

Differential Expression of Folate Receptor in Pituitary Adenomas¹

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ABSTRACT

Pituitary adenomas cause significant morbidity caused by compression of regional structures or the inappropriate expression of pituitary hormones. However, little is known about the molecular changes that contribute to the development of these tumors. To investigate these changes, we recently used cDNA microarray analysis to identify several genes with altered expression patterns in pituitary adenomas. The folate receptor (FR α) was significantly overexpressed in clinically nonfunctional (NF) adenomas but not in functional adenomas (adrenocorticotropic hormone, growth hormone, and prolactin). FR α is a high affinity folate transporter that is overexpressed by other tumors and could provide a growth advantage to cells that express it. Analysis of FR α expression by Western blotting confirmed that FR α protein was specifically overexpressed in NF tumors. The FR α was capable of binding folates from measurements of [³H] folic acid binding, indicating that the overexpressed receptor was properly folded and may mediate vitamin uptake. Comparison of protein and specific [³H] folic acid binding levels in subtypes of NF adenomas suggested that the immunohistochemically negative adenomas produced more properly folded FR α than adenomas that stained positively for anterior pituitary hormones. Finally, immunohistochemistry demonstrated that FR α was specifically expressed in NF adenoma cells. These results demonstrate that overexpression of FR α mRNA by NF pituitary adenomas results in production of properly folded FR α protein, may mediate vitamin transport, and could potentially facilitate the growth of these tumors.

INTRODUCTION

Pituitary adenomas account for ~10% of intracranial tumors. They cause significant morbidity by compression of regional structures and the inappropriate expression of pituitary hormones (1, 2). Functional tumors, such as GH³ and ACTH adenomas, give rise to severe life-threatening clinical syndromes, such as Acromegaly or Cushing's disease, and PRL adenomas result in impaired reproduction. NF pituitary adenomas account for ~30% of pituitary tumors (3). The term NF reflects the fact that these tumors do not cause clinical hormone hypersecretion (3–6). The NF tumors are uniquely heterogeneous (Table 1). They typically are quite large and cause hypopituitarism or blindness from regional compression (1). Despite the lack of clinical hormone hypersecretion, immunocytochemical staining for hormones reveals evidence for hormone expression in \leq 79% of these tumors, and we refer to these as immunohistochemically positive

(NF+). The remainder is negative for hormone expression (6, 7), and these are referred to as immunohistochemically negative (NF-). Cell culture studies also demonstrate that some NF tumors secrete hormone *in vitro* (8).

Unlike the functional pituitary tumors, there is no available effective medical therapy for the NF tumors, and only a better understanding of the molecular biology of these tumors will provide needed medical treatment options.

Although pituitary tumors are mostly benign, 5–35% of them are locally invasive. A small number exhibits a more aggressive course, infiltrating dura, bone, and sinuses, and are highly aggressive. A smaller number are truly malignant, *i.e.*, metastasize outside the central nervous system.

Molecular genetic studies have demonstrated that these tumors are monoclonal in origin (9, 10). A minority is part of an autosomal dominant syndrome, multiple endocrine neoplasia type 1, which is associated with mutations in the multiple endocrine neoplasia type 1 tumor suppressor gene. Others are associated with loss of heterozygosity on 11q13 chromosome (11–14). Interestingly, FR α maps to 11q13, and amplification of this region in some carcinomas is associated with overexpression of the FR (15). It is not known whether chromosomal abnormalities of the FR gene locus are associated with changes in folate expression in pituitary tumors.

A dominant mutation occurs in the *Gas* gene in ~30% of somatotrophinomas, but this mutation is rare in other pituitary tumors (4, 16, 17). Regarding the nonfunctional tumors, reduced levels of expression of the retinoid X receptor, estrogen receptor, and thyroid hormone receptor have been found and are thought to contribute to abnormal thyroid hormone regulation of α -subunit production in these tumors. The relevance to pituitary tumorigenesis is unknown (18–21). The EGFR is overexpressed in 80% of NF adenomas and virtually undetectable in functional adenomas. NF tumors in culture proliferate in response to epidermal growth factor administration and up-regulate EGFR mRNA (22). Because this finding was reported several years ago, no further elaboration of the role of EGFR in pituitary pathogenesis has been published.

In a previous study (23), we used cDNA microarray analysis and RT-qPCR to compare expression profiles of 7075 genes in the normal pituitary with that in different adenomas, including NF-, PRL-producing adenomas, GH-producing adenomas, and ACTH-secreting adenomas. In those experiments, we found that the FR α gene was significantly overexpressed in NF- adenomas.

The FR α (gp38) is a glycosylphosphatidylinositol-anchored membrane protein that transports reduced folates into cells (24–27). Folate is an essential vitamin that plays a critical role as a cofactor in cellular one carbon metabolism and is required for the synthesis of DNA precursors, cellular growth, and development (28, 29). FR α is normally only expressed in few tissues, including placenta, kidney, and lung (30), but is intensely expressed in choroid plexus (27). However, FR α is highly overexpressed in tumors of epithelial origin, including ovarian, renal cell, breast, colorectal carcinomas, anaplastic ependymomas, and choroid plexus tumor (31–33). Whether cellular overexpression of the FR α confers a growth advantage to tumors by facilitating the acquisition of folate is unclear. However, the high affinity

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³ The abbreviations used are: GH, growth hormone; ACTH, adrenocorticotropic hormone; EGFR, epidermal growth factor receptor; PRL, prolactin; NF, nonfunctioning; TSH, thyroid stimulating hormone; LH, luteinizing hormone; TNE, [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA]; Mab, monoclonal antibody; RT-qPCR, reverse transcription-real time quantitative PCR; IHC, immunohistochemistry; FSH, follicle-stimulating hormone; FR, folate receptor.

Table 1 Classification of NF adenomas by cell of origin

Cell type	Hormone expression	% of NF tumors
Null cell	None	17%
Oncocytoma	None	6%
Silent corticotroph	ACTH	8%
Silent somatotroph	GH	3%
Gonadotrophs	Intact LH/FSH or subunits	40–79%

of FR for folate and its selective overexpression in tumors provides a unique opportunity for directed chemotherapy and radiopharmaceutical delivery, *e.g.*, folic acid analogues and conjugates, such as 5,10-dideazatetrahydrofolic acid, are directly cytotoxic and therapeutically effective against some types of tumors. Because there are currently no effective chemotherapeutic agents against NF adenomas, these analogues may provide a novel medical treatment for NF tumors primarily or for residual disease.

In this report, we characterize the expression of FR α in NF+ and NF–, PRL, GH, and ACTH pituitary adenomas. FR α mRNA expression in NF+ pituitary adenomas was determined by RT-qPCR to complement our previous report in which we revealed that FR α mRNA was overexpressed in NF– tumors. Furthermore, FR α protein expression levels in the tumors were quantified by Western blotting, folic acid binding assay, and IHC. The identification of FR α may further elucidate the pathways of pituitary oncogenesis and provide effective therapeutic treatment to pituitary tumors.

MATERIALS AND METHODS

Patients and Tumor Characterization. Thirty-nine sporadic pituitary adenomas were obtained from patients at Emory University Hospital after transphenoidal surgery (Table 2). Portions of the surgical specimens were frozen in liquid nitrogen and stored at –80°C. The remaining tumor was processed for routine histology and IHC. Informed consent for inclusion in this study was obtained. Five normal pituitary glands obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) were used as controls.

RNA Extraction and RT-qPCR. Tumor tissue was washed in ice-cold PBS to remove blood. Total RNA was extracted from normal pituitaries (100–200 mg) or pituitary adenomas (30–200 mg) using the TRIzol reagent protocol (Life Technologies, Inc., Gaithersburg, MD). RT-qPCR was performed as described (23). Briefly, the human FR α primers used were: (a) sense strand 5' GAACGCCAAGCACCACAAG 3' and (b) antisense strand 5' GGTCGACACTGCTCATGCAA 3'. RNase (18S rRNA) was used as internal control (PE Applied Biosystems). Total RNA (5 μ g) of each sample was reverse transcribed in 20 μ l using 150 ng of random prime hexamers, 0.5 mM deoxynucleotide triphosphate, and 50 units of Superscript reverse transcriptase as recommended by the manufacturer (Life Technologies, Inc.).

The reverse transcriptase reaction products were diluted in water (10–100-fold for candidate genes and 10,000-fold for 18S rRNA) and subjected to PCR according to PE Applied Biosystems' recommendations, with few modifications. All PCR reactions were cycled in the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems) at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of the PCR reactions was determined from the dissociation curve analysis. Standard curves for FR α and 18S rRNA were performed each time the genes were analyzed. All PCR products were in the geometric phase of PCR amplification. The quantity of the specific genes obtained from standard curves was normalized to that of the 18S rRNA of the same sample. All PCR reactions were performed at least in duplicate. Fold difference was determined as the ratio of the normalized value of each tumor sample to the mean of the five normalized values of the normal pituitaries.

Sample Preparation for Protein Assay. Pituitary tissue samples (20–210 mg) were washed with ice-cold PBS to remove blood and other extracellular material and homogenized in 5 ml of PBS by hand in a dounce homogenizer, followed by a Polytron homogenizer. The sample was then centrifuged at 2500 \times g for 10 min at 4°C, and the pellet was solubilized by incubation in 1

ml of TNE with 1% Triton X-100 on ice for 20 min. Protein concentration was determined using the bicinchoninic acid protein assay.

Western Blot Analysis. Equal amounts of total protein from each sample (10 μ g) were loaded into a 15% acrylamide-SDS gel, and proteins were resolved by electrophoresis. The proteins were then transferred into nitrocellulose, and FR α was detected using a polyclonal antihuman FR α antibody. The antibody was produced by rabbits immunized with a recombinant FR α -glutathione S-transferase fusion protein. The FR α protein was visualized by chemiluminescence on Biomax ML film (Kodak) using an antirabbit IgG as the secondary antibody and the enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia). The absorbance of the FR α protein bands was measured using Bio-Rad imaging densitometer model GS-670 with the Molecular Analyst program. After subtraction of the background for each film, the absorbance value of each tumor sample indicated the FR α protein expression/10 μ g of total protein. The fold difference in expression was determined by dividing the corrected absorbance of each sample by the mean of the five absorbance values determined for the normal pituitary controls.

Quantitation of FR α by Folic Acid Binding. Because folic acid is bound very tightly by the FR [Kd = 0.4 nM (34)], specific binding of this ligand can be used to estimate the amount of this receptor in a cell or cellular extract. Pituitary samples were diluted with TNE +1% Triton X-100 to a protein concentration of \geq 0.25 mg/ml. The final protein concentration of each sample was verified by bicinchoninic acid assay after dilution. Total folic acid binding

Table 2 Clinical and pathological characteristics of adenomas from patients used in this study

Sex, age of patient, and a brief description of tumor type are given.

Patient ID	Sex, age	Clinical features/tumor size	IHC
93	M, 57	NF, visual loss, cavernous sinus invasion, 1.5 cm	Neg. ^a
74	M, 67	NF, hypogonadism, 2.5 cm	Neg.
164	M, 35	NF, visual loss, 4 cm	Neg.
153	M, 72	NF, hypogonadism, 2 cm	Neg.
155	M, 66	NF, headache, visual loss, hypocortisolism, 1.5 cm	Neg.
104	F, 61	NF, visual loss, 1.5 cm	Neg.
105	F, 48	NF	Neg.
75	M, 60	NF, visual loss, 2 cm	Neg.
68	M, 74	NF	Neg.
191	F, 44	NF	Neg.
65	F, 54	NF	FSH 1+, LH 2+
77	M, 67	NF	FSH 2+
174	F, 53	NF	FSH 1+, LH 1+
143	M, 60	NF	FSH 1+, LH 1+, ACTH 1+
89	M, 62	NF	FSH 2+
91	M, 56	NF	FSH 2+, TSH 1+
100	F, 65	NF	FSH 1+, LH 1+, ACTH 2+, PRL+, GH1+
112	F, 58	NF	FSH 3+, PRL 2+, LH 2+
69	M, 67	NF	FSH 2+, LH 3+
60	F, 90	NF	FSH 1+, LH 2-3+
198	M, 58	NF	FSH 1+, LH 1+
208	F, 47	NF	LH1+
138	M, 60	NF	FSH 1-2+, LH 1-2+
183	M, 36	Hyperprolactinemia, visual loss, 2 cm	PRL 3+
192	M, 41	Hyperprolactinemia, visual loss, 3 cm	PRL 3+
151	M, 52	Hyperprolactinemia	PRL 3+, GH 1+
240	M, 39	Hyperprolactinemia	PRL 3+
244	F, 40	Hyperprolactinemia	PRL 3+
213	M, 24	Hyperprolactinemia	PRL 3+
123	M, 30	Acromegaly, 2 cm	GH 3+, TSH 3+, FSH 3+, LH 3+
196	F, 69	Acromegaly, 2 cm	ND ^b
168	F, 39	Acromegaly, 1 cm	GH 3+, PRL 2-3+
218	F, 33	Acromegaly	GH 3+, PRL 2-3+
145	M, 34	Acromegaly	GH 3+, PRL 2-3+, TSH 1+
232	F, 39	Cushing's disease	ACTH 2+
137	F, 41	Cushing's disease	ND
239	F, 55	Cushing's disease, cav sinus invasion	ACTH 4+
126	F, 29	Cushing's disease, 1.6 cm	ACTH 3+
233	M, 11	Cushing's disease	ACTH 3+, FSH 1+

^a Neg., immunostaining for GH, ACTH, PRL, FSH, LH, and TSH were negative.

^b ND, not determined.

was determined using [^3H]folic acid purified as described previously (35). Briefly, samples (300 μl) were incubated with [^3H]folic acid (50 nM) at 37°C for 1 h in shaking water bath. After cooling on ice for 5 min, unbound ligand was removed by incubating with 1 ml of a dextran-coated charcoal solution (8 mM dextran and 80 mM activated charcoal in TNE) on ice for 15 min, followed by centrifugation for 25 min at 3000 $\times g$ at 4°C. Aliquots of the supernatant were counted by scintillation counting to determine the total [^3H]folic acid binding. Nonspecific binding in each sample was measured by quantifying [^3H]folic acid binding in the presence of a 750-fold excess of unlabeled folic acid. Average percentage of nonspecific radioactivity bound was 1.06 ± 0.13 (SD) of total radioactivity. Specific binding was determined by subtracting the nonspecific value from the total binding.

Immunohistochemical Characterization of Pituitary Adenomas. FR α expression in pituitary adenomas was assessed by IHC using LK26, a Mab to FR α (36). LK26 was commercially available through Signet Pathologies, Inc. (Dedham, MA).

Five- μm -thick frozen tissue sections were cut, mounted on gelatin-coated slides, air dried, and fixed in cold acetone for 10 min at 4°C. Sections were then treated with 0.3% H_2O_2 for 15 min at room temperature to block endogenous peroxidase and incubated with normal horse serum for 30 min at room temperature. Tissue sections were further blocked from endogenous biotin or biotin-binding proteins by incubating in Avidin/Biotin solution (Vector, Burlingame, CA) for 15 min before binding of Mab LK26 (0.5 $\mu\text{g}/\text{ml}$ PBS and 1% BSA) for 12–18 h at 4°C. Sections from an ovarian tumor were used as a positive control. As a negative control, the ovarian cancer sections were incubated in PBS and 1% BSA without antibody for 12–18 h at 4°C. Sections were then washed and incubated with biotinylated secondary antibody (VECTASTAIN Elite avidin-biotin complex kit; Vector) for 30 min at room temperature, followed by avidin-biotin-horseradish peroxidase complex. Diaminobenzidine (liquid 3,3'-diaminobenzidine substrate-chromogen system; DAKO Corp., Carpinteria, CA) was used to visualize the final reaction product. Sections were counterstained with Harris hematoxylin. Adenomas were graded blindly by a neuropathologist from 0 to 4 for intensity of FR α immunoreaction.

Immunohistochemical Characterization of Hormones in Pituitary Adenomas. Tissue sections were treated with 0.3% H_2O_2 for 15 min at room temperature to block endogenous peroxidase, after which time they were incubated at room temperature with an antibody directed toward ACTH (polyclonal, 1:12,000; DAKO), FSH (polyclonal, 1:24,000; DAKO), GH (monoclonal, 1:200; BioGenex, San Ramon, CA), LH (monoclonal, 1:6400; BioGenex), PRL (monoclonal, 1:160; BioGenex), or TSH (monoclonal, 1:1600; BioGenex). Antibodies were detected using the avidin-biotin complex method, using diaminobenzidine as the chromogen. Adenomas were graded blindly by a neuropathologist from 0 to 4 for intensity of staining for each peptide hormone.

Statistical Methods. All data from Western blot analysis, folic acid binding measurement, and RT-qPCR were tested for normality and homoscedasticity (ANOVA). Because none of the assumptions for parametric testing could be met, including transformation of the data, all of the dependent variables were analyzed by nonparametric alternatives with Kruskal-Wallis one-way ANOVA on ranks. To determine differences between groups, the Mann-Whitney test, a nonparametric, pairwise comparison procedure, was used. Analyses were calculated using SPSS for Windows. Differences were considered significant at a probability $<5\%$ ($P < 0.05$).

RESULTS

Tumor Classification. The clinical and pathological characteristics of the 39 adenomas used in this study are listed in Table 2. Ten of the NF adenomas were not positive with anterior pituitary hormone histochemistry and were designated immunohistochemically negative (NF $-$) tumors. Thirteen NF tumors stained with one or more anterior pituitary hormones and were designated immunohistochemically positive (NF $+$). Six were classified as PRL, five as GH, and five as ACTH-positive adenomas. With the exception of two with cavernous sinus invasion, all tumors were noninvasive as defined by histological and radiological criteria. Other clinical features related to the tumor are noted in Table 2. Five normal pituitary controls were obtained

from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases.

FR α mRNA Expression in NF $+$ Adenomas. We have reported previously (23) the relative expression level of FR α mRNA in NF $-$, PRL, GH, and ACTH-secreting pituitary adenomas as determined by cDNA microarray analysis and RT-qPCR. In this study, the level of FR α mRNA expression in NF $+$ adenomas was measured by RT-qPCR and compared with controls also. These results are shown in Fig. 1. The level of overexpression of FR α mRNA ranged from 17- to 174-fold greater than the mean of the control samples. The difference between these adenomas and controls was significant at $P = 0.001$.

Expression of FR α Protein. Whether the high levels of FR α mRNA resulted in overexpression of FR α protein was investigated with Western blotting of 39 pituitary tumors and 5 normal pituitaries using a highly specific polyclonal antibody against this receptor. This antibody was produced by rabbits immunized with a recombinant FR α -glutathione *S*-transferase fusion protein and has been shown to recognize the same protein as the Mab LK26 (data not shown). The results for two normal pituitaries (N1 and N3), one NF $+$ adenoma (#65), and 9 NF $-$ adenomas are shown in Fig. 2A and for the 13 NF $+$ adenomas are shown in Fig. 2B. Two PRL-secreting adenomas (PRL = #183 and 192) and 3 GH-secreting adenomas (GH = #123, 196, and 168) are shown in Fig. 2C, whereas 4 ACTH-secreting (ACTH = #232, 137, 239, and 126) adenomas are shown in Fig. 2D. Adenoma #65, which was NF $+$, was included in all of the blots to provide a basis of comparison for the different gels. These results show that the majority (7 of 10 tumors showing FR α expression) of the NF $-$ tumors and all of the NF $+$ tumors overexpress FR α relative to the normal pituitaries and functional adenomas.

The levels of FR α expression in each adenoma and normal pituitary controls were quantified by densitometry. The fold difference was the ratio of the absorbance signal of each sample to the mean of that of controls. The overexpression of FR α in the eight NF $-$ tumors (Fig. 2A) was by 3–39-fold and in the NF $+$ tumors (Fig. 2B) was by 5–71-fold compared with controls. Only one of the hormone-secreting adenomas showed any overexpression of FR α , and that was at a relatively low level (2–3-fold) compared with the nonfunctional tumors.

The results of the Western blot analysis are summarized in a box plot shown in Fig. 3. The horizontal line in each box represents the median value of FR α expression of each adenoma group and controls. The box represents the 25th and 75th percentile range of scores. The 10

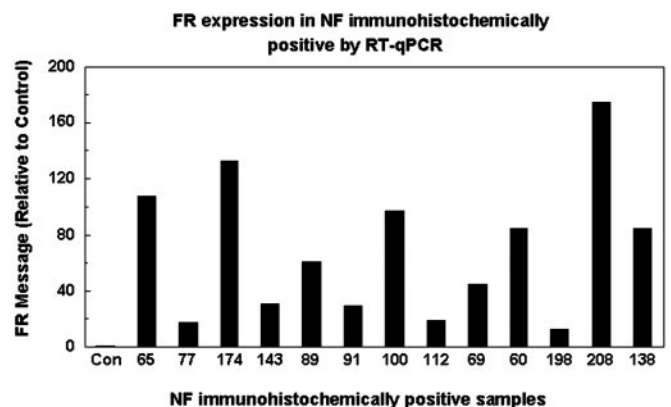


Fig. 1. Relative expression levels of FR α mRNA in NF $+$ adenomas by RT-qPCR. Expression levels of each tumor were normalized to the 18 S RNase of the same sample. Fold difference was the ratio of the normalized value of each sample to controls as described in "Methods and Materials." Con, 5 normal controls; 65–138, NF $+$ adenomas. There was a significant overexpression of FR α mRNA in 13 NF $+$ adenomas from 17- to 174-fold compared with the controls ($P = 0.001$). Notably, sample 208 exhibited the highest FR α mRNA expression among the 13 samples (174-fold).

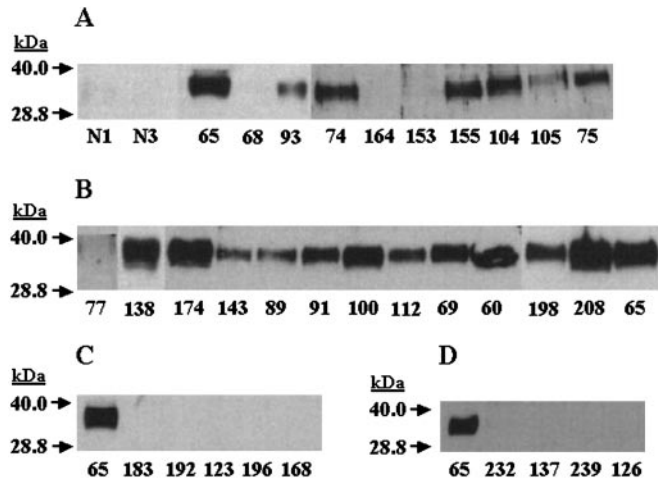


Fig. 2. A–D, Western blot of FR α expression in pituitary adenomas. Total protein (10 μ g) of each sample was separated by a 15% SDS-PAGE. Immunodetection was carried out using a polyclonal antibody, rabbit antihuman FR α IgG, as described in “Methods and Materials.” Fig. 1A showed FR α expression in 2 normal pituitaries, 1 NF+ (65), and 9 NF– adenomas. Fig. 1B showed FR α expression in 13 NF+ adenomas. Fig. 1C showed FR α expression in 1 NF+ (65), 2 PRL (183 and 192), and 3 GH-secreting adenomas (123, 196, 168), whereas Fig. 1D showed 1 NF+ (65) and 4 ACTH-secreting adenomas (232–126).

NF– samples showed an average increase of 19-fold compared with controls, whereas the 13 NF+ showed a mean increase of 36-fold relative to controls. The 6 PRL, 5 GH, and 5 ACTH-secreting adenomas showed no increase compared with controls. The Kruskal-Wallis test on ranks of the median values of FR α protein showed there were significant differences between tumor groups ($\chi^2 = 29.639$, degrees of freedom = 5, $P < 0.05$). The Mann-Whitney test showed there was a significant difference between NF– compared with controls ($P = 0.014$), PRL ($P = 0.009$), GH ($P = 0.014$), and ACTH-secreting adenomas ($P = 0.007$). Furthermore, there was a significant difference between NF+ compared with controls ($P = 0.001$), NF– ($P = 0.041$), PRL ($P = 0.001$), GH ($P = 0.001$), and ACTH-secreting adenomas ($P = 0.001$). However, there was no significant difference between PRL, GH, and ACTH-secreting adenomas compared with controls.

To investigate the nature of the relationship between the levels of FR α mRNA and protein in the NF adenomas, these two parameters were compared directly. As shown in Fig. 4, the levels of FR α mRNA and protein correlated well in NF+ adenomas (closed circles, solid line, $r = 0.89$). However, in NF– adenomas, the correlation between these parameters was much less (open circles, dashed line, $r = 0.57$), suggesting that the expression of FR α mRNA may be differentially controlled in these tumors.

Assessment of FR α Binding Capacity. The Western blot analysis indicates the relative amount of a protein in the tissue or cell but does not provide any information about its functional potential. To determine whether the overexpressed FR α protein in the adenomas was properly folded and had the potential to transport folates, specific binding of folic acid was measured in the various tumors for which sufficient tissue was available (37 pituitary tumors and 5 normal pituitaries). In the 5 normal pituitary controls, folic acid binding was 0.9–4.8 pmol/mg protein, with a mean of 2.2 pmol/mg protein. In the 10 NF– samples, binding ranged from 1.6 to 136.1 pmol/mg protein, which was 0.7–62-fold greater than the mean of the controls. The 13 NF+ adenomas bound between 7.6 and 242.7 pmol/mg protein, which was 3–110-fold higher than the mean of control samples. Folic acid binding was very low (0.02–2.1 pmol/mg protein) in the 5 PRL, 5 GH, and 4 ACTH-secreting adenomas.

The folic acid binding results for each of the tumor types compared with the control samples are shown in Fig. 5. As in Fig. 3, the horizontal line in each box represents the median value of folic acid binding of each adenoma group and controls. The box represents the 25th and 75th percentile range of scores, whereas the vertical lines indicate the highest and lowest values for each tumor type. The mean binding of 10 NF– samples was 22-fold higher than controls, whereas the mean binding of 13 NF+ adenomas was 29-fold higher than controls. The differences in the median values of folic acid binding between all groups of tumors were significant by the Kruskal-Wallis test ($\chi^2 = 30.762$, degrees of freedom = 5, $P < 0.05$). The Mann-Whitney test showed there was a significant difference between NF– group compared with controls ($P = 0.007$), PRL ($P = 0.003$), GH ($P = 0.003$), and ACTH-secreting adenomas ($P = 0.002$). Furthermore, there was a significant difference between NF+ compared with controls ($P = 0.001$), PRL ($P = 0.001$), GH ($P = 0.001$), and ACTH-secreting adenomas ($P = 0.001$). Notably, there was also a significant difference of binding in PRL ($P = 0.047$)-secreting adenomas compared with controls. However, the differences between GH, ACTH-secreting adenomas, and controls and that between NF– and NF+ samples were not significant.

To evaluate the relationship between the level of FR α expression and the folic acid binding capacity, these two parameters were compared in the NF+ and NF– samples. This comparison, which is shown in Fig. 6, indicates that there is a weak relationship between the FR α protein levels (derived from Fig. 2) and folic acid binding (derived from Fig. 5) for the NF+ adenomas (closed circles, solid line, $r = 0.58$). In fact, if the NF+ sample with the highest FR α level and binding (#208, marked with an asterisk in Fig. 6) is excluded from the comparison, the relationship is even weaker (dotted line, $r = 0.28$). The relationship between FR α levels and binding capacity was stronger in the NF– adenomas (open circles, dashed line), with $r = 0.79$.

Immunohistochemical Analysis of FR α Expression. IHC analysis was performed in the human pituitary tumors to determine the cellular and subcellular localization of FR α overexpression. Frozen tissue sections from 5 NF–, 6 NF+, 2 PRL, 3 GH, and 2 ACTH-secreting adenomas and two normal anterior pituitary glands were

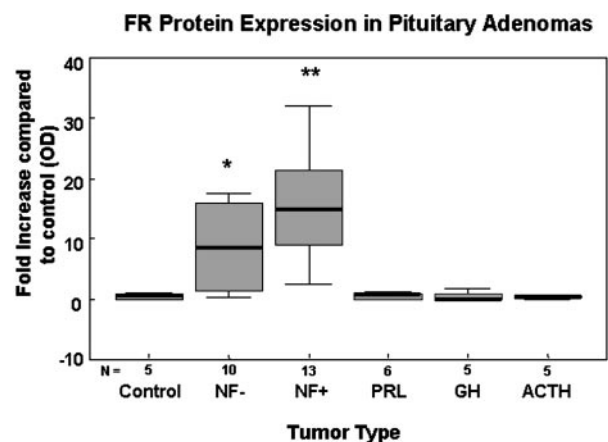


Fig. 3. Box plots representing the FR α protein expression by adenoma subtypes, by Western blot. A horizontal line in each box represents the median value of FR α protein expression of each group. Box, the 25th and 75th percentile range of scores. Whiskers, the highest and lowest values. Numbers of pituitaries tested in each group were: $n = 5$ for controls, $n = 10$ for NF–, $n = 13$ for NF+, $n = 6$ for PRL, $n = 5$ for GH, and $n = 5$ for ACTH-secreting adenomas. *, in NF– adenomas, FR α was significantly overexpressed compared with controls ($P = 0.014$), PRL ($P = 0.009$), GH ($P = 0.014$), and ACTH-secreting adenomas ($P = 0.007$). **, in NF+ adenomas, FR α was significantly overexpressed compared with controls ($P = 0.001$), NF– ($P = 0.041$), PRL ($P = 0.001$), GH ($P = 0.001$), and ACTH-secreting adenomas ($P = 0.001$).

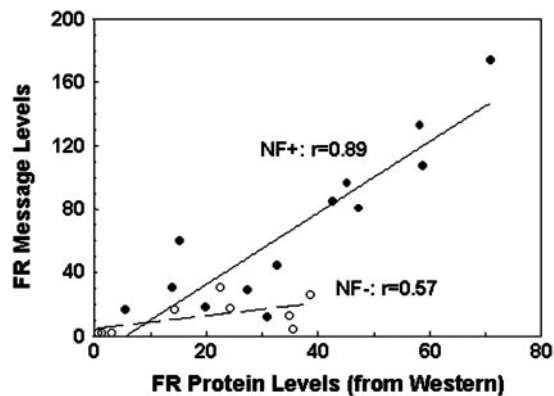


Fig. 4. Correlation between the levels of FR α mRNA and protein in the NF adenomas. *Solid line*, the levels of FR α mRNA in function of the protein levels in NF+ adenomas (\bullet , *solid line*, $r = 0.89$). *Dashed line*, the levels of FR α mRNA in function of the protein levels in NF- adenomas (\circ , *dashed line*, $r = 0.57$).

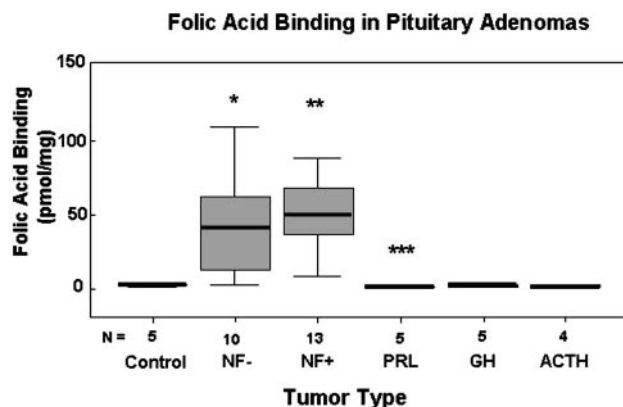


Fig. 5. Box plots representing folic acid binding to FR α by adenoma subtypes. A *horizontal line* in each *box* represents the median value of folic acid binding to FR α of each group. *Box*, the 25th and 75th percentile range of scores. *Whiskers*, the highest and lowest values. Numbers of pituitaries tested in each group were: $n = 5$ for normal pituitary controls, $n = 10$ for NF-, $n = 13$ for NF+, $n = 5$ for PRL, $n = 5$ for GH, and $n = 4$ for ACTH-secreting adenomas. *, in NF- adenomas, folate binding was significantly different from controls ($P = 0.007$), PRL ($P = 0.003$), GH ($P = 0.003$), and ACTH-secreting adenomas ($P = 0.002$). **, in NF+ adenomas, folate binding was significantly different from controls ($P = 0.001$), PRL ($P = 0.001$), GH ($P = 0.001$), and ACTH-secreting adenomas ($P = 0.001$). ***, in PRL adenomas, folate binding was significantly different from controls ($P = 0.047$).

used for these analyses. Three specimens of ovarian adenocarcinoma served as a positive control for FR α IHC because this tumor type consistently expresses high levels of FR α . All of the ovarian adenocarcinomas showed strong luminal and membranous immunoreactivity for FR α and moderate cytoplasmic staining (Fig. 7A, *arrow*). The ovarian adenocarcinomas showed no immunoreactive staining when the primary antibody (LK26) FR α was replaced with normal PBS (Fig. 7B), indicating that there was no nonspecific staining from the secondary antibody.

All 5 of the NF- adenomas showed FR α expression by IHC. Immunostaining was seen diffusely in the cytoplasm of tumor cells in these adenomas and varied from strong in four tumors (100% of cells; Fig. 7D) to moderate in one tumor (50% of cells; Fig. 7E). No staining was noted within other cellular constituents of the adenoma, such as vascular structures or supporting stromal elements. The 6 NF+ adenomas that showed focal weak staining for hormones (Fig. 7F, stained for LH, *arrow*) expressed high levels of FR α (Fig. 7G, stained with LK26). Strong cytoplasmic expression was also seen in five of these NF+ tumors (100% of cells), and more than moderate staining was seen in one tumor (80% of cells). Cytoplasmic FR α expression in

these NF adenomas was much greater than that seen in normal (nonneoplastic) anterior pituitary glands, which showed only focal weak staining of pituitary cells (Fig. 7C, *arrow*).

In the seven functional adenomas analyzed (2 PRL, 2 ACTH, and 3 GH adenomas), FR α expression was either totally absent (2 GH tumors, 0% of cells) or only minimally detected (1–5% of cells in 2 PRL and three other tumors). As in Fig. 7, H and I, adenomas (PRL) that showed strong staining for PRL hormone (Fig. 7H) showed very light staining for FR α (Fig. 7I, stained with LK26).

DISCUSSION

Previous microarray and RT-qPCR analyses demonstrate that FR α mRNA is overexpressed by nonfunctional pituitary adenomas (NF-; Ref. 23). In this study, we demonstrate by RT-qPCR that NF+ also overexpressed FR α mRNA and that the majority of NF tumors contain high levels of FR α protein, indicating that the overexpressed message is actively translated by the NF adenomas. Consistent with our findings for mRNA, FR α expression was limited to the NF adenomas and not observed in hormone-secreting adenomas or normal pituitary tissue. The level of overexpression was variable, with the NF+ adenomas exhibiting a larger range of expression (5–71-fold over controls) than the NF- tumors (3–39-fold over controls). These levels are fairly comparable with those reported previously for a variety of tumors (based on mRNA levels; Ref. 30) and a large collection of ovarian tumors (based on protein levels; Ref. 37). Although FR α is overexpressed in other tumors of epithelial origin (30), this report is the first to document this in pituitary tumors.

Comparison of the levels of overexpression of FR α mRNA and protein (Fig. 4) revealed that there was a somewhat different relationship between these parameters in the NF+ and NF- adenomas. The strong correlation found in NF+ samples indicates that the level of message largely determines how much FR α is produced by these adenomas. Conversely, increased mRNA production does not result in a corresponding increase in FR α protein expression in the NF- adenomas. These results suggest that translation, or some other post-translational process needed to produce mature FR α , is limiting in the NF- samples. These other processes could include folding into the appropriate native conformation, addition of the glycosylphosphatidyl inositol anchor, or addition and remodeling of the N-linked carbohydrates found on this receptor. Any of these processes could be compromised if there were specific mutations in the FR α sequence that

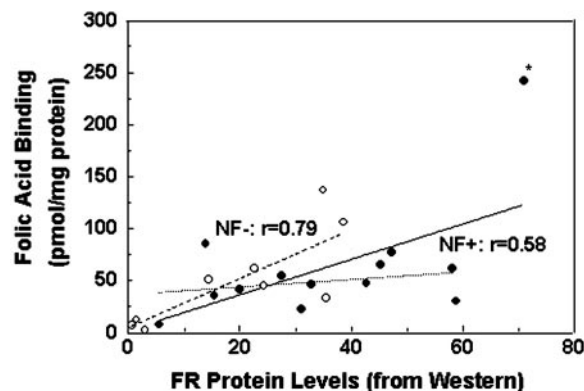


Fig. 6. Correlation between the levels of FR α expression and folic acid binding capacity. *Solid line*, the folic acid binding (derived from Fig. 5) in function of the FR α protein levels (derived from Fig. 3) in the NF+ adenomas (\bullet , *solid line*, $r = 0.58$). When the NF+ sample with the highest FR α level and binding (208, marked with *) is excluded from the comparison, the relationship is even weaker (*dotted line*, $r = 0.28$). *Dashed line*, the folic acid binding in function of the FR α protein levels in the NF- adenomas (\circ , *dashed line*), with $r = 0.79$.

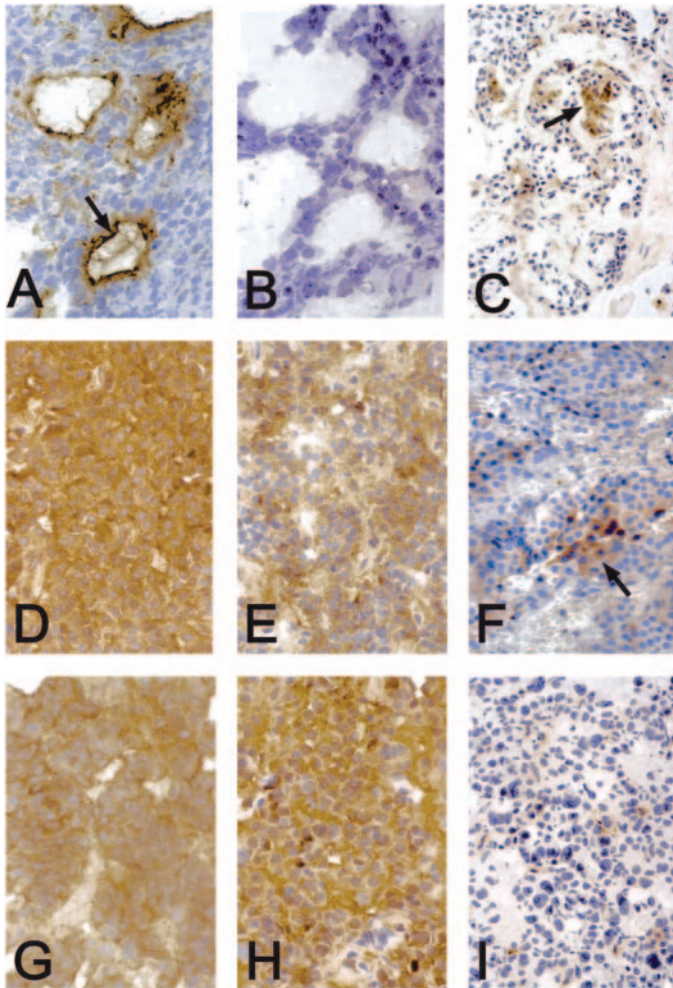


Fig. 7. IHC demonstrating cytoplasmic FR α in anterior pituitary gland, NF pituitary adenomas, and functional pituitary adenoma. Ovarian adenocarcinoma served as a positive control for FR α receptor IHC. Strong immunoreactivity for FR α was present on the luminal membrane of ovarian adenocarcinoma glands and, to a lesser extent, within the cytoplasm (A; arrow). Ovarian adenocarcinoma did not show any immunoreactivity when the primary antibody directed against FR α was replaced with PBS (B; negative control). Normal (nonneoplastic) anterior pituitary gland showed focal weak staining for FR α in glandular cells but none in stromal cells (C; arrow). NF adenomas that did not show any evidence of hormone production by IHC were all immunoreactive for FR α , with the intensity of staining various from strong (D) to moderate (E). Immunoreactivity for FR α was limited to the cytoplasm of adenoma cells and not present in stromal cells or blood vessels. NF adenomas that showed only focal, weak immunoreactivity for hormones (IHC for LH shown by arrow in F) also showed strong cytoplasmic expression FR α (G). Pituitary adenomas, such as a prolactinoma showing cytoplasmic immunoreactivity for PRL by IHC (H), did not show appreciable cytoplasmic FR α (I).

alter specific residues needed for these events. Studies in which specific mutations introduced to affect glycosylphosphatidylinositol (38) or oligosaccharide (39, 40) in addition show that compromising these modifications can decrease the amount of FR α produced. However, there have been no studies to determine whether FR α overexpressed by tumors frequently contains mutations. Whether this contributes to the low levels of FR α protein in NF $-$ adenomas with higher amounts of FR α mRNA has not been determined. Furthermore, why the NF $+$ and NF $-$ are different in the relationship between FR α message and protein levels cannot be explained because of the lack of understanding of the molecular differences between these tumors.

Introduction of FR α into cells that normally do not express this receptor allows them to grow in low (nanomolar) concentrations of folate (41, 42) and form larger tumors when injected into nude mice (43). Thus, the overexpression of FR α by various tumors may facilitate the growth of these cancers. However, that FR α actually allows

tumor cells to acquire more folate than their normal counterparts that do not express this receptor has not been demonstrated. Because the ability of FR α to transport folates cannot be assessed with fixed or frozen tissue, we were unable to determine whether the FR α overexpressed by the NF pituitary adenomas was actually functional. However, we could measure the ability of the receptor to bind folates as a measure of the functional potential of the overexpressed FR α . All of the NF adenomas that showed significant FR α protein by Western blotting (Fig. 3) also exhibited an increase in specific [3 H]folic acid binding relative to normal pituitary samples (Fig. 5). This finding indicates that at least some portion of the FR α expressed by these samples is capable of binding folates and potentially mediating the uptake of elevated levels of this vitamin necessary to support the accelerated growth of the tumor cells.

There was a reasonable correlation between the levels of FR α protein and folic acid binding in the NF $-$ samples (Fig. 6), indicating that the molecular machinery in these adenomas was capable of processing additional receptors to the conformation needed for ligand binding. The NF $+$ adenomas appeared to differ in this regard. In these tumors (except for one sample with extremely high FR α expression), folic acid binding increased little as more FR α was made. This could result from limited capacity in one or more steps in the post-translational processing of this receptor. If this is the case, which cannot be determined based on the results presented here, then it would suggest an interesting difference between the NF $-$ and NF $+$ adenomas. Alternatively, reduced affinity of the more highly expressed FR α could explain the reduced folic acid binding seen in the NF $+$ adenomas with more of this receptor. Comparison of the binding characteristics of FR α overexpressed in ovarian adenocarcinomas with that from normal ovary revealed no significant differences in these receptors (44). Thus, there is no precedent to support a substantial difference in the binding affinities of tumor and normal tissue FR α .

FR α was expressed by all of the tumor cells of NF adenomas (Fig. 7) by immunohistochemical analysis, and these data were supported by the Western blotting and folate binding assay. Thus, the overexpression of FR α was a characteristic shared by all of the cells of the adenoma rather than a subset of them. Because overexpression is uniform, the amount of FR α in a portion of the adenoma will indicate the degree of expression in the entire tumor. The intensity of the immunohistochemical staining varied somewhat between the NF groups (strong in all NF $+$ but only 75% of NF $-$ samples). However, there were no significant differences in the staining patterns between the NF $-$ and NF $+$ adenomas.

FR α is normally only expressed in a few tissues in the body, including kidney, placenta, choroid plexus, and lung (27–30). Thus, the overexpression of FR α by a tumor provides a fairly specific marker of this tissue. Coupled with the fact that this receptor binds its ligands with extremely high affinity (45), this has led to the development of numerous cancer therapies that target the tumor through the overexpressed FR α . These include immunotherapy with Mab against this protein (46) and chemotherapy with numerous cytotoxic agents conjugate to folic acid (47). Imaging methods to visualize FR-expressing tumors using folate-conjugated metal chelates are also being developed (48). The finding that FR α is highly overexpressed in NF pituitary adenomas but not normal pituitary cells suggests that these tumors could be diagnosed or treated with these methods. The enhanced sensitivity of FR α labeling for neuroimaging in patients with NF adenomas could be especially helpful in imaging microadenomas, ectopic adenomas, or tumor invasion in the cavernous sinus. FR α labeling may also provide a means of differentiating between postoperative changes and residual adenoma after surgery. Finally, FR α imaging may provide an additional means for targeting NF tumors for radiosurgical treatment or image-guided NF tumor resection.

The level of expression of FR α in ovarian tumors has been asso-

ciated with disease grade and response to therapy (37, 49). In both cases, higher expression was associated with a less favorable outcome. We have found that the level of FR α expression in the NF pituitary adenomas varies significantly. If overexpression of this receptor plays a similar role in these tumors as in ovarian cancer, then FR α expression may be useful as a prognostic factor for tumor aggressiveness (*i.e.*, cavernous sinus or dural invasion and propensity for recurrence) and potential development and response to chemotherapies for NF pituitary tumors.

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