A key axis of the N-WASP signaling pathway promotes distant metastasis in pancreatic cancer: LOXL2-FAK-N-WASP

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Directed by Professor Joon Seong Park

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ABSTRACT

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid malignancies; however, a specific mechanism underlying its metastatic progression is not established. Herein, we investigated whether the neural Wiskott-Aldrich syndrome protein (N-WASP) plays a role in PDAC progression. We found that N-WASP is markedly expressed in primary pancreas samples from patient with PDAC. Noteworthy, N-WASP positive patients had a much higher incidence of distant metastasis than the N-WASP negative group. RNA sequencing analysis further revealed that N-WASP is a mediator of epithelial–mesenchymal transition (EMT). In addition, N-WASP knockdown in pancreatic cancer cells significantly inhibited cell invasion, migration, and EMT. We also observed that the lysyl oxidase-like 2 (LOXL2) and focal adhesion kinase (FAK) were positively associated with the N-WASP-mediated response, thereby modulating EMT and invadopodia. Furthermore, depletions of both N-WASP and LOXL2 significantly reduced the incidence of liver and lung lesions in orthotopic mouse models of pancreatic cancer. These results clarify a new role for N-WASP signaling associated with LOXL2/FAK in EMT and invadopodia that regulates intercellular communication of cancer cells to promote pancreatic cancer metastasis. These findings may aid in the development of therapeutic strategies against pancreatic cancer.

Keywords: pancreatic cancer, neural wiskott-aldrich syndrome protein, metastasis, epithelial–mesenchymal transition, invadopodia

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I. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is known as a fatal disease. Despite advanced early screening and diagnosis, the overall 5-year survival rate remains unchanged as early recurrence rate, local recurrence, and distant metastasis tend to be high even after surgery. Cancer metastasis is a multistep process that relies on local invasion, intravasation, transport, extravasation, and colonization so tumor cells can migrate from their primary site and form secondary tumors at distant sites. In particular, the epithelial–mesenchymal transition (EMT) is an important step to promote metastasis in epithelium-derived carcinoma[1-6]. EMT transcription factors can also induce the formation of specialized subcellular structures, called “invadopodia,” that invade the local extracellular matrix (ECM) and play a key role in metastasis formation[1,7-11]. EMT allows carcinoma cells to dissociate from each other, but also provides them the ability to degrade the ECM for single-cell invasion and initiate the metastatic cascade. The critical initial steps in pancreatic cancer metastasis involve the detachment and invasion of cancer cells into the surrounding tissues, which require changes to their adhesive and migratory properties. These changes occur in part through cell polarization and with the extension of actin-rich membrane structures in the direction of the movements, such as filopodia, lamellipodia, or invadopodia observed in invasive cancer cells[9,12-15]. However, to date, the process of EMT-

induced invadopodia in pancreatic cancer remains to be elucidated.

Recent studies suggest that the focal adhesion kinase (FAK) is involved in cancer progression by mediating the signals of integrins and other cell surface receptors that are known to play important roles in the regulation of cell migration. The formation of membrane protrusions, which are crucial for cell motility, is controlled by the rearrangement of the actin cytoskeleton. FAK, which is crucial for focal adhesion turnover, inhibits ECM proteolysis. Nevertheless, the specific role of FAK in the regulation of invadopodia formation and function in cancer cells remains unclear[16-21].

Members of the Wiskott-Aldrich syndrome protein (WASP) family are involved in mechanisms that regulate cell morphology, such as invadopodia formation and cell growth, and are correlated with certain cancer phenotypes. In particular, high levels of neural (N)-WASP, which is ubiquitously expressed, are positively correlated with progression and/or poor outcomes in lung, pancreatic, cervical cancer, hepatocellular, and esophageal squamous cell carcinoma[22-26]. In previous studies, we confirmed that N-WASP plays an important role in the formation of distant metastases and that its signals are regulated by FAK. Hence, the FAK/N-WASP pathway may represent a valuable therapeutic target to prevent cancer progression and distant metastasis. Noteworthy, recently we reported that lysyl oxidase-like 2 (LOXL2) is important for regulating EMT in pancreatic cancer[27]; however, the exact mechanism underlying LOXL2-mediated distant metastasis in pancreatic cancer has not been fully characterized. Herein, we investigated whether N-WASP expression is associated with LOXL2 by examining the development of pancreatic cancer using *in vitro* and *in vivo* models. We found that N-WASP mediates cancer progression by linking LOXL2 and FAK signals, which suggests that the LOXL2/FAK/N-WASP signaling axis is a key mediator of tumor metastasis through regulation of invadopodia. Taken together, these findings may provide new foundation for the development of enhanced therapeutic strategies to tackle pancreatic cancer.

II. MATERIALS AND METHODS

1. Patients

A total of 81 patients who underwent radical curative resection for pancreatic cancer from June 2002 to December 2012 at the Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, were enrolled in the study. Among them, three patients were excluded due to poorly preserved tissue samples, incomplete clinicopathologic data, or loss to follow-up. Patients were followed every 3 months during the first 12 months, and then every 6 months after the first year. The study protocol was approved by the Institutional Review Board at Gangnam Severance Hospital, Yonsei University of Korea (3-2014-0153) and the Institutional Animal Care and Use Committee of the institution (2019-0104), and complied with the Declaration of Helsinki. Informed consent was obtained from all participants.

2. Cell culture

MIA PaCa-2, PANC-1, BxPC-3, and AsPC-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the respective guidelines. Cells were cultured in Dulbecco's Modified Eagle Medium or RPMI 1640 medium (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (Biowest) and 1% antibiotic-antimycotic (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37℃.

3. Small interfering RNA (siRNA) assay

For gene knockdown, the following siRNAs were purchased from Bioneer (Daejeon, KR): siLOXL2 (#4017-1; sense 5′-AGAUUCCGGAAAGCGUACA-3′ and antisense 5′-UGUACGCUUUCCGGAAUCU-3′) and siN-WASP (#8976-1; sense 5′-GUGCAUUAAUGGAAGUGAU-3′ and antisense 5′-AUCACUUCCAUUAAUGCAC-3′). Transfection was conducted using Invitrogen Lipofectamine RNAiMAX (Thermo

Fisher Scientific) according to the manufacturer's instructions. Cells were harvested and processed 48–72 h post-transfection.

4. Antibodies and Western blot

To prepare the lysates, frozen pancreatic cancer tissues and cells were washed with ice-cold phosphate buffer solution (PBS) and lysed in RIPA lysis buffer. Proteins were separated by sodium dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes, blocked in 5% skim milk, and incubated with respective primary antibodies (1:1,000 dilution). Anti-N-WASP, phospho-N-WASP, and anti-LOXL2 from Abcam (Cambridge, UK); anti-vimentin and anti-snail (Cell Signaling Technology, Danvers, MA, USA); anti-N-cadherin and anti-E-cadherin (BD Bioscience, Franklin Lakes, NJ, USA); anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA); and anti-γ-tubulin (Sigma-Aldrich, St. Louis, MO, USA) were used. The membranes were washed twice with Tris-buffered saline with Tween 20 buffer (TBST), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:7,000 dilution; Cell Signaling Technology) in TBST with 3% skim milk. Bound antibody was probed using ECL solution (Bio-Rad, Hercules, CA, USA). Chemiluminescent signals were captured using X-ray films (AGFA, Mortsel, Belgium). All experiments were performed in triplicate.

5. RNA isolation and quantitative polymerase chain reaction (qPCR)

Cells and tissues were collected, and RNA was isolated using TRIZOL Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Of the total isolated RNA, 0.2 μg was analyzed by reverse transcriptase PCR using one-step RT-PCR kit (iNtRON Biotechnology, Seongnam, Korea). The first-strand cDNA was synthesized with 1 μg of RNA as a template, using the RT-qPCR cDNA Synthesis Kit (Intron), according to the manufacturer's instructions. RT-qPCR was performed using Applied Biosystems SYBR qPCR reaction mix (Thermo Fisher Scientific). Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method with *Gapdh* as reference gene.

6. Migration and invasion assays

For scratch wound migration assay, transfected cells were plated at $1.2–1.4\times10^4$ on a 24-well plate. Wound scratches were made 24 h after plating. Images of migrated cells were taken every 24 h using a microscope. For invasion assays, 8-μm pore size wells in a Transwell system (Corning, Corning, NY, USA) were coated for 1 h with Matrigel (1:50; Corning) at room temperature. Transfected cells (2×10^4) were seeded on the apical side of the Transwell chamber (24-well insert) in serum-free media, and growth media was added to the lower compartment. The cells were allowed to invade for 24 h. The remaining cells on the top of the chamber were gently scraped off using wetted cotton swabs. The cells that had invaded the basal side were fixed in methanol for 10 min, stained with 0.2% crystal violet, and then washed multiple times with 3'DW. The migration and invasion assays were performed in triplicate and repeated three times independently.

7. Immunocytochemistry

Transfected cells were fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 30 min. Cells were washed with PBS thrice and incubated with 1% bovine serum albumin for 1 h at room temperature. Cells were incubated with primary antibodies (LOXL2, N-WASP, ARP2, and phalloidin iFlour-488 [1:100–1:1000]; all from Abcam) overnight at 4 ℃, and then the cells were incubated at room temperature with Alexa Fluor 488- or 555-conjugated secondary antibodies (1:1000). FluoroShield mounting medium with DAPI (Abcam) was used. Immunostained cells were observed under a LMS780 fluorescence confocal microscope (Carl Zeiss, Oberkochen, Germany).

8. Invadopodia formation assay

FITC-gelatin invadopodia assays were performed using the commercially available QCM Gelatin Invadopodia Assay kit (Millipore, Burlington, Ma, USA) as per the manufacturer's procedure. Briefly, 8-well chamber slides were coated with poly-L-lysine for 20 min at room temperature, washed with PBS, fixed with glutaraldehyde for 15 min, and washed

with Dulbecco's PBS three times. After washing, a fluorescein gelatin:unlabeled gelatin mixture (1:5) was applied to each well and incubated at room temperature for 10 min. Substrates disinfected with 70% ethanol in sterile water were incubated at room temperature for 30 min and protected from light. To eliminate residual free aldehydes, growth media were added to each well, incubated at room temperature for 30 min, and protected from light. Cells were plated in wells at 20–80% confluence and incubated for 24 h. Afterwards, slides were fixed with 3.7% formaldehyde in Dulbecco's PBS for 30 min at room temperature. Blocking or permeabilization buffer for fluorescent staining was added for 1 h at room temperature. Then, the slides were incubated in TRITC-phalloidin and DAPI in fluorescent staining buffer for 1 h. The chamber was removed and the slide was covered with mounting media. Imaging of invadopodia and ECM degradation was performed using a LMS780 fluorescence confocal microscope (Carl Zeiss).

9. Knockdown stable cell lines

Five different short hairpin RNAs (shRNA) targeting N-WASP and a control non-targeting shRNA cloned in lentiviral vectors (pLKO.1-Puro) were purchased from Sigma-Aldrich. For the production of lentiviruses and transfection to HEK293 cells, Lipofectamine 3000 (Invitrogen) was used. PANC-1 cells were infected with the collected and purified viral particles; cells were stably selected and maintained with 2 μg/mL puromycin treatment. The shRNA sequence that sufficiently knocked down N-WASP expression was GCACAACTTAAAGACAGAGAA (TRCN0000123061; Sigma-Aldrich).

10. Immunoprecipitation

To analyze protein binding, cell lysates were incubated with anti-FAK or anti-ARP2 antibodies overnight at 4 ℃, and immune complexes were pulled down with protein A/G magnetic beads (MerckMillipore, Burlington, MA, USA). The interacted proteins were eluted in a denaturing SDS sample buffer and analyzed by Western blotting as described above.

11. Pancreatic cancer orthotopic model

All animal studies were approved by the animal ethics committee of the Yonsei University College of Medicine (approval #2019-0104). Female BALB/c nude mice (6-week-old) were purchased from Orient Bio (Seongnam, Korea). The pancreas was surgically exposed through an abdominal excision under anesthesia upon intraperitoneal injection of Alfaxan (25 mg/kg). Human pancreatic cancer cells were inoculated directly into the pancreas of the mice using a 30 G needle (BD Biosciences). PANC-1 cells (1×10^6) were mixed with serum-free Dulbecco's Modified Eagle medium and Matrigel (1:1) and injected in a volume of 50 μL. To prevent cancer cells from leaking, the excision was closed after covering the injection site with the surge cell. After this procedure, the mice were warmed and monitored until conscious, and then placed in HEPA-filtered cages with access to food and water. After 12 weeks, the mice were euthanized and examined for tumor spreading through macroscopic and microscopic observations of the pancreas, spleen, liver, and lungs was performed by hematoxylin and eosin staining.

12. Immunohistochemical (IHC) staining

Serial sections (5 μm) of each tissue block were adhered to poly-L-lysine-covered slides and incubated at 62 °C for 60 min. After deparaffinization and rehydration, the sections were heated in 10 mM citrate buffer (pH 6.0) for 15 min and stained with an antibody. Normal pancreas tissue within the block was used as internal positive control. Immunohistochemical staining was categorized as negative, “1+,” “2+,” or “3+” in high-power fields (200×) according to the staining intensity. Positivity was assigned for the scores “2+” and “3+.” The staining intensity was assessed by two pathologists who were blind to the clinical outcomes.

13. Statistical analysis

Unpaired *t*-test and analysis of variance were performed to compare two or more groups,

respectively. The differences were considered statistically significant at $p<0.05$. The tests were performed using Prism 8.01 (GraphPad Software, San Diego, CA, USA).

III. RESULTS

1. N-WASP is highly expressed in pancreatic cancer cell lines and tissues

To elucidate the effects of N-WASP and its underlying molecular mechanisms, N-WASP expression was analyzed in several pancreatic cancer cell lines (MIA PaCa-2, PANC-1, AsPC-1, and BxPC-3) and 81 primary PDAC samples. N-WASP was detected in all pancreatic cell lines (Fig. 1A) and was found to be overexpressed in PDAC tissues as compared with normal pancreatic tissue (Fig. 1B–E).

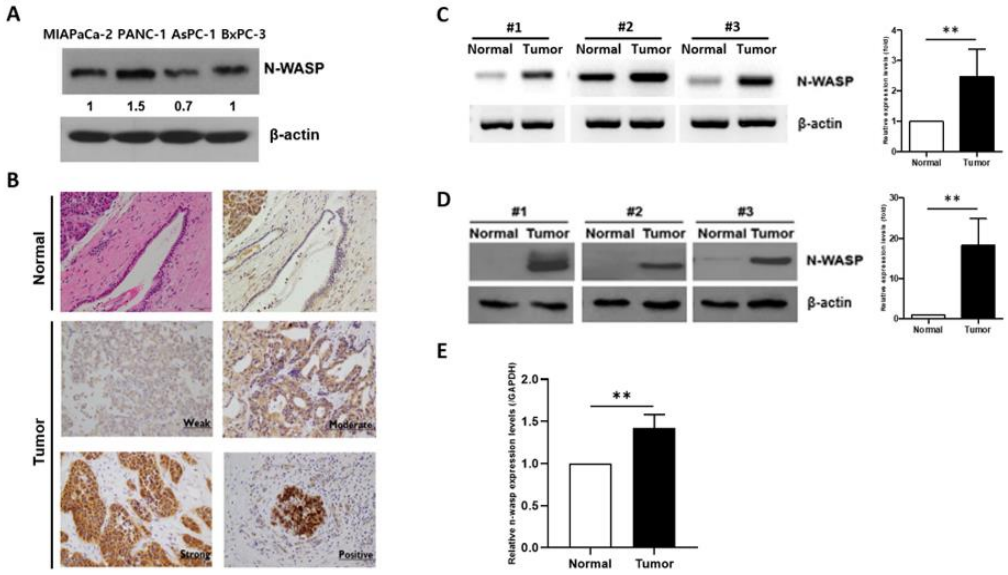

Figure 1. N-WASP is highly expressed in pancreatic cancer cell lines and primary PDAC tissues

(A) N-WASP detection in pancreatic cancer cell lines by Western blot. (B) Immunohistochemical staining of N-WASP in human pancreatic tissues. (C) Relative mRNA expression of N-WASP in human pancreatic cancer tissues. (D) N-WASP detection in human pancreatic cancer tissues by Western blot. (E) Relative N-WASP expression in

human pancreatic cancer tissues. All data are presented ± standard deviation. **p<0.01 by t-test.

2. Clinical characteristics and patterns of recurrence according to N-WASP status

Patient characteristics, including N-WASP expression, were retrospectively evaluated (Table 1). No significant difference in T stage, N stage, tumor stage, cell differentiation, perineural invasion, and lymphovascular invasion was observed among patient groups based on the N-WASP expression status, as determined by immunohistochemistry. Nevertheless, N-WASP positive patients had more distant metastasis (p=0.004) than the N-WASP negative group (Table 2).

Table 1. Clinical characteristics of the patients according to N-WASP expression

	N-WASP negative (n=56)	**N-WASP positive (n=25)**	***p*-value**
Age	63.66±10.15	64.24±9.56	0.925
Sex			
Men	26 (46.4%)	14 (56%)	0.426
Women	30 (53.6%)	11 (44%)	
T stage [a]			
T1	9 (16.1%)	4 (16.0%)	0.414
T2	36 (64.3%)	17 (68%)	
T3	11 (19.6%)	3 (12%)	
Unknown	0 (0%)	1 (4%)	
N stage [a]			
N0	19 (33.9%)	6 (24%)	0.645
N1	28 (50%)	15 (60%)	

	N-WASP negative (*n*=56)	N-WASP positive (*n*=25)	*p*-value
N2	9 (16.1%)	4 (16%)	
Stage [a]			
IA	5 (9.1%)	1 (4%)	0.561
IB	8 (14.5%)	2 (8%)	
IIA	6 (10.9%)	2 (8%)	
IIB	27 (49.1%)	15 (60%)	
III	9 (16.4%)	4 (16%)	
Unknown	0 (0%)	1 (4%)	
Perineural invasion			
Positive	39 (69.6%)	20 (80%)	0.622
Negative	14 (25%)	4 (16%)	
Unknown	3 (5.4%)	1 (4%)	
Lymphovascular invasion			
Positive	22 (39.3%)	15 (60%)	0.223
Negative	31 (55.4%)	9 (36%)	
Unknown	3 (5.4%)	1 (4%)	
Cell Differentiation			
Well	11 (19.6%)	2 (8%)	0.168
Moderate	39 (69.6%)	16 (64%)	
Poor	5 (8.9%)	5 (20%)	
Undifferentiated	1 (1.8%)	2 (8%)	

Abbreviation: N-WASP, neural Wiskott-Aldrich syndrome protein.

[a] According to the 8th edition of the American Joint Committee on Cancer staging system.

Table 2. Recurrence pattern between N-WASP negative and positive patients

	N-WASP negative (*n*=46)	N-WASP positive (*n*=17)	*p*-value
Local metastasis	27 (58.7%)	3 (17.6%)	0.004
Distant metastasis	19 (41.3%)	14 (82.4%)	

Abbreviation: N-WASP, neural Wiskott-Aldrich syndrome protein

3. N-WASP knockdown inhibits EMT and reduces motility and invasiveness of pancreatic cancer cells

To evaluate whether N-WASP affects the phenotype of pancreatic cancer cells, N-WASP was knocked down in PANC-1 and AsPC-1 cells using targeted siRNA. Cells lacking N-WASP had significantly higher expression of E-cadherin, whereas snail and vimentin were significantly reduced (Fig. 2A). Moreover, wound healing assays revealed that N-WASP-knockdown cells were unable to efficiently close the wound gap (Fig. 2B and F), suggesting that N-WASP is important for cell migration. Furthermore, transwell assays revealed nearly 70% reduction in cell migration and 50% reduction in cell invasion in N-WASP siRNA-transfected PANC-1 and AsPC-1 cells (Fig. 2C and E). Hence, inhibition of N-WASP can suppress PDAC progression.

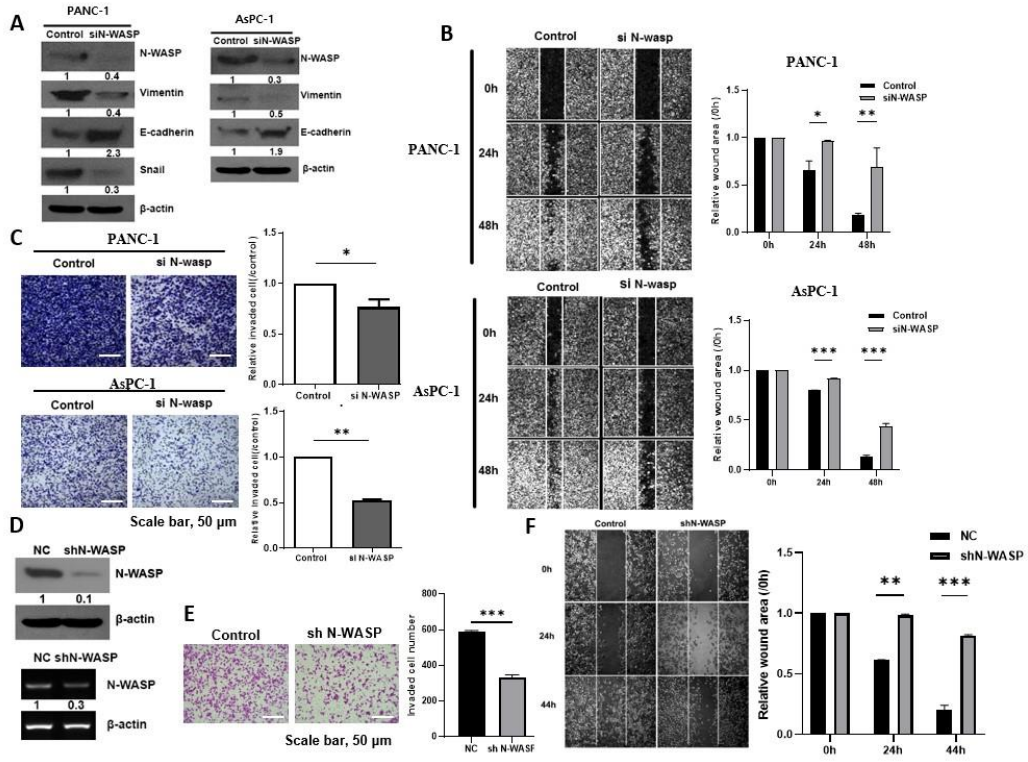

Figure 2. Impact of N-WASP on EMT, migration, and invasiveness of pancreatic cancer cells

(A) PANC-1 and AsPC-1 cells were transfected with negative control (NC) or N-WASP siRNAs. Western blot analysis was performed for EMT markers. (B) Cell monolayers transfected with negative control or N-WASP siRNAs were scraped with a razor blade and then incubated in a fresh medium containing 5% fetal bovine serum. After incubation for the indicated time points, the number of cells that migrated across the wound was counted. Representative photographs and wound area are presented ± standard deviation $^{**}p<0.01$, $^{*}p<0.05$. (C) Transwell migration and invasion assays of PANC-1 and AsPC-1 cells transfected with negative control or N-WASP siRNAs. (D) Stable knockdown of N-WASP using shRNA in PANC-1 as detected by Western blot (upper) and qPCR analysis (lower). (E) Transwell invasion assay and (F) wound healing migration assay of PANC-1 cells stably transfected with N-WASP shRNA.

4. Identification of potential target genes of N-WASP in pancreatic cancer

To investigate the underlying mechanism of N-WASP in promoting PDAC progression, we performed RNA-sequencing of PANC-1 cells lacking N-WASP and compared their expression pattern with that of untransfected cells. Several genes showed more than 2-fold difference in expression compared with shControl cells (Fig. 3A and B). Gene set enrichment analysis further revealed that N-WASP enrichment was associated with genes in “HALLMARK EMT” and “HALLMARK Cell migration” (Fig. 3C). Moreover, gene ontology analysis associated the differentially expressed genes with cell migration and EMT (Fig. 3D and E). Among the lower expressed genes of shN-WASP cells, many renowned genes associated with EMT and cell migration were identified (*e.g.*, *GPC4*, *CCL5*, *CCL2*, *CTHRC1*, *CX3CL1*, *ICAM1*, *SPARC*, *BMP2*, *SNAI3*, and *WNT11*). These findings were verified by qPCR after knockdown of N-WASP in PANC-1 cells (Fig. 3F).

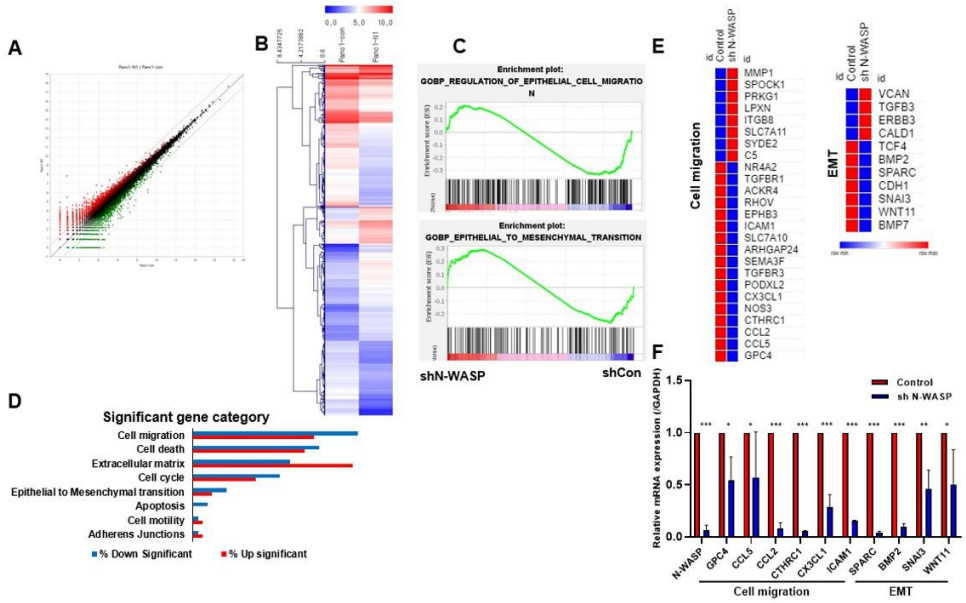

Figure 3. N-WASP expression is positively correlated with PDAC development

(A, B) Heatmap showing the relative expression of N-WASP and genes positively correlated with N-WASP in eight common solid tumors according to RNA sequencing data. Red, high expression; blue, low expression. (C) Gene set enrichment analysis plots indicating that high expression of genes involved in EMT and cell migration were significantly positively correlated in N-WASP expression. (D) Functional annotations of significantly downregulated and upregulated genes (with fold change >2 and $p<0.05$). Bars show the fold enrichment of pathways that were significantly downregulated or upregulated in N-WASP from advanced stages *vs*. early stages. (E) Heatmap for selected pathways showing Z-score of genes that were differentially expressed. (F) Relative mRNA expression of genes involved in cell migration and EMT quantified in PANC-1 cells by qPCR.

5. FAK-dependent phosphorylation of N-WASP is regulated by LOXL2

Our previous studies showed that the FAK/LOXL2 axis is important for regulating EMT and metastasis in PDAC. To clarify whether LOXL2 affects N-WASP, we first evaluated the correlation of FAK-dependent phosphorylation of N-WASP by LOXL2. FAK-knockdown cells exhibited significantly reduced expression of phosphorylated N-WASP (p-N-WASP) (Fig. 4A). In addition, LOXL2-knockdown cells exhibited significantly reduced expression of phosphorylated FAK (p-FAK) and p-N-WASP in PANC-1 cells. In agreement with these results, AsPC-1 cells overexpressing LOXL2 had increased expression of p-FAK and p-N-WASP (Fig. 4B). Indeed, LOXL2 knockdown impaired the interaction between N-WASP and FAK (Fig.4C). These findings demonstrate that the FAK/N-WASP axis in pancreatic cancer cells is significantly associated with LOXL2 expression.

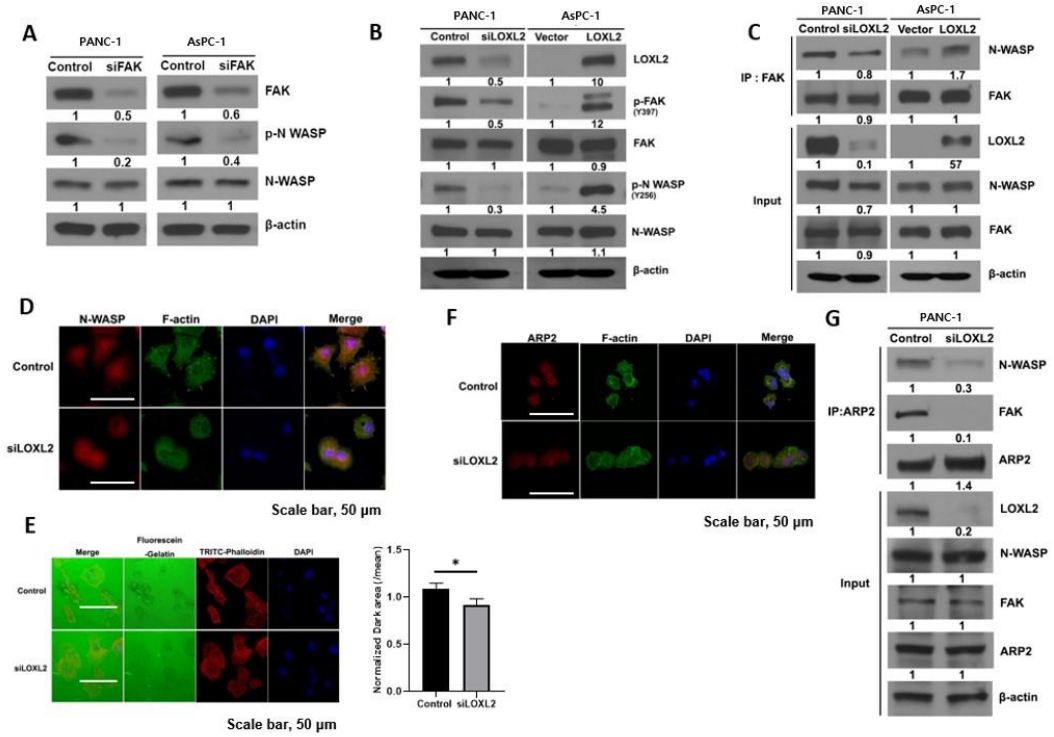

Figure 4. FAK-dependent phosphorylation of N-WASP is regulated by LOXL2

(A) PANC-1 and AsPC-1 cells were transfected with negative control or FAK siRNA. Western blot analysis was performed to assess EMT markers. (B) Cells transfected with LOXL2 siRNA or pcDNA-LOXL2 expressing vector were assessed by Western blotting. β-actin was used as loading control. (C) Immunoprecipitation (IP) of endogenous FAK and N-WASP. Lysates from siLOXL2-transfected PANC-1 cells were immunoprecipitated with anti-FAK antibody and analyzed by Western blotting. (D) Immunofluorescence staining of N-WASP (red) is affected by LOXL2 silencing. Green and blue fluorescent signals indicate the F-actin cytoskeleton and nuclei. (E) Invadopodia formation assay. Dark areas of gelatinase activity (black) reveal invadopodia-mediated degradation. Red and blue fluorescent signals indicate the F-actin cytoskeleton and nuclei. Matrix degradation was quantified (right) by measuring the dark area of five individual fields; results are normalized and represented as mean ± standard deviation. *p=0.01 by unpaired t-test. (F)

Validation of translocation of ARP2 upon LOXL2 silencing. Immunofluorescence staining for endogenous ARP2 (red) and F-actin cytoskeleton (green) (G) Immunoprecipitation of endogenous ARP2 with N-WASP and FAK in LOXL2 siRNA transfected cells.

6. LOXL2 influences the ability of N-WASP to regulate the morphology, invasiveness, and EMT of pancreatic cancer cells

Invadopodia formation is essential for invasion and metastasis. Emerging evidence revealed a critical role for cortactin in invadopodia formation, as well as in promoting cell motility and invasion (Fig. 4D and 4E). Furthermore, knockdown of LOXL2 expression impaired the interaction between N-WASP and ARP2 (Fig. 4F and 4G). These results suggest that inhibition of the LOXL2/FAK/N-WASP axis may prevent the progression of PDAC by regulating the cell morphology and invadopodia formation.

To further explore the detailed molecular mechanisms by which the LOXL2/N-WASP axis inhibits EMT, we transfected PANC-1 cells with siRNAs targeting N-WASP and LOXL2. Cells lacking both N-WASP and LOXL2 showed lower levels of EMT markers (N-cadherin, vimentin, snail) compared with siN-WASP or siLOXL2 cells (Fig. 5A). Moreover, silencing of both N-WASP and LOXL2 significantly repressed the invasiveness (Fig. 5B) and migration (Fig. 5C) of PANC-1 cells. These data suggest that the LOXL2/N-WASP axis is involved in pancreatic cancer cell migration, invasion, and morphology change.

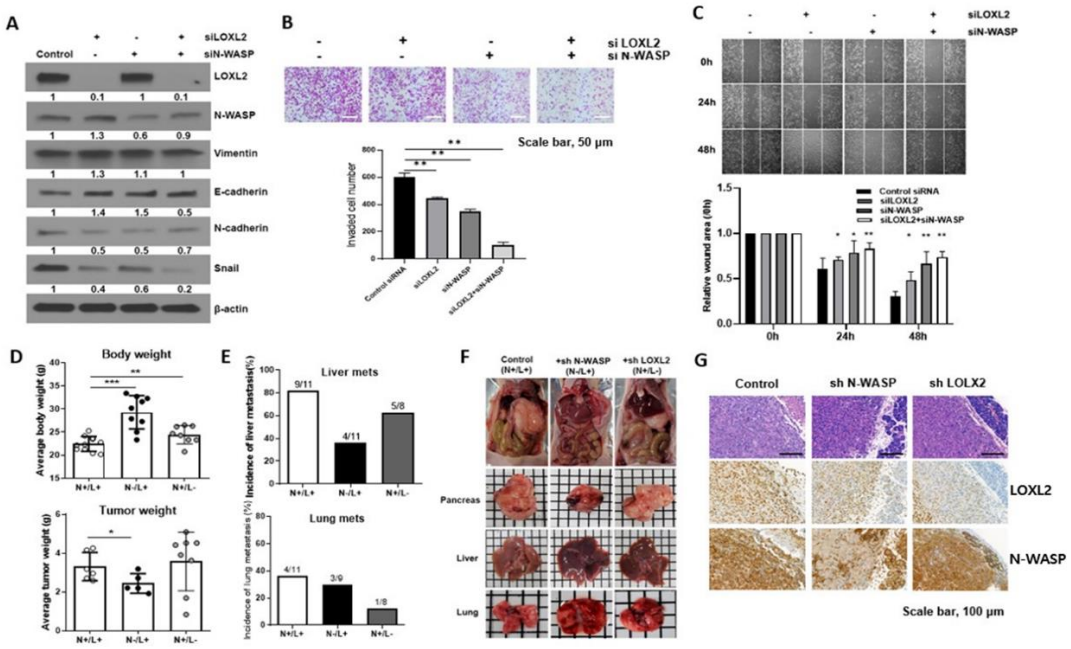

Figure 5. Inhibition of N-WASP reduces metastasis of pancreatic cancer to the liver and lungs

PANC-1 cells were transfected with LOXL2 and N-WASP siRNA. (A) Western blotting was performed to assess EMT markers. (B) Transwell invasion assay and (C) wound healing migration assay. (D) Average body weight and tumor weight of orthotopic pancreatic cancer mice. Control shRNA (N+/L+), N-WASP shRNA, and LOXL2 shRNA transfected PANC-1 cells (1×10^6) were orthotopically injected into the pancreas of nude mice (n=5 per group). Tumor weight and body weight were measured after the mice were euthanized at 12 weeks. (E) Percentage of mice with lung and liver metastases in each group. (F) Representative images of the tumors and orthotopic mice from each group. (G) Microscopic features of the primary tumor in an orthotopic model confirmed by immunohistochemical staining (N-WASP, LOXL2). Top: hematoxylin and eosin staining, ×200.

7. Inhibition of N-WASP impairs the metastatic potential of pancreatic cancer *in vivo*

To confirm whether the LOXL2/N-WASP axis impacts on the progression of pancreatic cancer *in vivo*, PANC-1 cells transfected with shN-WASP or a control vector were injected into the pancreas of nude mice. On day 84 following the orthotopic modeling, the body weight of mice bearing shN-WASP tumors was remarkably higher than that of the control group. Notably, the average tumor weight was lower in the shN-WASP group (Fig. 5D), and these animals had fewer liver and lung lesions than the animals with PANC-1-derived tumors. Hence, these findings suggest that N-WASP performs a substantial function in pancreatic cancer metastasis and proliferation (Fig. 5D and E). Further analysis of the microscopic features of the primary tumors confirmed that the PANC-1-derived tumors expressed both LOXL2 and N-WASP (Fig. 5G). Noteworthy, LOXL2 expression in the shN-WASP group was similar to that in the PANC-1 control group, whereas N-WASP was similar or slightly decreased in shLOXL2 group (Fig. 5G). These results suggest that LOXL2 directly regulates, to some extent, the expression of N-WASP in pancreatic cancer *in vivo*.

IV. DISCUSSION

In this study, we demonstrated that the LOXL2/N-WASP signaling axis contributes to the invasion and metastasis of pancreatic cancer cell. Our *in vitro*, *in vivo*, and clinical results showed that N-WASP is overexpressed in both pancreatic cancer lines and human pancreatic cancer tissues. Moreover, high expression of N-WASP is associated with increased distant metastasis and poor prognosis.

There are five known members of the WASP family: WAVE1–3, N-WASP, and WASP. Among these, N-WASP and WAVE-1 are important for invadopodia formation and ECM degradation, respectively. Thus, there are distinct pathways that activate branched actin assembly for invadopodia formation[28-36]. Invadopodia are subcellular structures that are selectively invasive as compared with non-invasive cancer cells and are involved in a pivotal process in cancer invasion[7,13,37]. Notably, we showed that N-WASP promotes the proliferation and migration of pancreatic cancer cells, as N-WASP increased colony formation *in vitro* and distant metastasis *in vivo*, where deletion of N-WASP inhibited cell proliferation and migration. Moreover, high levels N-WASP were found to be correlated with higher distant metastasis potential. Our *in vivo* study showed that N-WASP is essential for the invasion and metastasis of pancreatic cancer. Analysis of the relationship of N-WASP expression with clinicopathologic features of pancreatic cancer further suggested that higher N-WASP levels are correlated with distant metastasis after surgery.

Invadopodia and podosomes share many common functional and molecular characteristics, including dependence on Src kinase and branched actin assembly, as well as common molecular components, including focal adhesion proteins, integrins, and proteases. Many molecules that localize to focal adhesions are found in invadopodia or podosomes, including cytoskeletal proteins such as alpha-actinin, VASP, and zyxin, integrin linkers such as vinculin and talin, and signaling molecules such as FAK, paxillin, p130Cas, and ERK[38-41]. In our previous study, we showed that LOXL2 expression is higher in human pancreatic cancer cells than in normal pancreas tissues. Moreover, the higher expression of

LOXL2 the pancreatic cancer had, significantly greater was its potency for distant metastasis[27].

Moreover, positive modulation of the FAK and Src pathways by LOXL2 has been reported, which contributes to cell migratory behavior. Recently, Baker *et al*. showed that LOXL2 activates fibroblasts through integrin-mediated FAK activation for tumor cell invasion and metastasis[42]. Furthermore, LOXL2 plays an integral role in EMT promotion and invasiveness of pancreatic cancer cells. Our *in vitro* study indicated that LOXL2 activates EMT-like processes in pancreatic cell lines, which are associated with invasive and migratory properties. Moreover, LOXL2 positively contributes to the activation of FAK/Src and influences the expression of CDH1, Snail, and L1CAM, which are all related to EMT and invasiveness of pancreatic cancer cells.

This study has several limitations. The number of patients for the clinical sample used in this study was small. Using a larger cohort of patients may provide more definitive conclusions. In addition, additional investigations are needed to explore in further detail the underlying mechanisms of N-WASP for promoting distant metastasis, as well as on its molecular partners other than LOXL2 and FAK.

V. CONCLUSION

Notwithstanding, we gathered *in vitro* and *in vivo* evidences that demonstrate that N-WASP is effectively involved in the regulation of distant metastasis of pancreatic cancer and identified the LOXL2/FAK/N-WASP axis as an important mechanism involved in the metastasis process. In particular, we showed that N-WASP has an important role in the expression of genes associated with cell migration and EMT. Moreover, we proved a correlation between the expression of N-WASP and LOXL2 in pancreatic cancer and distant metastasis. Taken together, the LOXL2/N-WASP axis may represent a valuable candidate for developing new therapeutic strategies to prevent distant metastasis in pancreatic cancer.

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ABSTRACT(IN KOREAN)

췌장암의 원격 전이에 영향을 미치는 N-WASP 신호 전달 경로 [LOXL2-FAK-N-WASP]

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김 형 선

췌장암은 예후가 나쁘고 재발과 전이가 많은 악성 종양 중의 하나로 알려져 있다. 그러나 췌장암의 재발과 전이에 관련된 메커니즘은 아직까지 명확하게 규명되어 있지 않다. 이 연구에서는 췌장암의 전이에 관련된 메커니즘 중 Neural Wiskott-Aldrich Syndrome Protein (N-WASP)의 역할에 대해서 알아보고자 하였다. 먼저 N-WASP의 발현이 췌장암 환자에서 증가되어 있다는 것을 확인하였고 N-WASP의 발현이 높은 췌장암 환자에서 N-WASP의 발현이 낮은 췌장암 환자보다 수술 후 재발 시 원격 전이의 비율이 높은 양상을 보였다. 또한 췌장암 세포주에서 N-WASP의 발현을 억제하였을 때 유의미하게 세포의 침습, 이동성, 상피간엽이행이 줄어드는 것을 볼 수 있어서 N-WASP가 Epithelial mesenchymal transition(상피간엽이행, EMT)의 조절자로의 역할을 한다는 것을 새로이 확인할 수 있었다. 그리고 N-WASP의 상위 조절 인자로 LOXL2(Lysyl oxidase-like 2)와 FAK(Focal adhesion kinase)가 관여한다는 것도 확인하였는데, in vitro실험에서 LOXL2-FAK-N-WASP의 메커니즘을 억제한 결과 EMT와 Invadopodia도 영향을 받았으며 in vivo실험에서 N-WASP와 LOXL2의 발현을 각각 억제한 결과 췌장암 세포주로 만든 Orthotopic mouse model에서 의미 있게 간과 폐의 전이가 감소하였다. 이것으로 LOXL2와 연관된 N-WASP 신호전달과정이 췌장암의 전이 과정을 촉진시키는 EMT와 Invadopodia 과정과 연관되어 있음을 보여주었다. 이러한 결과는 췌장암의 원격전이와 연관된 메커니즘을 규명하고, 이를 토대로 치료방침의 결정에 도움을 줄 새로운 타겟 연구에 도움을 줄 수 있다.

핵심되는 말 : 췌장암, neural wiskott-aldrich syndrome protein, 전이, 상피간엽 이행, invadopodia

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