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TNK1 Deficiency Promoted Cell Migration, Invasion Assay and Effects on MAPK and PI3K/Akt Signalling Pathways

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Abstract: **PRC is a part of the male reproductive system that helps make and store seminal fluid. In adult men, a typical prostate is about 3 centimetres long and weighs about 20 grams. It is located in the pelvis, below the urinary bladder and in front of the rectum. Most of the nRTKs are localised in the cytoplasm. TNK1/Kos1 is a 72-KDa NRPTK located on the human chromosome 17p13.1. TNK1/Kos1 is a member of the Ack family. The main function of non-receptor protein-tyrosine kinase (nRTKs) is to participate signal transduction in activated T-cells and B-cells in the immune system. To investigate the role of TNK1 in PC-3 cells, TNK1 deficient cells were used in the study. The results showed that TNK1 deficiency, migration and invasion were enhanced in the absence of functional TNK1. Immunoblotting results revealed decreased Akt phosphorylation level and increased Erk phosphorylation level. These results suggest that TNK1 might contribute to the maligancy of tumor cells, therefore, TNK1 might be a potential prognosis factor for metastatic prostate cancer.**

Keywords: **PRC / TNK1 / Migration / Invasion / PC-3 cells.**

1. INTRODUCTION

1.1 Prostate cancer (PRC):

Is the second most frequent tumour in males in the industrialized world [1-2]. An increase in this disease has been detected over the past few decades, probably in part due to a greater use of prostate-specific antigen (PSA) testing without ruling out the influence of unknown factors [3]. This higher rate of incidence is associated with an increase in the number of patients with the localized disease (gland and surrounding anatomical area) at diagnosis, permitting greater disease control by surgery and radiotherapy (RT). The classification in degrees of risk for localized disease and prognostic algorithms enables local treatment options to be offered with or without hormone therapy, which is currently the standard treatment [4].

PRC, the cells of these prostate glands mutate into cancer cells. PRC is classified as an adenocarcinoma, or glandular cancer, that begins when normal semen-secreting prostate gland cells mutate into cancer cells. The peripheral zone is the region of the prostate gland where the adenocarcinoma is most common. Initially, small clumps of cancer cells remain confined to otherwise normal prostate glands, a condition known as carcinoma in situ orprostatic intraepithelial neoplasia (PIN). The protein ZIP1 is responsible for the active transport of zinc in prostate cells. One of zinc's important roles is to change the metabolism of the cell in order to produce citrate, an important component of semen.

1.2 Tyrosine kinases:

Tyrosine kinase is an enzyme that is able to convert a phosphate group from ATP to a protein with a cell. It functions as an "on" or "off" switch in a lot of cellular functions. Tyrosine kinases are a subclass of protein kinase. The phosphate set is attached to the amino acid tyrosine on the protein. Tyrosine kinases are a subset of the larger category of protein kinases that attach phosphate sets to other amino acids (serine and threonine). Phosphorylation of proteins by kinases is a substantial mechanism in communicating signals within a cell (signal transduction) and regulating cellular activity, such as cell division. Protein kinases may mutate, become adhesive are in the "on" position, and cause unregulated cell growth, which is a necessary move for the evolution of cancer.

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Receptor tyrosine kinases (RTKs) are the upper-affinity cell surface receptors for a lot of the polypeptide growth factors, cytokines, andhormones. Of the 90 unique tyrosine kinase genes identified in the human genome, 58 encode receptor tyrosine kinase proteins [5]. Receptor tyrosine kinases have not only been seen to have key organizers of normal cellular processes, but also to be a crucial role in the evolution and progression of varying types of cancer [6]. Receptor tyrosine kinases are part of a larger family of protein tyrosine kinases, consist of a receptor tyrosine kinase protein that contains a transmembrane domain, as long as the non receptor tyrosine kinases do not possess transmembrane domains [7].

1.3 Non-receptor tyrosine kinases (NRTKs):

RTKs organs, for instance cell's growth, proliferation, differentiation, adhesion, migration and apoptosis are critical components in the organization of the immune system. Tnk1 is a 72-KDa non–receptor protein tyrosine kinase (NRPTK) located on the human chromosome 17p13.1 and has been involved in the organization of apoptosis, cell growth, nuclear factor-κB, and Ras [8,9]. In mice, two gene products (Tnk1a and Tnk1b) are prophesied to be produced by an alternate link and the utilization of distinct polyadenylation signals from the murine Tnk1 locale in chromosome 11 (which is compatible to human chromosome 17p13.1 [10]. While extracting amino acid sequences from the Tnk1a transcript is a 72-KDa protein, when splicing from exons 8 to 9 does not occur, an alternate splicing gene producer (Tnk1b) is produced.

2. MATERIALS AND METHODS

2.1 Reagents, antibodies and others:

- DMEM Medium (from HyClone Labs, USA)

-0.25% Trypsin (from Gibco, USA)

- FBS (Fetal Bovine Serum from Gibco, USA)

- DMSO ((Dimethyl sulfoxide from Sigma, USA)

- G418

- Penicillin / streptomycin

- PVDF membrane (polyvinylidene fluoride from Millipore Immobion, USA)

- RIPA buffer (Beyotime, China)

- Primary antibody Beta-actin (Beverly, MA, USA)

- Primary antibody TNK1 (tyrosine non receptor kinases) (Beverly, MA, USA)

- Primary antibody Vimentin (Beverly, MA, USA)

- Primary antibody Beta-catenin (Beverly, MA, USA)

- Secondary antibody, anti-mouse and anti-rabbit HRP-conjugated antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA)

- X-ray film (Kodak, USA)

- Cell culture plates 10cm and 3.5cm (Thermo Scientific, China.)

2.2 Apparatus:

- Cell culture incubator (Thermo scientific, Model 311, USA)

- Advanced Research Microscope (Model Nikon Eclipse 80i, Japan)

- Inverted microscope (Olympus, Japan)

- Electrophoresis and membrane transfer apparatus (Biorad, USA)

- Refrigerators (Electrolux, France)

- -80 ^{o}C and -20 ^{o}C freezers (Hair, China

- Sonicator (Scientz, Model Jy92-II, USA)

- Centrifuge (Eppendorf, Model 5810k, USA)

- Nitrogen Tank (Thermo scientific, Model 8147, USA)

- Heating block (Mixing Black Model MB-102, Bioer, China)

- Ice-maker (XUEKE, China)

2.3 Cells and cell line:

PC-3 human prostate cancer cell lines PC-3 human prostate cancer cell lines are one of the cell lines utilized in the research of prostate cancer. These cells are helpful in the investigation of biochemical change in prostate cancer cells and in evaluating their response to chemotherapeutic factors. Furthermore, they have been used to create cancers under dermis in the mice for the aim to get a model of the cancer environment in the framework of the organism.

Description: PC-3, DU145 and LNCaP cells are the most commonly used prostate cancer cell lines in the laboratory. PC-3 cells have high metastatic potential, while DU145 and LNCaP cells have moderate and low metastatic potential respectively [11]. The PC-3 cell line was created in 1979 from the bone metastasis of class IV prostate cancer in a 62-year-old Caucasian male. These cells do not respond to the androgen. PC-3 cells have been reduced in testosterone-5-alpha activity of the enzyme acid phosphatase [11]. Moreover, karyotype analysis showed that PC-3 cells are almost three-ploidy, and contain 62 chromosomes. They have common features of tumour cells with the epithelial origin, such as junction complexes, numerous microvilli, abnormal mitochondria, abnormal nuclei and nucleoli, annulate lamellae, and lipoidal bodies. As shown that in (Fig. 1).

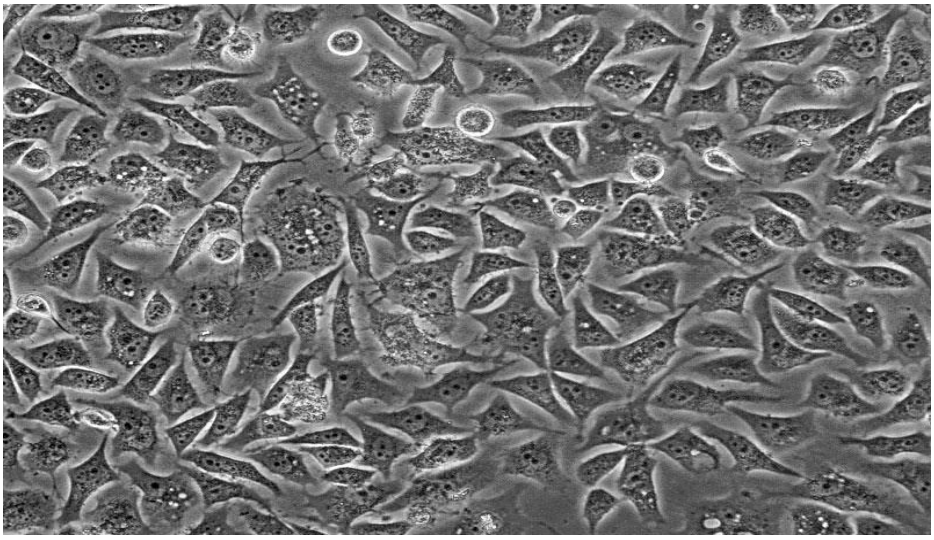

Fig 1: Morphology of PC-3 cells. The image was obtained from website https://commons. wikimedia.org/wiki/File: PC-3-cell.jpg

2.4 Migration assay:

PC-3 human cancer cells were cultured in complete DMEM with 10% FBS + antibiotic under conditions described before. In the assay day, cells were washed twice with 1X PBS. Then, cells were conference around 70% change the medium to free DMEM without FBS + antibiotic and store in the incubator 24 hours [12]. On the second day prepare the 24 plate (24-well format with 8 µm pore size) with a Transwell migration membrane before starting to account the cells, adding to the lower compartment 700 µl of DMEM without 10% FBS + antibiotic and put the transwell membrane in the well and adding 200 µl of DMEM without 10% FBS + antibiotic to the transwell membrane in the well and store in the incubator for one hour at least. Previously stored cells were taken out from the incubator and prepare to account the the cells on the chamber slide, After centrifugation change the medium and suspend the pipette up and down gently, then take 900 µl from the cells transfer to microtube + 100 µl of PBS or free medium without 10% FBS + antibiotic ,take 10 µl of the sample to account it on the chamber slide under the microscope, take the average of the numbers of the cells to get a convenient cell number is 3×10^4, take out the plate from the incubator and take new wells to adding to the lower compartment 700 µl of DMEM with10% FBS+ antibiotic and put the transwell membrane add to it.

After had got on the cell number + free medium until to get the total volume, sample is 200 µl (cells + free medium without 10% FBS + antibiotic) adding to the bottom of the membrane, all these steps inside the hood, store in the incubator 24 hours at 37 ^{o}C and no CO_2. On the third day, after 24 hours take out the plate from the incubator wash the membrane by 1X PBS one time and quickly fixed by 4% formaldehyde for 5 minutes, then wash again the membrane by 1X PBS for twice and adding methanol 100% for 20 minutes, and then wash again the membrane by 1X PBS for twice and stained with 1% Crystal Violet in 20% methanol for 20 minutes too, in the end wash the membrane by 1X PBS for many times until to be clear membrane and dry it with a cotton swab, cut the membrane gently and fix it on the slide of microscope to take images to the membrane . Finally, the membranes were observed under the microscope at 10X magnification to quantify migrated cells [12].

2.5 Invasion assay:

The methodology used in this assay is similar with transwell - migration assay but adding in the upper chamber 70 µl of Matrigel® at 1mg/ml and gelling incubated at least one hour at 37 ^{o}C before getting the cells on the seeding. Then, follow the same protocol that of transwell-migration assay as described before in the transwell-migration assay [12].

2.6 SDS-Page:

2.6.1 Preparation of gel solutions:

8 % separating gel was prepared as shown in Table:

Table 1: Recipe for 8% SDS-PAGE stacking gel (10 ml)

Reagents	Volume (ml)
ddH_2O	4.6
30 % acrylamyde	2.7
1.5 M Tris buffer (pH 8.8)	2.5
10 % SDS	0.1
10 % AP	0.1
TEMED	0.006

5 % separating gel was prepared as shown in Table:

Table 2: Recipe for 5% separating gel solution (6ml)

Reagents	Volume (ml)
ddH_2O	4.1
30% acrylamyde	1.0
1.5 M Tris buffer (pH 8.8)	6.8
10% SDS	0.6
10% AP	0.6
TEMED	0.006

2.6.2 Protein separation:

To cast 1.5 mm thickness of the gel, put the long plate to the short plate and fixed together in the gel matrix. 7 ml to the 8% concentration of the gel solution was added between the plate in the matrix gel and add the isopropanol for the surface of the gel, then keep it for one hour in the room temperature and make sure do not move it, and the gel was washed with distilled water. And then to prepare the upper gel 2.5 ml of the concentration of the gel was added into the first gel, and put the comb on top of the concentration of the gel, then keep it for half hour in the room temperature and make sure do not move it. When the gel will be ready, the gel plates take off from the shelf, and then put the gel plate into the electrophoresis tank that containing 1X electrophoresis buffer. Equal amounts of cell lysis to load into the gel, and the electrophoresis be run at 80 V, Am more than 300 for 10 minutes to make sample level equal, and then follow by 110 V, Am more than 300 for 2 hrs to separate proteins.

2.7 Membrane transfer:

2.7.1 Buffer preparation:

10X transfer buffer preparation:

Table 3: 10X Transfer Buffer (1L)

Reagents	Weight (g)	Final concentration (M)
Trizma base	30.3	0.25
Glycine	144	1.92

The chemicals dissolve in distilled water to a final volume of 1L, pH 8.3.

1 X transfer buffer preparation:

Table 4: 1X Transfer Buffer (1L)

Reagents	Volume (ml)
Methanol	200
10X transfer buffer	100
ddH_2O	700

2.7.2 Membrane transfer:

After electrophoresis, have been separated gel plates, and the concentration of the gel was decreased. The bottom right-hand corner of the gel was blunted for orientation to carry the gel from the plate or glass, and then soak the gel in the transfer buffer. At the same time, cut a piece of PVDF membrane convenient to the size of the gel and soak in transfer buffer. Then put the gel in the transfer cassette. To put the transfer cassette in the electrophoresis tank containing 1X membrane transfer buffer. And then put the tank in the ice box, and the membrane transfer was running at 80 V, Am more than 300 for 10 minutes to make sample level equal at the gel convenient with the membrane, and then follow by 110 V, Am more than 300 for 2 hrs.

2.8 Wester blot:

2.8.1 Buffer preparation:

Tris-buffered saline (10X TBS):

Table 5: 10X TBS Buffer (2L) pH 7.6

Reagents	Weight (g)	Final concentration (mM)
Trisbase	48.4	10
NaCl	160	150

The reagents are dissolved in 2 L-distilled water and pH 7.5 with HCl (hydrochloride) to increase PH, and NaoH (Sodium hydroxide) to decrease pH.

2. TBST buffer: 900 ml 10X TBS buffer with 1 ml Tween-20 (1X TBS buffer containing 0.05% of Tween-20).

3. 5% blocking buffer (5g to 100 ml) of BSA or nonfat dry milk are dissolved in TBST buffer, stored at 4°C.

4. Primary antibody diluent buffer 5%: 0.5g of BSA is dissolved in 100ml TBST buffer, stored at 4°C.

5. Secondary antibody diluent buffer 5%: 0.5g of nonfat dry milk is dissolved in 100 ml TBST buffer, stored at 4°C.

2.8.2 Wester blot:

At the end of the transfer, after finish the transmembrane process takes the membrane from the cassette, put into a small container, and then wash with 10 ml of TBST buffer for a short time (15 minutes). The container was then placed on the shaker and block the membrane with 5% blocking buffer (5g to 100 ml) of BSA or nonfat dry milk are dissolved in TBST buffer at room temperature for at least 2 hours. Then wash the membrane for half hour three times with TBST buffer at room temperature. To investigate for the protein, incubate the membranes with primary antibodies (1:2000 dilution) overnight at

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4°C plus to the movement. The next day, wash the membrane for 1 hour three times (10 mins each) with TBST buffer at room temperature and incubate with secondary antibody (1:20000 dilution) at room temperature for 2 hrs plus to the movement on the shaker. After widely washing for 1 hour three times (10 mins each) with TBST buffer at room temperature, put the membrane in the autoradiography cassette, exposed to X-film, and developed in the dark room.

3. RESULTS

3.1 TNK1 deficiency promoted cell migration:

MTT assay and clonogenic assay results indicated that TNK1 promoted cell growth and tumerigenesis. To examine whether TNK1 affects cell migration, transwell assay was performed. The results showed that TNK1 deficiency promoted cell migration (Fig. 2).

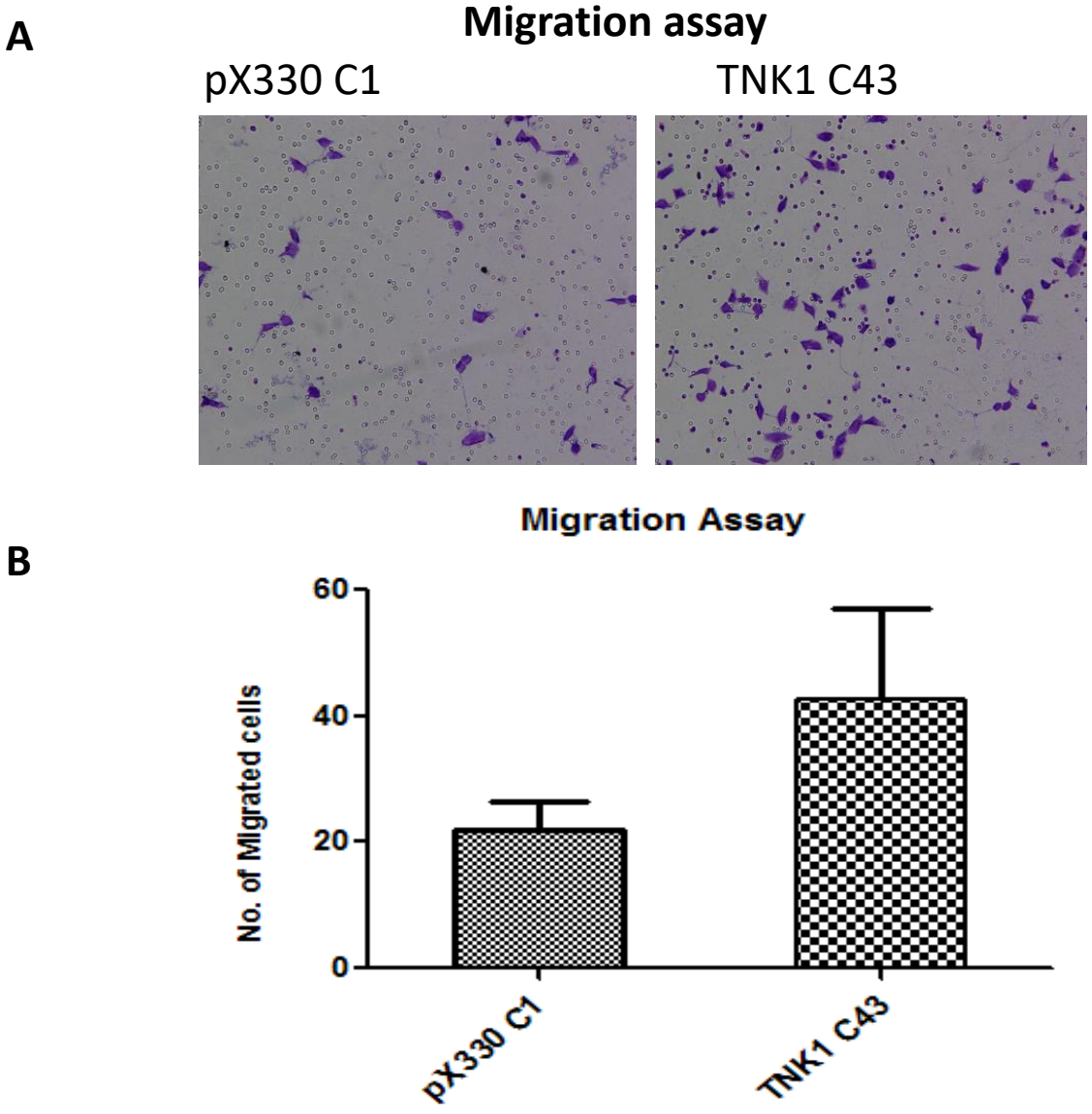

Fig 2: TNK1 deficiency promoted cell migration. After cells reach 95% confluence in 10 cm plates, then starved the cells with a free medium for 24 hours, prepared the plate, put transwell membrane in 700 µl free medium and added 200 µl free, incubated at least 1 hour, cells were trypsinized, 3×10^4 cells were seeded into transwell membrane and incubated for 24 days. (A). Migration assay results. Images one represents wells of each clone that was taken. (B). Quantitative analysis of migration assay. The experiments were preformed two times and the results were reproducible.

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3.2 TNK1 deficiency enhanced cell invasion:

The results showed that in the control cell PX330 C1 compared to the cell clone TNK1-3 C43. The results showed TNK1 deficiency promoted for the prostate cancer cells (Fig. 3).

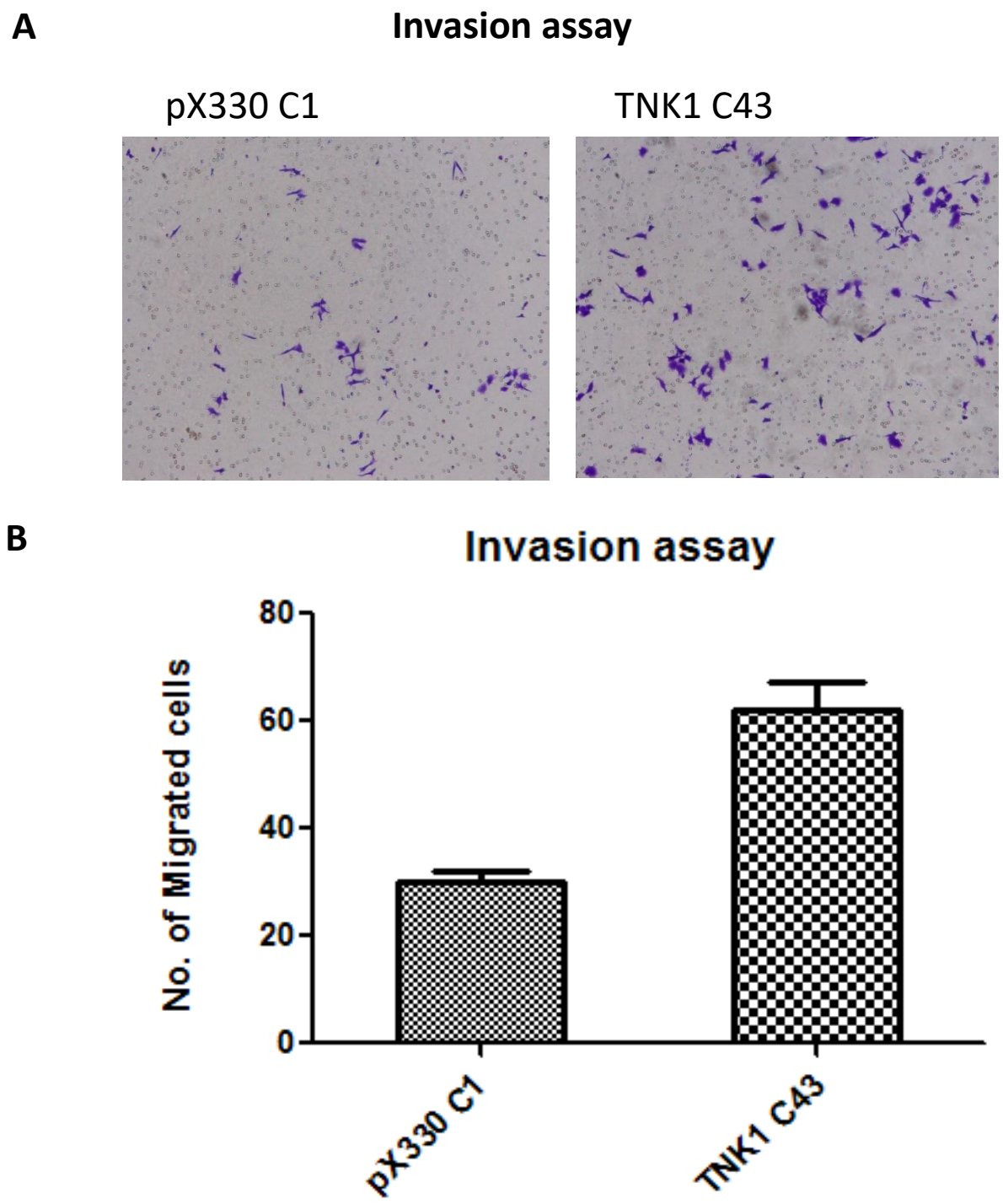

Fig 3: TNK1 deficiency stimulated cell invasion. After cells reach 95% confluence in 10 cm plates, then starved the cells with a free medium for 24 hours, prepared the plate, put transwell membrane in 700 µl free medium, added 70 µl matrigel desolved 10 times, incubated at least 1 hour, cells were trypsinized, and 10×10^4 cells were seeded into transwell membrane then incubated for 24 days. (A). Invasion assay results. Images one represent wells of each clone was taken. (B). Quantitative analysis of migration assay. The experiments were preformed two times and the results were reproducible.

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3.3 The effect of TNK1 on MAPK and PI3K/Akt signalling pathways:

Based on the prefered data, TNK1 promoted cell survival, and inhibited cell migration and invasion. Since MAPK and P13k/Akt signalling pathways are the most common pathways to regulate cell survival, proliferation, and migration, it is worth to know whether the effect of TNK1 is mediated by MAPK and P13k/Akt signalling pathways. To address this issue, Western blot experiments were preformed and the results showed that decreased Akt phosphorylation level and increased Erk phosphorylation level compared to control cells, but the difference was not significant (Fig. 4).

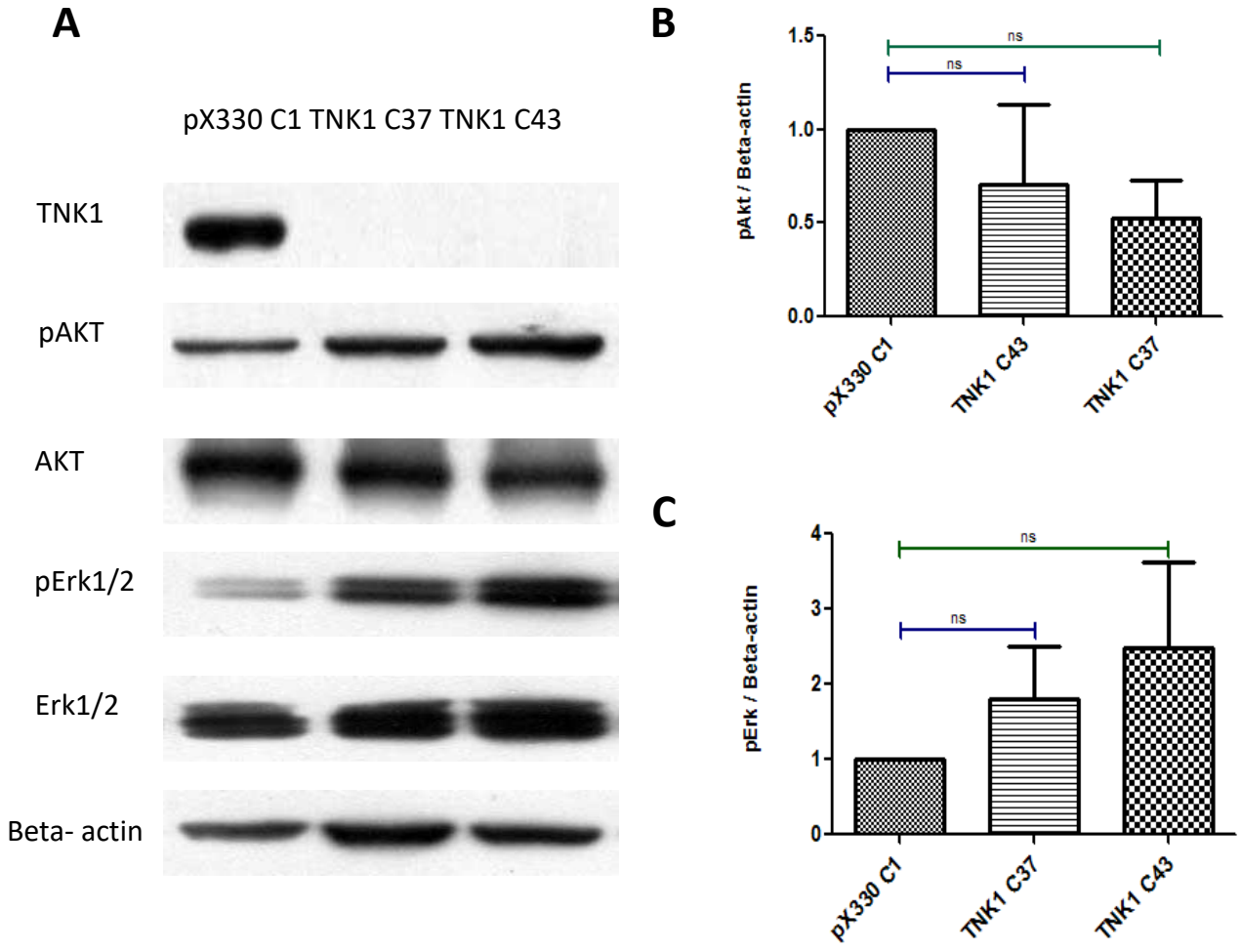

Fig 4: TNK1 deficiency effects on MAPK and PI3K/Akt signalling pathways in P-C3 cells. After cells reach 95% confluence in 10 cm plates, cells were trypsinized, and then quantified the TNK1 protein level with the lysis and loading buffer. The cell lysate samples were resolved by SDS-PAGE followed by the membrane transfer. Loaded cells volumes around 20 µl cells and blotting. The experiments were preformed two times and the results were reproducible. The amount of protein was determined using ImageJ® programme. (A). Western blot results. (B). Quantitative analysis of pAkt/Beta-actin. (C). Quantitative analysis of pErk/Beta-actin. The experiments were preformed three times and the results were reproducible. ***P<0.001, **P<0.01, *P<0.05, ns<not.

4. DISCUSSION

In 2014 Ee Lyn Ooi and colleagues showed that TNK1 is abundantly expressed in hepatocytes and is localized in the cytoplasm during the resting condition. TNK1 shifts its localization to the PM during cellular response to type I IFN. This shift to the PM is accompanied by phosphorylation of the TNK1 at amino acid Y277. The immunoprecipitation assays also demonstrated that TNK1 associates with the IFNAR complex through Tyk2 [13]. Extended further observations with published data provides evidence that shows that the TNK1 plays an important role in the inhibition of cancer growth.

This study showed that TNK1 deficiency in PC-3 cells in cell migration and invasion the results are decreased. TNK1 deficiency in PC-3 cells decreased Akt phosphorylation level and increased Erk phosphorylation level.

In this study, TNK1 deficiency slightly decreased Akt phosphorylation level and increased Erk phosphorylation level but the difference was not significant, in vitro, in cell migration and invasion results decreased, where 3×10^4 cell number was used in the Transwell migration assay, and the 10×10^4 cell number was used and the Matrigel 70 µl in the Transwell invasion assay. Suggesting that TNK1 might be a potential therapeutic target in metastatic prostate cancer.

TNK1 deficiency slightly decreased Akt phosphorylation and increased Erk phosphorylation but the difference was not significant, based on the results Akt and Erk are not significant according to the Jnk literature [14]. In the PC-3 cells TNK1 can inhibited not very bad for the tumor TNK1 can inhibit the malignant phenotype tumor cells. These results are not only simple to prove TNK1 ability on the inhibited prostate cancer and increased cell migration and invasion.

REFERENCES

[1] Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. Cancer Incidence and Mortality Worldwide: IARC Cancer Base GLOBOCAN. 2012. 1: 11.

[2] José Expósito, Isabel Linares, Isabel Castillo, Miguel Martínez, Pilar Vargas, Ismael Herruzo, José Antonio Medina,Amalia Palacios, Eloísa Bayo, Francisco Peracaula, Javier Jaén, José Antonio Sánchez, and María José Ortiz. Evaluation of the utilization of external radiotherapy in the treatment of localized prostate cancer in Andalusia, Spain. Radiat Oncol. 2015. 10: 265.

[3] Chamie K, William SB, Hu JC. Population-based assessment of determining treatments for prostate cancer. JAMA Oncol. 2015. 1: 60-67.

[4] Heidenreich A1, Bastian PJ2, Bellmunt J3, Bolla M4, Joniau S5, van der Kwast T6, Mason M7, Matveev V8, Wiegel T9, Zattoni F10, Mottet N11; European Association of Urology. Guidelines on prostate cancer. Arnhem (The Netherlands): European Association of Urology (EAU). 2014. 65:467-479.

[5] Robinson DR, Wu YM, Lin SF. "The protein tyrosine kinase family of the human genome". Oncogene. 2000. 19: 5548–5557.

[6] Zwick E, Bange J, Ullrich A. "Receptor tyrosine kinase signalling as a target for cancer intervention strategies". Endocr. Relat. Cancer. 2001. 8: 161 173.

[7] Hubbard SR, Till JH. "Protein tyrosine kinase structure and function". Annu. Rev. Biochem. 2000. 69: 373–398.

[8] Hoehn GT, Stokland T, Amin S. Tnk1: a novel intracellular tyrosine kinase gene isolated from human umbilical cord blood CD34+/Lin-/CD38- stem/progenitor cells. Oncogene 1996. 12: 903–913.

[9] Azoitei N, Brey A, Busch T, Fulda S, Adler G, Seufferlein T. Thirty-eight-negative kinase1 (TNK1) facilitates TNFα-induced apoptosis by blocking NF-Kb activation. Oncogene 2007. 26: 6536–6545.

[10] Hoare K, Hoare S, Smith OM, Kalmaz G, Small D, May WS. Kos1, a nonreceptor tyrosine kinase that suppresses Ras signalling. Oncogene 2003. 22: 3562–3577.

[11] Kaighn, M.E.; K.S. Narayan; Y. Ohnuki; J. F. Lechner; L.W. Jones. "Establishment and characterization of a human prostatic carcinoma cell line (PC-3).". Invest Urol. 1979. 17: 16-23.

[12] Guan J-L. Cell Migration. Developmental Methods and Protocols. Totona, New Jersey: Human Press Ink. 2005. 353-359.

[13] Ee Lyn Ooi,a Stephanie T. Chan,a Noell E. Cho,a Courtney Wilkins,b Jessica Woodward,b Meng Li,c Ushio Kikkawa,d Timothy Tellinghuisen,e Michael Gale, Jr.,b and Takeshi Saitoa,b,1. Novel antiviral host factor, TNK1, regulates IFN signaling through serine phosphorylation of STAT1. Proc Natl Acad Sci U S A. 2014. 111: 1909–1914.

[14] Isabet T, Montagnac G, Regazzoni K, Raynal B, El Khadali F, England P, Franco M, Chavrier P, Houdusse A, Menetrey J.Literature: JNK-interacting protein, leucine zipper II,The structural basis of Arf effector specificity: the crystal structure of ARF6 in a complex with JIP4. EMBO Journal. 2009. 28:2835-2845.