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The correlation among Claudin-9, Tyrosine kinase-2, and Signal transducers and activators of transcription-3 expressions in non-functioning pituitary adenoma and invasiveness.

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Abstract

BACKGROUND: Deeper studies on the pathological mechanism associated with invasiveness of non-functioning pituitary adenoma (NFPA) is imperative to find better treatments. This research was preliminarily conducted to investigate the correlation between the expression of Claudin-9 (CLDN9), Tyrosine kinase-2 (TYK2), Signal transducers and activators of transcription-3 (STAT3) and invasiveness in NFPA to illustrate the pathological mechanism.

METHODS: Clinical data and surgical specimens of 12 patients with NFPA were collected and divided into invasive and non-invasive NFPA groups, comprising six patients for each group. CLDN9, TYK2 and STAT3 transcription and expression levels in the NFPA tissues of the two groups were detected by quantitative real-time polymerase chain reaction (qRT-PCR), Western blotting (WB) and immunohistochemistry (IHC). The lentiviral plasmid transfection technique was used to develop a rat pituitary tumour GT1-1 cell line null control group (NC) and CLDN9-overexpressed experimental group (OE-CLDN9), and TYK2 and STAT3 transcription levels in the NC and OE-CLDN9 cell groups were detected using qRT-PCR.

RESULTS: The CLDN9 and STAT3 expressions were significantly higher in invasive than in non-invasive NFPA tissues, whereas the TYK2 expression in invasive NFPA tissues was significantly lower than that in non-invasive NFPA ($p < 0.001$); The STAT3 upregulated ($p < 0.001$) and the TYK2 downregulated ($p < 0.01$) after the CLDN9 overexpression.

CONCLUSION: Upregulated CLDN9 may increase the NFPA invasiveness through STAT3. In addition, low TYK2 expression might enhance the invasiveness in NFPA, which needs further studies to confirm. These results could provide a promising research leads for targeted treatment of NFPA.

ORIGINAL ARTICLE

INTRODUCTION

Non-functioning pituitary adenoma (NFPA) is a common type of pituitary adenoma accounting for approximately 25%-35% of cases (Zhu *et al.* 2018). However, performing total surgical resection is difficult because it frequently invades the cavernous sinus, optic nerve and other important adjacent structures, resulting in a postoperative recurrence rate of 58% (Guo *et al.* 2019; Zhu *et al.* 2018). Effective drugs or other therapeutic approaches are lacking since the pathological mechanisms associated with NFPA invasiveness are not well understood (Guo *et al.* 2019).The efficacy and safety of radiotherapy are also unfavourable (Fu *et al.* 2016). Therefore, further studies on the molecular mechanism of NFPA associated with invasiveness and associated with genes is essential in finding better therapeutic approaches.

Claudins (CLDNs), including 27 members, are essential for maintaining cellular stability and polarity as a structural component of the tight junction (TJ) (Liu *et al.* 2021). Previous studies revealed that aberrant expression (up or downregulation) of CLDN proteins could promote or inhibit malignant progression of various tumours (Kwon MJ 2013; Tabariès S and Siegel PM 2017). Therefore, the aberrant expression of CLDNs is considered as one of the important mechanisms for the malignant progression of tumours (Lu *et al.* 2021). In a previous study, we found, by gene microarray technology and gene ontology (GO) analysis, high CLDN9 expression in pituitary adenoma (Hong *et al.* 2014). In studies on the mechanisms associated with CLDN protein and tumor invasiveness, it has been found that CLDN9 can play a role in promoting malignant tumor growth through some important signaling pathways. Among them, the relationship between CLDN and TYK2/STAT3 signaling pathway has been reported in some literature. For example, Liu *et al.* found that CLDN9 promotes tumor invasive growth and metastasis through the TYK2/STAT3 signaling pathway in liver tumor cell lines (Liu *et al.* 2019). In addition, Sun *et al.* also showed that CLDN17 promotes liver tumor migration and invasion by affecting TYK2 and STAT3 (Sun *et al.* 2018). Therefore, the authors hypothesized that CLDN9, TYK2 and STAT3 may play a common role in the invasive growth of NFPA.

TYK2 and STAT3 are important members of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway (including four JAK members [JAK1, JAK2, JAK3, and TYK2] and seven STAT members [STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6]), which serves as a "bridge" for conveying intra- and extracellular information and is one of the central pathways in cancer research (Bousoik E and Montazeri Aliabadi H 2018; Wöss *et al.* 2019). Its abnormal expression or activation has been associated with the occurrence and development of various tumours (Meng *et al.* 2020; Owen *et al.* 2019; Tu *et al.* 2011). In this study, the correlation among CLDN9, TYK2 and STAT3 expressions in NFPA and invasiveness was analysed. In addition, the correlation between the overexpressed CLDN9 in NFPA and TYK2 and STAT3 was also initially discussed.

MATERIALS AND METHODS

NFPA tissue specimens

The NFPA tissue specimens were obtained from 12 patients with NFPA hospitalised in the Department of Neurosurgery at the People's Hospital of Xinjiang Uygur Autonomous Region for endoscopic transsphenoidal pituitary tumour resection from June 2020 to May 2021. Basic information, preoperative hormone levels, preoperative pituitary magnetic resonance imaging (MRI) and postoperative pathological examination results were collected and divided into invasive and non-invasive NFPA groups based on their preoperative imaging and intra-operative information, comprising six patients for each group. A total of seven men and five women, aged 27–82 (average, 49.3±16.7) years, were included.

Inclusion criteria: ① no preoperative treatment; ② no history of endocrine disease or long-term hormone administration; ③ with clinical manifestations, preoperative endocrine hormone and postoperative pathological immunohistochemical results all supporting the diagnostic criteria of NFPA; and ④ tumour tissues obtained by the same operator

Exclusion criteria: ① patients with recurrent PA; ② incomplete data and ③ a history of other tumours

Diagnostic criteria: Invasive PA was diagnosed based on any of the following criteria: ① preoperative MRI of Knosp classification (Even-Zohar N and Greenman Y 2022; Micko *et al.* 2015) grade IV; ② preoperative MRI of Knosp classification grade III and intraoperative visible invasive growth towards the paracavernous sinus or saddle base bone or the suprasellar area. This study was conducted following the Declaration of Helsinki (www.wma.net/en/30publications/10policies/b3/index.html).

Storage of pituitary adenoma tissue specimens

Specimens for quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting (WB) were frozen at −80 °C and stored in liquid nitrogen, and those for immunohistochemistry (IHC) were fixed in 4% formaldehyde, embedded in paraffin and stored at the pathology department of our hospital.

Cell culture and plasmid transfection

As gonadotropin cell tumours are the most common NFPA in clinical practice (Even-Zohar N and Greenman Y 2022), this study used the rat pituitary tumour GT1-1 cell line (Procell China Wuhan

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Life Sciences Co., Ltd.) as NFPA cells for the experiments. The GT1-1 cell line was cultured in the basal medium (Procell China Wuhan Life Sciences Co., Ltd.) containing 10% foetal bovine serum (Gibco Shanghai Suer Biotechnology Co., Ltd.) + 1% of 100 U/mL penicillin and streptomycin in an incubator at 37 °C with 5% CO_2. The CLDN9 plasmid and null plasmid (U-Bio, China) were transfected into GT1-1 cells using the lipofectamine 3000 transfection kit (Invitrogen, USA), and the CLDN9 overexpressed experimental group (OE-CLDN9) and the null cell control group (NC) were constructed. 1.5*10^5 cells in each group were placed in three culture dishes and incubated in the same way for 48 h.

qRT-PCR

The total RNA for each group of tissues and cells was extracted using the Trizol kit (Invitrogen, USA) and reverse transcribed into cDNA following the instructions of the reverse transcription kit (Thermo Fisher Scientific, USA). qRT-PCR was then performed using the SYBR Green PCR kit (All-Style Gold Biotechnology, Beijing, China). The reaction conditions were as follows: pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 10 s, annealing extension at 60 °C for 30 s and 40 cycles in total. Relative changes in mRNA transcription levels were calculated using the comparative cycle method (2−ΔΔCt), and data were normalized by beta-actin. The primers used in this study are shown in the table below.

WB

Tumor tissues of each group were lysed in RIPA buffer (Beijing Solabao Technology Co., Ltd.) supplemented with protease and phosphatase inhibitors. Protein samples were quantified using the BCA Protein Assay Kit (Beijing Solabao Technology Co., Ltd.) and heated at 100 °C for 10 min. Then, they were separated by SDS–PAGE and transferred to PVDF membranes (Millipore agent-Shanghai Yubo Biotechnology Co., Ltd.) where 5% milk in TBST (TBS with 0.05% Tween 20) was used as the blocking buffer. After incubation for 1 h, primary antibodies (Rabbit polyclonal anti-CLDN9 antibody, 1:10,000, NOVUSBIO, USA; Rabbit polyclonal anti-TYK2, STAT3 antibodies, 1:10,000, Wuhan Abletech Biotechnology Co.) were incubated overnight at 4 °C in the blocking solution. The membrane was washed three times with Tris-buffered saline (Beijing Solabao Technology Co., Ltd.), each time for 10 min. horseradish peroxidase (HRP)-labeled secondary antibodies (Goat anti-rabbit lgG, 1:2000, Beijing ZhongShan JinQiao biotechnology Co.) were incubated in TBST for 2 h at room temperature. Bands were detected by the ECL western-blotting substrate (BioRad, 1705061). Images were analysed using the Image-Pro Plus 6.0 (Media Cybernetics).

Table.

Gene	Sequences
β-actin	Forward 5´-CCCATCTATGAGGGTTACGC-3´
	Reverse 5´-TTTAATGTCACGCACGATTTC-3´
CLDN9	Forward 5´-CCTTTCGACCTTGGCCTGGAT-3´
	Reverse 5´-GGGGGAGAACATCAAAGGGG-3´
TYK2	Forward 5´-CAGCCCCGTGTTCTGGTATG-3´
	Reverse 5´-GAAAGGACGCCTCTGTCTCC-3´
STAT3	Forward 5´-GGAGAAACAGGATGGCCCAA-3´
	Reverse 5´-ATCCAAGGGGCCAGAAACTG-3´

IHC

Formalin-fixed, paraffin-embedded sections (5-μm) were placed in 68 °C incubator (Shanghai Boxun Medical Biological Instrument Co., Ltd.) for 45 min for baking and dewaxed with xylene and ethanol. Then, 3% of hydrogen peroxide was added dropwise to the tissue and incubated at room temperature for 20 min to quench endogenous peroxide activity. A closed buffer prepared with 5% foetal bovine serum (Gibco Shanghai Suer Biotechnology Co., Ltd.) and PBS buffer were added dropwise to the tissues and placed in an incubator at 37 °C for 30 min. The primary antibodies (Rabbit polyclonal anti-CLDN9 antibody, 1:200, NOVUSBIO, USA; Rabbit polyclonal anti-TYK2,STAT3 antibodies, 1:200, Wuhan Abletech Biotechnology Co.) diluted with the closed buffer added to corresponding slides, incubated overnight at 4 °C. After several washes with PBS, slides were incubated with HRP-conjugated secondary antibodies (Goat anti-rabbit lgG, 1:2000, Beijing ZhongShan JinQiao biotechnology Co.) for 30 min at room temperature. The prepared DAB color developer (Beyotime, Shanghai) was added. They were observed under a microscope and rinsed with tap water when the color reached optimum. Then, which were stained in haematoxylin (Beijing Solaibao Technology Co., Ltd.) for 1–2 min. The sections were dehydrated with gradient ethanol (Beijing Solabay Technology Co., Ltd.), sealed with neutral gum (Beijing Solabay Technology Co., Ltd.), fixed and photographed. Image Pro Plus 6.0 (Media Cybernetics) was used to convert the images into grayscale images, and the optical density at each point of the images was accumulated to obtain the integrated optical density (IOD) for each group of slices, and then divided by the area of the target distribution area to obtain the average optical density (AOD), and the computed AOD values indicated the expression level of the candidate proteins, which were analyzed quantitatively.

Statistical analysis

Data were statistically analysed and plotted using the SPSS 25.0 and GraphPad Prism 8.00 software. Co-normality test by SW method and expressed as mean ± standard deviation ($\bar{x}$±SD), the Student's t-test

Tab. 1. Correlation between the expression of CLDN9, TYK2 and STAT3 in NFPA and age

Indicators	Methods	Age (years)		t	p
		<50	>50		
CLDN9	qRT-PCR	1.71 ± 0.75	1.57 ± 0.58	0.35	0.731
	WB	1.04 ± 0.43	1.06 ± 0.23	-0.11	0.918
	IHC	3147.22±1056.78	3234.06±930.42	-0.15	0.883
TYK2	qRT-PCR	0.66 ± 0.42	0.65 ± 0.39	0.05	0.964
	WB	0.65 ± 0.16	0.69 ± 0.23	-0.42	0.683
	IHC	5984.72±1001.58	5281.06±878.64	1.29	0.225
STAT3	qRT-PCR	2.29 ± 1.45	2.50 ± 1.66	-0.23	0.824
	WB	0.88 ± 0.26	0.84 ± 0.36	0.22	0.830
	IHC	4520.22±1833.43	4943.06±1923.22	-0.39	0.705

was performed for two-way comparisons between groups, two-way analysis of variance was performed for multiple comparisons and $p < 0.05$ were considered statistically significant.

RESULTS

Expression of CLDN9, TYK2 and STAT3 in NFPA was not correlated with age

Among the 12 NFPA patients, 6 were aged >50 years and 6 were aged <50 years, with an average of (49.3±16.7) years. Statistical analysis showed that the expression of CLDN9, TYK2 and STAT3 in NFPA was not correlated with age ($p > 0.05$) (Table 1).

CLDN9 expression is upregulated in invasive NFPA tissues

The qRT-PCR results showed that the mRNA level of CLDN9 in invasive NFPA tissues was significantly higher than that in non-invasive NFPA tissues, and the differences between the two groups were statistically significant ($p < 0.001$) (Table 2 and Figure 1A). WB and IHC results showed that the CLDN9 expression in invasive NFPA tissues was significantly higher than in non-invasive NFPA tissues and CLDN9 protein was mainly expressed in the nucleus, the expression differences between the two groups were statistically significant ($p < 0.05$, $p < 0.001$, respectively) (Table 2 and Figure 1B-C).

TYK2 expression is down-regulated in invasive NFPA tissues

The qRT-PCR results showed that the mRNA level of TYK2 in invasive NFPA tissues was significantly lower than in non-invasive NFPA tissues, with a statistically significant difference between the two groups ($p < 0.001$) (Table 3 and Figure 2A). WB and IHC results showed that the TYK2 expression in invasive NFPA tissues was significantly lower than in the non-invasive NFPA tissues, and TYK2 was mainly expressed in the cytoplasm and slightly expressed in the extracellular matrix, the expression difference between the two groups was statistically significant ($p < 0.05$, $p < 0.01$, respectively) (Table 3 and Figure 2B-C).

Tab. 2. CLDN9 expression level in two groups of NFPA

Group	qRT-PCR	WB	IHC
Non-invasive	1.04 ± 0.03	0.85 ± 0.17	2294.50 ± 154.92
Invasive	2.23 ± 0.23	1.24 ± 0.34	4086.78 ±188.79
t	12.63	2.45	17.98
p	<0.001	0.032	<0.001

Tab. 3. TYK2 expression level in two groups of NFPA

Group	qRT-PCR	WB	IHC
Non-invasive	1.02 ± 0.02	0.78 ± 0.19	6380.89 ± 714.23
Invasive	0.29 ± 0.13	0.57 ± 0.12	4884.89 ± 468.32
t	13.82	2.26	4.29
p	<0.001	0.047	0.002

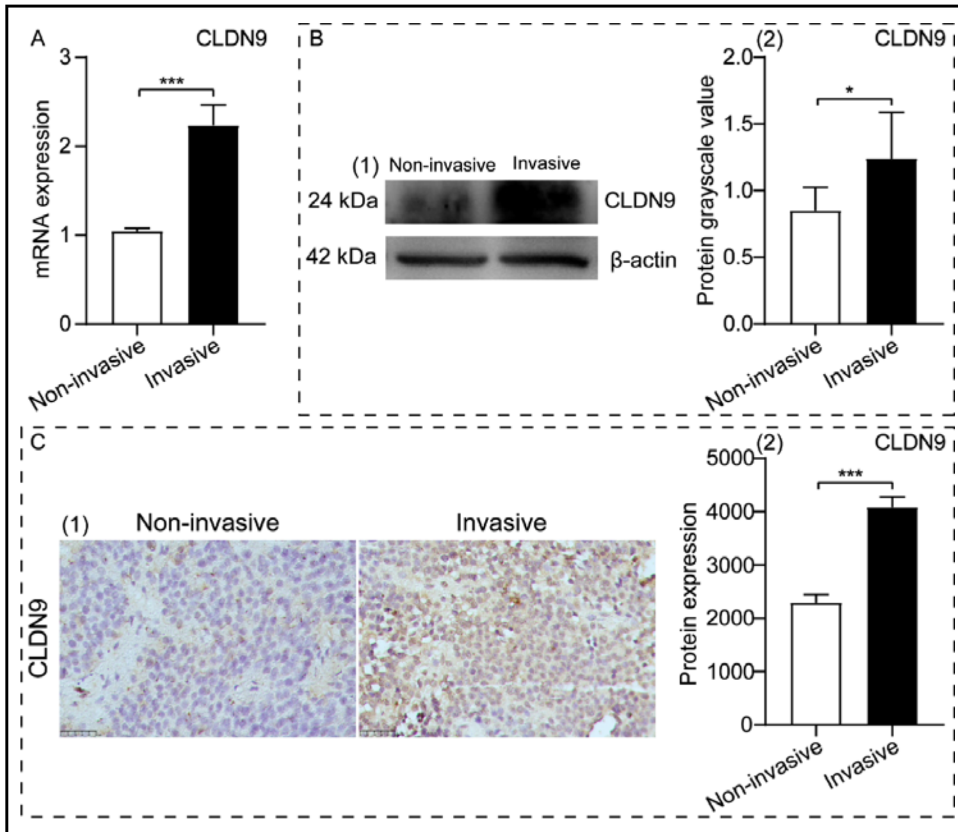

Fig. 1. The expression levels of CLDN9 in NFPA tissues. **A:** The relative mRNA expression of CLDN9 detected using qRT-PCR. **B:** The CLDN9 protein expression detected using WB. **C:** The CLDN9 protein expression detected using IHC (400×), and brown staining represents CLDN9 high expression. (Note: *: $p < 0.05$; ***: $p < 0.001$ vs, non-invasive group)

STAT3 expression is upregulated in invasive NFPA tissues

The qRT-PCR results showed that the mRNA level of STAT3 in invasive NFPA tissues was significantly higher than that in non-invasive NFPA tissues, with statistically significant difference between the two groups ($p < 0.001$) (Table 4 and Figure 3A). WB and IHC results showed that the STAT3 expression in invasive NFPA tissues was significantly higher than that in non-invasive NFPA tissues, and STAT3 was mainly expressed in the cytoplasm and nucleus, with statistically significant difference ($p < 0.05$, $p < 0.001$, respectively) (Table 4 and Figure 3B-C).

STAT3 expression is up-regulated and TYK2 expression is down-regulated after CLDN9 overexpression

The CLDN9 levels in both cell groups were detected using qRT-PCR after the null plasmid (NC) and CLDN9 (OE-CLDN9) plasmid transfection, and results showed that the expression in the OE-CLDN9 group was significantly higher than NC group ($p < 0.001$) (Table 5 and Figure 4A), indicating that the CLDN9 transfection was successful. Next, TYK2 and STAT3 levels in the two cell groups were detected by qRT-PCR, and results showed that the STAT3 transcription levels in the OE-CLDN9 group was significantly higher than in the NC group ($p < 0.001$) (Table 5 and Figure 4B), and the TYK2 transcription levels in the OE-CLDN9 group was significantly lower than in the NC group ($p < 0.01$) (Table 5 and Figure 4C).

DISCUSSION

NFPA is a benign tumour but its invasive nature makes it one of the most challenging neurosurgical conditions to treat (Zhang *et al.* 2017). Consequently, research related to the invasiveness of NFPA is one of the most popular in the field of neurosurgery. The development and progression of NFPA is a complex process involving activation of proto-oncogenes, inactivation of oncogenes, hormonal stimulation, increasing growth factors, and abnormalities in cell signalling pathways as with the other tumours (Sapochnik *et al.* 2016; Yang Q and Li X 2019). In a previous study, we identified CLDN9 as a gene associated with pituitary adenomatosis, with higher expression in pituitary adenomas than in normal pituitary gland (Hong *et al.* 2014). In this study, further research was conducted with the hope of providing a valuable research base for the pathological mechanisms of NFPA invasiveness.

Abnormal expression of CLDNs proteins, as an important structure for maintaining the epithelial barrier, is closely associated with increased tumorigenesis and aggressiveness (Zhang *et al.* 2019). For example, CLDN3/4 is highly expressed in prostate cancer, and the aggressiveness and survival of prostate cancer cells is reduced after CLDN3/4 is knocked out (Liu *et al.* 2021). Moreover, the aberrant expression of CLDNs in tumours is tissue-dependent, with differential expression abnormalities in tumours at different tissue origins (Lu *et al.* 2017), such as high

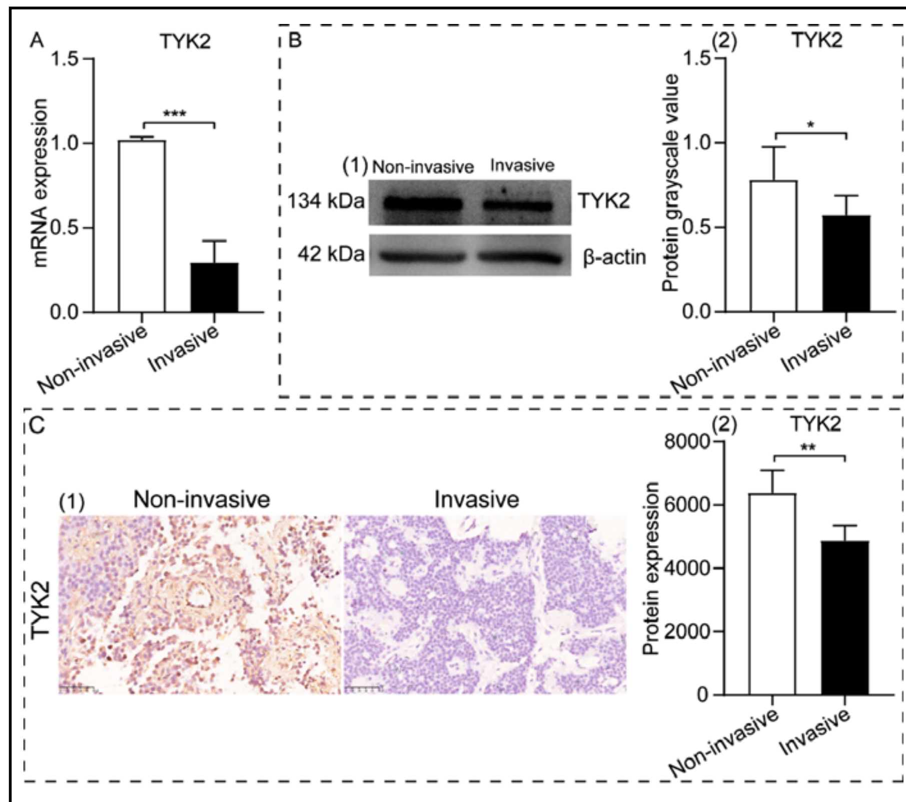

Fig. 2. The expression levels of TYK2 in NFPA tissues. **A:** The relative mRNA expression of TYK2. **B:** The TYK2 protein expression detected using WB. **C:** The TYK2 protein expression detected using IHC (400×), and brown-yellow staining represents TYK2 high expression. (Note: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs, non-invasive group)

CLDN1 and low CLDN2 expression in plasma papillary carcinomas (Lu *et al.* 2021). Conversely, CLDN1 is lowly expressed and CLDN2 is highly expressed in the endometrium-like hyperplastic tissues (Lu *et al.* 2021). However, only a few studies have been reported on the association between CLDNs and pituitary adenoma invasiveness. In the present study, CLDN9 was highly expressed in invasive NFPA than that in non-invasive NFPA, which were consistent with our previous results obtained in the gene microarray and GO analysis (Hong *et al.* 2014). In addition, Kim *et al.* also found that high CLDN9 expression promotes NFPA invasiveness through bioinformatics (Kim YH and Kim JH 2019). Combined with these findings, CLDN9 overexpression is associated with increased NFPA invasiveness. The upregulation of CLDN9 protein expression may disturb the normal composition ratio of TJ components, resulting in changes in their integrity and function, because of increased cell gaps and tumour cell proliferation through the "relaxed" cell gaps (Lu *et al.* 2021), contributing to the increased NFPA invasiveness. In addition, aberrantly expressed CLDNs promote tumour aggressiveness and distant metastasis by inducing epithelial-to-mesenchymal transition (EMT) (Hashimoto I and Oshima T 2022). Therefore, knockdown of highly expressed CLDN9 may be a promising potential therapeutic approach for NFPA.

STAT3, as an important member of the signal transduction pathway, transduces signals from outside the cell into the cell (Okabe *et al.* 2021). STAT3 is phosphorylated in a variety of tumours in response to its upstream protein TYK2, which appears aberrantly expressed and promotes tumour aggressiveness in a TYK2/STAT3 signalling axis pattern (Derecka *et al.* 2012). For example, high expression or sustained activation of TYK2 and STAT3 is observed in ovarian cancer, hepatocellular carcinoma, lung cancer and hematologic tumours, further increasing their aggressiveness (Wöss *et al.* 2019). However, the present study demonstrated that STAT3 was highly expressed and TYK2 was lowly expressed in invasive than in non-invasive NFPA. The

Tab. 4. STAT3 expression level in two groups of NFPA

Group	qRT-PCR	WB	IHC
Non-invasive	1.02 ± 0.01	0.69 ± 0.30	3278.62±1180.48
Invasive	3.78 ± 0.57	1.03 ± 0.18	6184.67± 840.77
t	11.90	2.38	4.91
p	<0.001	0.038	<0.001

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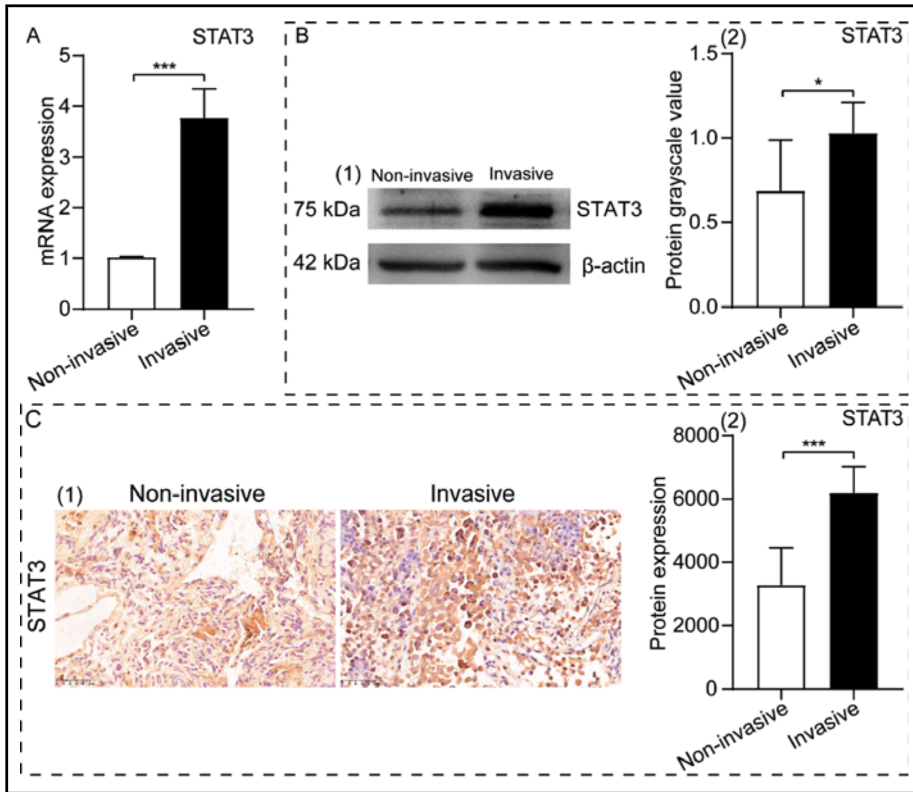

Fig. 3. The expression levels of STAT3 in NFPA tissues. **A:** The relative mRNA expression of STAT3. **B:** The STAT3 protein expression detected using WB. **C:** The STAT3 protein expression detected using IHC (400×), and brown-yellow staining represents STAT3 high expression. (Note: *: $p < 0.05$; ***: $p < 0.001$ vs, non-invasive group)

high STAT3 expression in invasive NFPA is consistent with that of the previously reported findings. For example, Shen and Feng *et al.* found that STAT3 was highly expressed in invasive NFPA and suggested that high STAT3 expression was associated with increased NFPA invasiveness (Feng *et al.* 2016; Shen *et al.* 2021). Zhou *et al.* also found that STAT3 expression was upregulated in NFPA and growth hormone pituitary adenoma as compared with the normal pituitary gland and S3I-201 (a STAT3 inhibitor) that inhibited the growth of pituitary adenoma cells (Zhou *et al.* 2015). The mechanism by which highly expressed STAT3 promotes NFPA invasiveness may be that it is phosphorylated and then dimerized in homo- or heterodimerization and transferred to the nucleus, regulating the transcription of the corresponding genes and thus affecting important functions such as cell survival, proliferation or apoptosis (Feng *et al.* 2016). It has also been suggested in the literature that STAT3 may increase the invasiveness of pituitary adenomas by enhancing the function of mitochondria (Liu *et al.* 2021). Combined with these findings, the high STAT3 expression was suggested to may be associated with increased NFPA invasiveness, and reduced STAT3 may be the promising target for the NFPA treatment.

Surprisingly, TYK2 expression was lower in invasive than in non-invasive NFPA. A further literature review found that low TYK2 expression in some tumour samples or sections is also considered as a marker of poor prognosis (Uhlen *et al.* 2017). For example, downregulated TYK2 expression has been reported in the literature, contributing to the local metastasis of breast cancer (Sang *et al.* 2012). A meta-analysis on hepatocellular carcinoma than a normal or high TYK2 expression was suggested to be associated with longer patient survival (Wang *et al.* 2019). It was also reported that TYK2 can act as immunosurveillance, promoting apoptosis and anti-tumour proliferation by mediating cytokines, such as interferons and interleukin-12 (IL-12), in addition to its oncogenic role (Leitner *et al.* 2017). Furthermore, JAKs may bind to various cytokine receptors and activate different STATs, thus initiating

Tab. 5. CLDN9, STAT3, and TYK2 expression levels in the two groups of cells

Group	CLDN9	STAT3	TYK2
NC	1.46 ± 0.50	0.64 ±0.13	1.03 ± 0.14
OE-CLDN9	4680.02 ± 646.42	0.87± 0.12	0.85 ± 0.12
F	943.30	42.99	13.64
p	<0.001	<0.001	0.004

Note: NC is the null control group,OE-CLDN9 is the CLDN9-overexpressed experimental group.

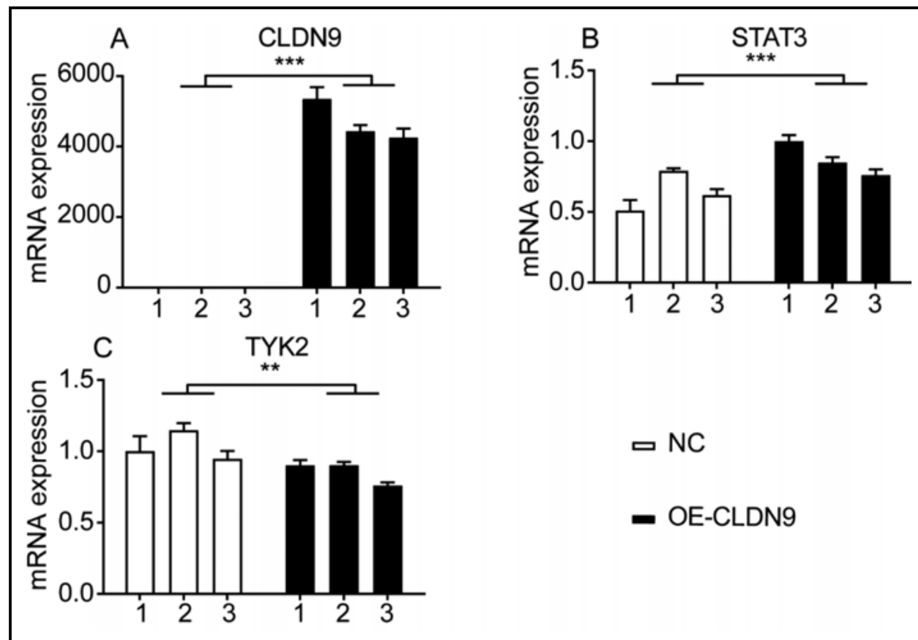

Fig. 4. Construction of CLDN9-overexpressed pituitary tumour GT1-1 cells line and the STAT3 and TYK2 expressions in two cell groups. **A-C:** The relative mRNA expression of CLDN9, STAT3 and TYK2 (respectively) in two cell groups detected using qRT-PCR. (Note: **: $p < 0.01$; ***: $p < 0.001$ vs, NC group)

unique JAK/STAT signalling axes, as indicated in the relevant literature (Bousoik E and Montazeri Aliabadi H 2018; Coskun *et al.* 2013). For example, the IL-2 receptor increases breast cancer progression by initiating the JAK2/STAT1 and STAT5 signalling pathways (Bousoik E and Montazeri Aliabadi H 2018). IL-4 plays a role in tumours, such as cervical, ovarian and liver cancers by initiating the JAK1,3/STAT6 signalling axis (Bousoik E and Montazeri Aliabadi H 2018).

Feng *et al.* suggested that STAT3 mediates IL-6 and increases the invasiveness of NFPA through the IL-6/JAK2/STAT3 signalling pathway (Feng *et al.* 2016). Additionally, tumour cells can resist apoptosis by upregulating the anti-apoptotic gene BCL-2, and the STAT1/STAT3 upregulates the BCL-2 expression or other members in a TYK2-dependent or TYK2-independent manner (Wöss *et al.* 2019). STAT3 may also be activated by other pathways, such as EGFR and SRC, but not only by JAK (Bousoik E and Montazeri Aliabadi H 2018). Based on these literature reports, we considered that TYK2 may play an immunosurveillance and anti-tumour proliferation role in NFPA, and low TYK2 expression may be associated with NFPA invasiveness and poor prognosis. Furthermore, in NFPA TYK2 and STAT3 may be mediated by different cytokines to initiate different signalling axes rather than the TYK2/STAT3 signalling axis, or be associated with more complex mechanisms. This assumption needs to be confirmed by further study.

Studies revealed that CLDNs contribute to tumourigenesis and progression by participating in cellular signaling pathways (Zhang *et al.* 2018). For example, the low expression of CLDN3 in colon cancer promotes its aggressiveness by inducing a STAT3-dependent Wint/β-catenin signalling pathway (Ahmad *et al.* 2017). The high CLDN9/CLDN17 expression and the promotion of hepatocellular carcinoma aggressiveness may be due to the aberrant expression or activation of the TYK2/STAT3 signalling pathway (Liu *et al.* 2019; Sun *et al.* 2018).

In the present study, the overexpressed CLDN9 pituitary tumour GT1-1 cell lines were constructed to observe whether there was any relationship between CLDN9, which was highly expressed in NFPA, and TYK2 and STAT3. The results showed that STAT3 expression was upregulated and TYK2 expression was downregulated after CLDN9 overexpression in the pituitary tumour GT1-1 cell line, which was consistent with the NFPA tissue experiments. Studies revealed that aberrantly expressed CLDNs induce EMT, which is essential for promoting tumour invasion and proliferation, through various activated signalling pathways (Yu *et al.* 2019). For example, CLDN12, which is highly expressed in lung squamous carcinoma, induces EMT through activated TYK2/STAT1, thereby promoting the aggressiveness of lung squamous carcinoma (Sun *et al.* 2019). Highly expressed STAT3 in breast cancer promotes its metastasis through the induction of EMT (Kim *et al.* 2019). Therefore, STAT3 is considered as a regulator of EMT (Xu *et al.* 2020). In tumours, STAT3, which is activated in response to the corresponding cytokines, is transferred to the nucleus and binds to the proto-oncogene c-Myc, which in turn binds to the snail gene, causing its activation (Shen *et al.* 2021), and the activated snail gene upregulates the CLDN9 expression (Kim YH and Kim JH 2019), as a regulator of gene expression in CLDNs (Escudero-Esparza *et al.* 2011), further induces EMT, thereby increasing tumour aggressiveness (Escudero-Esparza *et al.* 2011; Lu *et al.* 2021). Numerous studies have reported that EMT can contribute to invasiveness in pituitary adenomas (Jia *et al.* 2015; Mertens *et al.* 2015; Wang *et al.* 2018). Therefore, we consider

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that highly expressed CLDN9 in NFPA may induce EMT through being aberrantly expressed or activated STAT3, thereby causing NFPA invasiveness, and further studies are needed to confirm this.

To our best knowledge, this is the first study conducted on the correlation between TYK2 expression in NFPA and invasiveness. Additionally, this study has limitations such as small sample size and simplicity of the experiment. Next, we will further investigate the exact role of TYK2 in NFPA through more sample-based and rational experiments, and the relationship between low expression of TYK2 and prognosis of NFPA patients.

CONCLUSION

The upregulated CLDN9 and STAT3 expression in NFPA were associated with invasive growth of NFPA, and CLDN9 may promote NFPA invasiveness through STAT3. Low TYK2 expression in NFPA may be associated with poor prognosis, a finding that requires further investigation. Results of the present study provide preliminary research clues for further studies on the NFPA invasiveness mechanism, which is expected to be a promising therapeutic target for invasive NFPA.

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