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EGFL7-blocking antibody inhibit pituitary adenoma proliferation and invasion

Abstract

Objective: To study the effect of EGFL7-blocking monoclonal antibody inhibiting the proliferation and invasion of mouse adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas (ACTHomas) AtT-20 cells.

Methods: After treated with different concentration of EGFL7-blocking monoclonal antibody for 24, 48, 72 h, the proliferation of AtT-20 cells was determined by MTS. Simultaneously, ACTH secretion of AtT-20 cells by ELISA and the mRNA expression of invasion related genes such as E-cadherin, snail, MMP-2 and MMP-9 by Real-time PCR were detected.

Results: EGFL7-blocking monoclonal antibody could effectively inhibit the proliferation and ACTH secretion of AtT-20 cells in a dose-dependent and time-dependent manner. At the same time, EGFL7-blocking monoclonal antibody significant down-regulated the mRNA level of snail, MMP-2 and MMP-9 in AtT-20 cells.

Conclusion: EGFL7-blocking monoclonal antibody could inhibit mouse pituitary adenoma AtT-20 cells proliferation and invasion.

Keywords: EGFL7, monoclonal antibody, AtT-20 cells, proliferation, invasion

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Qian Liu, Jianpeng Wang, Hua Gao, Yazhuo Zhang
Beijing Neurosurgical Institute, Beijing Tiantan Hospital, China

Correspondence: Yazhuo Zhang, Beijing Neurosurgical Institute, Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China, Email zyz2004520@yeah.net

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Abbreviations: EGFL7, Epidermal growth factor-like domain 7; GH, growth hormone; PRL, prolactin; ACTH, adrenocorticotropic hormone; ACTHomas, adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas; GHomas, GH-secreting pituitary adenoma; CNS, central nervous system

Introduction

Pituitary adenoma are one of the most common nervous system tumors. Most pituitary adenomas have the function of secreting distinct pituitary hormones such as growth hormone (GH), prolactin (PRL) and adrenocorticotropic hormone (ACTH).[1] Cushing's disease accounts for 5% - 10% of hormone secreting pituitary adenomas, due to ACTH-secreting pituitary adenomas (ACTHomas) leads to hypercortisolemia. However, the mechanism of ACTHomas is still unclear.[2] The pituitary adenoma invasion is an important factor associated with the clinical prognosis. Endothelial cell derived-secreted factor epidermal growth factor-like domain 7 (EGFL7), plays an important role in angiogenesis.[3] Previously, we reported that increased EGFL7 expression is correlated with the clinical progression, poor prognosis, and tumor grade in hormone-producing pituitary adenomas.[4–6]

However, the effect of EGFL7 in ACTHomas remains to be completely elucidated. Therefore, the present study aimed to investigate the effect of inhibition of EGFL7 on proliferation and ACTH secretion in mouse ACTHomas AtT-20 cells. In vitro administration of anti-EGFL7 antibodies could effectively inhibit the proliferation and ACTH secretion of AtT-20 cells in a dose-dependent and time-dependent manner. At the same time, EGFL7-blocking monoclonal antibody significant down-regulated the mRNA level of E-cadherin, snail and vimentin in AtT-20 cells. Taken together, these findings suggest that EGFL7 might serve as a potential novel biomarker for ACTHomas.

Material and methods

Cell culture

Mouse ACTHomas AtT-20 cells were purchased from ATCC and cultured in F12 medium (containing 2.5% fetal bovine serum, 15% horse serum, 100 units/mL penicillin, 100 units/mL streptomycin), cultured in an incubator containing 5% carbon dioxide at 37 ℃ for 48 hours. Once every 72 hours replaced the culture medium.

Cell proliferation assay

AtT-20 cells in logarithmic growth stage were placed in 96-well microplates, after 24 hours of culture, various concentrations of EGFL7 monoclonal antibodies were added, and the final concentrations were (10, 50 and 100 μg/mL). The normal human IgG was used as a control. Each concentration was parallel to 3 wells, and the treatment was continued for 24, 48 and 72 hours. 20 μL MTS solution was added to each well with 100 μL culture medium to culture at 37 ℃ for 4 h. The absorbance value at the wavelength of 490 nm was measured under the microplate reader.

ELISA assay

AtT-20 cells in logarithmic growth stage were placed in 96-well microplates, after 24 hours of culture, various concentrations of EGFL7 monoclonal antibodies were added. The supernatant of AtT-20 cells treated with 10, 50 and 100 μg/mL monoclonal antibody was stored at - 80 ℃ for ELISA to detect the level of active ACTH using ACTH RapidBio ELISA kit, which performed as described previously.[6]

qRT-PCR

Total RNA was extracted from frozen tumor samples and normal anterior pituitaries using Trizol. Firststrand cDNA synthesis was

generated using the kit according to the manufacturer's instructions.[7] qRT-PCR primer sets are listed in Table 1.

Table 1 RT-PCR primer list

Gene name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
E-cadherin	TAGAGGGTCACCGCGTC	GGGCTGGAGTCTGAACT
Snail	CAATCGGAAGCCTAACTA	CAGATGAGCATTGGCAGCG
MMP-2	AGACATACATCTTTGCTG	CTTGAAGAAGTAGCTGTG
MMP-9	CCTGGAGACCTGAGAACCAAT	CTTGAAGAAGTAGCTGTG
GAPDH	GGTGGTCTCCTCTGACTTCAA	CCAAATTCGTTGTCATACCAG

Statistical analysis

SPSS 13.0 statistical software was used for data analysis. The data are expressed as mean ± standard deviation. The differences between groups were analyzed by two independent sample t-test and variance (ANOVA). The value $p < 0.05$ was considered statistically significant.

Results

Anti-EGFL7 antibody inhibited the proliferation of AtT-20 cells

We previously demonstrated that attenuation of EGFL7 expression inhibits the growth of hormone-producing pituitary adenomas progression and invasion.[4,5] Therefore, to further evaluate the effects and therapeutic potential of inhibition of EGFL7 in ACTHomas, MTS experiments were performed with a commercially available antibody that specifically binds to the EGFL7 protein. As shown in Figure 1, in ACTHomas 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.

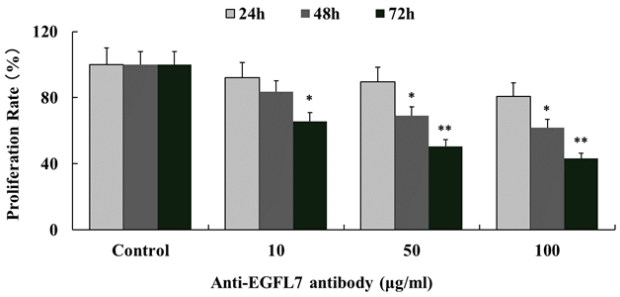

Figure 1 Anti-EGFL7 antibody inhibited the proliferation of AtT-20 cells by MTS. *P<0.05, **p<0.01 versus normal human IgG control.

EGFL7-blocking antibody inhibited the ACTH secretion from AtT-20 cells

Furthermore, the levels of secreted ACTH in the culture supernatant from AtT-20 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 2. Treatment of AtT-20 cells with 50 and 100 μg/mL anti-EGFL7 antibodies for 72 h significantly reduced the level of secreted ACTH as compared with normal human IgG control. The serum ACTH levels were reduced to 120±25, 89±19, and 74±13 pg/mL in response to 10, 50, and 100 μg/mL of anti-EGFL7 antibodies, respectively (Figure 2) ($p < 0.01$).

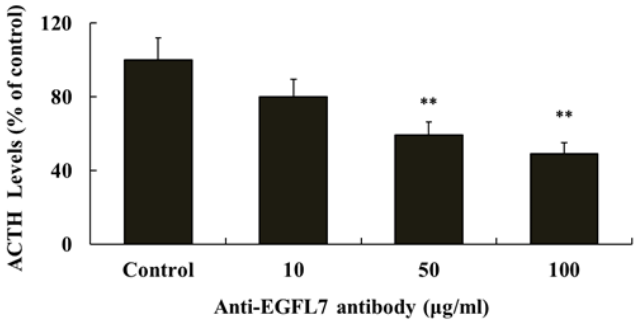

Figure 2 Downregulation of EGFL7 inhibited the ACTH secretion from AtT-20 cells by ELISA. **p<0.01 versus normal human IgG.

EGFL7-blocking antibody down-regulated the mRNA level of invasion related genes in AtT-20 cells

In order to determine whether EGFL7 monoclonal antibody affects the invasion of AtT-20 cells, we detected the effect of EGFL7 monoclonal antibody on the expression of invasion related genes of AtT-20 cells by qRT-PCR. Treatment of AtT-20 cells with 10 and 50 μg/mL anti-EGFL7 antibodies for 24 h, without affecting the cell proliferation activity, significantly inhibited the mRNA expression levels of snail, MMP-2 and MMP-9 and induced the mRNA expression levels of E-cadherin ($p < 0.01$) (Figure 3).

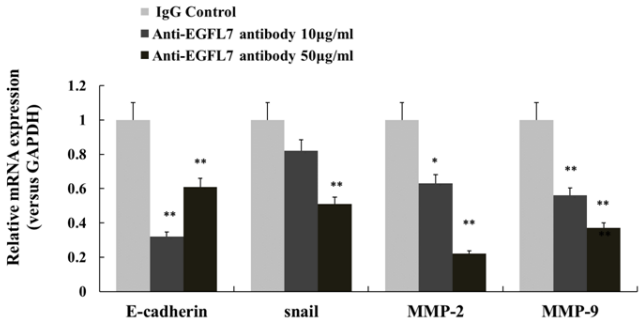

Figure 3 Anti-EGFL7 antibody inhibited the invasion of AtT-20 cells by RT-PCR. *P<0.05, **p<0.01 versus normal human IgG control.

Discussion

Tumor arise in anterior pituitary gland is pituitary adenoma, which are the most common central nervous system tumor.[8] Most pituitary adenomas are functional in nature, which secreting PRL, GH or ACTH.[9–11] In our group, for the first time we found that, compared to non-invasive GH-secreting pituitary adenoma (GHomas), the expression of EGFL7 protein in invasive GHomas was markedly increased. The expression of EGFL7 was significantly associated with the histologic subtypes, tumor volume, invasiveness and recurrence of GHomas via Notch2/DLL3 signaling pathway.[4,5] Furthermore, our published data[5] indicated knockdown of EGFL7 expression suppresses GH3 and GT1-1 cells proliferation and invasion in vitro and inhibits human GHomas growth *in vivo*. The data suggest that as a Notch agonist, EGFL7 may potentially be an appropriate novel molecular target for future development of GHomas medical therapy.

In this study, our finding suggests that EGFL7 may contribute to the regulation of proliferation and ACTH secretion in mouse ACTHomas AtT-20 cells. Furthermore, anti-EGFL7 antibodies inhibit the mRNA expression levels of invasion related genes snail and MMP-2/MMP-9 without affecting the cell proliferation activity.

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Taken together, EGFL7 may serve as a potential novel therapeutic target for ACTHomas.

Conclusion

EGFL7-blocking monoclonal antibody could inhibit mouse pituitary adenoma AtT-20 cells proliferation and invasion. These findings raised the possibility that EGFL7 might serve as a useful biomarker to assess ACTHomas invasion and prognosis or a potential therapeutic target for ACTHomas treatment.

Acknowledgments

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Conflicts of interest

The authors declare that they have no competing interests.

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