Received: 17.11.2013 / **Accepted:** 28.03.2014
DOI: 10.5137/1019-5149.JTN.9812-13.1

Increase in Folate Receptor Alpha Expression in Nonfunctional Pituitary Adenomas

Fonksiyonel Olmayan Hipofiz Adenomlarında Folat Reseptörü Alfa Ekspresyonunda Artış

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ABSTRACT

AIM: Human pituitary adenomas account for 10% of intracranial tumours and occur in about 20% of the population. They cause hypopituitarism or the compression of adjacent regional structures. However, little is known about the molecular pathogenesis that contributes to the development of these tumours.

MATERIAL and METHODS: In this study, we investigated the relationship between the expression level of folate receptor alpha and some clinical factors (endocrine, age, gender, repeated operation or not, mean diameter of tumour and invasiveness) of pituitary adenomas. Real-time fluorescent quantitative reverse transcriptase polymerase chain reaction was used to determine the expression of folate receptor alpha mRNA in pituitary adenomas and normal pituitaries. Folate receptor alpha protein levels were quantified using Western blot analysis and Immunohistochemistry.

RESULTS: We found folate receptor alpha mRNA and protein were significantly upregulated in clinically nonfunctional pituitary adenomas compared to functional tumours. For nonfunctional tumours, folate receptor alpha expression was much higher in the invasive group than in non-invasive group.

CONCLUSION: These results suggest that the overexpression of folate receptor alpha mRNA and protein by nonfunctional pituitary adenomas may facilitate the growth of these tumours. Potentially, this finding could be exploited to develop innovative molecular targeted diagnosis and treatment for human nonfunctional pituitary adenomas.

KEYWORDS: Pituitary adenoma, Folate receptor alpha, Invasiveness, Targeted therapy

ÖZ

AMAÇ: İnsan hipofiz adenomları intrakraniyal tümörlerin %10'unu oluşturur ve popülasyonun yaklaşık %20'sinde görülür. Hipopitüitarizm veya bölgesel komşu yapıların kompresyonuna yol açarlar. Ancak bu tümörlerin gelişmesine katkıda bulunan moleküler patogenez hakkında çok az bilgi vardır.

YÖNTEM ve GEREÇLER: Çalışmada hipofiz adenomlarında folat reseptörü alfa ekspresyonu seviyesi ile bazı klinik faktörler (endokrin, yaş, cinsiyet, tekrarlanan ameliyat olup olmaması, tümörün ortalama çapı ve invaziv olup olmaması) arasındaki ilişki incelendi. Folat reseptörü alfa mRNA'sının hipofiz adenomları ve normal hipofizlerde ekspresyonunu belirlemek üzere gerçek zamanlı floresan kantitatif revers transkriptaz polimeraz zincir reaksiyonu kullanıldı. Folat reseptörü alfa protein seviyeleri Western blot analizi ve immünohistokimya teknikleri kullanımıyla ölçüldü.

BULGULAR: Folat reseptörü alfa mRNA ve proteininin fonksiyonel tümörlere göre, klinik olarak nonfonksiyonel hipofiz adenomlarında önemli ölçüde yukarı düzenleme gösterdiğini bulduk. Nonfonksiyonel tümörlerde folat reseptörü alfa ekspresyonu invaziv grupta, noninvaziv gruba göre çok daha yüksekti.

SONUÇ: Elde edilen sonuçlar folat reseptörü alfa mRNA ve proteininin nonfonksiyonel pitüiter adenomlar tarafından aşırı ekspresyonunun bu tümörlerin büyümesini kolaylaştırabileceğini düşündürmektedir. Bu bulgu potansiyel olarak insan nonfonksiyonel hipofiz adenomlarının yeni moleküler hedefe yönelik tanı ve tedavilerini geliştirmek üzere kullanılabilir.

ANAHTAR SÖZCÜKLER: Hipofiz adenomu, Folat reseptörü alfa, İnvazivlik, Hedeflenmiş tedavi

INTRODUCTION

Human pituitary adenomas comprise 10% of all brain tumours and occur in about 20% of the population. They present with symptoms as a result of their size and location, or the inappropriate expression of pituitary hormones, or both of these effects combined (1, 2, 9,12). Clinically functional pituitary tumours, such as growth hormone (GH), ACTH (adrenocorticorticotropic hormone) and prolactin (PRL) adenomas, generally secrete a significant amount of growth hormone, prolactin or adrenocorticotropic hormone and therefore give rise to severe life-threatening clinical

syndromes such as acromegaly or Cushing's disease or result in impaired reproduction. Approximately 30% of all pituitary adenomas are termed nonfunctional pituitary adenomas due to their lack of clinical hormone hypersecretion (13). Clinically, nonfunctional (NF) pituitary adenomas are usually large at the time of diagnosis because clinical features are not apparent until tumour mass effects occur. There are four treatment options for pituitary tumours: (i) observation, (ii) neurosurgery, (iii) radiation therapy, and (iv) medical therapy. Unfortunately, although several clinical studies have been carried out, currently there is no medical treatment or specific imaging technique for nonfunctional pituitary adenomas. It is therefore important to identify potential biological markers as diagnostic and therapeutic targets of NF pituitary tumours. Folate receptor (FR) is a glycoprotein that could efficiently transfer folic acid in that it binds to folate with high affinity. The FR genes are located on chromosome 11q13.3-13.5, a region frequently deleted in pituitary adenomas (7,14) and commonly amplified in carcinomas of the head and neck and breast (21). There are three isoforms of FR that include FRα, FRβ and FRγ. FRα and FRβ anchor in cell membranes with glycosyl-phosphatidylinositol (GPI) but type γ is primarily a secretory protein due to lack of an efficient signal for GPI modification (23). FRα is the major isoform mediating folate transport and many studies suggest that FRα is absent or poorly expressed in most normal tissues but is vastly over expressed in some tumours which are especially differentiated from epithelial tissues (19). Previously, C.O. Evans first reported that FRα is robustly upregulated in nonfunctional pituitary adenomas, but not in functional adenomas (5,6). In addition, the upregulation of FRα in pituitary tumour gonadotroph cells promotes cell proliferation in part through the NOTCH pathway (26). However, there is scarcely any specific research about the relationship between the expression level of FRα and some clinical factors (age, gender, repeated operation or not, mean diameter of tumour and invasiveness) of pituitary adenomas. The goal of this study was therefore to test the hypothesis that the expression of FRα is associated with some clinical factors of pituitary adenomas. Hence, we focused on the analysis of FRα mRNA and protein levels as well as the implications of these findings.

MATERIAL and METHODS

Patients and Tissue

Fifty-seven tumour tissue specimens from patients with pituitary adenomas were obtained from the Nanjing Medical University affiliated with Wuxi No.2 Hospital from 2009 to 2012. There were 32 NF adenomas, 9 ACTH adenomas, 8 GH adenomas and 8 PRL adenomas. Twenty-eight tumour samples were taken during the surgery and frozen in liquid nitrogen and then stored at -80°C until use. These were analysed with RTPCR, Western blot and immunohistochemical analysis. The remaining tumour samples embedded in paraffin were obtained from the pathology department of Wuxi No.2 Hospital and these were only analysed using immunohistochemistry staining. Three normal human pituitary tissue specimens were obtained through an organ donor program. As regards the mean diameter of the tumours, we classified NF tumours into three parts (there were 5 cases with a diameter of 4 cm) as determined by magnetic resonance imaging. Invasive adenomas were defined as Knosp classification grade III and IV. The infiltration of bones and cavernous sinus or encasement of sinus structures observed during surgery also indicated invasive adenomas. Besides, the patients were classified into 4 groups according to their ages (<40,40-49,50-59,>59). All samples were collected and handled according to national guidelines. All procedures were approved by Ethics Committee of Nanjing Medical University Affiliated with Wuxi No.2 Hospital.

RNA Extraction and Real-Time Quantitative PCR

Real-time fluorescent quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the expression of FRα mRNA in pituitary adenomas. Tumour tissues and normal pituitary samples were washed in ice-cold phosphate buffer solution (PBS) to remove blood and reduced to powder using a liquid nitrogen-cooled vessel. Total RNA was extracted from the normal pituitaries or pituitary adenomas using the Trizol reagent protocol (TianGen). The first-strand cDNA was synthesized by using PrimeScript first-strand cDNA synthesis kit (TaKaRa), and PCR was performed using the following primers: Human FRα (sense strand 5′- AGGTGCCATCTCTCCACAGT-3′, antisense strand 5′-GAGGACAAGTTGCATGAGCA-3′, cDNA amplicon size: 135 bp, Tm: 60°C). Human GAPDH (sense strand 5′-TTAAAAGCAGCCCTGGTGAC-3′, antisense strand 5′- CTCTGCTCCTCCTGTTCGAC-3′, amplicon size: 138 bp, Tm: 60°C). All PCR reactions were cycled using SYBR Green RT-PCR in an ABI Prism 7900 System (ABI Applied Biosystems) at 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 35 s. The specificity of the PCR reactions was determined from the dissociation curve analysis. The quantity of the specific genes obtained from standard curves was normalized to that of the GAPDH of the same sample. Fold difference was determined as the ratio of the normalized value of each tumour sample to the mean of the three normalized values of the normal pituitaries. All PCR reactions were performed at least in triplicate.

Western Blot Analysis

FRα protein levels were quantified using Western blot analysis. The tissues were washed twice in PBS and then cut into small pieces. Lysis buffer was added and the tissue was ground into semiliquid. After that, the lysates were centrifuged at 15,000 revolutions per minute under 4°C for 15 min twice. Equal amounts of total protein from each sample (10 μg) were loaded into a 12% acrylamide sodium dodecyl sulphate gel, and proteins were resolved by electrophoresis. The proteins were then transferred onto a polyvinylidene fluoride membrane that was blocked with 5% non-fat milk powder for 1 hour at room temperature. The membrane was incubated in a FRα antibody (Epitomics, Abcam ID: ab125030, diluted 1: 2000) overnight at 4°C. The FRα protein was visualized

by chemiluminescence on film (Kodak) using an anti-rabbit IgG as the secondary antibody conjugated to horseradish peroxidase and the enhanced chemiluminescence Western blotting analysis system. The absorbance of the FRα protein bands was measured using the Bio-Rad imaging densitometer model ChemiDoc XRS+ with the Image Lab program. After subtraction of the background for each film, the absorbance value of each sample indicated the FRα protein expression/10 μg of total protein. Each experiment was repeated three times.

Immunohistochemistry Straining

Cryostat sections (Eight-micrometer-thick) were prepared from frozen tissue and fixed in 4% paraformaldehyde for 15 min. Any endogenous peroxidase activity was blocked with 0.3% peroxide for 10 minutes. The slides were incubated in normal rabbit serum for 1 hour and then incubated overnight in the primary FRα anti-body (Santa Cruz Biotechnology, sc-16386, diluted 1:50) at 4°C. After thorough rinsing in PBS, the slides were incubated with secondary antibodies conjugated to horseradish peroxidase for 1 hour. Colour was developed using the 2,4- diaminobutyric acid (DAB) substrate according to the manufacturer's instructions and the slides were then counterstained with haematoxylin for 30 seconds. To ensure antibody specificity, five NF pituitary adenomas as control slides were incubated with PBS instead of the primary antibody. Immunointensity was scored independently by three investigators according to the following scale: negative (0), low (1+), intermediate (2+), or high (3+).

Statistical Analysis

The one-way analysis of variance (ANOVA) was used to evaluate any significant differences of FRα protein and mRNA between the individual groups. An independent-samples t test was used to determine the relationship between FRα expression levels and tumor invasiveness. The correlation between FRα expression and the other clinical factors were evaluated by use of the Mann-Whitney test and one-way ANOVA. Values of $p < 0.05$ were considered statistically significant. The Statistical Package for the Social Sciences version 13.0 was used for statistical analyses.

RESULTS

FRα mRNA Expression in Human Pituitary Adenomas and Normal Pituitary Glands

The expression of FRα mRNA in 28 pituitary adenomas and 3 normal pituitary glands was determined by qRTPCR, and the results are shown in Figure 1. Using one-way ANOVA, we found that FRα mRNA expression was significantly different between the different kinds of pituitary adenomas and normal pituitary glands ($p < 0.05$). The FRα mRNA level was markedly elevated in the NF pituitary adenomas compared to the other kinds of pituitary tumours and normal pituitary glands ($p < 0.05$, Figure 2).

Expression of FRα Protein

Whether the high levels of FRα mRNA resulted in overexpression of FRα protein was investigated with Western blotting of 28 pituitary tumours and 3 normal pituitaries. In western blot analysis of those pituitary tumour tissues, seventeen NF tumour samples appeared to have up-regulated expression of FRα. One of the four ACTH-secreting adenomas exhibited very weak FRα protein expression and we could not find obvious expression of FRα in the rest of the samples including three normal pituitary tissues (Figure 3A-D). After normalising the FRα protein to that of β- actin, we found that there were significant differences in FRα expression in the NF pituitary tumour tissues compared to functional pituitary

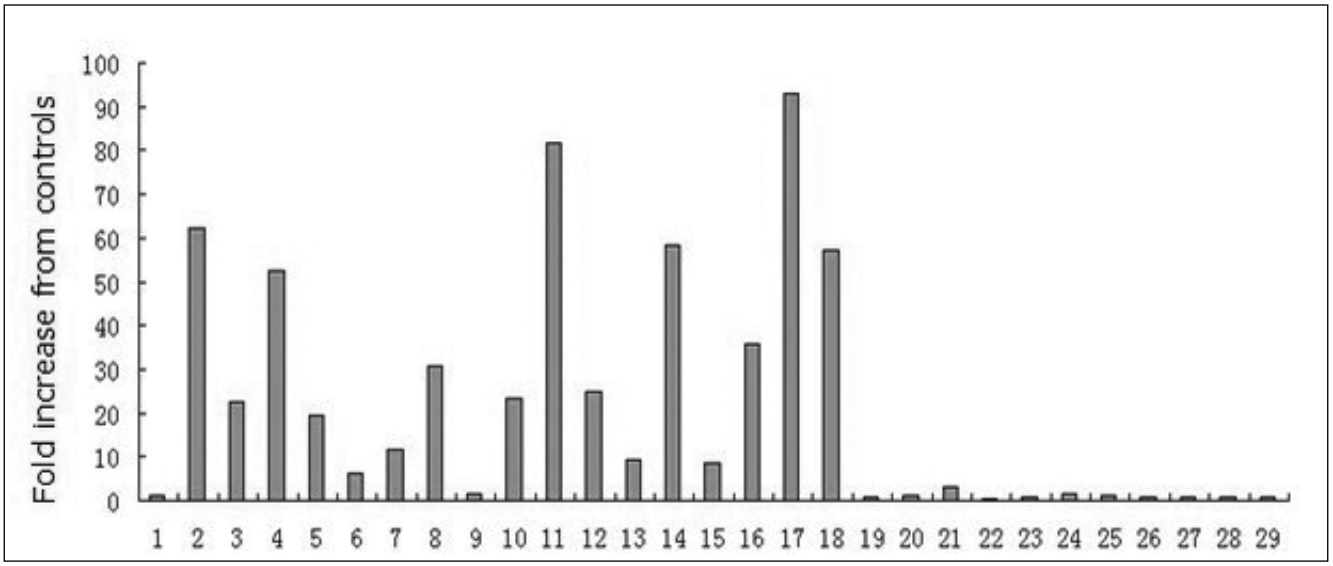

Figure 1: Expression of FRα (Folate Receptor α) mRNA in 28 pituitary adenomas and 3 normal pituitary glands. Here 1 is the mean of the three normalized values of the normal pituitary glands as control, and 2-18 NF (nonfunctional), 19-22 ACTH (Adrenocorticorticotropic Hormone), 23-25 GH (Growth Hormone) and 26-29 PRL (Prolactin) secreting adenomas.

adenomas and normal pituitary gland tissues ($p < 0.05$, Figure 4). FRα protein was significantly overexpressed in NF pituitary tumours but not in functional adenomas or normal pituitary glands.

Immunohistochemistry Localisation of FRα Expression

FRα was expressed in 30 of 32 (93.8%) NF pituitary adenomas. The degree of immunostaining was 3+ in 6 cases, 2+ in 14 cases, 1+ in 10 cases and 0 in 2 cases. FRα was predominantly expressed in the cytoplasm and on the membrane of the tumor cells (Figure 5A, B). Immunostaining was not observed in the functional adenomas or normal glands except in one 1+ ACTH tumour (Figure 5C-F). Using one-way ANOVA, we found that the expression level of FRα was significantly higher in NF pituitary tumour tissues than in functional pituitary adenomas or normal pituitary glands ($p < 0.05$). In addition, there was no significant difference of FRα expression among the 3 kinds of functional pituitary adenomas or normal pituitary glands ($p > 0.05$). The intensity of the FRα-positive staining corresponded to the FRα immunoblot results.

Correlation Between FRα and Some Clinical Factors in NF Pituitary Adenomas

Using the Mann-Whitney test and one-way ANOVA, we found that the expression of FRα had no correlation with age, gender, presence or absence of repeated surgery and mean diameter of the tumours ($p = 0.637$, $p = 0.662$, $p = 0.826$ and $p = 0.247$ respectively). However, FRα expression level was remarkably higher in invasive NF tumours than in noninvasive NF tumours ($p < 0.05$, Figure 6).

DISCUSSION

Folic acid is an essential component involved in cellular 1-carbon transfer reactions that form the basis of DNA

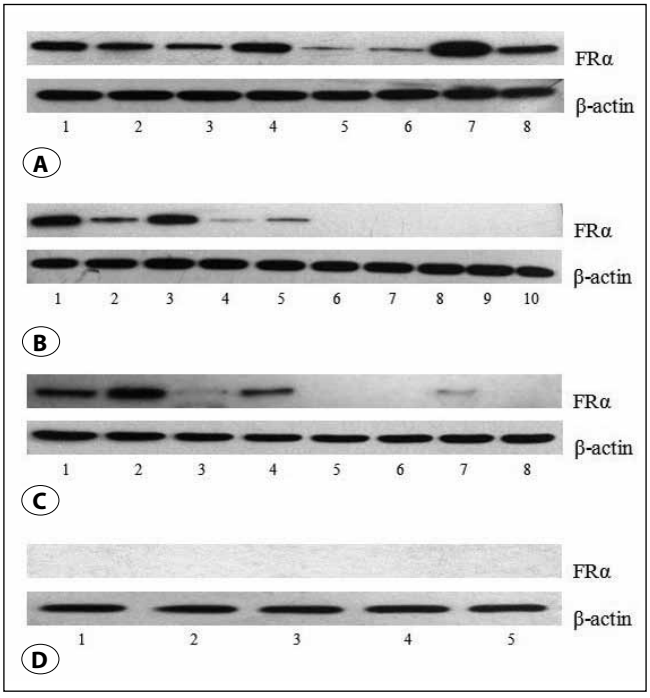

Figure 3: Western blots of FRα (Folate Receptor **A)** protein and housekeeping gene β-actin expression in pituitary tumour and normal pituitary glands. A showed FRα expression in 8 NF (nonfunctional) pituitary tumours (lanes 1-8). **B)** showed FRα expression in 5 NF pituitary tumours (lanes 1-5) and 5 functional pituitary adenomas (GH (Growth Hormone), lanes 6-8. PRL (Prolactin), lanes 9-10). **C)** showed FRα expression in 4 NF pituitary tumours (lanes 1-4) and 4 functional pituitary adenomas (ACTH (Adrenocorticorticotropic Hormone), lanes 5-8). **D)** showed expression of FRα in 2 functional pituitary adenomas (PRL, lanes 1-2) and 3 normal pituitary tissues (lanes 3-5).

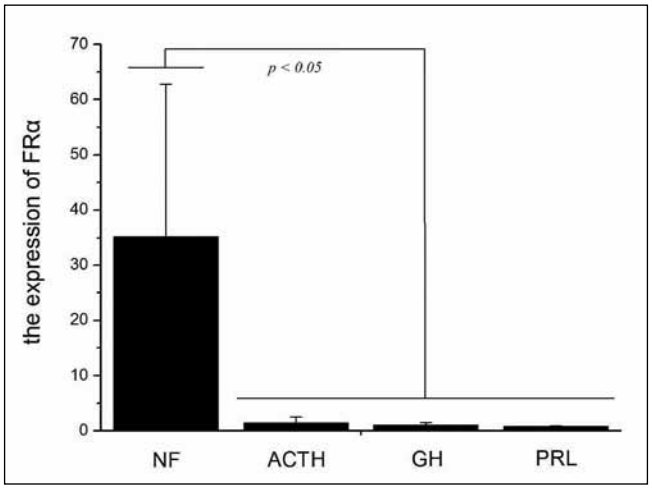

Figure 2: The ratio of the normalized value of each tumour sample to the mean of the three normalized values of the normal pituitaries. FRα (Folate Receptor α) mRNA expression was significantly higher in NF (nonfunctional) pituitary tumour tissues than in functional pituitary adenomas.

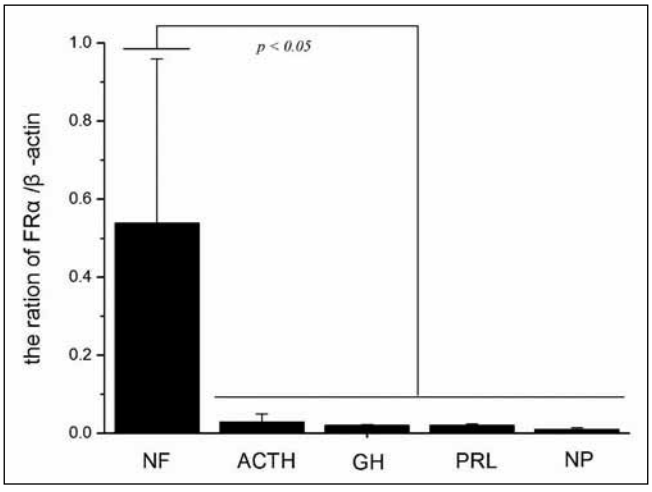

Figure 4: Relative quantities of the genes are presented as the ratios of the intensities of the FRα (Folate Receptor α) blots against the housekeeping gene β-actin, showing that FRα expression was significantly higher in NF (nonfunctional) pituitary tumour tissues compared to functional pituitary adenomas and normal pituitary gland tissue.

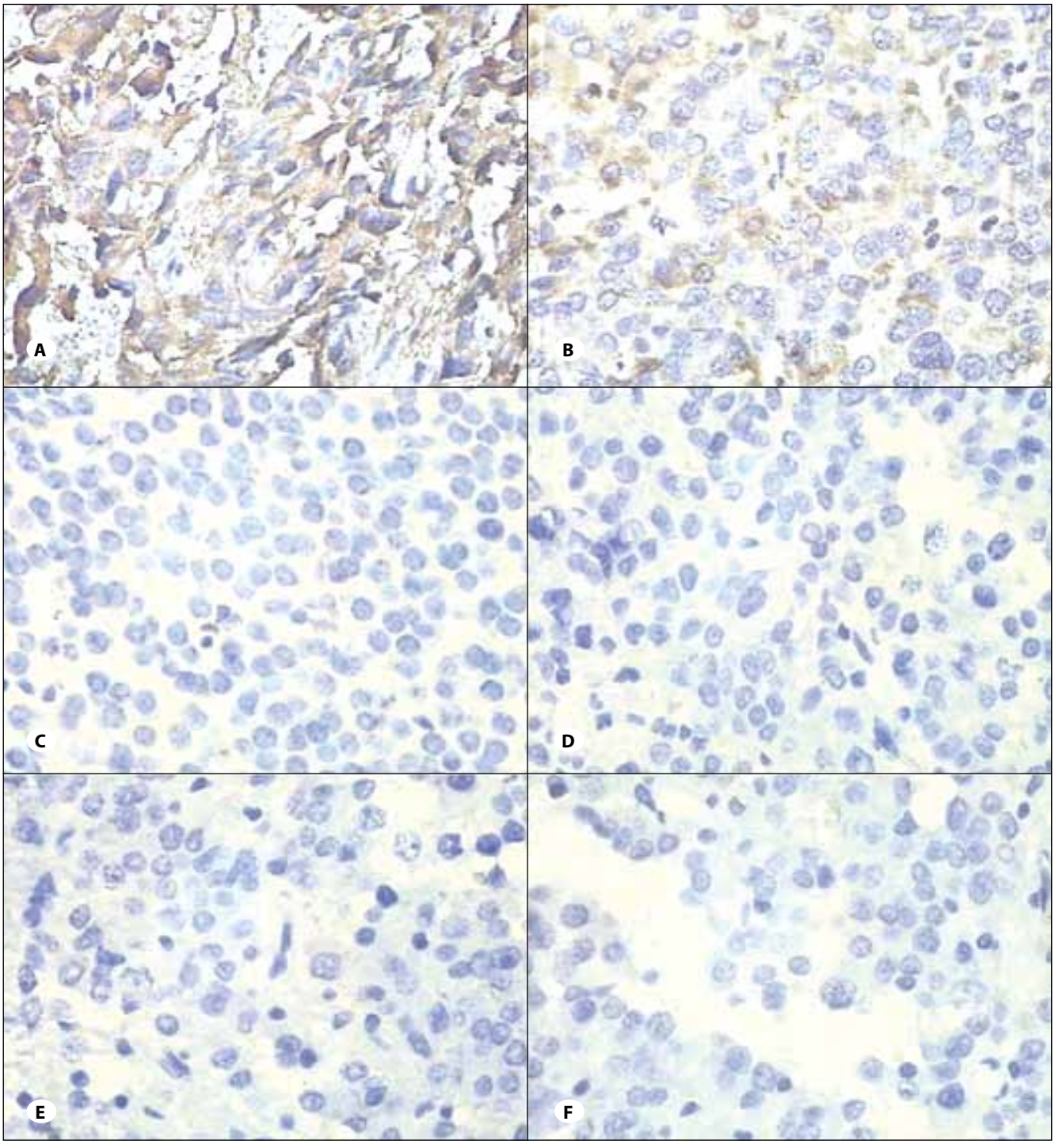

Figure 5: Expression and location of FRα (Folate Receptor α) in pituitary adenomas and normal pituitary glands (haematoxylin, 800×). **A)** Strong immunoreactivity for FRα in the cytoplasm and on the cellular membrane of NF (nonfunctional) pituitary adenomas. **B)** Part of the NF pituitary tumours showed intermediate immunoreactivity for FRα. **C)** ACTH (Adrenocorticorticotropic Hormone) secreting adenomas did not show appreciable FRα. **D)** GH(Growth Hormone) secreting adenomas did not show appreciable FRα either. **E)** PRL (Prolactin) secreting adenomas also did not show appreciable FRα. **F)** NF pituitary adenomas did not yield any immunoreactivity when the primary antibody directed against FRα was replaced with phosphate-buffered saline.

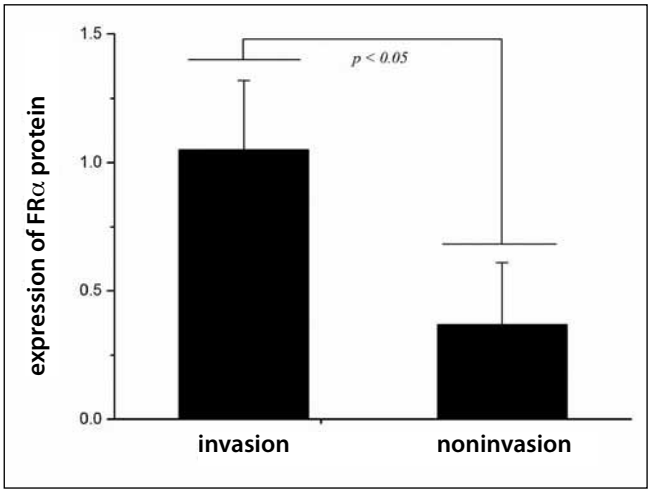

Figure 6: The expression of FRα (Folate Receptor α) protein in invasive and noninvasive NF (nonfunctional) pituitary adenomas. The FRα expression level was remarkably higher in invasive NF tumours than in noninvasive NF tumours.

synthesis and replication (22). The effect of folate receptor is mainly the transport of folic acid into cells by endocytosis and the transport efficiency is nearly 1000 times higher than all the other folate carriers (25). Therefore, FRα overexpressed in some organizations and especially tumour tissues may improve the transport efficiency of folic acid and it is beneficial to cell proliferation. In the early stage of neoplasia, FRα could be overexpressed and result in stimulating the cells to repair the DNA that was damaged. Meanwhile, when the DNA undergoes more cell damage than it can repair, FRα will be continuously expressed and finally it will form a particular cellular environment that is beneficial to tumorigenesis and tumour progression (3). Some studies claim that the introduction of FRα into cells that normally do not express this receptor allows them to grow in low concentrations of folate (15,17) and form larger tumors when injected into nude mice (4). Thus, the overexpression of FRα by various tumors may facilitate the growth of these cancers. Although it is biologically plausible that folate internalization could lead to promotion of cell proliferation by FRα, it is also possible for FRα to stimulate cell proliferation through other pathways. Hough found that upregulated FRα was associated with two proliferative pathways based on statistical analysis of real-time quantitative PCR data in a panel of 39 microdissected ovarian carcinomas (11). In addition, Evans claimed that FRα regulated pituitary tumour cell proliferation through mechanisms involving the NOTCH pathway (26). Our neuroscience center has studied the overexpression of the Notch3 receptor in NF pituitary tumours (27) and now we are further investigating how NOTCH3 and FRα interact with each other. In this study, we found that the FRα mRNA and protein were significantly overexpressed in NF tumour samples but not in functional adenomas or normal pituitary glands. These results are consistent with the previous reports (5,6). We also found that the FRα mRNA and protein expression was specifically increased in most of the invasive NF pituitary tumours. As we know, the majority of invasive tumours have faster speed of cell division and growth rate. Meanwhile, the overexpression of FRα in tumour tissues could improve the transport efficiency of folic acid and it is beneficial to the cell proliferation. Our data therefore indicate that overexpression of FRα in NF tumours is associated with invasiveness of pituitary adenomas and this might be used for evaluation of the invasiveness level of NF pituitary adenomas. Besides, the expression of FRα had no significant relationship with age, gender, whether the surgery was repeated or not and mean diameter of the tumours. NF pituitary adenomas are generally difficult to diagnose till they are large enough to cause tumour mass effects such as headaches, cranial nerve palsy, hypopituitarism, or visual field defects, because they lack clinical hormone hypersecretion (10). When the tumours involve the cavernous sinus, they are quite difficult to remove totally and the relapses must be taken seriously. Unlike functional pituitary tumours, there are no available medications or specific imaging technique to detect NF pituitary adenomas. Therefore, it is particularly crucial to identify potential biological markers as diagnostic and therapeutic targets of NF pituitary tumours. Molecular targeted treatment for human tumours has been attracting more and more attention since the molecular level study of tumours goes deeper. Currently, some chemotherapy drugs have little effect due to the lack of effective concentration. Meanwhile, most drugs always result in increased side effects. The high affinity of FRα for folate and its selective overexpression in tumors provides a unique opportunity for directed chemotherapy and radiopharmaceutical delivery. Therefore, FRα is one of the most promising biomarkers of targeted diagnosis and therapy for the patients with NF pituitary adenomas. As we know, there are many series of receptors expressed in the surface of tumor cells, which are able to mediate an internalizing effect by specifically connecting with corresponding ligands. These receptors are potential targets for drugs combined with conjugates so that delivery of the drug-conjugate compounds can be targeted to tumor cells. A folate-drug conjugate generally contains three parts that are folate or pterin, the drug carrier and the drug. Ni claim that cellular uptake of FR-targeted liposomal daunorubicin (f-L-DNR) in KB oral carcinoma cells, Chinese hamster ovary (CHO-FR-beta), and KG-1 human acute myelogenous leukemia cells were 9.4-, 40-, and 4.6-fold higher than non-targeted liposomal DNR (L-DNR), respectively. The cytotoxicity of fL-DNR in KB and CHO-FR-beta cells was 18 times and 49 times higher than L-DNR, respectively (18). Moreover, a wide range of imaging molecules have been conjugated to folate and evaluated for FRα targeting, including 67Gadeferoxamine-folate (16), 99mTc-DTPA-folate (24), 99mTc-EC20-folate (20) and so on. In a recent clinical study, intravenous injection of 99mTc-EC20-folate resulted in a sensitivity of 81% and specificity of 83% in patients with NF pituitary adenomas (8).

CONCLUSION

FRα was significantly overexpressed in NF pituitary adenomas but not in functional tumours (GH, ACTH, PRL) or normal

pituitary glands. For NF tumours, FRα expression was much higher in the invasive group than in the non-invasive group so it may play an important role in the development and progression of invasive NF pituitary adenomas. However, the expression of FRα had no significant relevance with age, gender, repeated operation or not and mean diameter of tumour. Potentially, these results could be exploited to develop innovative molecular targeted diagnosis and treatment for human NF pituitary adenomas.

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