Research Article

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Anti-c-myc efficacy block EGFL7 induced prolactinoma tumorigenesis

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Abstract: Resistance to Dopamine agonists therapy is still a key factor that hinders the clinical treatment of prolactinoma. Consequently, a large number of investigations have been carried out to identify novel therapeutic targets. Our previous studies have suggested that the epidermal growth factor-like domain 7 (EGFL7) plays a crucial role in tumorigenesis of pituitary adenomas via EGFR/AKT/MAPK signaling pathway. In the present research, we found a positive staining of c-myc intimately associated with high-level EGFL7 in invasive prolactinoma compared to non-invasive prolactinoma and the normal pituitary gland. Meanwhile, PI3K/Akt and MAPK signaling cascades closely related to the activation of c-myc. Therefore, this research was conducted to explore the cooperation effect of c-myc and EGFL7 in prolactinoma. The inhibition of c-myc with anti-c-myc antibodies significantly reduced the proliferation, PRL secretion and invasion of rat prolactinoma MMQ cells. Notably, down regulation c-Myc by in vitro administration of anti-c-Myc antibodies could significantly depress EGFL7 induced MMQ cell proliferation, PRL secretion and invasion. An anti-c-Myc antibody could block EGFL7 induced Akt activation, but the expression of p-ERK was not altered by an anti-c-Myc antibody. Thus, our results suggest that anti-c-myc efficacy could block EGFL7 induced prolactinoma tumorigenesis via inhibited Akt activation in MMQ cells.

Keywords: EGFL7; c-myc; Cooperation; Prolactinoma; Tumorigenesis.

1 Introduction

Prolactinoma are the most common tumors of pituitary adenoma with hyperprolactinemia [1]. Treatment with a dopamine agonist is effective in the inhibition of prolactin (PRL) secretion and tumor growth [2]. However, dopamine agonist-resistant to prolactinoma is still a key factor hindering prolactinoma treatment [3]. Therefore, novel and efficacious therapies are desired for these patients.

As a proto-oncogene, c-myc enables cells grow infinitely and has been reported to initiate or accelerate tumorigenesis in a variety of tissues [4]. Prior studies have revealed that c-myc over-expression has an effect to turn tumor cells into malignant phenotypes by activating a cancer gene or deactivating its suppressors [5-6]. This upregulation proceeds through multiple mechanisms, ranging from gene amplification, cellular senescence, to mutation of upstream signal pathways [7]. This research aims to examine the c-myc over-expression levels in prolactinoma associated with the high level of EGFL7.

Previously, we found EGFL7 played the tumorigenesis role in pituitary adenomas [8-9]. Over-expression of EGFL7 is observed in growth hormone-producing pituitary adenomas [9] and prolactinoma [10]. Attenuation of EGFL7 expression Inhibits the tumor growth and invasion in vivo and in vitro experiments [9]. Furthermore, EGFL7 can act as a potential novel viable target for prolactinoma.

Our previous results showed that the MAPK and PI3K/AKT signaling pathway is a dominant driver in EGFL7 induced tumor growth and invasiveness [8,11]. Meanwhile, the PI3K/Akt and MAPK signaling cascades closely related to activation of c-myc [12]. Therefore, the current
study reveals the c-myc and EGFL7 cooperation effect in prolactinoma. It was found that the EGFL7 expression level was positively correlated with expression of c-myc in prolactinomas by using Tissue microarray (TMA). Using MTT, ELISA and Transwell assay, c-Myc-blocking antibody could block EGFL7 induced prolactinoma tumorigenesis. Down-stream signaling pathways studied investigated the biological process that is related to the phosphorylation of AKT.

2 Materials and Methods

2.1 Patients and specimens

Tissue samples from 126 patients with prolactinomas were provided by the biobank of Beijing Neurosurgical Institute [8]. All these patients had an endoscopic trans-sphenoidal operation between December 2012 and January 2014 at Tiantan Hospital, Beijing, China. There were 51 invasive specimens and 75 non-invasive specimens. The following invasive PAs diagnostic criteria were adopted: 1) Knosp classification grade III-IV tumors and Hardy classification III-IV; 2) Tumor cells confirmed via pathology as invading sellar bone or adjacent dura mater; 3) Tumor cells invading the sphenoid sinus cavity or peripheral vascular and nerve. We also collected anterior pituitaries glands from six organ donors whose deaths were irrelevant to neurological or endocrine diseases. For the sake of rooting out the possibility of incidental pathologies, all the normal anterior pituitaries were pathologically examined by immunohistochemistry. The study obtained approval from the Ethics Committee of Beijing Tiantan Hospital.

2.2 Immunohistochemistry

The immunohistochemical (IHC) SABC method was performed as depicted previously [9]. Prolactinoma and pituitary gland specimens were paraffin-embedded and put in 10% formaldehyde, and tissue sections of 4 μm were obtained through a standard hematoxylin-eosin (H&E) stain for histopathological evaluation. IHC analysis with rabbit recombinant monoclonal c-myc (1:100, cat. ab32072; Abcam) and mouse monoclonal anti-EGFL7 antibody (1:100, cat. sc-101349; Santa) were conducted on the sections by random, automated, slide staining system Leica BOND-III (Leica Biosystems, Wetzlar, Germany). A Bond Polymer Refine Detection system (Leica Biosystems) was adopted to visualize the primary antibodies while an Aperio AT2 digital scanner (Leica Biosystems) was utilized to examine the expression of immunostained slides. The negative control sections received identical treatment other than omitting the primary antibody. Moreover, endothelial cells as a positive control obtained desirable results.

2.3 Cell culture

A rat prolactinoma cell MMQ was provided by American Type Culture Collection. MMQ cells were cultured in F12K medium added with 15% horse serum and 2.5% fetal bovine serum as well as 100 units/mL penicillin and streptomycin in a humidified air containing 5% CO2 at 4°C.

2.4 Cell proliferation assay

The proliferation of MMQ cells was assessed by an MTS assay following the manufacturers’ protocols. Briefly, MMQ cells were seeded into 96-well microplates and cultured in the medium mentioned above with 50 ng/mL recombinant human EGFL7 (rhEGFL7) or 10 μg/mL anti-c-myc antibodies for 72 h. Subsequently, a 20 μL MTS solution (Promega, Madison, WI, USA) was supplemented to each well with a 100 μL culture medium for four hours. The absorbance was assessed at 490 nm with a multi-detection microplate reader (Tecan Infinite® M200 pro). All experiments were performed in triplicate.

2.5 ELISA assay

A PRL RapidBio ELISA kit (West Hills, CA, USA) was used to evaluate the PRL secretions from MMQ cells as described previously [13]. According to the standard curve, standard density and OD, a linear regression equation was used to calculate the concentration of PRL. The color intensity of the reaction product was proportional to the concentration of PRL and was detected by the same multi-detector microplate reader mentioned in the cell proliferation test.

2.6 Western blot analyses

A DC Protein Assay (Bio-Rad) was applied to measure the concentrations of cell lysate protein [14]. Laemmlı method was employed to resolve the same amounts of protein (40 μg) from each cell lysate. The proteins were transferred
onto a 0.45-mm nitrocellulose membranes (Bio-Rad) which was sealed at 4°C and incubated overnight with the primary antibody. All the antibodies appeared as follows: anti-EGFL7 antibody (19291-1-AP, Proteintech), anti-p-ERK(ser42/44) antibody (#4696, CST), anti-AKT antibody (#4685, CST), anti-ERK antibody (#4685, CST) and GAPDH (Sigma) antibody, horseradish peroxidase-labeled secondary antibodies (Santa Cruz Bio.) Enhanced chemiluminescence was employed to visualize the membranes, and density measurements were performed by Amersham Imager 6000 (GE, American).

2.7 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The purified total RNA (1μg) was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using a Revert Aid First-Strand cDNA Synthesis Kit (Thermo Shandon) based on the manufacturer’s protocol. The primer details of genes were described in Table 1. GAPDH was used as an endogenous control for normalizing the level of the target genes. qRT-PCR was performed on an ABI 7500 Fast Real-Time PCR System using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). A comparative CT method was used to calculate fold-change in the differential expression of each gene (2^{ΔΔCT} method). PCR conditions: 50°C 20 min, 95°C 10 min, then 95°C 10 secs to 60°C 1 min, 40 cycles.

2.8 Statistical analysis

All data were expressed as the mean ± standard deviation (SD) of at least three independently performed experiments. Statistical analysis was conducted by SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was adopted to analyze the statistical significance of the differences among three groups, followed by a least significant difference post-hoc test to get individual P values. P value <0.05 was deemed as statistically significant.

3 Results

3.1 The positive staining of c-myc was intimately associated with high-level EGFL7 in invasive prolactinoma

Firstly, the expression level of c-myc and EGFL7 was measured by using tissue microarray in invasive prolactinomas in comparison with normal pituitary and non-invasive prolactinomas. As illustrated in Figure 1, the positive staining of c-myc in invasive prolactinoma was significantly higher than that in non-invasive prolactinoma. The result showed that the average expression level of c-myc protein in invasive prolactinoma vs. non-invasive prolactinoma was about 2.05-fold increased (Figure 2A). In addition, total expression level of EGFL7 has been determined and presented in Figure 2B. As the result shown in Figure 2B, noticeably higher expression of EGFL7 was observed in invasive prolactinoma than non-invasive prolactinoma and normal pituitary. In addition, the correlation of overexpression of c-myc and EGFL7 was also analyzed. As illustrated in Figure 2C, the positive staining of c-myc was intimately associated with high-level EGFL7 in invasive prolactinoma (r=0.411; P<0.05. n=126).

3.2 Down regulation c-Myc depress EGFL7 induced cell proliferation and PRL secretion in MMQ cells

This research studied the role c-myc played in EGFL7-induced MMQ cell proliferation and PRL secretion in an effort to better understand the biological function of c-myc in the EGFL7 signaling pathway. Our previous investigation indicated that knockdown EGFL7 effectively inhibited pituitary adenoma cell growth [8-9]. In this study, MMQ cells were incubated with 50 ng/mL rhEGFL7 protein or PBS for 72 h. As shown in Figure 3, 50 ng/mL rhEGFL7 significantly promote cell proliferation (Figure 3A, 1.88±0.37 fold) and PRL secretion (Figure 3B, 1.51±0.32 fold). To further clarify whether c-myc is involved in EGFL7 induced cell growth, MMQ cells transfected with or without rhEGFL7 were performed with 10 pg/mL anti-c-myc antibodies. As shown in Figure 3A and 3B, down regulation c-Myc by anti-c-myc antibodies could depress
Figure 1: Overexpression of c-myc and EGFL7 in invasive prolactinoma. Representative staining of a TMA was showed. c-myc and EGFL7 positive staining was significantly higher in invasive prolactinoma than that in non-invasive prolactinoma and normal pituitary. scale bar=60 µm.

Figure 2: Positively correlated of c-myc and EGFL7. H-scores of staining of TMA expressed as Mean ± SD. Both c-myc (A) and EGFL7 (B) overexpression in invasive prolactinoma. *P<0.05, **P<0.01 vs. non-invasive prolactinoma. In addition, (C) c-myc expression levels were significantly positively correlated with EGFL7 expression levels (r=0.411; P<0.05).
EGFL7 induced cell proliferation and PRL secretion in MMQ cells.

### 3.3 Down regulation c-Myc could depress EGFL7 induced cell invasion

As our above results indicated, knockdown EGFL7 effectively inhibited the invasion and migration of pituitary adenoma. Thus, we measured the effect of down regulation c-Myc in EGFL7 induced cell invasion gene expression of MMQ cells. After incubation with or without rhEGFL7 and with anti-c-myc antibodies in MMQ cells, the mRNA level of invasion related genes N-cadherin, MMP2, MMP-9 and vimentin were measured by RT-PCR experiments. As shown in Figure 4, 50 ng/mL rhEGFL7 significantly enhance the level of MMQ cell invasion related genes N-cadherin (4.6 ± 1.2 fold), MMP-2 (3.8 ± 0.9 fold), MMP-9 (1.8 ± 0.3 fold) and vimentinin (2.9 ± 0.7 fold), and 10 μg/mL anti-c-myc antibodies could significantly inhibit cell invasion gene expression. Meanwhile, down regulation c-Myc by anti-c-myc antibodies could depress EGFL7 induced the level of cell invasion related genes in MMQ cells.

### 3.4 anti-c-Myc antibody could block EGFL7 induced Akt activation

The MAPK and PI3K/AKT signaling pathway are dominantly involved in EGFL7-induced tumor invasiveness. There are some studies reporting that EGFL7 is capable of interacting with AKT and ERK, by modulating EGFR signaling[15][16]. Meanwhile, PI3K/Akt and MAPK signaling cascades are closely related to the activation of c-myc[12]. Thus, the present study explored whether the downregulation of c-Myc affects EGFL7-induced AKT and ERK activation. Protein extracts from MMQ cell transfected with or without 50 ng/mL rhEGFL7 and with 10 μg/mL anti-c-myc antibodies were detected by western blot, while the phosphorylation status of Akt and Erk in MMQ cells were measured. As seen in Figure 5, after 3 days’ treatment with 50 ng/ml rhEGFL7, it was observed that Akt (Figure 5A and Figure 5B) and Erk (Figure 5A and Figure 5C) phosphorylation levels in MMQ cells were significantly activated by rhEGFL7 in comparison with PBS control. However, anti-c-Myc antibody could block EGFL7 induced Akt activation, but the expression of p-ERK did not alter by anti-c-Myc antibody. Thus, our results suggest that anti-c-myc efficacy could block EGFL7 induced prolactinoma tumorigenesis via inhibited Akt activation in MMQ cells.

### 4 Discussions

The proteins c-myc and EGFL7 have been previously reported to be potent markers of tumor development in a wide range of solid tumors [17-20]. It was observed that the expression of c-myc had a sharp upregulation at a relatively late stage of prostate cancer, suggesting that correlated with tumor invasion and metastasis [21]. Many pieces of evidence revealed that anti-EGFL7 inhibited tumor growth and enhanced the antiangiogenesis [22-23]. Our previous results indicated a significantly higher expression of EGFL7 in hormone-secreting pituitary adenomas, which was associated with poor prognosis and invasiveness of tumor cells [8-9]. So far, very few studies have investigated the correlation of c-myc and EGFL7 with tumorigenesis and invasion clinicopathological parameters in prolactinoma.
Therefore, the current study is probably a pioneering study with the aim to explore the association between them. The study has investigated the correlation of c-myc and EGFL7 proteins with invasion in prolactinoma by IHC on tissue microarrays. The data demonstrated that both c-myc and EGFL7 were over-expressed in invasive prolactinoma in comparison with the non-invasive prolactinoma and the normal pituitary tissues. Meanwhile, the positive staining of c-myc was strongly linked with high-level EGFL7 in invasive prolactinoma. Thus, c-myc and EGFL7 was further demonstrated to be potential and potent biological markers for the detection of invasive prolactinoma.

The EGFL7 protein sequence is composed of an amino-terminal signal peptide domain, an EMI, and two centrally located EGF-like domains [24]. Predecessor research results have indicated EGFL7 binds to EGFR with
EGF-like domains. Our previous study showed that EGFL7 knockdown has an effect on EGFR signaling cascades [8]. EGFR led to auto-phosphorylation of tyrosine residues with the result that a multitude of downstream signaling pathways were activated, containing protein kinase B (AKT/PKB) and mitogen-activated protein kinase (MAPK) pathways [25]. Meanwhile, PI3K/Akt and MAPK signaling cascades closely related to activation of c-myc [26]. To further investigate the cooperation effect of c-myc and EGFL7 in prolactinoma, MMQ cells were incubated with a 50 ng/mL recombinant human EGFL7 (rhEGFL7) and anti-c-myc antibodies were added for down-regulation c-myc level. We compared the cell ability, PRL releasing and invasion gene expression of MMQ cells. The results after the two treatments with rhEGFL7 suggested that cell growth and invasion were inhibited. Moreover, down regulation of c-Myc by anti-c-myc antibodies could depress EGFL7 induced cell proliferation and invasion in MMQ cells (Figure 1-4). To further clarify the effect of c-myc knockdown on EGFL7 induce signaling cascades, we detected the AKT and ERK activation from MMQ cells transfected with or without rhEGFL7 were performed with 10 μg/mL anti-c-myc antibodies. Interestingly, the EGFL7 induced activation of AKT was significantly suppressed by c-myc antibody, but activation of ERK was not changed (Figure 5). These results with both treatments showed the positive interaction of c-myc and EGFL7 in biological function of MMQ cells and may correlate with Akt activation.

In the present study, we report for the positive interaction between c-myc and EGFL7 in prolactinoma. We also demonstrated that down regulation c-Myc with antibody could depress EGFL7 induced cell proliferation, PRL secretion and invasion of MMQ cells. In brief, our finding suggests that anti-c-myc efficacy block EGFL7 induced prolactinoma tumorigenesis. Thus, c-myc and EGFL7 may serve as a potential novel viable target for treatment of prolactinomas.

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

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