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Anti-EGFL7 antibodies inhibit rat prolactinoma MMQ cells proliferation and PRL secretion

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Abstract: Prolactinoma is the most frequently diagnosed pituitary tumors. Dopamine agonists (DAs) are recognized as first-line therapy; however, approximately 10% patients will develop resistance to DAs therapy. Consequently, a large number of investigations have been carried out to identify novel therapeutic targets. Recently, studies have suggested that epidermal growth factor-like domain 7 (EGFL7) can promote tumor growth, invasion, and angiogenesis. We previously reported that overexpression of EGFL7 might play a crucial role in hormone-producing pituitary adenomas. In the present study, we now demonstrated a significantly higher protein expression of EGFL7 in prolactinoma compared with the normal pituitary gland. However, inhibition of EGFL7 with anti-EGFL7 antibodies significantly reduced the proliferation and PRL secretion of rat prolactinoma MMQ cells. Notably, *in vitro* administration of anti-EGFL7 antibodies significantly induced MMQ cells apoptosis in a dose-dependent manner. In conclusion, our finding suggests that EGFL7 is significantly overexpressed in prolactinoma and inhibition of EGFL7 with antibodies promoted MMQ cells apoptosis and inhibited PRL secretion. Thus, EGFL7 may serve as a potential novel therapeutic target for prolactinomas.

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Keywords: EGFL7; Antibody; Prolactinoma; Proliferation; PRL secretion.

1 Introduction

Prolactinoma is most frequently associated with pathological hyperprolactinemia, which accounts for approximately 60% of all pituitary adenomas [1]. Prolactinomas secrete an excess of prolactin (PRL), which results in hypogonadism, galactorrhea, and infertility [2]. Dopamine agonists (DAs) remain the first-line therapies for prolactinomas through suppression of PRL secretion and inhibition of tumor growth [3]. The majority of cases with prolactinomas initially respond efficiently to dopamine agonists therapy; however, approximately 10% of cases with prolactinomas will eventually develop resistance to the dopamine agonist therapy or intolerance to their adverse effects [4]. Therefore, novel and efficacious therapies are desired for these patients.

Epidermal growth factor-like domain 7 (EGFL7), an endothelial cell derived-secreted factor plays an essential role in physiologic angiogenesis [5]. Over-expression of EGFL7 is observed in several human solid tumors, including malignant gliomas, breast cancer and colon cancers [6-9]. Moreover, EGFL7 levels are significantly associated with pathologic characteristics [10, 11]. Previously, we reported that increased EGFL7 expression is correlated with the clinical progression, poor prognosis, and tumor grade in hormone-producing pituitary adenomas [12, 13]. However, the effect of EGFL7 in prolactinoma remains to be completely elucidated.

Therefore, the present study aimed to investigate the tissue expression of EGFL7 in prolactinoma in comparison with the normal pituitary gland. The study further evaluated the effect of inhibition of EGFL7 on proliferation and PRL secretion in rat prolactinoma MMQ cells. Using, tissue microarray we demonstrated a significant overexpression of EGFL7 in a prolactinoma. Moreover, treatment with anti-EGFL7 antibodies exhibited significant inhibition of proliferation and PRL

secretion in rat prolactinoma MMQ cells. Notably, *in vitro* administration of anti-EGFL7 antibodies induced MMQ cells apoptosis in a dose-dependent manner. Taken together, these findings suggest that EGFL7 might serve as a potential novel biomarker for prolactinoma.

2 Materials and Methods

2.1 Patients and specimens

Tissue samples from 12 patients with prolactinomas were obtained from the biobank of Beijing Neurosurgical Institute. All these patients underwent endoscopic transsphenoidal surgery between December 2012 and January 2014 at Tiantan Hospital, Beijing, China. We also received normal human anterior pituitaries from six organ donors that died of non-neurological and non-endocrine diseases. To exclude the possibility of incidental pathologies, all the normal anterior pituitaries were histologically examined using immunohistochemistry. This study was approved by the Ethics Committee of Beijing Tiantan Hospital, and written informed consent was obtained from each patient.

2.2 Immunohistochemistry

The immunohistochemical (IHC) SABC method was performed as described previously[12]. Prolactinoma and pituitary gland specimens were fixed in 10% formaldehyde and embedded in paraffin, and tissue sections of 4 μm were stained with standard hematoxylin-eosin (H&E) stain for histopathological evaluation. IHC analysis with mouse monoclonal anti-EGFL7 antibody (1:200, 2H2 sc-101349; Santa Cruz Biotechnology, California, USA) was performed on the sections using Leica BOND-III (Leica Biosystems, Wetzlar, Germany) automated, random, and continuous-access slide staining system and heat-induced epitope retrieval at a high pH (3 min). The primary antibodies were visualized by Bond Polymer Refine Detection system (Leica Biosystems). The expression of immunostained slides was examined by Aperio AT2 digital scanner (Leica Biosystems). The negative control sections were treated identically with the omission of the primary antibody. Moreover, endothelial cells were used as the positive control, and all controls provided satisfactory results.

2.3 Cell culture

The rat prolactinoma, MMQ pituitary adenoma cell line was obtained from the American Type Cell Collection. MMQ cells were cultured into an F12K medium (Invitrogen, Carlsbad, CA) supplemented with 2.5% fetal bovine serum, 15% horse serum, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2. The culture medium was replaced every other day.

2.4 Cell proliferation assay

Effect of different concentrations of anti-EGFL7 antibodies (10-100 μg/mL) on the proliferation of MMQ cells was assessed by MTS assay following the manufacturers' protocols. The normal human IgG was used as a control. Briefly, MMQ cells were seeded into 96-well microplates, and cultured in the medium mentioned above containing an incremental concentration of anti-EGFL7 antibodies (0-100 μg/mL) for 24, 48, and 72 h. Subsequently, 20 μL MTS solution (Promega, Madison, WI, USA) was added to each well with 100 μL culture medium for four h at 37 °C. The absorbance was measured at 490 nm with a multi-detection microplate reader (Tecan Infinite® M200 pro, Tecan Group AG, Männedorf, Schweiz). All experiments were performed in triplicate.

2.5 Apoptosis assay

Apoptosis in MMQ cells was quantitatively determined using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit. Briefly, MMQ cells were treated with increasing concentration of anti-EGFL7 antibodies for 72 hours. After treatment MMQ, cells were harvested, washed with PBS and resuspended in 100 μL of binding buffer. A total of 5 μL of Annexin V-FITC was added to each well and incubated for 10 min at 25°C. 10 μL of PI was added in the cells and incubated for another 15 min in the dark. Next, 400 μL binding buffer was added to each well before analysis. Samples were analyzed by imaging flow cytometry (ImageStream X MarkII, Amnis, USA).

2.6 ELISA assay

The PRL secretions from MMQ cells treated with increasing concentration of anti-EGFL7 antibody (0-100 μg/mL) were measured using a rat PRL RapidBio ELISA kit (West Hills,

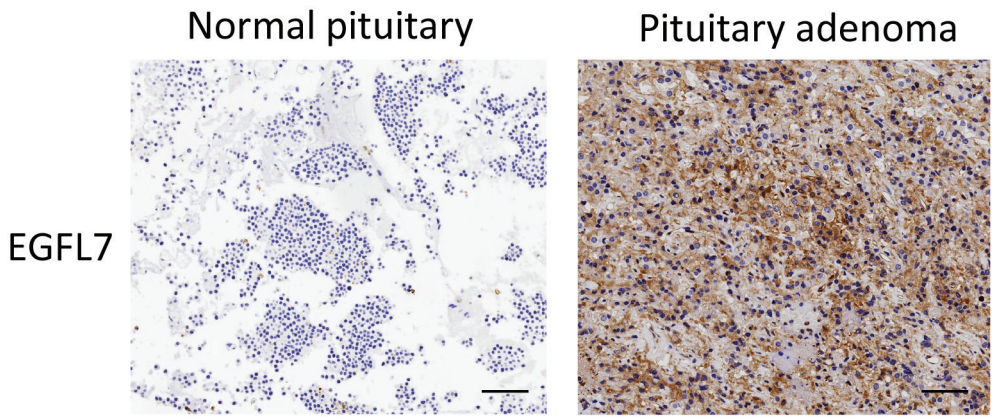

Figure 1: Overexpression of EGFL7 in prolactinoma tissues. Representative EGFL7 staining of TMA showed a significantly higher expression of EGFL7 in the cytoplasm of prolactinoma than in normal pituitary tissue. scale bar=200 μm.

CA, USA) performed as described previously [14]. The color intensity of the reaction product (proportional to the concentration of PRL) was measured using a multi-detection microplate reader (Tecan Infinite® M200 pro, Tecan Group AG, Männedorf, Schweiz). The concentration of PRL was calculated from the standard curve using a straight-line regression equation from the standard curve with the standard density and the OD value.

2.7 Statistical analysis

All data were expressed as the mean ± standard deviation (SD) of at least three independently performed experiments. All statistical analysis was performed using the SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The statistical significance of the differences among three groups was analyzed by one-way analysis of variance (ANOVA), followed by a least significant difference post-hoc test to obtain individual P values. The value $p < 0.05$ was considered statistically significant.

3 Results

3.1 Overexpression of EGFL7 in prolactinoma tissues

The tissue expression levels of EGFL7 in prolactinoma in comparison with normal pituitary tissue were determined using tissue microarrays and IHC staining. As shown in Figure 1, EGFL7-positive expression was predominantly detected in the cytoplasm. Noticeably higher expression of EGFL7 was observed in prolactinoma than normal pituitary.

3.2 Anti-EGFL7 antibody inhibited the proliferation of MMQ cells

We previously demonstrated that attenuation of EGFL7 expression inhibits the growth of hormone-producing pituitary adenomas progression and invasion [12, 13]. Therefore, to further evaluate the effects and therapeutic potential of inhibition of EGFL7 in prolactinoma, MTS experiments were performed with a commercially available antibody that specifically binds to the EGFL7 protein. As shown in Figure 2, in rat prolactinoma MMQ cells, treatment with increasing concentrations of the anti-EGFL7 blocking antibody led to significant decreases in cell proliferation compared with normal IgG control in a time-dependent manner.

3.3 EGFL7-blocking antibody inhibited the PRL secretion from MMQ cells

Furthermore, the levels of secreted PRL in the culture supernatant from MMQ cells were also assayed by ELISA using an antibody against the EGFL7 protein. The most representative results after treatment with EGFL7-blocking antibody for 72 h are shown in Figure 3. Treatment of MMQ cells with 50 and 100 μg/mL anti-EGFL7 antibodies for 72 h significantly reduced the level of secreted PRL as compared with normal human IgG control. The serum PRL levels were reduced to 12.56 ± 2.5, 9.29 ± 1.9, and 8.37 ± 1.3 ng/mL in response to 10, 50, and

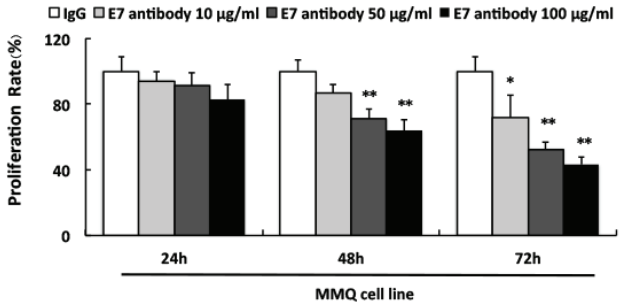

Figure 2: Anti-EGFL7 antibody inhibited the proliferation of MMQ cells by MTS. **P<0.05, **p<0.01* versus normal human IgG control.

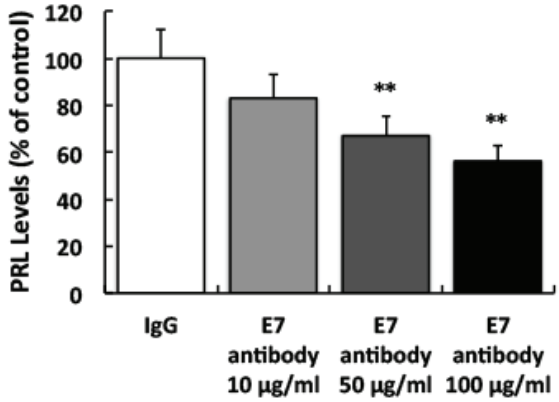

Figure 3: Downregulation of EGFL7 inhibited the PRL secretion from MMQ cells by ELISA. ***p<0.01* versus normal human IgG.

100 μg/mL of anti-EGFL7 antibodies, respectively (Figure 3) ($p < 0.01$).

3.4 EGFL7-blocking antibody induce the apoptosis in MMQ cells

Apoptosis is determined to be responsive to the anti-cancer activities of EGFL7-blocking antibody. Consequently, using Annexin V-FITC/PI staining assay we further evaluated the effect of anti-EGFL7 treatment on apoptosis of MMQ cells by imaging flow cytometry. As shown in Figure 4A and C, the level of apoptosis of MMQ cells increased to 14.6%, 26%, and 47.5%, respectively, corresponding well with the increasing concentrations of anti-EGFL7 antibody (10, 50, and 100 μg/mL) for 72 h. A significant dose-dependent increase in the percentage of induced apoptotic cells was indicated. Conversely, normal human IgG control failed to increase the apoptotic rate in MMQ cells. Furthermore, evaluation of cell morphology and fluorescent staining confirmed standard features of living (AnnV−PI−), early apoptotic (AnnV+PI−) and late apoptotic (AnnV+PI+) cells (Figure 4B). Expression of apoptotic-related proteins was assessed using Western blot. As shown in Figure5, treatment with EGFL7-blocking antibody for 72 h did not affect the expression of Caspase-8 in MMQ cells; however, the activation of caspase-9 and -3 were noted. These results suggested that the apoptosis induced by EGFL7-blocking antibody was through an intrinsic pathway in MMQ cells.

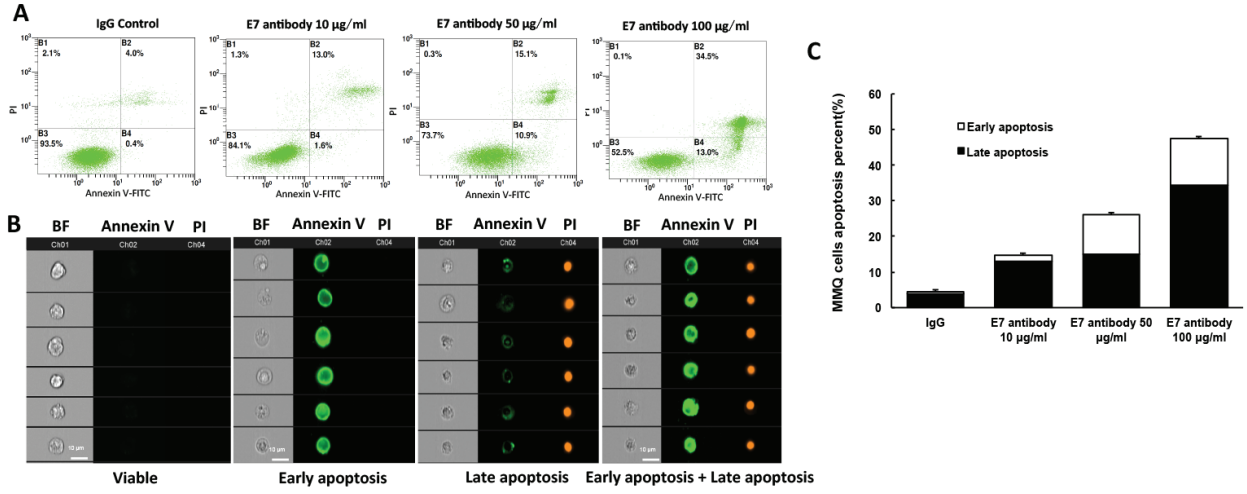

Figure 4: EGFL7-blocking antibody induced the apoptosis in MMQ cells. (A) Dot plot shows viable, early apoptotic, late apoptotic, and necrotic cell populations (AnnV−/PI−, AnnV+PI−, AnnV−/PI+, and AnnV+PI+, respectively). (B) Representative images of each distinct cellular subset. n=4; scale bars = 10 μm. (C) Percent apoptotic MMQ cells (early apoptotic + late apoptotic) at 72 hours.

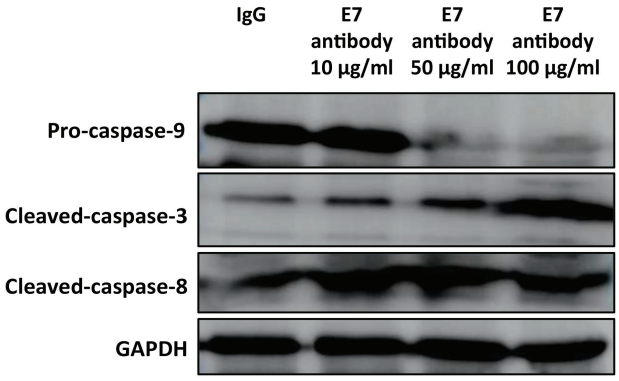

Figure 5: EGFL7-blocking antibody-induced apoptosis was dependent on the activation of caspase-9 and -3. MMQ cells treated with or without EGFL7-blocking antibody for 72 hours at indicated concentrations.

4 Discussion

The vascular endothelial growth factor (VEGF) has been validated as an effective anticancer target which mediates tumor angiogenesis [15]. Particularly, probing different angiogenic factors and improving the currently used anti-angiogenic therapy is a reasonable strategy for tumor therapy. Besides, as a vascular-restricted extracellular matrix protein and due to its association with tumor angiogenesis, EGFL7 has emerged as a potent potential target [16]. Therefore, the present study investigated the tissue expression level of EGFL7 in prolactinoma and normal pituitary gland. As demonstrated by the tissue microarray results, there was a significantly higher expression of EGFL7 in prolactinoma compared with normal pituitary tissue. These finding suggested that EGFL7 may play an oncogenic role in prolactinoma and can serve as a potential therapeutic target.

Several studies have revealed the benefits of blocking EGFL7 in tumor models [12, 17, 18]. Recent studies demonstrated that the combination of an anti-EGFL7 antibody and an anti-VEGF enhanced the anti-angiogenic activities and survival prolongation associated with anti-VEGF monotherapy [17]. In the present study, we evaluated the efficacy of antibodies against EGFL7 treatment in rat prolactinoma MMQ cells. Interestingly, there was a significant reduction in the MMQ cell proliferation by blocking of EGFL7 function with anti-EGFL7 monoclonal antibodies. As increased PRL secretion from anterior pituitary lactotrophs leads to various health-related complications including hypogonadism; importantly, we also observed that antibodies against EGFL7 treatment significantly reduced the PRL levels from MMQ cells. These results are consistent with our previous findings which demonstrated that EGFL7 plays a significant role in the progression of hormone-secreting pituitary adenomas.

As imaging flow cytometry combines the statistical power and fluorescence sensitivity of standard flow cytometry with the spatial resolution and quantitative morphology of digital microscopy [19]. In this study, we used the imaging flow cytometry technique to determine the potential effect of EGFL7-blocking treatment on cell viability. The results showed that after exposure to anti-EGFL7 antibodies, strong Annexin V-FITC and PI staining were identified in the cytoplasm and nucleus of the MMQ cells. This evidence suggested that EGFL7 blocking promoted MMQ cells apoptosis.

The major limitation of the present study was small sample size due to non-availability of prolactinoma tissue samples. Consequently, further research may be needed to validate our findings.

In conclusion, EGFL7 may contribute to the regulation of proliferation and PRL secretion in rat prolactinoma MMQ cells through induction of cell apoptosis. Taken together, our finding suggests that EGFL7 is significantly overexpressed in prolactinoma and inhibition of EGFL7 with antibodies promoted MMQ cells apoptosis and inhibited PRL secretion. Thus, EGFL7 may serve as a potential novel therapeutic target for prolactinomas.

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Conflict of interest: The authors declare that they have no competing interests.

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