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NTNG1 Modulates Progressions of Prostate Cancer Cells through JAK/STAT Signalling Pathway

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KEYWORDS Janus Kinase. Ki67. Netrin G1. Prostate Cancer. Signal Transducer. Activator of Transcription 3

ABSTRACT Netrin-G1 (NTNG1) is a glycosyl-phosphatidylinositol-affixed synaptic adhesion molecule that participates in carcinoma developments. However, its underlying mechanism in regulating prostate cancer (PCa) is uncertain. NTNG1 mRNA and protein expressions in prostate cancer tissue samples and cells were demonstrated to be promoted using RT-qPCR and western blot. Thereafter, using CCK-8, NTNG1 overexpression accelerated PCa cell viability while suppressed NTNG1 restrained cell viability. Additionally, transwell results indicated that migratory and invasive abilities of PCa cells were also facilitated by overexpressed NTNG1 but inhibited with NTNG1 suppression. Furthermore, using RT-qPCR, Janus kinase 1 (JAK1) has been detected to be upregulated by NTNG1 upregulation but suppressed with NTNG1 downregulation. Moreover, JAK1, JAK2, signal transducer and activator of transcription 3 (STAT3), Ki67 and E-cadherin protein expressions were also suppressed by the knockdown of NTNG1 but elevated with NTNG1 overexpression. In PCa cells, NTNG1 acted as an oncogene through activating JAK/STAT3 signalling pathway.

INTRODUCTION

Cancer has been commonly described as an aggressive disease in which cells multiply and grow without any control and movement restriction. Cancer progression characteristic is associated with activated cell invasiveness and migration that are frontrunner traits to mortality among cancer patients (van de Merbel et al. 2018). Prostate cancer (PCa) is the most aggressive malignancy distressing males. Currently, a wide variation of prostate cancer incidences has been reported with higher rates experienced in nationalities from western countries at an approximated rate of 70 cases in every 100,000 and 17 annually while lower rates of 11 cases in every 100,000 and 6 annually of PCs have been reported in East Asia (Reulen et al. 2017). Some studies have reported that low risk PCa has seen a nearly seventy percent increase with the prostate-specific antigen (PSA) examination (Garisto and Klotz 2017). However, PCa incidences in recent times continue to increase haphazardly especially in prostate cancer patients at moderately late medical stage in China (Liu X et al. 2019; Ren et al. 2013). PCa has been normally termed as an age related disease that is unnoticed among youthful patients (Bleyer et al. 2020). PSA blood screening has been employed in several prostate cancer stages of management, including testing and evaluation of upcoming risks of PCa growth, diagnosis following local treatment and management in disease progressions (Nguyen-Nielsen and Borre 2016). Moreover, some studies have investigated the mechanisms that promote cell invasion and migration via several pathways to establish therapeutic measures and understand PCa pathogenesis (Aghdam et al. 2019; Barrett et al. 2017). For example, Uegaki et al. have reported that downregulated Ral GTPase-activating protein enhanced prostatic epithelial cell invasiveness and advancement from intraepithelial neoplasia to cancer throughout prostate tumorigenesis (Uegaki et al. 2019). Furthermore, another study has revealed that silenced Carbonic Anhydrase 1 (CA1) exosomes-regulated proteins in prostate cancer cells were responsible for metabolism, biogenesis, cell element arrangement and immunity, which also accelerated cell proliferation, migration and invasion (Banova Vulic et al. 2019). The androgen receptor regulatory pathway has also been studied in PCa with its suppressing effect triggering

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castration resilient prostate cancer cells to enzalutamide after a mixture of phospholipase Cμ knockdown and GANT61 (Sun et al. 2019). It has also been reported that Nuclear Factor of Activated T Cells 1 knockdown represses PCa cells proliferation, migration, invasion, and Warburg Effect (Liu Y et al. 2019). Downregulated Krüppel like factor 4 expression was found to be related to aggressiveness of PCa and KLF4 upregulation restricted the proliferation, wound healing and migration of PCa cells by provoking cell cycle fix and expression of Ecadherin (Zhang N et al. 2019).

Netrin-G1 (NTNG1) is a glycosyl-phosphatidylinositol-affixed synaptic adhesion molecule that encodes a preproprotein processed into a secreted protein containing eukaroytic growth factor (EGF)-like areas (Zhang Q et al. 2016). It mediates cell matrix adhesion and significantly upregulated in most homo sapiens' tissues including the prostate and interacts with several proteins. However, its interaction with JAK/STAT signalling is not yet known with regards to PCa and also its role in PCa progression has not been established. Several studies have analysed the specific role of JAK/STAT pathway in progression or regulation of various malignancies (Aghajani et al. 2019; Shati and El-Kott 2019; Xu et al. 2019). In this study, the researchers examined the functional roles of NTNG1/JAK1-mediated JAK/STAT axis and its underlying mechanism in the promotion of cell migration and invasion in PCa cells. This study envisages that NTNG1 might be a novel therapeutic marker for PCa and its clinical pathogenesis.

Objectives

This study aimed to explore impacts of NTNG1 in regulating PCa cell viabilities, migration and invasiveness and its functions in modulating JAK1, JAK2, STAT3, Ki67 and E-cadherin protein expressions.

Experimental

Main Reagents

The main reagents used for this study were RPMI-1640 tissue medium (Gibco, USA), FBS (Gibco), penicillin and streptomycin (Gibco), Keratinocyte-SFM (Gibco), bovine pituitary extricate (Merck, USA), epidermal growth factor (Sigma Aldrich, USA), si-NTNG1 (Genelily Bio-Tech, Shanghai, China), si-NC (Genelily Bio-Tech), pc-DNA 3.1 vector (Novobio Biotechnology, Shanghai, China), Lipofectamine 6000 (Beyotime, Shanghai, China), TRIzol reagent (TaKaRa, Japan), Beyort™ III cDNA first chain synthesis kit (Beyotime), Beyofast™ SYBR Green QPCR Mix (2X, High ROX) (Beyotime), RIPA (Beyotime), anti-NTNG1 (1:1000, ab153834, Abcam, UK), anti-JAK1 (ab133666), anti-JAK2 (ab108596), anti-STAT3 (ab109085), anti-Ki67 (ab92742), anti-E-cadherin (ab76055) and anti-GAPDH (ab128915), HRP-labelled Goat Anti-Rat IgG (Beyotime) chemiluminescent reagent (Millipore, WBKLS0100), CCK-8 (Beyotime), Matrigel (Clontech, USA), methanol (Sigma Aldrich), and crystal violet (Thermo Scientific, USA).

MATERIAL AND METHODS

Tissue Samples

Recent PCa tissues (n=51) and normal adjacent tissue (n=51) specimens were obtained from the patients having PCa at Urology Department, Hainan Cancer Hospital from 2018.9.2 to 2019.3.1. The entire collection of samples (PCa tissues with respective normal tissues at 2 cm apart with PCa tissues) was preserved by direct storage in liquid nitrogen at minus 80°C to extract RNA and proteins. All the participants provided cognisant permission to undergo the study and the utilisation of human tissues for experimental purposes and research protocol was sanctioned and endorsed by the Ethics Committee of Hainan Cancer Hospital, Hainan. This study strictly abided by the Helsinki declaration throughout the experiments.

Cell Culture

The researchers purchased the cell lines for prostate cancer (PC3, LNCaP, and DU145) and human prostate epithelial cell line (PrEC) from the Chinese Academy of Medical Science cell bank (Shanghai, China). Culturing of PC-3, LNCaP, DU145 was done in RPMI-1640 medium (Gibco, USA) added with ten percent FBS (Gibco) and 200 U/millilitre penicillin together with

200 U/millilitre streptomycin (Gibco) and PrEC cells were multiplied in Keratinocyte-SFM (Gibco) added with 0.05 mg/mL bovine pituitary extricate (Merck, USA) and 5 ng/mL epidermal growth factor (Sigma Aldrich, USA) at 37°C and five percent CO_2. The researchers followed the manufacturer's guideline to perform all cell culture procedures.

Cell Transfection

The siRNAs of NTNG1 (si-NTNG1) and negative control siRNA (si-NC) were synthesised by Genelily BioTech (Shanghai, China). The pcDNA 3.1 vectors from Shanghai Novobio Biotechnology (Shanghai, China) were used to create overexpressed NTNG1. LNCaP and DU145 cells were plated onto six-well plates at 37°C for 24 hours and then quickly filled with si-NTNG1, pc-NTNG1 or coordinated controls by Lipo6000 (Beyotime, Shanghai, China) following manufacturer's instructions. RT-qPCR was performed to assess RNA expressions.

RT-Qpcr

Total RNAs were removed from PCa tissues and cells by employing TRIzol reagent (TaKaRa, Japan). Subsequently, reverse transcription of RNA to cDNA was carried out using a Beyort™ III cDNA first chain synthesis kit (Beyotime). PCR was conducted on an Applied Biosystems Vii7 RT-qPCR instrument (ABI, USA) with Beyofast™ SYBR Green QPCR Mix (2X, High ROX) (Beyotime). GAPDH was for normalisation and the $2^{-\Delta\Delta CT}$ method was applied for calculating NTNG1 and JAK1 mRNA expressions. The experiments were conducted in triplicates and the primer arrays were recorded as follows:

Name	*Sequences*
NTNG1 (F)	5'-ATGTATTTGTCAAGATTCCT-GTCG-3'
NTNG1(R)	5'-GAACACCAGGGGGCTGGC-3'
JAK1 (F)	5'-GGATGGACTATTTGGGTTCTC-3'
JAK1(R)	5'-CACTTGGTGTTCACTCTCA-3'
GAPDH (F)	5'-GGCATGGACTGTGGTCATGAG-3'
GAPDH (R)	5'-TGCACCACCAACTGTTAGC-3'

Western Blot Analysis

LNCaP and DU145 cells were gathered and lysed using RIPA (Beyotime) for isolating total protein. Thereafter, proteins were detached by ten percent SDS-PAGE electrophoresis followed by transferring to PVDF membranes (Millipore USA). Membranes were congested in five percent defatted milk and then were nurtured with primary antibodies anti-NTNG1 (1:1000, ab153834, Abcam, UK), anti-JAK1 (1:1000, ab133666), anti-JAK2 (1:1000, ab108596), anti-STAT3 (1:1000, ab109085), anti-Ki67 (1:1000, ab92742), anti-E-cadherin (1:1000, ab76055) and anti-GAPDH (1:1000, ab128915) at 4°C overnight. Then, washing was done five to six times the following day and membranes were nurtured with HRP-labelled Goat Anti-Rat IgG (1:900, Beyotime) at 25 °C for 1 hour. The washing was repeatedly done and incubation with chemiluminescent reagent (Millipore, WBKLS0100) for 5 minutes, an ECL imaging system (Tanon, Tanon-5200) assisted in viewing of blotting results and the protein levels were computed by Image J software (NIH, USA).

Cell Viability Assay (CCK-8 Assay)

LNCaP and DU145 cells were grown on 96-well plates (5×10^3 cells/well) at 37 °C for 24 hours, 48 hours and 72 hours. Thereafter, CCK-8 (10μl, Beyotime) was added and cultivated with cells for 1 hour. Afterwards, absorbance was evaluated at 450nm using Infinite F50 (Tecan, Switzerland). The experiment was run triplicate.

Transwell Assay

Transwell assay examined cell invasion and migratory ability. Transwell chambers (Corning, USA) with or without 2 mg/mL Matrigel (Clontech, USA) were applied for invasiveness and migration, respectively. PCa cells (1×10^5 cells) were planted into the upper 24-well compartments with serum-free medium. RPMI- 1640 substrate having twenty percent FBS was supplemented to the lower compartments as a chemoattractant. The cells on the upper surface of the film were detached after 24 hours, and the cells on the lower surface were repaired with one hundred percent methanol (Sigma Aldrich), disco-

loured with 0.05 percent crystal violet (Thermo Scientific, USA) and their photos taken under a microscope. The number of migrated cells was recorded from 3 separate experiments.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (USA) and GraphPad Prism 9 (USA). To measure statistical significance between two groups, Student's t test was used while one way ANOVA was employed for three or more groups. The mean ± SD was used to express the data or a particular value from at least three independent experiments. $P < 0.05$ was a representative statistically significant criterion. All experiments were run in triplicates

RESULTS

NTNG1 Upregulated in PCa Tissues and Cell Lines

To explore functions of NTNG1, its mRNA expression was detected in PCa tissues and normal prostate tissues. RT-qPCR results indicated that NTNG1 expression was higher in PCa tissues than normal ones (Fig. 1A, $P<0.05$). Beyond that, NTNG1 mRNA and protein expressions in PCa cells and normal cells were analysed, indicating that its mRNA and protein expressions were upregulated in DU145, PC3 and LNCaP cells in comparison with PrECs (Figs. 1B, C and D, $^{**}P<0.05$). These results showed upregulation of NTNG1 in PCa cells, suggesting

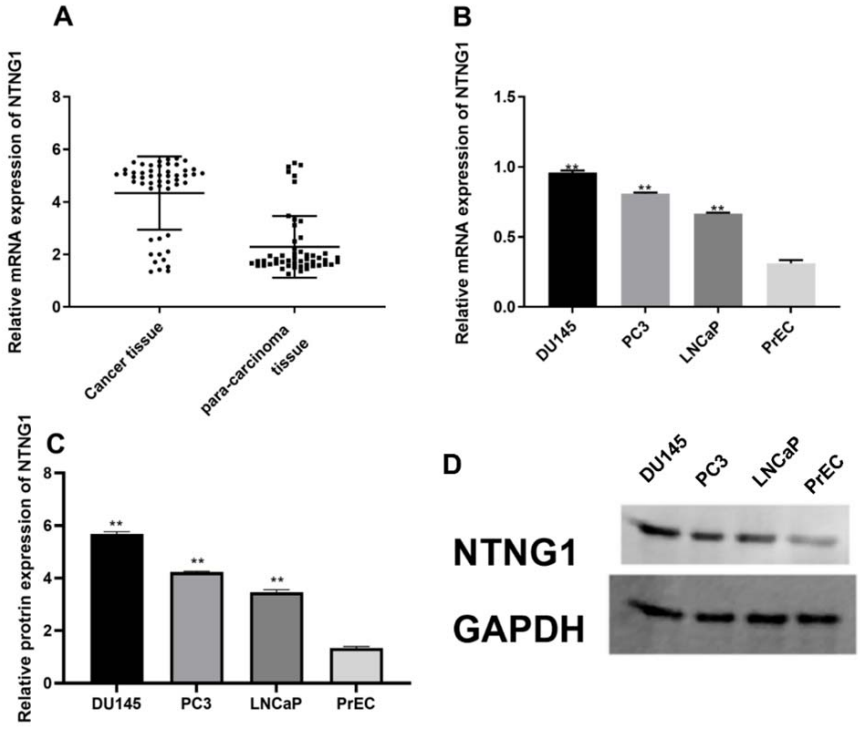

Fig. 1. NTNG1 was upregulated in PCa cells A) NTNG1 mRNA expressions in PCa tissue samples and normal ones, $^{**}P<0.05$ B) NTNG1 mRNA expressions in LNCaP, PC3, DU145 and PrEC cells were analyzed by RT-qPCR, $^{**}P<0.05$. C, D) LNCaP protein expressions in LNCaP, PC3, DU145 and PrEC cells were checked with western blot, $^{**}P<0.05$
Source: Authors

that NTNG1 might play oncogenic roles in PCa cells.

NTNG1 Modulated PCa Cell Viability, Migration and Invasiveness

To further analyse impacts of NTNG1 on PCa cells, DU145 cells were selected for NING1 suppression while LNCaP cells was chosen for NTNG1 overexpression. Afterwards, suppressed NTNG1 hampered DU145 cell viability while NTNG1 overexpression facilitated LNCaP cell viability (Figs. 2A and B, $^{**}P<0.05$). Beyond that, migratory and invasive abilities of PCa cells were evaluated, indicating that the upregulated NTNG1 accelerated migratory ability and invasiveness of LNCaP cells (Figs. 2C and D, $^{**}P<0.05$). Meanwhile, NTNG1 suppression restrained migration and invasiveness of DU145 cells (Figs. 2E and F, $^{**}P<0.05$). The results suggested that knockdown of NTNG1 inhibited PCa cell viabilities, invasiveness and migratory abilities while overexpressed NTNG1 accelerated PCa cell progression.

NTNG1 Interplayed with JAK/STAT Signalling Pathway in PCa Cells

To explore signalling pathways with NING1 in PCa cells, JAK1 expressions in PCa tissues were examined, indicating that JAK1 was upregulated in PCa tissues compared to adjacent normal ones (Fig. 3A, $^{**}P<0.05$). Furthermore, interactions between NTNG1 and JAK1 were evaluated, indicating that JAK1 mRNA expressions were suppressed with NTNG1 suppression, while NTNG1 overexpression increased JAK1 mRNA expres-

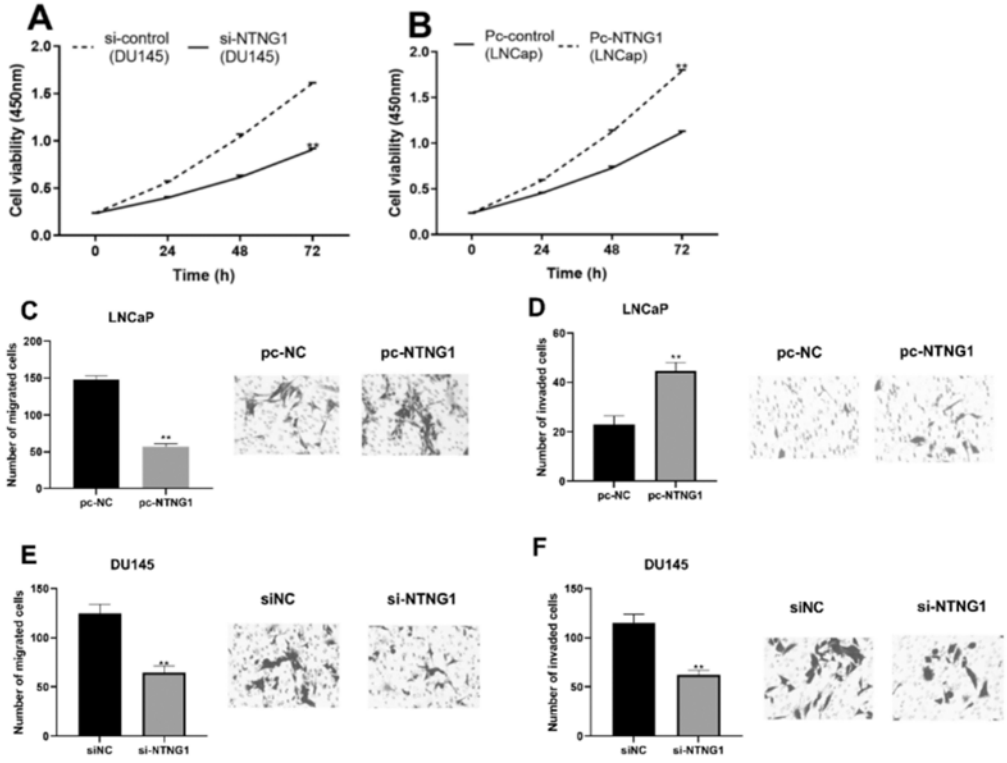

Fig. 2. NTNG1 mediated PCa cell viabilities, migration and invasiveness. A, B) DU145 cell viabilities with siNTNG1 and LNCaP cell viabilities with pcNTNG1 were examined using CCK-8, $^{**}P<0.05$. C, D) LNCaP cell migratory and invasive abilities with NTNG1 suppression were examined using transwell, $^{**}P<0.05$. E, F) Transwell examined migratory ability and invasiveness of DU145 cells after NTNG suppression, $^{**}P<0.05$.
Source: Authors

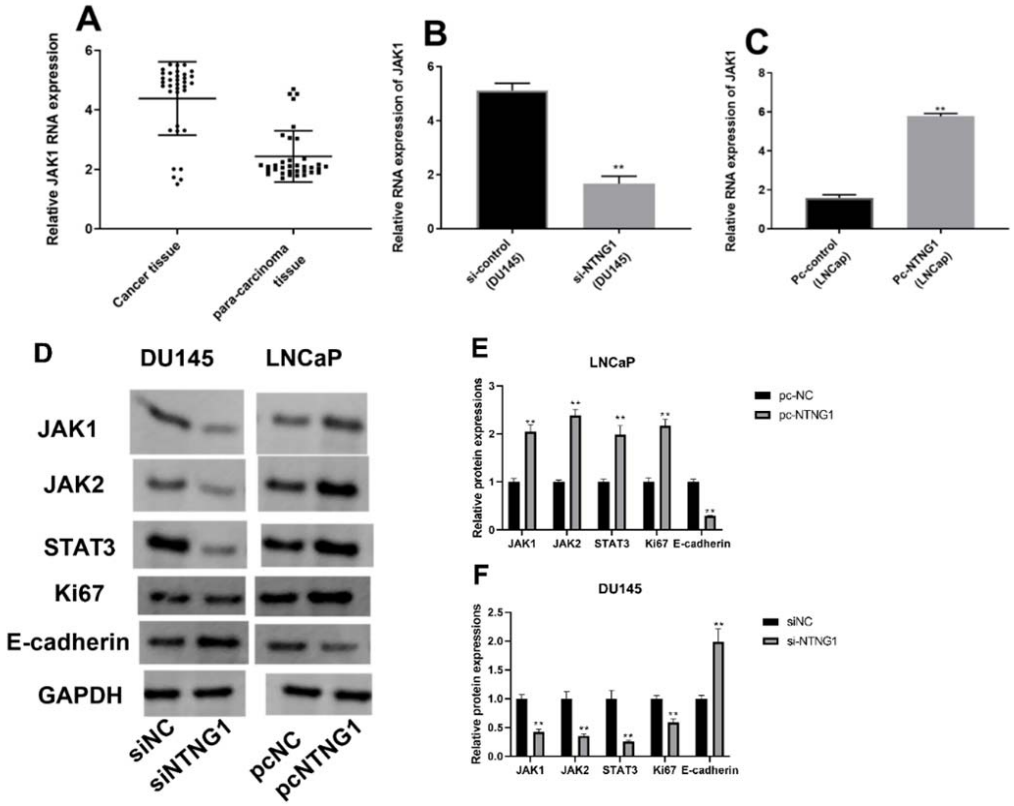

Fig. 3. NTNG1 activated JAK/STAT signaling pathway in PCa cells A) JAK1 mRNA expressions in PCa tissues and normal ones, **P<0.05. B, C) JAK1 mRNA expressions with NTNG1 overexpression and NTNG1 suppression were analyzed by RT-qPCR, **P<0.05. D, E, F) western blot assays detected JAK1, JAK2, STAT3, Ki67 and E-cadherin protein expressions with NTNG1 overexpression and NTNG1 downregulation in PCa cells, **P<0.05
Source: Authors

sions (Figs. 3B and C, **P<0.05). Furthermore, proteins related to JAK/STAT signalling pathway were analysed, indicating that JAK1, JAK2 and STAT3 protein expressions were elevated with NTNG1 upregulation, which were all downregulated by suppressed NTNG1 (Figs. 3D, E and F, **P<0.05). Moreover, proteins related to PCa cell proliferation and migration were examined. Results of western blot showed that Ki67 protein expression was increased by NTNG1 upregulation but its expression was downregulated with NTNG1 suppression (Figs. 3D, E and F, **P<0.05). Meanwhile, E-cadherin protein expression was prompted by NTNG1 downregulation, which was suppressed with NTNG1 upregulation (Figs. 3D, E and F, **P<0.05).

DISCUSSION

While PCa has been the only male exclusive disease, its prevalence and related deaths have increased in the past ten years and PCa has been one of the prominent causes of sarcoma linked deaths (Sehn 2018). Although extraordinary progress has been made in PCa in areas of oncogenes or malignant tumour repressor gene alteration, the timely detection and therapy of PCa with abnormal protein manifestation and detec-

tion of cancer stem cells are also critical (Intasqui et al. 2018). Thus, an immediate in-depth understanding about molecular mechanisms related to PCa clinicopathogenesis and progressions is required to come up with important biomarkers that will be useful for creation of applicable novel proven therapeutic approaches for PCa. Therefore, researchers have paid increasing attention to study the influence of mRNAs, microRNAs and long noncoding RNAs in regulating PCa developments(Christoph et al. 2018;Dominska et al. 2017; Yin et al. 2019). However, biological roles of NTNG1 in cancers have been seldom explored. For instance, Sho et al. identified that NTNG1 played an oncogenic function for predicting cancer relapse in colorectal cancer stages II and III (Sho et al. 2017). In this study, NTNG1 expressions were upregulated in PCa tissues and cells. Furthermore, NTNG1 downregulation restrained DU145 cell viabilities and decreased migratory and invasive capacities while its overexpression accelerated LNCaP cell viabilities and elevated migratory and invasive abilities. These detections indicated that NTNG1 might act as an oncogene in PCa progression. The JAK/STAT network played an important function in cell proliferation, segregation and apoptosis. Recent studies have indicated that the abnormality of JAK/STAT pathway has been consistently implicated in numerous cancers, including PCa (Das et al. 2017). In this study, the researchers attempted to observe the impacts of NTNG1 overexpression on JAK/STAT signalling pathway within the perspective of prostate cancer as therapeutic molecules. JAK1 was also investigated on PCa progressions and its interactions with NTNG1. According to previous studies, JAK/STAT3 signalling pathway has been verified to accelerate PCa development and blocking STAT3 activation could restrain PCa progression (Dambal et al. 2020). Moreover, activation of JAK/STAT3 signalling pathway also promoted oncogenic impacts of LINC00473 on accelerating PCa cells proliferation (Xing et al. 2020). In this study, JAK1 was elevated in PCa tissues and its expressions were increased with an increase of NTNG1, suggesting that NTNG1 upregulated the expression of JAK1 in PCa cells. The study also showed that NTNG1 overexpression prompted JAK1, JAK2, STA3, Ki67 protein expressions and decreasing E-cadherin proteins, while suppressed NTNG1 reversed its effects on protein expressions. The investigation revealed that impacts of NTNG1 on modulating PCa progressions were achieved through activating the JAK/STAT signalling pathway in vitro.

CONCLUSION

NTNG1 was upregulated in PCa cells and accelerated PCa progressions through activating JAK/STAT signalling pathway, suggesting that NTNG1 might be a promoter in PCa progressions. However, studies in vivo and clinical stage are requested to get more knowledge.

RECOMMENDATIONS

NTNG1 mRNA and protein expressions were upregulated in PCa tissue samples and cell lines. Overexpressed NTNG1 accelerated PCa cell viabilities, migration and invasiveness while downregulated NTNG1 suppressed viabilities, migration and invasiveness of PCa cells. Moreover, JAK1 was also upregulated in PCa tissues, which was also upregulated by NTNG1 overexpression and suppressed by NTNG1 downregulation. Moreover, JAK1, JAK2, STAT3, Ki67 and E-cadherin protein expressions were also upregulated with NTNG1 overexpression and downregulated by NTNG1 suppression.

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None

CONFLICT OF INTEREST

None

ABBREVIATION LIST

NTNG1: netrin G1
JAK: Janus kinase
STAT3: Signal transducer and activator of transcription 3
PCa: prostate cancer

CCK-8: Cell Counting Kit-8
PSA: prostate-specific antigen
CA1: Carbonic Anhydrase 1
FBS: foetal bovine serum
ECL: Electrochemiluminescence
KLF4: Krüppel like factor 4
RIPA: Radio Immunoprecipitation Assay

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