2-fold change when compared with the reference sample.

Postoperative follow up

Cushing's disease remission was defined as clinical improvement of hypercortisolism associated with normal postoperative 24-h urinary cortisol measures throughout the first year after transsphenoidal surgery or continued need for corticosteroid replacement at the same period. Recurrence was defined as elevated 24-h urinary cortisol samples associated with clinical symptoms of hypercortisolism, after the first year of surgery.

Statistical analysis

Statistic analysis was performed with the SPSS (version 13.0; SPSS Inc., Chicago, IL) and MedCalc (version 9.4.2.0; MedCalc Software, Mariakerke, Belgium) software. Continuous and categorical variables were compared by Mann-Whitney rank-sum test and χ^2 or Fisher's exact test when appropriate, respectively. Univariate analysis was performed using Kaplan-Meier curves and log-rank test statistics for comparison. $P \leq 0.05$ was considered significant.

Results

Of the 76 patients with corticotroph tumors, 65 had microadenomas (85.5%) and 11 had macroadenomas. Forty-five of 65 microadenomas (69%) were visualized by pituitary MRI. The median size of visible tumors was 7 mm. In the remaining 20 cases, the diagnosis was established by a central to periphery ACTH gradient on inferior petrosal sinus sampling, consistent with an ACTH-secreting pituitary tumor.

The majority of the corticotroph adenomas (80.3%) were classified as Hardy grade 1, 9.2% as Hardy grade 2 and 10.5% as Hardy grade 3. Pathological diagnosis of ACTH-secreting pituitary adenoma was confirmed in 50 of 76 patients (65.8%). Lack of pathology confirmation (normal pituitary tissue, necrosis, insufficient material for immunohistochemical analysis) was observed in 26 cases. Immediate postoperative morning serum cortisol levels of no more than 2 μg/dl were observed in 47.2% of the patients.

After the first transsphenoidal surgery, 24 of 76 patients (31.6%) did not achieve remission. Among the 52 patients who achieved remission and were followed for more than 1 yr, 13 (25%) patients had Cushing's disease recurrence. The median time to recurrence was 60 months (range, 18–180 months).

FGFR4 genotype in Cushing's disease patients and controls

FGFR4 genotype (Gly/Gly^{388}, Gly/Arg^{388}, and Arg/Arg^{388}) frequencies were similar between patients (60.5, 36.8, and 2.7%, respectively) and controls (56.3, 34, and 9.7%, respectively) ($P = 0.17$). The distribution of genotypes in both groups was consistent with Hardy-Weinberg equilibrium. The carriers of arginine allele in heterozygous or homozygous state (Gly/Arg^{388} and Arg/Arg^{388}) were grouped and compared with Gly/Gly^{388} for statistical analysis.

Remission/recurrence *vs.* molecular and clinic-pathologic features

Remission vs. *persistent disease*

The age, gender, tumor size, and frequency of the Gly/Gly^{388} genotype were not statistically different between cured and noncured patients. A significantly higher frequency of persistent disease was observed among the patients with Hardy grade 3 tumors (75%) when compared with patients with Hardy grade 1 and grade 2 tumors ($P = 0.006$) (Table 1).

TABLE 1. Association between different variables and early postoperative outcome after the first transsefenoidal surgery for Cushing's disease

Variables	Remission	Persistent disease	*P*
Age (yr) (n = 76)			
Median	31	28.5	NS
Range	12–60	13–52	
Gender (n = 76)			
Female	43 (72%)	17 (28%)	NS
Male	09 (56%)	07 (44%)	
Tumor size (n = 76)			
Microadenomas	44 (68%)	21 (32%)	NS
Macroadenomas	08 (73%)	03 (27%)	
Tumor classification (n = 76)			
Hardy grade 1	43 (70%)	18 (30%)	
Hardy grade 2	07 (100%)	00	0.006[a]
Hardy grade 3	02 (25%)	06 (75%)	
Pathology confirmation (n = 76)			
Positive	38 (76%)	12 (24%)	0.049[a]
Negative	14 (54%)	12 (46%)	
FGFR4 G388R (n = 76)			
Gly/Gly^{388}	33 (72%)	13 (28%)	NS
Gly/Arg^{388} and Arg/Arg^{388}	19 (63%)	11 (37%)	
Postoperative cortisol (n = 72)			
≤2 μg/dl	32 (94%)	02 (6%)	0.0001[a]
>2 μg/dl	16 (42%)	22 (58%)	

Gly/Gly^{388}, Homozygous state for glycine allele at codon 388 of FGFR4; Gly/Arg^{388} and Arg/Arg^{388}, heterozygous and homozygous state for arginine allele at codon 388 of FGFR4, respectively; NS, statistically not significant.

[a] χ^2 test.

Postoperative variables analyses showed that pathology confirmation of ACTH-producing adenoma and low values of cortisol in the first 2 wk after surgery were significantly associated with remission. Although 76% of patients with pathology-confirmed ACTH tumor had Cushing's disease remission, only 54% of patients without tumor confirmation achieved remission. In addition, 94% of the patients with low cortisol levels (≤2 μg/dl) in the early postoperative achieved remission, whereas only 42% of the patients with cortisol of no more than 2 μg/dl had postoperative remission (Table 1).

Recurrence vs. *long-term remission*

Cushing's disease recurrence rate was significantly higher in male patients (56% of recurrence in men *vs.* 19% in women). Hardy grade 3 tumors were also as-

sociated with a higher likelihood of disease recurrence (100 *vs.* 43% in grade 2 and 19% in grade 1 tumors; $P = 0.017$). In addition, lack of histopathology tumor confirmation (50 *vs.* 16%) and elevated levels of cortisol in the early postoperative period (50 *vs.* 12%) were also associated with higher recurrence rates (Table 2).

The analysis of postoperative recurrence according to the *FGFR4* genotypes showed a significantly reduced disease-free survival in the patients homozygous for FGFR4 Gly^{388} allele (hazard ratio of 6.91; 95% confidence interval of 1.14–11.26; $P = 0.028$) (Fig. 3). Male gender ($P = 0.036$), lack of pathology confirmation ($P = 0.009$), and cortisol levels more than 2 μg/dl in the early postoperative period ($P < 0.001$) were also significant predictors of Cushing's disease relapse in the univariate analysis (Fig. 3).

TABLE 2. Association between different variables and long-term postoperative evolution in patients with Cushing's disease who achieve long-term remission after the first transsefenoidal surgery

Variables	Long-term remission	Recurrence	*P*
Age (yr) (n = 52)			
Median	33	30	NS
Range	15–60	12–48	
Gender (n = 52)			
Female	35 (81%)	08 (19%)	0.033[b]
Male	04 (44%)	05 (56%)	
Tumor size (n = 52)			
Micro	35 (79%)	09 (21%)	NS
Macro	04 (50%)	04 (50%)	
Hardy modified classification (n = 52)			
Grade 1	35 (81%)	08 (19%)	
Grade 2	04 (57%)	03 (43%)	0.017[a]
Grade 3	00 (0%)	02 (100%)	
Pathology confirmation (n = 52)			
Positive	32 (84%)	06 (16%)	
Negative	07 (50%)	07 (50%)	0.026[b]
FGFR4 G388R (n = 52)			
Gly/Gly^{388}	21 (64%)	12 (36%)	
Gly/Arg^{388} and Arg/Arg^{388}	18 (95%)	01 (5%)	0.018[b]
Postoperative cortisol (n = 48)			
≤2 μg/dl	28 (88%)	04 (12%)	0.01[b]
>2 μg/dl	08 (50%)	08 (50%)	

Gly/Gly^{388}, Homozygous state for glycine allele at codon 388 of FGFR4; Gly/Arg^{388} and Arg/Arg^{388}, heterozygous and homozygous state for arginine allele at codon 388 of FGFR4, respectively; NS, statistically not significant.

[a] χ^2 test;

[b] Fisher's exact test.

FGFR4 *expression analysis*

Eighteen corticotroph tumors were shown to be *Pit-1* mRNA negative and therefore available for quantitative real-time RT-PCR *FGFR4* mRNA analysis. Four of 18 tumors were macroadenomas and two had invasive signals (Table 3). *FGFR4* overexpression was detected in 8 of 18 (44%) corticotrophinomas. Age, gender, tumor size, invasive signals, *FGFR4* genotype, and postoperative cortisol levels were not statistically different between patients with low/normal or high *FGFR4* expression. Nevertheless, all patients who did not achieve remission had high levels of *FGFR4* mRNA expression, whereas 75% of the patients who achieved remission had normal/low *FGFR4* expression levels ($P = 0.009$) (Table 3). Among the 18 patients available for expression studies, only one case of recurrence was registered, probably because of the shorter follow up of this group (median follow up of 29 months).

Discussion

Cushing's disease is a rare disease associated with considerable morbidity and mortality (1, 2, 25). The majority of affected patients are adult females and most of the tumors are non-invasive microadenomas (1, 28). Transsphenoidal surgery for selective removal of corticotroph adenoma is still the treatment of choice for Cushing's disease, although high rates of treatment failure are reported (1, 4, 6, 8). A second transsphenoidal approach is associated with even higher rates of failure to cure (3, 29). Considering this, in our study, we have included only data from the first surgical approach, which was associated with a postoperative remission rate of 68.4%.

Among the preoperative factors assessed in this cohort of patients with Cushing's disease, we found a significant association between tumor invasion signs (Hardy grade 3) and persistent Cushing's disease. When evaluating the postoperative variables, lack of pathology confirmation of the tumor and high levels of cortisol in the early postoperative period were also associated with a poorer outcome. These findings are in accordance with previous studies (1, 30). In addition, variables associated with Cushing's disease recurrence were a high Hardy grade, male gender, lack of pathology confirmation of tumor, and cortisol levels more than 2 μg/dl in the early postoperative period. Pecori-Giraldi *et al.* (31) compared Cushing's disease in female and male patients and reported a worse prognosis in males (recurrence of 32% in male *vs.* 15% in female). Higher and earlier recurrence rates were also demonstrated in patients with lack of pathology confirmation of ACTH tumor (30). Furthermore, patients with postoperative serum cortisol more than 2 μg/dl were 2.5 times more likely to have a recurrence than patients with serum cor-

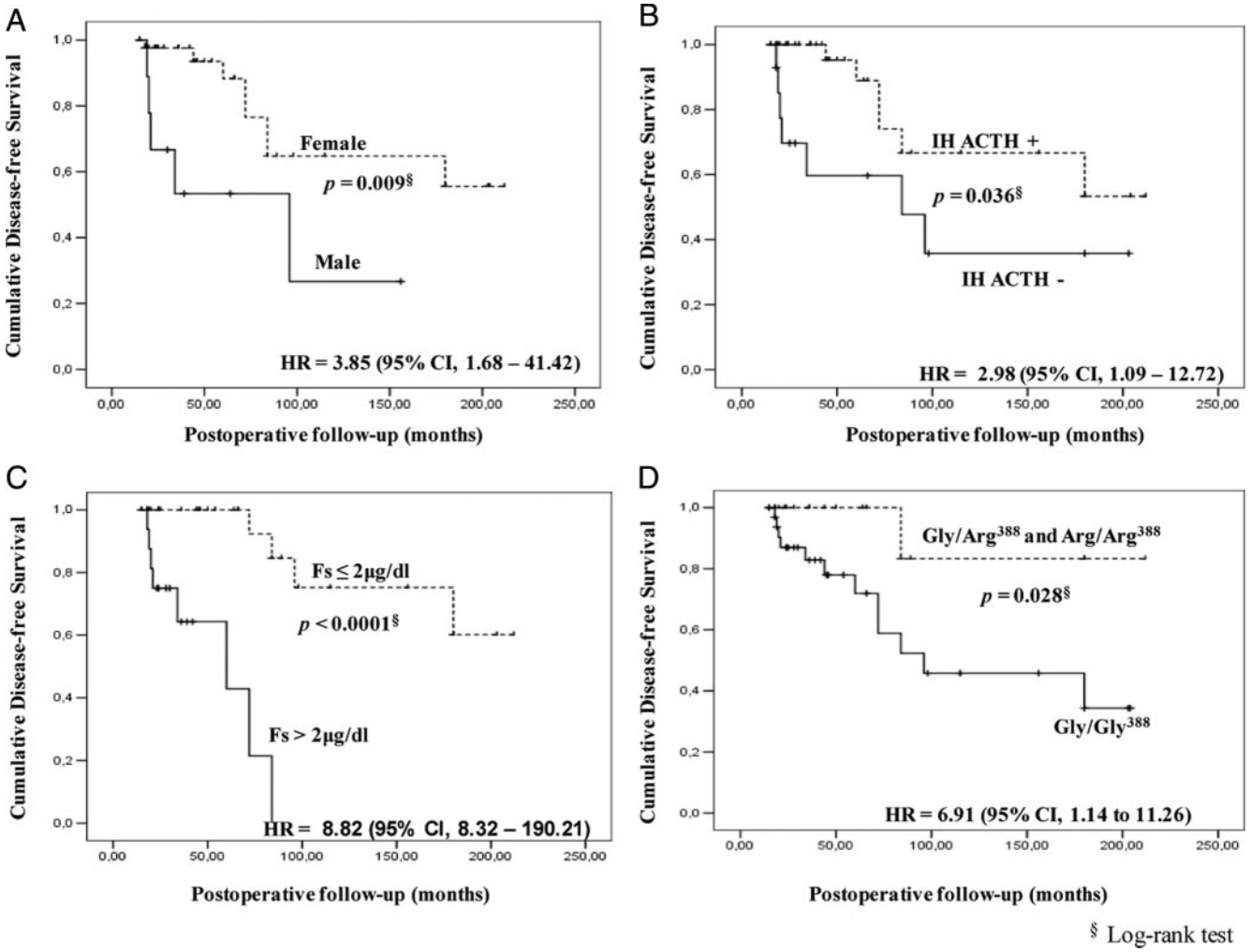


FIG. 3. Univariate analysis of disease-free survival in Cushing's disease patients with postoperative remission. Cumulative proportion of patients with relapse was stratified by female or male sex (A), confirmation or lack of pathology confirmation of ACTH tumor (B), postoperative cortisol of no more than 2 μg/dl (C), and Gly/Gly^{388}- or Arg-carrying (Gly/Arg^{388} and Arg/Arg^{388}) genotypes (D). Small crosses indicate censored observations. Log-rank analysis was used to establish statistical significance between curves.

tisol less than or equal to 2 μg/dl (7). In agreement with previous studies (6–8), the rate of recurrence among the 52 patients who achieved remission was 25% (13 of 52) after a median follow-up period of 60 months.

Some corticotroph tumors had an aggressive behavior, and signs of invasion of nearby structures are occasionally found. In accordance with a previous report (2), we demonstrate here that tumor invasiveness in corticotroph pituitary adenomas is inversely related to favorable outcome. Genetic factors that play a role in the development of corticotrophinomas or that may influence their behavior remain to be determined. The difficulty in studying these tumors also resides on the fact that corticotroph tumors are rare and small lesions so, therefore, with little material available for molecular study. *FGFR4* expression appears to have an important role in the pituitary tumor development and/or progression. The expression of intact *FGFR4* or its isoform pdt-FGFR4 was not identified in normal pituitary, but pdt-FGFR4 was demonstrated by RT-PCR in 40% of pituitary tumors (17). Immunohistochemical studies detected cytoplasmic expression of FGFR4 in more than 80% of gonadotroph tumors and nonfunctioning null cell but in less than 10% of prolactinomas. GH- and ACTH-producing tumors showed a positive staining for FGFR4 in 50% of the cases (16). An association between pdt-FGFR4 expression and larger tumors (16) and invasive signals (21, 32) has also been found.

Our data indicate an association between increased *FGFR4* mRNA expression and lower remission rates of hypercortisolism after transsphenoidal surgery. All patients who did not achieve remission had high levels of *FGFR4* mRNA expression, whereas 75% of the patients who achieved remission had normal/low *FGFR4* expression levels Nevertheless, we did not find any association between *FGFR4* overexpression and clinical features such as age, gender, tumor size, and invasiveness signs (Hardy grade). Although these findings are in agreement with pre-

TABLE 3. Association between different variables and *FGFR4* expression in corticotroph pituitary tumors

Variables	Low/normal FGFR4	High FGFR4	*P*
Age (yr) (n = 18)			
Median	33	25	
Range	15-54	13-34	NS
Gender (n = 18)			
Female	09 (60%)	06 (40%)	
Male	01 (33%)	02 (67%)	NS
Tumor size (n = 18)			
Micro	08 (57%)	06 (43%)	
Macro	02 (50%)	02 (50%)	NS
Hardy (n = 18)			
Grade 1	08 (62%)	05 (38%)	
Grade 2	02 (67%)	01 (33%)	
Grade 3	00 (0%)	02 (100%)	NS
FGFR4 G388R (n = 18)			
Gly/Gly^{388}	06 (60%)	04 (40%)	
Gly/Arg^{388} and Arg/Arg^{388}	04 (50%)	04 (50%)	NS
Postoperative cortisol (n = 17)			
≤2 μg/dl	04 (67%)	02 (33%)	
>2 μg/dl	05 (45%)	06 (55%)	NS
Early outcome (n = 17)			
Remission	09 (75%)	03 (25%)	
Persistent disease	00	05 (100%)	0.009[a]

Gly/Gly^{388}, Homozygous state for glycine allele at codon 388 of FGFR4; Gly/Arg^{388} and Arg/Arg^{388}, heterozygous and homozygous state for arginine allele at codon 388 of FGFR4, respectively; NS, statistically not significant.

[a] Fisher's exact test.

vious studies (15, 17), Qian *et al.* (16) demonstrated a positive correlation between *FGFR4* overexpression determined by a semiquantitative score and tumor size from a series with various pituitary tumors subtypes, including 17 ACTH-producing adenomas with Cushing's syndrome. Moreover, Morita *et al.* (21) did not find a correlation between the presence of *FGFR4* expression and tumor size in 45 GH-producing tumors, but an association between *FGFR4* expression and cavernous sinus invasion was observed. In our study, we were not able to show a significant association between *FGFR4* expression and invasive signs of ACTH tumors because only two cases were classified as Hardy grade 3. However, both cases had high *FGFR4* expression levels.

Activated FGFR4 stimulates several intracellular signaling pathways that control proliferation, differentiation, migration, and cell survival. These pathways include the signaling cascade of MAPK and phosphatidylinositol 3-kinase (18). The pdt-FGFR4 isoform is constitutively phosphorylated, and it has been demonstrated *in vitro* and *in vivo* that cells transfected with this isoform had greater capacity of matrix invasion *in vitro* and *in vitro* (32).

Additionally, an *FGFR4* functional polymorphism at codon 388 (G388R) has been shown to be possibly associated with human cancer prognosis. Conflicting data regarding the influence of Arg^{388} allele and prognosis of human tumors have been described previously. The Arg^{388} allele has been associated with a worse evolution in several human tumors (19, 22–24, 33). It has been suggested that this is caused by a Gly^{388} allele tumor suppressor activity (34). However, others studies do not observed the same association with prognosis, even assessing the same tumor types (35, 36). In this study, we assessed the influence of *FGFR4* G388R genotype in Cushing's disease remission rate and disease-free survival and found a significant association between Gly/Gly^{388} genotype and reduced disease-free survival in univariate analysis. A multivariate analysis to evaluate the polymorphism as an independent prognostic factor is hampered by the number of events in the study. Interestingly, a previous study evidenced that the Gly/Gly^{388} genotype was associated with an increased risk of bladder cancer recurrence (37). A higher risk of oral cavity carcinoma was also correlated with the Gly/Gly^{388} genotype (38). These discrepancies can be explained by the possibility of a tissue-specific effect of the polymorphism (39). Another possible bias is the small number of patient in the recurrence group (37). In our series, the number of patients in the relapse group was also small (n = 13). In addition, we found no differences in G388R genotype frequencies between Cushing's disease patients and controls, and *FGFR4* expression in corticotroph tumors did not correlate with the *FGFR4* G388R genotype. The presence of the Arg^{388} allele in heterozygous or homozygous state has been associated with increased levels of FGFR4 in breast cancer cell lines and in malignant gliomas (22, 40) but not in normal lung tissue (24).

In conclusion, the *FGFR4* Gly/Gly^{388} genotype was a significant predictor of postoperative recurrence of Cushing's disease. Additionally, high *FGFR4* expression levels were associated with a lower postoperative remission rate. Therefore, our data represent the first demonstration that genetic factors may influence the postoperative outcome of patients with Cushing's disease.

Acknowledgments

Address all correspondence and requests for reprints to: Luciana P. Brito, Unidade de Endocrinologia do Desenvolvimento, Laboratorio de Hormonios e Genetica Molecular LIM/42, Faculdade de Medicina da Universidade de Sao Paulo, Avenida Dr. Enéas de Carvalho Aguiar 155, Prédio dos Ambulatórios 2° Andar, Bloco 6, 05403-900 São Paulo, Brazil. E-mail: lucpbrito@gmail.com or mariafragoso@uol.com.br.

This work was supported by Fundacao de Amparo a Pesquisa do Estado de Sao Paulo Grant 06/52492-0 (L.P.B).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Hammer GD, Tyrrell JB, Lamborn KR, Applebury CB, Hannegan ET, Bell S, Rahl R, Lu A, Wilson CB** 2004 Transsphenoidal microsurgery for Cushing's disease: initial outcome and long-term results. J Clin Endocrinol Metab 89:6348–6357
2. **Dekkers OM, Biermasz NR, Pereira AM, Roelfsema F, van Aken MO, Voormolen JH, Romijn JA** 2007 Mortality in patients treated for Cushing's disease is increased, compared with patients treated for nonfunctioning pituitary macroadenoma. J Clin Endocrinol Metab 92:976–981
3. **Biller BM, Grossman AB, Stewart PM, Melmed S, Bertagna X, Bertherat J, Buchfelder M, Colao A, Hermus AR, Hofland LJ, Klibanski A, Lacroix A, Lindsay JR, Newell-Price J, Nieman LK, Petersenn S, Sonino N, Stalla GK, Swearingen B, Vance ML, Wass JA, Boscaro M** 2008 Treatment of adrenocorticotropin-dependent Cushing's syndrome: a consensus statement. J Clin Endocrinol Metab 93:2454–2462
4. **Bochicchio D, Losa M, Buchfelder M** 1995 Factors influencing the immediate and late outcome of Cushing's disease treated by transsphenoidal surgery: a retrospective study by the European Cushing's Disease Survey Group. J Clin Endocrinol Metab 80:3114–3120
5. **Newell-Price J, Bertagna X, Grossman AB, Nieman LK** 2006 Cushing's syndrome. Lancet 367:1605–1617
6. **Atkinson AB, Kennedy A, Wiggam MI, McCance DR, Sheridan B** 2005 Long-term remission rates after pituitary surgery for Cushing's disease: the need for long-term surveillance. Clin Endocrinol (Oxf) 63:549–559
7. **Patil CG, Prevedello DM, Lad SP, Vance ML, Thorner MO, Katznelson L, Laws Jr ER** 2008 Late recurrences of Cushing's disease after initial successful transsphenoidal surgery. J Clin Endocrinol Metab 93:358–362
8. **Sonino N, Zielezny M, Fava GA, Fallo F, Boscaro M** 1996 Risk factors and long-term outcome in pituitary-dependent Cushing's disease. J Clin Endocrinol Metab 81:2647–2652
9. **Asa SL, Ezzat S** 2009 The pathogenesis of pituitary tumors. Annu Rev Pathol 4:97–126
10. **Giacomini D, Páez-Pereda M, Theodoropoulou M, Labeur M, Refojo D, Gerez J, Chervin A, Berner S, Losa M, Buchfelder M, Renner U, Stalla GK, Arzt E** 2006 Bone morphogenetic protein-4 inhibits corticotroph tumor cells: involvement in the retinoic acid inhibitory action. Endocrinology 147:247–256
11. **Haedo MR, Gerez J, Fuertes M, Giacomini D, Páez-Pereda M, Labeur M, Renner U, Stalla GK, Arzt E** 2009 Regulation of pituitary function by cytokines. Horm Res 72:266–274
12. **Böhlen P, Baird A, Esch F, Ling N, Gospodarowicz D** 1984 Isolation and partial molecular characterization of pituitary fibroblast growth factor. Proc Natl Acad Sci U S A 81:5364–5368
13. **Li Y, Koga M, Kasayama S, Matsumoto K, Arita N, Hayakawa T, Sato B** 1992 Identification and characterization of high molecular weight forms of basic fibroblast growth factor in human pituitary adenomas. J Clin Endocrinol Metab 75:1436–1441
14. **Shimon I, Hinton DR, Weiss MH, Melmed S** 1998 Prolactinomas express human heparin-binding secretory transforming gene (hst) protein product: marker of tumour invasiveness. Clin Endocrinol (Oxf) 48:23–29
15. **Abbass SA, Asa SL, Ezzat S** 1997 Altered expression of fibroblast growth factor receptors in human pituitary adenomas. J Clin Endocrinol Metab 82:1160–1166
16. **Qian ZR, Sano T, Asa SL, Yamada S, Horiguchi H, Tashiro T, Li CC, Hirokawa M, Kovacs K, Ezzat S** 2004 Cytoplasmic expression of fibroblast growth factor receptor-4 in human pituitary adenomas: relation to tumor type, size, proliferation, and invasiveness. J Clin Endocrinol Metab 89:1904–1911
17. **Ezzat S, Zheng L, Zhu XF, Wu GE, Asa SL** 2002 Targeted expression of a human pituitary tumor-derived isoform of FGF receptor-4 recapitulates pituitary tumorigenesis. J Clin Invest 109:69–78
18. **Eswarakumar VP, Lax I, Schlessinger J** 2005 Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 16:139–149
19. **Streit S, Mestel DS, Schmidt M, Ullrich A, Berking C** 2006 FGFR4 Arg388 allele correlates with tumour thickness and FGFR4 protein expression with survival of melanoma patients. Br J Cancer 94: 1879–1886
20. **Yamada SM, Yamada S, Hayashi Y, Takahashi H, Teramoto A, Matsumoto K** 2002 Fibroblast growth factor receptor (FGFR) 4 correlated with the malignancy of human astrocytomas. Neurol Res 24:244–248
21. **Morita K, Takano K, Yasufuku-Takano J, Yamada S, Teramoto A, Takei M, Osamura RY, Sano T, Fujita T** 2008 Expression of pituitary tumour-derived, N-terminally truncated isoform of fibroblast growth factor receptor 4 (ptd-FGFR4) correlates with tumour invasiveness but not with G-protein α subunit (gsp) mutation in human GH-secreting pituitary adenomas. Clin Endocrinol (Oxf) 68: 435–441
22. **Bange J, Prechtl D, Cheburkin Y, Specht K, Harbeck N, Schmitt M, Knyazeva T, Müller S, Gärtner S, Sures I, Wang H, Imyanitov E, Häring HU, Knayzev P, Iacobelli S, Höfler H, Ullrich A** 2002 Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. Cancer Res 62:840–847
23. **Wang J, Stockton DW, Ittmann M** 2004 The fibroblast growth factor receptor-4 Arg388 allele is associated with prostate cancer initiation and progression. Clin Cancer Res 10:6169–6178
24. **Spinola M, Leoni V, Pignatiello C, Conti B, Ravagnani F, Pastorino U, Dragani TA** 2005 Functional FGFR4 Gly388Arg polymorphism predicts prognosis in lung adenocarcinoma patients. J Clin Oncol 23:7307–7311
25. **Arnaldi G, Angeli A, Atkinson AB, Bertagna X, Cavagnini F, Chrousos GP, Fava GA, Findling JW, Gaillard RC, Grossman AB, Kola B, Lacroix A, Mancini T, Mantero F, Newell-Price J, Nieman LK, Sonino N, Vance ML, Giustina A, Boscaro M** 2003 Diagnosis and complications of Cushing's syndrome: a consensus statement. J Clin Endocrinol Metab 88:5593–5602
26. **Bates AS, Farrell WE, Bicknell EJ, McNicol AM, Talbot AJ, Broome JC, Perrett CW, Thakker RV, Clayton RN** 1997 Allelic deletion in pituitary adenomas reflects aggressive biological activity and has potential value as a prognostic marker. J Clin Endocrinol Metab 82:818–824
27. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-$\Delta\Delta$C(T)) Method. Methods 25:402–408
28. **Savage MO, Besser GM** 1996 Cushing's disease in childhood. Trends Endocrinol Metab 7:213–216
29. **Rollin G, Ferreira NP, Czepielewski MA** 2007 Prospective evaluation of transsphenoidal pituitary surgery in 108 patients with Cushing's disease. Arq Bras Endocrinol Metabol 51:1355–1361
30. **Pouratian N, Prevedello DM, Jagannathan J, Lopes MB, Vance ML, Laws Jr ER** 2007 Outcomes and management of patients with Cushing's disease without pathological confirmation of tumor resection after transsphenoidal surgery. J Clin Endocrinol Metab 92:3383–3388.
31. **Pecori Giraldi F, Moro M, Cavagnini F** 2003 Gender-related differences in the presentation and course of Cushing's disease. J Clin Endocrinol Metab 88:1554–1558
32. **Ezzat S, Zheng L, Winer D, Asa SL** 2006 Targeting N-cadherin through fibroblast growth factor receptor-4: distinct pathogenetic and therapeutic implications. Mol Endocrinol 20:2965–2975
33. **Morimoto Y, Ozaki T, Ouchida M, Umehara N, Ohata N, Yoshida A, Shimizu K, Inoue H** 2003 Single nucleotide polymorphism in fibroblast growth factor receptor 4 at codon 388 is associated with prognosis in high-grade soft tissue sarcoma. Cancer 98:2245–2250
34. **Stadler CR, Knyazev P, Bange J, Ullrich A** 2006 FGFR4 GLY388 isotype suppresses motility of MDA-MB-231 breast cancer cells by EDG-2 gene repression. Cell Signal 18:783–794

35. **Spinola M, Leoni VP, Tanuma J, Pettinicchio A, Frattini M, Signoroni S, Agresti R, Giovanazzi R, Pilotti S, Bertario L, Ravagnani F, Dragani TA** 2005 FGFR4 Gly388Arg polymorphism and prognosis of breast and colorectal cancer. Oncol Rep 14:415–419
36. **Jézéquel P, Campion L, Joalland MP, Millour M, Dravet F, Classe JM, Delecroix V, Deporte R, Fumoleau P, Ricolleau G** 2004 G388R mutation of the FGFR4 gene is not relevant to breast cancer prognosis. Br J Cancer 90:189–193
37. **Yang YC, Lu ML, Rao JY, Wallerand H, Cai L, Cao W, Pantuck A, Dalbagni G, Reuter V, Figlin RA, Belldegrun A, Cordon-Cardo C, Zhang ZF** 2006 Joint association of polymorphism of the FGFR4 gene and mutation TP53 gene with bladder cancer prognosis. Br J Cancer 95:1455–1458
38. **Ansell A, Farnebo L, Grénman R, Roberg K, Thunell LK** 2009 Polymorphism of FGFR4 in cancer development and sensitivity to cisplatin and radiation in head and neck cancer. Oral Oncol 45:23–29
39. **Sibley K, Stern P, Knowles MA** 2001 Frequency of fibroblast growth factor receptor 3 mutations in sporadic tumours. Oncogene 20:4416–4418
40. **Mawrin C, Kirches E, Diete S, Wiedemann FR, Schneider T, Firsching R, Kropf S, Bogerts B, Vorwerk CK, Krüger S, Dietzmann K** 2006 Analysis of a single nucleotide polymorphism in codon 388 of the FGFR4 gene in malignant gliomas. Cancer Lett 239:239–245