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Cytoplasmic Expression of Fibroblast Growth Factor Receptor-4 in Human Pituitary Adenomas: Relation to Tumor Type, Size, Proliferation, and Invasiveness

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The pathogenesis of pituitary adenomas remains unknown. A pituitary tumor-derived (ptd) isoform of fibroblast growth factor receptor-4 (ptd-FGFR4) has been implicated in the neoplastic process. To further understand the expression of FGFR4 in sporadic human pituitary adenomas, we studied 137 pituitary adenomas of various types (102 adenomas from Japanese patients and 35 adenomas from Canadian patients) and 10 nontumorous pituitaries using a polyclonal antiserum that recognizes the C terminus of FGFR4 and analyzed possible relationships among expression of FGFR4, patient nationality, tumor type, size, invasion, and the labeling index of the proliferation marker Ki-67 using the MIB-1 antibody. Cytoplasmic expression of FGFR4 protein was observed in 57.8% of Japanese cases and 62.8% of Canadian cases. FGFR4 reactivity was absent in all 10 normal adenohypophysial tissues examined. FGFR4 expression in pituitary adenomas was restricted mainly to the cytoplasm, a pattern similar to that seen in rat pituitary cells transfected with human ptd-FGFR4 but different from that of cells transfected with wild-type FGFR4, which displayed membrane localization of staining. Protein from primary human adenomas migrated as a 65-kDa species consistent with the predicted size of ptd-FGFR4. FGFR4 protein expression was frequently found in adenomas containing GH, ACTH, or FSH/LH and was also found in null cell adenomas, but reactivity was relatively rare in prolactin-containing adenomas in both Japanese and Canadian groups. The expression of FGFR4 protein was stronger in macroadenomas than in microadenomas ($P = 0.02$) and high levels of FGFR4 expression (moderate or greater density staining) were more frequently observed in macroadenomas than in microadenomas ($P < 0.05$). High levels of FGFR4 expression also correlated significantly with the proliferation marker Ki-67 ($P = 0.002$) and tended (but not significantly) to be found in invasive tumors. These data are consistent with a role for ptd-FGFR4 in pituitary tumorigenesis in a majority of human pituitary adenomas. Moreover, detection of FGFR4 cytoplasmic staining may provide an ancillary diagnostic tool in the diagnosis of pituitary adenoma, particularly in equivocal cases. (*J Clin Endocrinol Metab* 89: 1904–1911, 2004)

THE DIAGNOSIS AND classification of pituitary adenomas have seen major progress in the last 25 yr (1). But the pathogenetic mechanisms underlying these common and potentially serious neoplasms remain unclear.

To identify the events leading to pituitary tumorigenesis, oncogenes have been extensively investigated (2). Activating mutations of the Gs-α gene have been implicated in a subset of sporadic GH-secreting pituitary adenomas (3). Common oncogenes in solid neoplasms, such as *ras*, are only exceptionally involved in invasive pituitary tumors (4). Equally, tumor suppressor genes have also been intensively studied (5). In pituitary adenomas, *p53* and *Rb* mutations were not detected by several groups (2). The absence of frequent genetic alterations in this type of neoplasms raised the possibility of epigenetic contributions. In particular, methylation changes in several cyclin dependent kinase inhibitors have been identified in pituitary adenomas (2).

The potential oncogenic contribution of fibroblast growth factors (FGFs) and their receptors (FGFRs) to pituitary tumorigenesis has been the subject of many studies (6, 7). FGFs and FGFRs are known to be important for a variety of biological processes, including mitogenesis, differentiation, development, angiogenesis, and tumorigenesis (8). FGF-2 (also known as basic or bFGF) is overexpressed by pituitary tumor cells with higher levels in more aggressive tumors (6).

The FGFs mediate their biological effects by binding to high-affinity tyrosine-kinase receptors, the FGFRs (9). Four distinct FGFRs (FGFR1, 2, 3, and 4) have been characterized. Each receptor is composed of three Ig-like extracellular domains, two of which are involved in ligand binding, a single transmembrane domain with a long juxtamembrane region, a split tyrosine kinase cytoplasmic domain, and a carboxy-terminal tail that contains tyrosines that are phosphorylated on ligand binding and recruit intracellular signaling proteins (9). The normal human adenohypophysis expresses mRNAs for FGFR1, FGFR2, and FGFR3 (10). Little if any FGFR4 reactivity has been identified in a limited number of normal

Abbreviations: FGF, Fibroblast growth factor; FGFR, FGF receptor; LI, labeling index; PRL, prolactin; ptd-FGFR4, pituitary tumor-derived FGFR4 isoform.

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human pituitaries. In contrast, unlike the normal pituitary, pituitary tumor cells frequently express FGFR4 mRNA sequences corresponding to the third Ig-like loop and the transmembrane and kinase domain (10, 11). This has been identified as due to a truncated receptor that results from diminished use of the 5′ upstream promoter (12) and alternative transcription initiation from a cryptic promoter (13). Introduction of this pituitary tumor-derived FGFR4 isoform (ptd-FGFR4) yields a constitutively phosphorylated protein with transforming properties (11). That this tumor-derived isoform is functionally implicated in pituitary tumorigenesis is suggested from transgenic studies in which targeted expression of ptd-FGFR4 in the pituitary recapitulates pituitary adenomas with morphologic features that mimic human pituitary adenomas (11).

To date there has been no study of FGFR4 expression in a large series of human pituitary adenomas and little is known about the clinicopathological correlations of ptd-FGFR4 expression in these tumors. In this study, therefore, we investigated the expression of FGFR4 in a series of 137 human pituitary adenomas of the various types and in 10 normal pituitary glands and compared these data with patient nationality, tumor type, size, invasiveness, and the labeling index of the proliferation marker Ki-67 antigen.

Materials and Methods

Patients and tissue preparation

Ten specimens of normal anterior pituitary gland were obtained at autopsy within 4 h postmortem. These samples were subjected to thorough histologic examination of the entire pituitary gland to ensure absence of any detectable pathology. One hundred thirty-seven pituitary adenomas were obtained at the time of pituitary surgery after written informed consent. These included 102 tumors from Tokushima University Hospital (Tokushima, Japan) and Toranomon Hospital (Tokyo, Japan) and 35 tumors from the University of Toronto (Toronto, Canada). All tissues were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. In some cases, a small amount of tumor was snap frozen in liquid nitrogen and stored at −70 C. Histological examination and immunohistochemistry for anterior pituitary hormones were performed using fixed tissues as previously described (1, 6, 10). The clinical and morphologic characteristics of the tumors examined are summarized in Table 1.

The tumors included GH-producing adenomas (29 cases), GH- and prolactin (PRL)-producing adenomas (8 cases), PRL-producing adenomas (27 cases), ACTH-producing adenomas with Cushing syndrome (17 cases), silent ACTH-producing adenomas (13 cases), FSH/LH-producing adenomas (23 cases), acidophil stem cell adenomas (4 cases), and null cell adenomas characterized by lack of immunoreactivity for all anterior pituitary hormones (16 cases). Tumor size and invasiveness were defined on the basis of preoperative radiological findings and operative findings using the modified Hardy criteria (14). Microadenomas were defined as tumors less than 10 mm in maximal diameter; in contrast, macroadenomas were larger than 10 mm. Tumor invasion was defined by evidence of bony destruction and/or tumor within the sphenoid and/or cavernous sinuses and/or into brain that was confirmed at the time of surgery. Thus, there were 39 microadenomas and 98 macroadenomas, and 86 noninvasive and 51 invasive adenomas. To examine potential geographic variations, these cases were divided into two groups based on the site of surgery in Japan or Canada (Table 2).

Materials for cell culture, protein isolation, and Western blotting

Rat pituitary GH4 cells were propagated in DMEM (Life Technologies, Inc., Grand Island, NY) with high glucose supplemented with 10% fetal bovine serum (Sigma, Oakville, Canada), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Twenty-four hours before transfection, cells were plated with DMEM containing 10% serum.

Plasmid constructs and transfections

Plasmids containing the coding region of human wild-type FGFR4 or ptd-FGFR4 cDNA were prepared and transfected as previously described (11).

Western blotting

Protein was extracted from frozen tissues and concentrations were determined by the protein method (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (50 μg) from whole-cell lysates were solubilized in 2× sodium dodecyl sulfate-sample buffer and separated on sodium dodecyl sulfate-8% polyacrylamide gel and transferred to nitrocellulose. Blots were incubated with polyclonal affinity-purified rabbit antiserum directed against the carboxy terminus of human FGFR4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:1500) as previously described (12). This antibody recognizes the common C terminus yielding a 110/90-kDa doublet corresponding to wild-type FGFR4 and a 65-kDa truncated product corresponding to ptd-FGFR4.

TABLE 1. Clinical and pathological characteristics in 137 pituitary adenomas

	No. of cases	Tumor size		Invasiveness	
		Macroadenoma	Microadenoma	Invasive	Noninvasive
Gender [n (%)]					
Male	65	55 (84.6)[a]	10 (15.4)	32 (49.2)[b]	33 (50.8)
Female	72	43 (59.7)[a]	29 (40.3)	19 (26.4)[b]	53 (73.6)
Tumor type [*n* (%)]					
GH (acromegaly)	29	24 (82.8)	5 (17.2)	16 (55.2)	13 (44.8)
GH/PRL (acromegaly)	8	3 (37.5)	5 (62.5)	1 (12.5)	7 (87.5)
PRL (prolactinoma)	27	16 (59.3)	11 (40.7)	11 (40.7)	16 (59.3)
ACTH (Cushing)	17	7 (41.2)	10 (58.8)	2 (11.8)	15 (88.2)
ACTH (silent)	13	9 (69.2)	4 (30.8)	5 (38.5)	8 (61.5)
ASCA (NF or hyper-PRL)	4	3 (75)	1 (25)	2 (50)	2 (50)
FSH/LH (NF)	23	22 (95.7)	1 (4.3)	11 (47.8)	12 (52.2)
Null cell (NF)	16	14 (87.5)	2 (12.5)	3 (18.7)	13 (81.3)
Total	137	98	39	51	86

GH, GH cell adenoma; GH/PRL, mixed GH cell-PRL cell adenoma; PRL, PRL cell adenoma; ACTH, ACTH cell adenoma; ASCA, acidophil stem cell adenoma; hyper-PRL, hyperprolactinemia; FSH/LH, FSH/LH cell adenoma; null cell, null cell adenoma; NF, nonfunctional.

[a] $P < 0.001$.

[b] $P < 0.01$.

TABLE 2. Cytoplasmic expression of FGFR4 in 137 pituitary adenomas from Japan and Canada

Endocrinological tumor type	Pathological tumor type	No. of cases	Cytoplasmic expression of FGFR4					Positive cases (≥ 1+)	High-expression cases (≥ 2+)
			–	1+	2+	3+	4+		
Japanese cases									
NF	FSH/LH	17	2	1	5	5	4	15 (88.2%)	14 (82.3%)
NF	Null cell	15	2	3	7	2	1	13 (86.6%)	10 (66.6%)
Silent	ACTH	11	2	4	4	0	1	9 (81.8%)	5 (45.4%)
Acromegaly	GH	18	7	8	3	0	0	11 (61.1%)	3 (16.6%)
Cushing	ACTH	13	6	2	0	1	4	7 (53.8%)	5 (38.4%)
Acromegaly	GH/PRL	8	6	1	1	0	0	2 (25%)	1 (12.5%)
Prolactinoma	PRL	20	18	2	0	0	0	2 (10%)	0
Total (Japan)		102	43	21	20	8	10	59 (57.8%)	38 (37.2%)
Canadian cases									
NF	FSH/LH	6	0	1	3	2	0	6 (100%)	5 (83.3%)
NF	Null cell	1	0	0	0	1	0	1 (100%)	1 (100%)
Silent	ACTH	2	0	1	1	0	0	2 (100%)	1 (50%)
Acromegaly	GH	11	3	2	5	1	0	8 (72.7%)	6 (54.5%)
Cushing's	ACTH	4	2	2	0	0	0	4 (50%)	0
Hyper-PRL	ASCA	4	2	2	0	0	0	2 (50%)	0
Prolactinoma	PRL	7	6	1	0	0	0	1 (14.2%)	0
Total (Canada)		35	13	9	9	4	0	22 (62.8%)	13 (37.1%)
Total (combined)		137	56	30	29	12	10	81 (59.1%)	51 (37.2%)

FSH/LH, FSH/LH cell adenoma; null cell, null cell adenoma; ACTH, ACTH cell adenoma; GH, GH cell adenoma; ASCA, acidophil stem cell adenoma; GH/PRL, mixed GH cell-PRL cell adenoma; PRL, PRL cell adenoma; NF, nonfunctional; hyper-PRL, hyperprolactinemia.

Immunohistochemistry

FGFR4 and Ki-67 antigen immunolocalization based on the labeled streptavidin biotin method were performed on sections from representative blocks of paraffin-embedded tissues used for pathology diagnosis. After deparaffinization and antigen retrieval using an autoclave oven technique, sections were incubated at 4 C overnight with Ki-67 mouse monoclonal antibody (clone MIB-1, 1:50 dilution; Immunotech, Marseilles, France) or with a polyclonal antiserum that recognizes the C-terminal tail of FGFR4, noncross-reactive with three other FGFRs (Santa Cruz Biotechnology Inc., C-16, 1:500 dilution). The biotinylated link antibody and peroxidase-labeled streptavidin were applied for 1 h each at room temperature. Sections were washed thoroughly in PBS between each of the immunostaining procedures. Antigen-antibody complexes were detected using the 3, 3′-diaminobenzidine/H_2O_2 reaction. The slides were counterstained lightly with hematoxylin or 1% methyl green and mounted for microscopic examination. The applications of these antibodies on formalin-fixed, paraffin-embedded materials, and their specificity have been described elsewhere (10, 11, 15). Sections incubated in PBS without the primary antibody served as negative controls. Furthermore, the specificity of all reactions for FGFR4 was verified by replacing the primary antibody with normal serum, examining negative control tissues, and preabsorbing primary antibody with purified FGFR4 peptide (Santa Cruz, antibody: peptide/6:1).

Assessment of FGFR4 and Ki-67 immunoreactivity

Each slide was examined by an observer blinded to the diagnosis and reviewed by a second blinded observer. Positive expression of FGFR4 was defined as exclusively strong staining. The sections were graded as to whether there was definite positive staining for FGFR4; a semiquantitative grade form (1+ to 4+) was then attributed to the positive cases to reflect the proportion of positive cells (1+: low-density staining, 2+: moderate-density staining, 3+: dense staining, and 4+: very dense staining). The Ki-67 labeling index (LI) was determined by counting the number of positive cells in a total of 1000 tumor cells observed in several representative high-power fields (×400).

Statistical analyses

Using StatView J-4.5 software (Abacus Concepts, Inc., Berkeley, CA), Mann-Whitney *U* test, χ^2 test, and Spearman's correlation coefficient by rank were performed to determine the significance of associations between different variables. The level of statistical significance was $P < 0.05$.

Results

Clinical data

As indicated in Table 1, macroadenomas and invasive adenomas were significantly more frequent in male (84.6, 49.2%, respectively) than in female patients (59.7, 26.4%, respectively) ($P < 0.001$, $P < 0.01$, respectively). However, there were no significant correlations among tumor size, invasion, and mean age (data not shown) at operation.

Immunohistochemical analysis of FGFR4 and Ki-67 LI

There was no appreciable immunostaining of FGFR4 or Ki-67 antigen in all 10 normal pituitary sample tissues. In pituitary adenomas, positive immunoreactivity for FGFR4 showed a cytoplasmic pattern without membrane or nuclear localization. When present, this reactivity was typically very intense throughout the cytoplasm of tumor cells of various types (Fig. 1, A–D). In addition, no immunoreactivity for FGFR4 was observed in nontumorous cells contained in surgical specimens. The data from the Japanese cases are summarized in Table 2. Cytoplasmic expression of FGFR4 was detected in 59 pituitary adenomas (57.8%). Thirty-eight of these (37.2%) showed high-level expression of FGFR4. FGFR4 reactivity was observed in FSH/LH cell adenomas (15 cases, 88.2%), null cell adenomas (13 cases, 86.6%), silent ACTH cell adenomas (nine cases, 81.8%), GH cell adenomas (11 cases, 61.1%), ACTH cell adenomas with Cushing syndrome (seven cases, 53.8%), and GH/PRL cell adenomas (two cases, 25%). FGFR4 reactivity was found only rarely in PRL cell adenomas (two cases, 10%). The frequency of high levels of FGFR4 expression within the individual tumor types displayed a similar trend to that of positive cases (Table 2).

Similarly, 22 of 35 (62.8%) pituitary adenomas from Canadian patients showed positive expression of FGFR4. Of these, 13 (37.1%) were determined to have high expression of

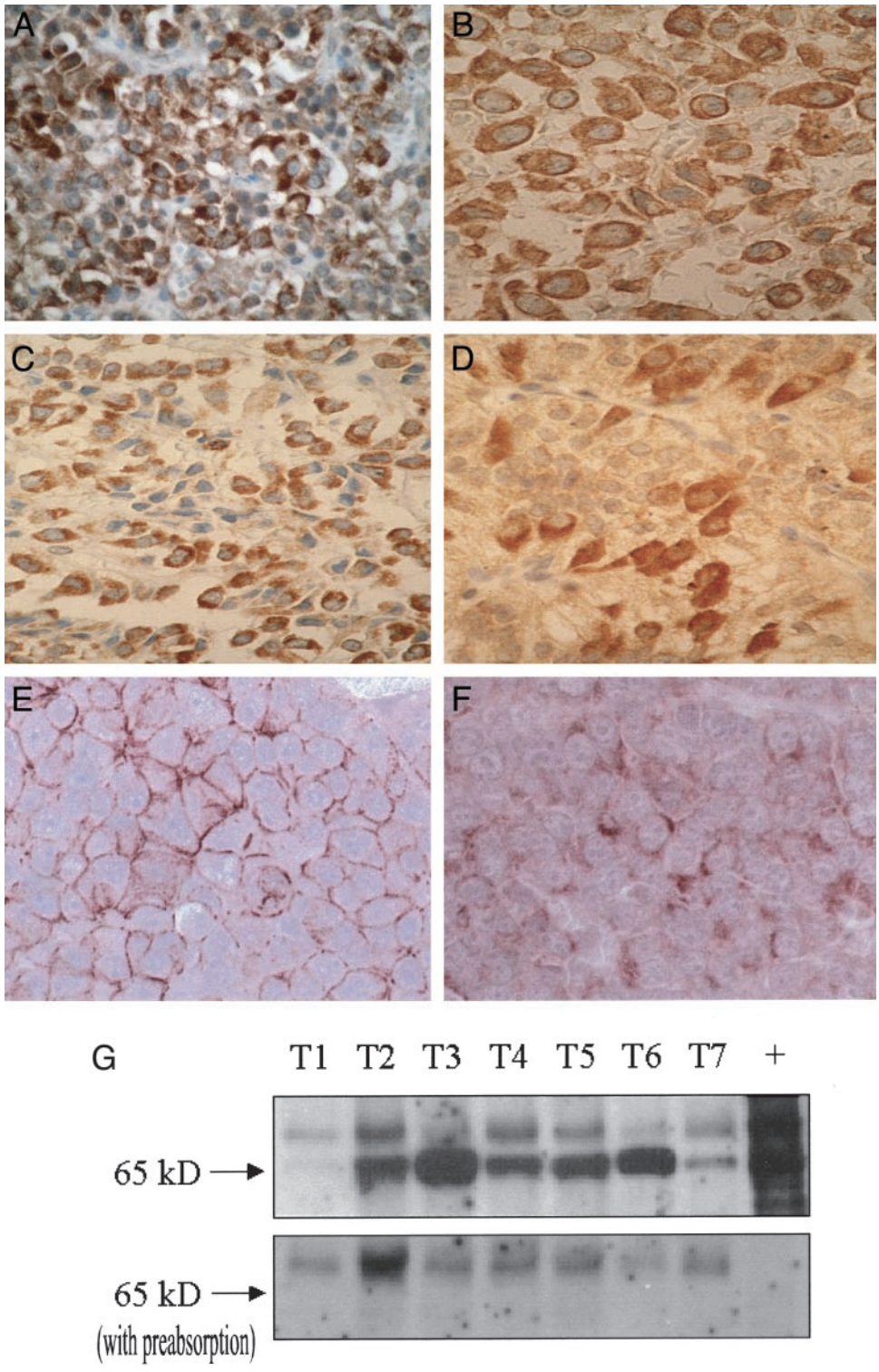

FIG. 1. Detection of FGFR4 reactivity in pituitary cells. A–D, Immunohistochemical localization of FGFR4 in human pituitary adenomas. C-terminal FGFR4 immunoreactivity is seen in the cytoplasm of tumor cells in varied tumor types. A, GH cell adenoma. B, ACTH cell adenoma. C, FSH/LH adenoma. D, Null cell adenoma. The immunoreactivity of FGFR4 is variable but always very intense throughout the cytoplasm (original magnification ×50–100). E and F, Immunohistochemical localization of FGFR4 and ptd-FGFR4 in transfected pituitary cells. Rat pituitary tumor-derived GH4 cells were stably transfected with full-length FGFR4 (E) or ptd-FGFR4 (F). Immunocytochemical examination was performed using an antibody that recognizes the C terminus of FGFR4. Note the predominant cytoplasmic pattern of staining in ptd-FGFR4 transfected cells, compared with the membrane reactivity in wild-type FGFR4-transfected cells. G, Western blot detection of FGFR4 in human pituitary adenomas. Protein lysates from seven human gonadotroph adenomas were electrophoresed and blotted with an antibody that recognizes the C terminus of FGFR4. The extreme right lane contains a positive control from HEK293 cells transiently transfected with ptd-FGFR4 (*upper panel*). Preabsorption of the primary antibody with purified antigen abolishes the lower 65-kDa protein (*lower panel*). The band migrating just above 65 kDa is a nonspecific species that is not abolished by preabsorption.

FGFR4. The frequency of FGFR4 positivity and high expression in each tumor type are detailed in Table 2.

In 110 Japanese and Canadian cases (excluding 27 PRL cell adenomas because of the low frequency of FGFR4 positive cases), the overall level of FGFR4 expression was significantly higher in macroadenomas (n = 82) than microadenomas (n = 28) ($P = 0$ 0.02, Fig. 2A). High expression of FGFR4 was observed more frequently in macroadenomas (44 cases, 54%) than in microadenomas (seven cases, 25%) ($P < 0.05$, Table 3). The overall expression of FGFR4 was not significantly different between invasive adenomas (n = 40) and noninvasive adenomas (n = 70) (Fig. 2B). However, high expression of FGFR4 tended to be present with greater frequency among cases with proven invasiveness (55%; n = 40),

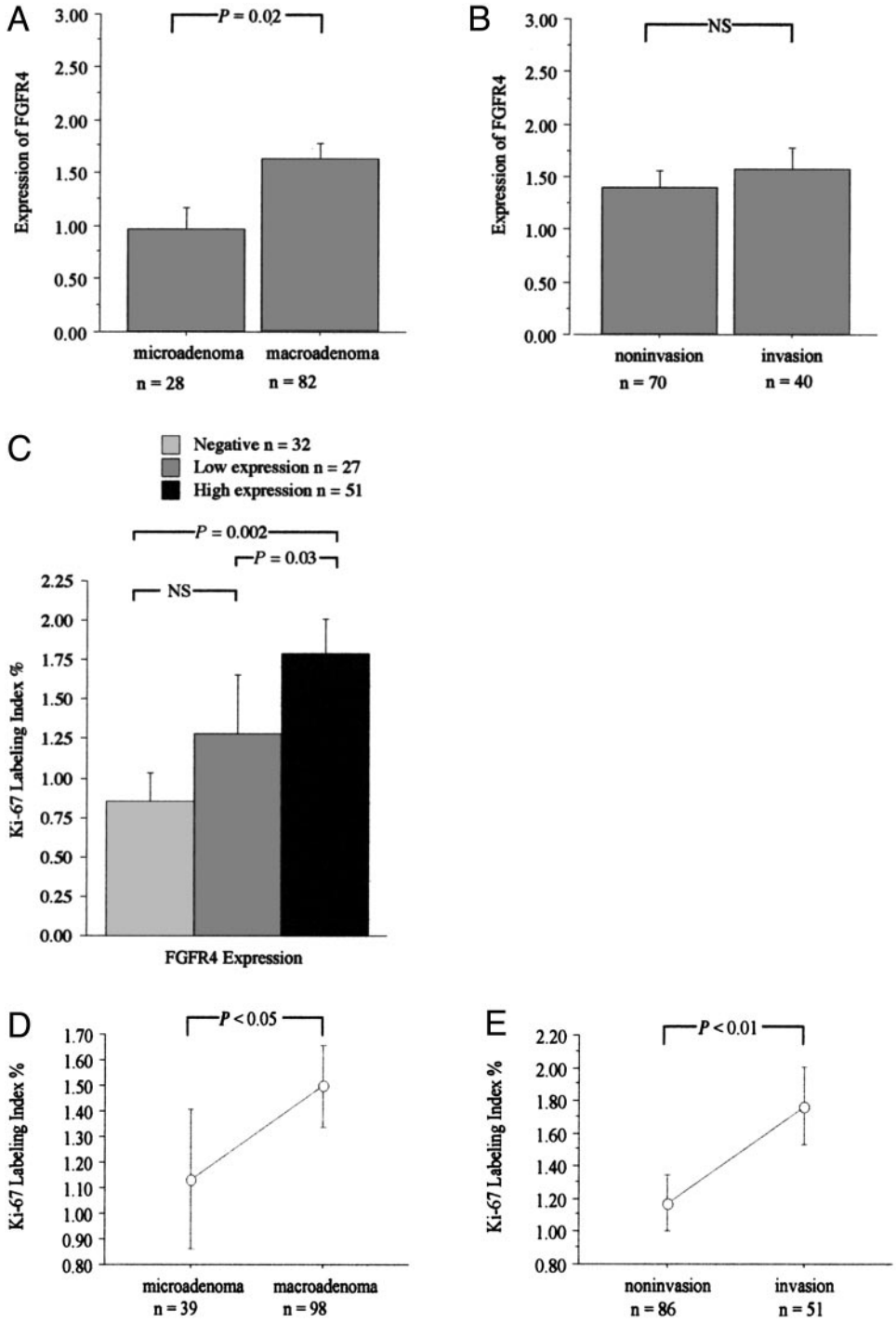

FIG. 2. Cytoplasmic expression levels of FGFR4 and Ki-67 LI in pituitary adenomas. A, The expression levels of FGFR4 in macroadenomas are significantly higher than those in microadenomas ($P = 0.02$). B, The expression level of FGFR4 in invasive adenomas is not significantly different from noninvasive adenomas. C, The mean Ki-67 LI (percent) was significantly higher in tumors that express high levels of FGFR4 than in the low FGFR4-expressing group, which in turn was higher than in the FGFR4-negative group of tumors ($P = 0.03$; $P = 0.002$, respectively). D and E, Ki-67 LI was higher in macroadenomas and invasive adenomas than in microadenomas and noninvasive adenomas ($P < 0.05$; $P < 0.01$, respectively).

compared with those that did not show features of invasiveness (41%; n = 70) (Table 3).

Immunoreactivity for Ki-67 antigen was detected in pituitary adenomas by positive nuclear staining for the antibody MIB-1. In 110 Japanese and Canadian cases (excluding 27 PRL cell adenomas), the mean Ki-67 LI (%) was significantly higher in adenomas that had high expression of FGFR4 (1.78 ± 0.2, n = 51) than in adenomas with low FGFR4 expression (1.28 ± 0.44, n = 27; $P = 0.03$) or in FGFR4-negative adenomas (0.86 ± 0.18, n = 32; $P = 0.002$) (Fig. 2C). Furthermore, the Ki-67 LI was higher in adenomas with low FGFR4 expression than in FGFR4-negative adenomas, although this trend did not reach statistical significance (Mann-Whitney *U* test, $P = 0.7$). By Spearman's rank correlation analysis, there was a significant positive correlation between Ki-67 LI and degree of FGFR4 expression ($P = 0.002$). In addition, positive correlations between high Ki-67 LI, tumor size, and invasiveness were identified. The mean Ki-67 LI was 1.50 ± 0.16 in macroadenomas (n = 98) *vs.* 1.13 ± 0.27 in microadenomas (n = 39) ($P < 0.05$). Furthermore, the

TABLE 3. Relationship between FGFR4 expression and tumor size and invasiveness in 110 pituitary adenomas

IHC of FGFR4	Tumor size		Invasiveness	
	Macroadenoma	Microadenoma	Invasive	Noninvasive
High-expression (≥ 2+)	44 (54%)	7 (25%)[a]	22 (55%)	29 (41%)
Low density (1+)	18 (22%)	9 (32%)	7 (18%)	20 (29%)
Negative (−)	20 (24%)	12 (43%)	11 (27%)	21 (30%)
Total	82 (100%)	28 (100%)	40 (100%)	70 (100%)

IHC, Immunohistochemistry.
[a] $P < 0.05$.

Ki-67 index was higher in invasive adenomas (1.76 ± 0.23; n = 51), compared with noninvasive (1.17 ± 0.17; n = 86; $P < 0.01$) (Fig. 2, D and E).

ptd-FGFR4 but not wild-type FGFR4 results in cytoplasmic pituitary staining

To investigate the nature of FGFR4 cytoplasmic staining in primary human pituitary adenomas, we compared the expression pattern of transfected ptd-FGFR4 with wild-type FGFR4 in pituitary cells. Because there is currently no available transfectable human pituitary cell line, we used the well-described rat pituitary GH4 cells. Overexpression of hFGFR4 in these cells resulted in a membrane pattern of staining (Fig. 1E) similar to that reported in HEK 293 cells (11). In contrast, overexpression of ptd-FGFR4 resulted in a cytoplasmic pattern of expression similar to that in primary human adenomas (Fig. 1F).

To further determine the identity of FGFR4 isoform resulting in cytoplasmic staining, we examined primary human adenomas by Western blotting. In a subset of seven human gonadotroph adenomas that demonstrated strong cytoplasmic staining, gel electrophoresis yielded a 65-kDa FGFR4 immunoreactive band that comigrated with that from cells transfected with ptd-FGFR4 (Fig. 1G). For comparison, full-length wild-type FGFR4 was shown previously to migrate as a 110/90 kDa-doublet (11).

Discussion

FGFRs have been implicated in tumorigenesis and have been shown to be overexpressed in comparison with normal tissues in a wide range of neoplasms including malignant melanoma, prostate cancer, glioblastoma multiforme, malignant astrocytoma, malignant salivary gland tumor, thyroid neoplasms, breast cancer, pancreatic cancer, and gastric carcinoma (16–24). Alterations in FGFR4 expression have been reported in breast cancer, ovarian tumors, pancreatic carcinoma, gastric carcinoma, and malignant astrocytoma (24–27).

FGFRs can be involved in tumorigenesis by overexpression, but they can also be implicated due to structural alterations. A truncated form of FGFR1 with only two Ig-like domains is overexpressed in some central nervous system glioblastomas (28). FGFR2 exon switching has been observed to accompany prostate cell transformation (29). Activating point mutations of FGFRs have been implicated in developmental defects such as skeletal dysplasias (30) and craniosynostotic syndromes (31, 32); in these cases, point mutations result in ligand-independent activation of FGFRs (33). Recently somatic mutations in FGFR2 and FGFR3 have been documented in human colorectal, bladder, and cervical malignancies (34, 35)

FGFR4 has not been considered a potent mitogen (36); however, other data suggest that it increases DNA synthesis as effectively as FGFR1 (37). Accelerated tumor progression and increased cellular motility have recently been associated with a naturally occurring FGFR4 polymorphism identified in patients with breast and colorectal cancers (38). Furthermore, FGFR4 overexpression mediated by hepatic nuclear factor 1α has been identified in pancreatic cancer (39).

We have previously shown that human pituitary adenomas express a novel N-terminally truncated isoform of FGFR4; this ptd-FGFR4 isoform has a distinctive cytoplasmic residence and is constitutively phosphorylated on tyrosine residues (11). Expression of ptd-FGFR4 in NIH3T3 cells results in increased cell proliferation and targeted expression of ptd-FGFR4 in the pituitary of transgenic mice results in tumor formation (11). Hence, we suggested that FGFR4 protein may be involved in pituitary tumorigenesis. Consistent with this hypothesis, we identified cytoplasmic reactivity indicative of FGFR4 expression in a majority of pituitary adenomas. In contrast, we found no appreciable immunostaining of FGFR4 in nontumorous pituitary tissues. These data support previous reports that this structural alteration of FGFR4, not wild-type FGFR4, may be more functionally relevant in pituitary neoplasia (10, 11).

The cytoplasmic localization of ptd-FGFR4 in transfected GH4 cells is now shown to be distinct from the membrane pattern seen in GH4 cells transfected with wild-type FGFR4. The pattern seen in human pituitary adenomas resembles that in cells expressing ptd-FGFR4. In addition, we have confirmed using Western blotting that human pituitary adenomas express a truncated 65-kDa protein that is immunoreactive for FGFR4; this size is the same as that shown for ptd-FGFR4 (11).

The expression of ptd-FGFR4 in pituitary adenomas did not correlate with the patient's nationality; we found FGFR4 positivity in 59 of 102 (57.8%) pituitary adenomas from Japanese patients and in 22 of 35 (62.8%) Canadian cases.

Our current data expand on our earlier description of the wide expression of FGFR4 in adenomas of different types (10, 11). Interestingly, the expression of FGFR4 was found at a high frequency in all hormone-containing and hormone-negative adenomas with the unique exception of PRL-containing adenomas; this included monohormonal lactotroph adenomas and plurihormonal GH- and PRL-containing adenomas in both Japanese and Canadian populations. The

reason for this lack of significant expression of FGFR4 in PRL-producing adenomas is not known but suggests a different set of pathogenetic mechanisms in their development. For example, *hst*/FGF-4 may have an important role in the pathogenesis of these adenomas (40). It should be emphasized that FGFR4 is not a specific receptor for FGF-4 signaling (41). Furthermore, other growth factor families including the TGFα/epithelial growth factor receptor family may play a critical role in PRL cell tumorigenesis (42). Thus, the pathogenesis of PRL-producing adenomas may be distinct from other types of pituitary adenoma.

In previous studies no correlation between expression of ptd-FGFR4 and clinicopathological data was identified (10, 11). These studies examined smaller numbers of cases. In this study we found a highly significant correlation between high levels of expression of FGFR4 and tumor size. FGFR4 showed a higher level of expression in macroadenomas than in microadenomas ($P = 0.02$), and high levels of expression of FGFR4 were observed more frequently in macroadenomas than in microadenomas ($P < 0.05$). We identified a significant correlation between the Ki-67 LI, tumor size, and the presence of invasion. As noted by some groups previously (15), we also identified a greater frequency of larger and more invasive tumors in male patients. The Ki-67 LI in macroadenomas and invasive adenomas was consistently higher than in microadenomas and noninvasive adenomas ($P < 0.05$, $P < 0.01$, respectively). These findings are in agreement with those in earlier reports (15, 43). Furthermore, we identified a correlation between expression of FGFR4 and Ki-67 LI in the vast majority of adenomas. The Ki-67 LI was higher in adenomas that had high levels of FGFR4 expression than in the other two groups that had low levels or were negative for FGFR4 ($P = 0.002$, $P = 0.03$, respectively). The Ki-67 LI also showed a higher level in tumors with low expression of FGFR4 than in the FGFR4-negative group. This finding, indicating a graduated increase in Ki-67 LI that correlates with FGFR4 expression, suggests that FGFR4 may be strongly associated with proliferation of pituitary tumor cells and that its increased expression may give tumors a growth advantage.

In conclusion, we found no appreciable immunoreactivity for FGFR4 in normal adult anterior pituitary glands. Cytoplasmic expression of FGFR4, consistent with expression of ptd-FGFR4, is common in the major types of pituitary adenomas with the singular exception of PRL-producing adenomas. High levels of expression of ptd-FGFR4 correlate with larger tumor size and higher Ki-67 proliferation index. Our current data support the premise that FGFR4 expression is an important and frequent event in pituitary tumorigenesis that plays an important role in cell proliferation and tumor progression. The lack of strict correlation with tumor invasiveness, however, suggests that this behavior likely involves FGFR4-independent and/or FGFR4-cooperative mechanisms.

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References

1. **Asa SL** 1998 Tumors of the pituitary gland. Atlas of tumor pathology. Series fascicle 22. Washington, DC: Armed Forces Institute of Pathology
2. **Asa SL, Ezzat S** 2002 The pathogenesis of pituitary tumors. Nat Rev Cancer 2:836–849
3. **Vallar L, Spada A, Giannattasio G** 1987 Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature 330:566–568
4. **Karga HJ, Alexander JM, Hedley-Whyte ET, Klibanski A, Jameson JL** 1992 Ras mutations in human pituitary tumors. J Clin Endocrinol Metab 174:914–919
5. **Alexander JM** 2001 Tumor suppressor loss in pituitary tumors. Brain Pathol 11:342–355
6. **Ezzat S, Smyth HS, Ramyar L, Asa SL** 1995 Heterogenous *in vivo* and *in vitro* expression of basic fibroblast growth factor by human pituitary adenomas. J Clin Endocrinol Metab 80:878–884
7. **Ezzat S** 2001 The role of hormones, growth factors and their receptors in pituitary tumorigenesis. Brain Pathol 11:356–370
8. **Powers CJ, McLeskey SW, Wellstein A** 2000 Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7:165–197
9. **Givol D, Yayon A** 1992 Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. FASEB J 6:3362–3369
10. **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
11. **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
12. **Ezzat S, Yu S, Asa SL** 2003 Ikaros isoforms in human pituitary tumors: distinct localization, deacetylation, and activation of the 5′ FGFR4 promoter. Am J Pathol 163:1177–1184
13. **Yu S, Asa SL, Weigel RJ, Ezzat S** 2003 Pituitary tumor AP-2α recognizes a cryptic promoter in intron 4 of fibroblast growth factor receptor 4. J Biol Chem 278:19597–19602
14. **Bradley KJ, Wass JA, Turner HE** 2003 Non-functioning pituitary adenomas with positive immunoreactivity for ACTH behave more aggressively than ACTH immunonegative tumours but do not recur more frequently. Clin Endocrinol (Oxf) 58:59–64
15. **Delgrange E, Trouillas J, Maiter D, Donckier J, Tourniaire J** 1997 Sex-related difference in the growth of prolactinomas: a clinical and proliferation marker study. J Clin Endocrinol Metab 82:2102–2107
16. **Ahmed NU, Ueda M, Ito A, Ohashi A, Funasaka Y, Ichihashi M** 1997 Expression of fibroblast growth factor receptors in naevus-cell naevus and malignant melanoma. Melanoma Res 7:299–305
17. **Giri D, Ropiquet F, Ittmann M** 1999 Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. Clin Cancer Res 5:1063–1071
18. **Morrison RS, Yamaguchi F, Bruner JM, Tang M, McKeehan W, Berger MS** 1994 Fibroblast growth factor receptor gene expression and immunoreactivity are elevated in human glioblastoma multiforme. Cancer Res 54:2794–2799
19. **Yamaguchi F, Saya H, Bruner JM, Morrison RS** 1994 Differential expression of two fibroblast growth factor-receptor genes is associated with malignant progression in human astrocytomas. Proc Natl Acad Sci USA 91:484–488
20. **Myoken Y, Myoken Y, Okamoto T, Sato JD, Kan M, McKeehan WL, Nakahara M, Takada K** 1996 Immunohistochemical study of overexpression of fibroblast growth factor-1 (FGF-1), FGF-2, and FGF receptor-1 in human malignant salivary gland tumours. J Pathol 178:429–436
21. **Shingu K, Fujimori M, Ito K, Hama Y, Kasuga Y, Kobayashi S, Itoh N, Amano J** 1998 Expression of fibroblast growth factor-2 and fibroblast growth factor receptor-1 in thyroid diseases: difference between neoplasms and hyperplastic lesions. Endocr J 45:35–43
22. **Yoshimura N, Sano H, Hashiramoto A, Yamada R, Nakajima H, Kondo M, Oka T** 1998 The expression and localization of fibroblast growth factor-1 (FGF-1) and FGF receptor-1 (FGFR-1) in human breast cancer. Clin Immunol Immunopathol 89:28–34
23. **Kornmann M, Beger HG, Korc M** 1998 Role of fibroblast growth factors and their receptors in pancreatic cancer and chronic pancreatitis. Pancreas 17:169–175
24. **Shin EY, Lee BH, Yang JH, Shin KS, Lee GK, Yun HY, Song YJ, Park SC, Kim EG** 2000 Up-regulation and co-expression of fibroblast growth factor receptors in human gastric cancer. J Cancer Res Clin Oncol 126:519–528
25. **Jaakkola S, Salmikangas P, Nylund S, Partanen J, Armstrong E, Pyrhonen S, Lehtovirta P, Nevanlinna H** 1993 Amplification of fgfr4 gene in human breast and gynecological cancers. Int J Cancer 54:378–382
26. **Ohta T, Yamamoto M, Numata M, Iseki S, Tsukioka Y, Miyashita T, Kayahara M, Nagakawa T, Miyazaki I, Nishikawa K, Yoshitake Y** 1995 Expression of basic fibroblast growth factor and its receptor in human pancreatic carcinomas. Br J Cancer 72:824–831
27. **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
28. **Eisemann A, Ahn JA, Graziani G, Tronick SR, Ron D** 1991 Alternative

splicing generates at least five different isoforms of the human basic-FGF receptor. Oncogene 6:1195–1202
29. **Yan G, Fukabori Y, McBride G, Nikolaropolous S, McKeehan WL** 1993 Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy. Mol Cell Biol 13:4513–4522
30. **Webster MK, D'Avis PY, Robertson SC, Donoghue DJ** 1996 Profound ligand-independent kinase activation of fibroblast growth factor receptor 3 by the activation loop mutation responsible for a lethal skeletal dysplasia, thanatophoric dysplasia type II. Mol Cell Biol 16:4081–4087
31. **Mangasarian K, Li Y, Mansukhani A, Basilico C** 1997 Mutation associated with Crouzon syndrome causes ligand-independent dimerization and activation of FGF receptor-2. J Cell Physiol 171:117–125
32. **Chun K, Siegel-Bartelt J, Chitayat D, Phillips J, Ray PN** 1998 FGFR2 mutation associated with clinical manifestations consistent with Antley-Bixler syndrome. Am J Med Genet 77:219–224
33. **Neilson KM, Friesel R** 1996 Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane, and kinase domains. J Biol Chem 271:25049–25057
34. **Cappellen D, De Oliveira C, Ricol D, de Medina S, Bourdin J, Sastre-Garau X, Chopin D, Thiery JP, Radvanyi F** 1999 Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet 23:18–20
35. **Jang JH, Shin KH, Park JG** 2001 Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. Cancer Res 61:3541–3543
36. **Wang JK, Gao G, Goldfarb M** 1994 Fibroblast growth factor receptors have different signaling and mitogenic potentials. Mol Cell Biol 14:181–188
37. **Vainikka S, Joukov V, Wennstrom S, Bergman M, Pelicci PG, Alitalo K** 1994 Signal transduction by fibroblast growth factor receptor-4 (FGFR-4). Comparison with FGFR-1. J Biol Chem 269:18320–18326
38. **Bange J, Prechtl D, Cheburkin Y, Specht K, Harbeck N, Schmitt M, Knyazeva T, Muller S, Gartner S, Sures I, Wang H, Imyanitov E, Haring HU, Knayzev P, Iacobelli S, Hofler H, Ullrich A** 2002 Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. Cancer Res 62:840–847
39. **Shah RN, Ibbitt JC, Alitalo K, Hurst HC** 2002 FGFR4 overexpression in pancreatic cancer is mediated by an intronic enhancer activated by HNF1α. Oncogene 21:8251–8261
40. **Shimon I, Melmed S** 1997 Genetic basis of endocrine disease: pituitary tumor pathogenesis. J Clin Endocrinol Metab 82:1675–1681
41. **Bellosta P, Iwahori A, Plotnikov AN, Eliseenkova AV, Basilico C, Mohammadi M** 2001 Identification of receptor and heparin binding sites in fibroblast growth factor 4 by structure-based mutagenesis. Mol Cell Biol 21:5946–5957
42. **McAndrew J, Paterson AJ, Asa SL, McCarthy KJ, Kudlow JE** 1995 Targeting of transforming growth factor-α expression to pituitary lactotrophs in transgenic mice results in selective lactotroph proliferation and adenomas. Endocrinology 136:4479–4488
43. **Thapar K, Kovacs K, Scheithauer BW, Stefaneanu L, Horvath E, Pernicone PJ, Murray D, Laws Jr ER** 1996 Proliferative activity and invasiveness among pituitary adenomas and carcinomas: an analysis using the MIB-1 antibody. Neurosurgery 38:99–107

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