Thesis for the Degree of Master of Science

Activity evaluation study of a mutational c-Src in NIH-3T3 cells

by

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The Graduate School

Pukyong National University

August 2019

Activity evaluation study of a mutational c-Src in NIH-3T3 cells

(유전자 변이형 c-Src의 NIH-3T3 세포에서의 활성평가 연구)

Advisor: Prof. Sun Joo Park

by

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A dissertation

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List of Abbreviations

A-loop	Activation-loop
Ala	Alanine
BCS	Bovine calf serum
c-Src	cellular-Src
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
HRP	Horseradish peroxidase
Lys	Lysine
MMP	Matrix metalloproteinase
N-WASP	Neural-Wiskott-Aldrich syndrome protien
PBS-T	Phosphate buffered saline-Tween 20
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

Phe	Phenylalanine
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RSV	Raus sarcoma virus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFKs	Src family kinases
SH	Src homology
Tyr	Tyrosine
v-Src	viral-Src

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Abstract

Cancers can be caused by oncogenes, and c-Src is one of the most common proto-oncogenes; it is observed in 40% of cancers. Activated c-Src contributes to cancer cell growth and invasion through structures known as “invadopodia”. To be activated, several residues in c-Src must undergo phosphorylation changes. Tyr416 and Tyr527 are important in this respect, and each residue acts as a positive or negative regulator. Replacing Tyr416 with Phe416 (Y416F, YF) results in c-SRC being continuously activated so that it acts like an oncogene. However, it is unclear which residue plays a pivotal role in the conformational change of c-Src. Recently, one paper reported on several simulations indicated that a mutation of Lys321 to Ala321 (K321A, KA) can lead to an imperfect conformational change of c-Src; however, it is unclear whether this works at the cellular level. We made KA-substituted c-Src DNAs and found that these mutants cause variation of the phosphorylation state, decrease migration, and inhibit invadopodia formation when transfected to NIH-3T3 mouse fibroblasts. From these results, we anticipate that the K321 residue might be used to find novel c-Src-related cancer therapies.

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Abstract

암은 암유전자에 의해 발생할 수 있으며, “c-Src”는 전체 암의 40% 에서 발견되는 대표적인 proto-oncogene 중 하나이다. 왈성화된 c-Src 는 암세포의 성장과 “invadopodia”라고 불리는 침투에 특화된 구조를 통하여 침투성에 기여한다. 활성화되기 위하여, c-Src 내 몇 개의 잔기는 반드시 인산화 변화를 겪어야한다. Tyr 416과 Tyr 527이 이러한 변화에서 중요하며 각각은 양성과 음성 조절자로 작용한다. Tyr 416을 Phe 416으로 치환하는 것 (Y416F, YF) 은 c-Src가 암유전자처럼 지속적으로 활성을 띄게 만들어준다. 하지만, 아직까지 어떠한 잔기가 c-Src의 구조적인 변화에서 중요한 역할을 하는지는 밝혀지지 않았다. 최근 한 논문에서 simulation을 통하여 Lys 321을 Ala 321으로 치환할 시 (K321A, KA), c-Src의 불완전한 구조변화를 일으킨다는 것이 밝혀졌다. 하지만, 이러한 변화가 실제로 세포내에서 일어나는지는 불확실하다.

따라서, 우리는 KA로 치환된 c-Src DNA를 제조하였고 이러한 돌연변이가 NIH-3T3 mouse fibroblast의 인산화 상태의 변화를 유도, 이동성 및 invadopodia 형성을 감소시킨다는 것을 확인하였다. 이러한 결과를 토대로 우리는 K321 잔기가 새로운 c-Src 관련 암의 치료에 사용될 수 있을 것이라 기대한다.

1. Introduction

1-1. c-Src

Cancer is a malignant tumor which is invasive and has motility. It has a variety of causes, including the activation of oncogenes. Src is one of the most common oncogenes (Guarino, 2010) and it encodes Src family kinase (SFK), which is known to function in cell proliferation, differentiation and motility. Src takes two principal forms: inactive-folded and active-open (Giannoni, Taddei, & Chiarugi, 2010).

Activated Src can be observed in 40% of cancers, and so many researchers have attempted to find anticancer drugs which target activated Src. However, Src inhibitors, such as dasatinib or saracatinib, have many side effects or can only be used in the early stages of cancer (Yeatman, 2004).

The fact that cancer can be caused by a virus was firstly shown with the Rous sarcoma virus (RSV) by Martin in the 1950s, and the single gene concerned was named Src by geneticists. Because this gene is from a virus, it is usually referred at as v-Src (viral-Src); other scientists identified the normal cellular homologue, c-Src (Martin, 2001).

Src is one of the SFKs, a nonreceptor membrane-associated kinase. There are eight other SFKs, including Fyn, Yes and Blk. SFKs have a common structure, with Src homology domains (SH domains) which have specific functions (Roskoski, 2004). Both c-Src and v-Src have almost the same composition, but the c-terminal tail is eliminated in v-Src (Fig. 1).

Fig. 1. Schematic presentation of c-Src (cellular–Src, a), v-Src (viral–Src, b) and c-Src (YF, c)

There are four kinds of SH domain in the SFKs. The SH1 domain (kinase domain) is responsible for the enzyme reaction, and the positive regulator Tyr416 residue is positioned in the SH1 domain. The SH2 domain controls

the interaction between phosphotyrosine motifs and Src or other proteins. The SH3 domain directs proline-rich motifs in intra- or intermolecular interactions (Koch, Anderson, Moran, Ellis, & Pawson, 1991). The SH4 domain has a myristoylation site with high variability (Resh, 1999).

The inactive form of c-Src has a folded shape, with phosphorylated Tyr527 bound to the SH2 domain. The Tyr416 residue in inactive c-Src is protected by an A-loop (activation loop). However, when Tyr527 is dephosphorylated and separated from the SH2 domain, Tyr416 is no longer in the A-loop and is phosphorylated by cis autophosphorylation. With these serial steps, folded c-Src turns into the activated form (Fig. 2). In v-Src, the c-tail region with Tyr527 is eliminated, so v-Src is continuously activated (Oneyama, Hikita, Nada, & Okada, 2008).

As mentioned, c-Src activation is closely related to cancer. However, it is still unclear which residue plays a pivotal role in c-Src activation. Recently, it has been shown that mutation of Lys321 to Ala321 can cause a conformational change in c-Src, which did not reach the perfect activation form through the several simulations (Yoon, Lee, Park, & Wu, 2018). However, it is unclear whether this effect occurs at the cellular level. Thus, we checked the inhibition effect of WT and YF c-Src substituted Lys321 to Ala (K321A) using an NIH-

3T3 mouse fibroblast cell line.

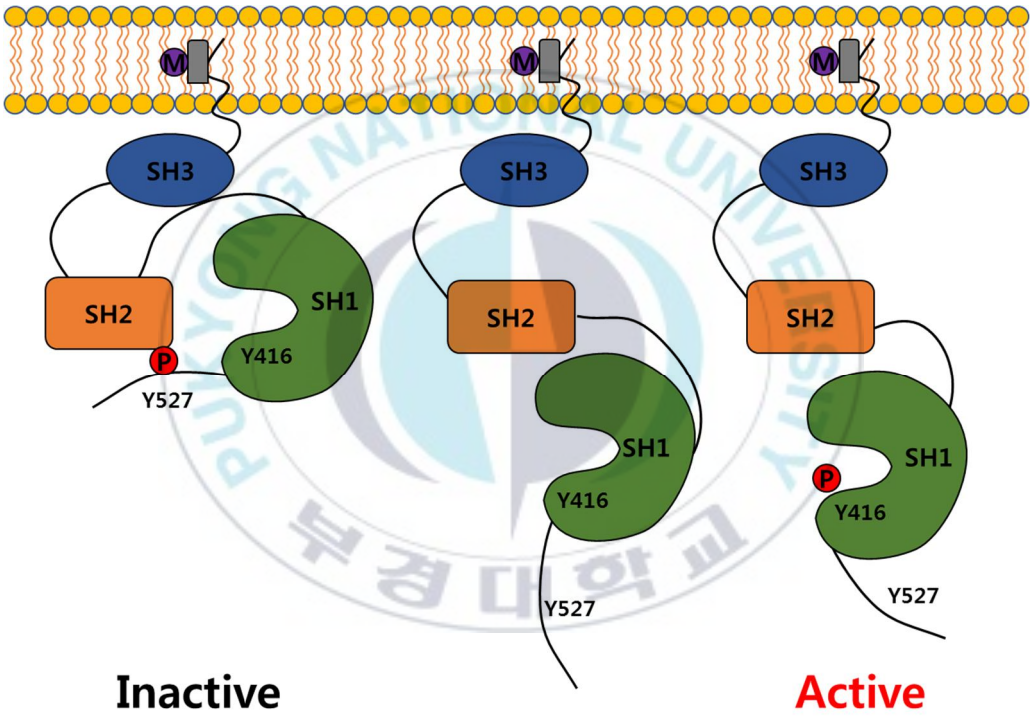


Fig. 2. Schematic presentation of conformational change, Inactive to Active state of c-Src

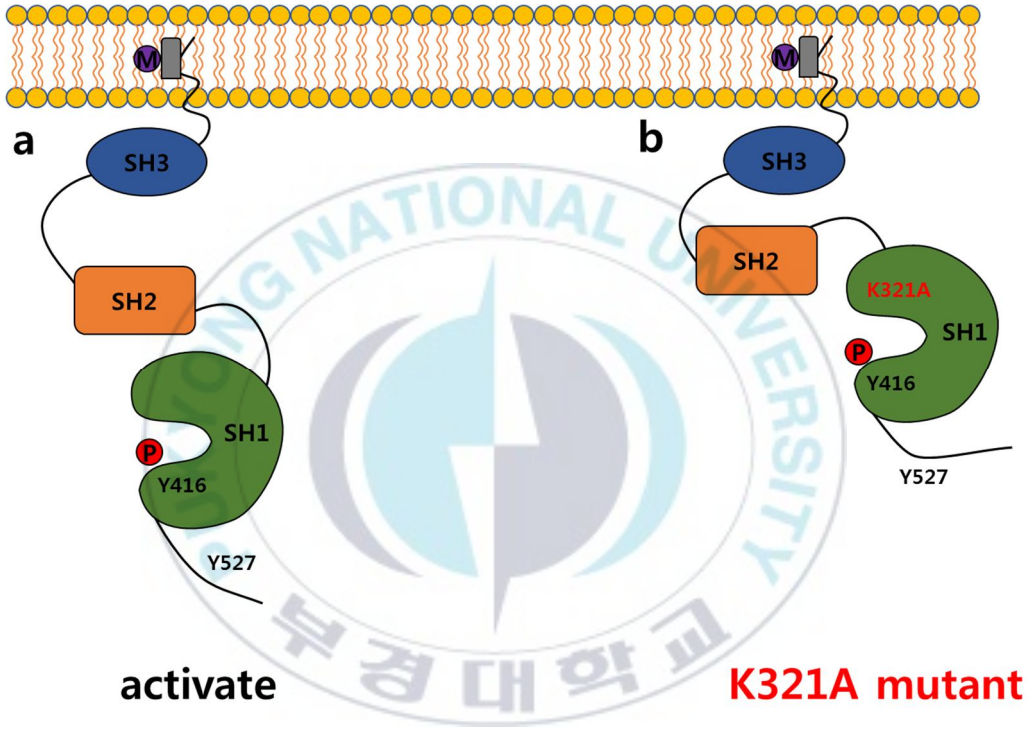


Fig. 3. Schematic presentation of activated (a) and K321A mutant c-Src (b)

1-2. Y527F (YF) c-Src

Both v-Src and c-Src can be activated continuously through a specific mutation on a residue (Fig. 1c). Substituting Tyr527 with Phe527 residue in c-Src eliminates the phosphorylation site and permits the continuous activation of Tyr416 (Hirai & Varmus, 1990). That is because the hydroxyl group in the Tyr is absent when Tyr527 turns into Phe527, allowing the phosphorylation of the 416 residue (Fig. 4).

In the 1960s, it was discovered that the RSV oncogene product ($pp60^{v\text{-}Src}$) could transform fibroblast cells, whereas its counterpart ($pp60^{c\text{-}Src}$) does not (Tipper et al., 1965). Both $pp60^{v\text{-}Src}$ and $pp60^{c\text{-}Src}$ are kinases that transfer phosphate to the tyrosine residue of receptor proteins. The phosphorylation of serine residues is the same in those two kinases, but phosphorylation of tyrosine is a little different; $pp60^{v\text{-}Src}$ has a phosphorylated Tyr416 residue (Clement, Taunton, Smart, & McNicol, 1981). In 1986, scientists discovered that Tyr527 is phosphorylated in $pp60^{c\text{-}Src}$ but is absent in $pp60^{v\text{-}Src}$ (Cooper, Gould, Cartwright, & Hunter, 1986). Finally, Y527F c-Src was first introduced in 1990 (Hirai et al. 1990).

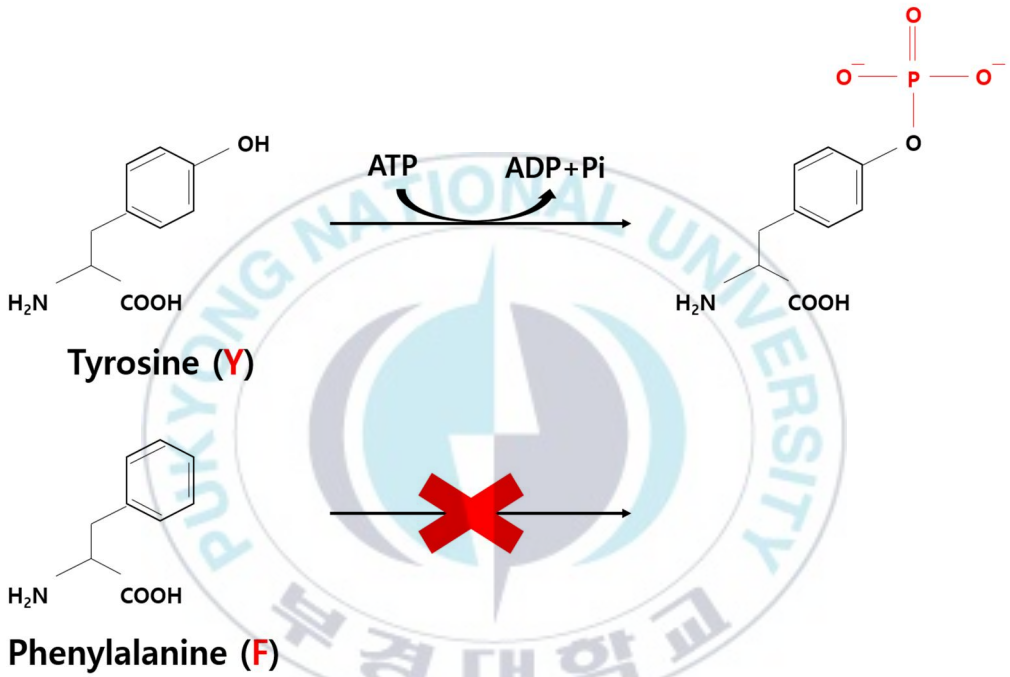


Fig. 4. Inhibition of phosphorylation at Phenylalanine

1-3. Transfection

Transfection is a procedure that introduces nucleic acids into cells to produce genetically modified cells. Transfection is used for studying for gene or protein function. The process of transfection includes opening transient pore of cell membrane which can cause morphological change of the target cells and allowing the genetic materials uptake. Introduced genetic materials can exist stable or transient in the cells (Recillas-Targa, 2006). In case of stable transfection, the genetic materials integrate directly to the host gene and have specific marker gene which can distinguish untransfected cells from transfected cells (Glover, Lipps, & Jans, 2005). In contrast to stable transfection, the genetic materials do not integrate into the host gene and can express for a short period (Stepanenko & Heng, 2017). There are three major type of transfection: biological, chemical and physical transfection. At first, biological transfection uses virus and retrovirus, such as adeno virus, is used for transfection (Woods et al., 2003). Biological transfection is usually known as transduction (Pfeifer & Verma, 2001). Second, chemical transfection uses cationic lipid, cationic polymer and calcium phosphate. Positive cationic compounds are combined with negative charged DNA and are attracted to cell membrane (Holmen, Vanbrocklin, Eversole, Stapleton, & Ginsberg,

1995). However, it is still elusive that how these compounds go inside of the cell membrane. Physical transfection includes micro injection, electroporation and particle delivery (Mehier-Humbert & Guy, 2005).

1-4. Vector

Vector is a DNA molecule which is used for transfection. Foreign DNA contained vector is called “recombinant DNA” and there are four types of vectors; plasmid, viral vector, cosmid and artificial chromosome. Plasmid is the most commonly known for vector (Remaut, Sanders, Fayazpour, Demeester, & De Smedt, 2006). Most plasmid vectors contain not only essential nucleotide sequences required for their use in DNA cloning but other things: a replication origin, a drug-resistance gene and multiple cloning sites. The replication origin (ORI) is a specific DNA sequence of 50 – 100 bp long and replication starts at this site. The drug-resistance gene is used for a selectable marker to distinguish plasmid transformed cell from not. Usually, ampicillin or kanamycin are used for these selectable markers. To clone target DNA in a plasmid vector the DNA must be produced and then inserted into the vector DNA. The restriction enzymes and DNA ligases are used for this

step and produce successful recombinant DNAs. Restriction enzymes are bacterial enzymes that recognize specific 4- to 8-bp sequences, called restriction sites, and then cleave both DNA strands at this site. The multiple cloning site is the region which these restriction sites are exists (Lodish et al. 2000).

1-5. Invadopodia

Mitogenic factors, such as growth factors, can induce cell movement. In the presence of growth factors, cells start to stretch its cytoplasm toward the growth factors. This stretching structure is called “lamellipodia”. The direction that lamellipodia is at the leading edge. Proteases decompose the extracellular matrix (ECM), and a new connection is formed between integrin and lamellipodia at the trailing edge. On the other side, proteases destroy the connection at the trailing edge. This sequence can move the cell toward the mitogenic factors (Uzman, 2003).

Cells can move not only horizentally but also vertically. However, there is a barrier (ECM) above the cells and cells have to intrude the ECM to move vertically. Simillar to lamellipodia, invadopodia are specific invasive structures.

Invadopodia are actin-rich structures which exist on the ventral side of the cell and penetrate into the ECM (Revach & Geiger, 2014). They are 0.5 to 2μm in width and are over 2μm long. They were first observed in the 1980s in RSV and, because of their feet like structure, they were called “Podosomes”. A few years later, Chen (Chen, 1989) discovered podosomes with a degradation activity and adhesive and invasive fuinctions and described them as “invadopodia”. Podosomes and invadopodia are found in normal cells, such as macrophages, endothelial cells, dentritic cells and osteoclasts as well as in cancer cells.

Invadopodia are complex and are composed of many molecules, including Src. Tks5 scaffolding protein, cortactin, neural Wiskott-Aldrich syndrome protein (N-WASP), Arp 2/3. Among theses, cortactin is an F-actin binding protein and is a critical component of the invadopodia. Cortactin can be phosphorylated by Src kinase and, in its phosphorylated form, releases cofilin which promotes Arp 2/3 polymerization (Revach & Geiger, 2014). N-WASP acts as an initiator for actin polymerization and is connected with the degradation acivity of invadopodia (Yamaguchi & Condeelis, 2007).

2. Material and methods

2.1. Antibodies

Phospho-Src Family (Tyr416), Phospho-Src Family (Tyr527) were purchased from Cell Signaling Technology (CST, USA). c-Myc (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Goat anti-rabbit IgG, polyclonal antibody (HRP conjugate) and Goat anti-mouse IgG, polyclonal antibody (HRP conjugate) were purchased from Enzo Life Sciences (Enzo, USA).

2.2 Polymerease chain reaction

Primers to specifically amplify the genes of c-Src WT (K321A) and c-Src YF (K321A) were used for following sequences. (Forward : 5'-GAAGAAGCTGAGGCATGAGGCGCTGGTGCAGTTGTATGCTG -3' Reverse : 5'-CAGCATATACAACTGCACCAGCGCCTCATGCCTCAGCTTCTTC -3')

Gene	Sequence
c-Src	atgggc agcaacaaga gcaagcccaa ggacgccagc cagcggcgcc gcagcctgga gccctcggaa aacgtgcacg gggcaggggg cgccttcccg gcctcacaga caccgagcaa gcccgcctcc gccgacggcc accgcgggcc cagcgccgcc ttcgtgccgc ccgcggccga gcccaagctc ttcggaggct tcaactcctc ggacaccgtc acctccccgc agagggcggg gcctctggca ggtggggtga ccacctttgt ggccctctat gactatgagt cacggacaga gactgacctg tccttcaaga aaggggagcg gctgcagatt gtcaataaca cgaggaaggt ggatgtcaga gagggagact ggtggctggc acactcgctg agcacgggac agaccggtta catccccagc aactatgtgg cgccctccga ctccatccag gctgaggagt ggtactttgg caagatcact agacgggaat cagagcggct gctgctcaac gccgagaacc cgagagggac cttcctcgtg agggagagtg agaccacaaa aggtgcctac tgcctctctg tatccgactt cgacaatgcc aagggcctaa atgtgaaaca ctacaagatc cgcaagctgg acagcggcgg tttctacatc acctcccgca cccagttcaa cagcctgcag cagctcgtgg cttactactc caaacatgct gatggcctgt gtcaccgcct cactaccgta tgtcccacat ccaagcctca gacccaggga ttggccaagg atgcgtggga gatcccccgg gagtccctgc ggctggaggt caagctgggc cagggttgct tcggagaggt gtggatgggg acctggaacg gcaccacgag ggttgccatc aaaactctga agccaggcac catgtcccca gaggccttcc tgcaggaggc ccaagtcatg aagaaactga ggcacgagaa actggtgcag ctgtatgctg tggtgtcgga agaacccatt tacattgtga cagagtacat gaacaagggg agtctgctgg actttctcaa gggggaaacg ggcaaatatt tgcggctacc ccagctggtg gacatgtctg ctcagatcgc ttcaggcatg gcctatgtgg agcggatgaa ctatgtgcac cgggaccttc gagccgccaa tatcctagta ggggagaacc tggtgtgcaa agtggccgac tttgggttgg cccggctcat agaagacaac gaatacacag cccggcaagg tgccaaattc cccatcaagt ggaccgcccc tgaagctgct ctgtacggca ggttcaccat caagtcggat gtgtggtcct ttgggattct gctgaccgag ctcaccacta agggaagagt gccctatcct gggatggtga accgtgaggt tctggaccag gtggagcggg gctaccggat gccttgtccc cccgagtgcc ccgagtccct gcatgacctt atgtgccagt gctggcggaa ggagcccgag gagcggccca ccttcgagta cctgcaggcc ttcctggaag actactttac gtccactgag ccacagtacc agcccgggga gaacctatag

Table. 1. mRNA sequence of c-Src (mouse)

Primer	Sequence	
K321A c-Src	Forward	5′-GAAGAAGCTGAGGCATGAGGCGCTGGTGCAGTTGTATGCTG-3′
	Reverse	5′-CAGCATATACAACTGCACCAGCGCCTCATGCCTCAGCTTCTTC-3′

Table. 2. Gene-specific primers used for PCR

2.3. Ligation and Plasmid DNA extraction

PCR product were inserted into pCMV Vector (Fig. 4) using ligation kit ver2.1 (Takara). 120 ng of insert DNA fragments and 100 ng of pCMV Vector and equal volume of ligation mixture were used for ligation. Ligation was conducted for 16°C, O/N using Wisetherm Heating Block (PMI-Labortechnik). Ligation products were transformed to competent cells (DH5α, Intron) and incubated on ice for 30min. After that, transfered to water bath and incubated for 1min at 42°C (heat-shock). Cells were recovered for 3min on ice and 1ml of LB medium were add for each cell. Finally, cells were incubated at the shacking incubator for 37°C, O/N. Plasmid DNAs were extracted from grown cells using Plasmid extraction kit, Midi (QIAGEN). Extracted DNAs were separated on redsafe (Intron) including 1% garose gel

for 40 min at 100V. The gels were photographed by UV illumination using Gene Flash (Syn gene bioimage).

2.4. Cell culture

NIH-3T3 cell line was cultured in 10 cm^2 dish and 6 cm^2 dish and maintained in DMEM supplemented with 10% bovine calf serum (BCS) and 100 μg streptomycin/penicillin per mL at 37°C in huminified atmosphere of 5% CO_2. Cells were subcultured by washing with 0.02% EDTA-PBS solution and detaching with 500 μl Trypsin 1-2 times every week at about 70-80% confluency.

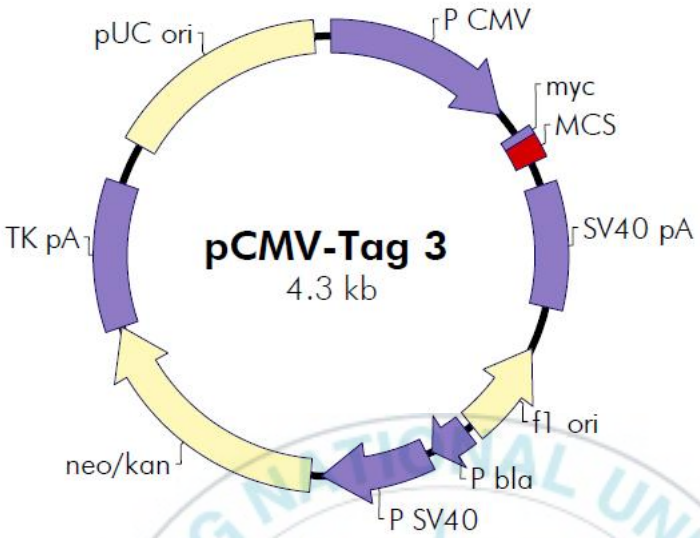


Fig. 5. Vector map of pCMV-Tag 3

Feature	Nucleotide Position
CMV promoter	1-602
C-myc tag	679-708
Multiple cloning site	709-782
SV40 polyA signal	859-1242
F1 origin of ss-DNA recplication	1380-1686
Bla promoter	1711-1835
SV40 promoter	1855-2193
Neomycin/kanamycin resistance ORF	2228-3019
HSV-thymidine kinase (TK) polyA signal	3020-3478
pUC origin	3607-4274

Table. 3. Nucleotide position of pCMV-Tag 3 vector

2.5. Transfection

To obtain transient expression, NIH-3T3 cells were plated at 70~80% confluence on a 6 cm^2 dish. Following day, 5 ㎍ of plasmid DNAs and 10 ㎕ P3000 (Invitrogen) in 250 ㎕ OPTI-MEM (Gibco) solutions were incubated for 10min and 7.5 ㎕ Lipofectamine 3000 (Invitrogen) in OPTI-MEM (Gibco) solutions were incubated for 5min. After incubation, solutions were mixed with equal volumes and incubated for 15 min. Incubated solutions were added to NIH-3T3 cells and incubated at 37°C for 6 h. Cells were transferred to growth medium and incubated for another 6 h at 37°C.

2.6. Sample preparation

Transfected NIH-3T3 cells were sereum starved for 16 h and stimulated with or without 50 ng/ml Recombinant Human PDGF-BB (Gibco) for 30 min. After stimulation, medium was discarded and washed with cold PBS two times. Cells were then lysed for 1 min on ice in 500 ㎕ of protease inhibitors

(5 mM Sodium orthovanadate, 1 mM Sodium orthovanadate, 1X cOmplete™,mini,EDTA-free protease inhibitor cocktail (Sigma-Aldrich) solution) contained RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH8.0)). Lysates were sonicated with Vibra cell (Sonics) for 30 sec and were centrifused for 12,000 rpm.

2.7. Western Blot analysis

Lysates were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred onto a PVDF (Polyvinylidene fluoride) membrane (Millipore), blocked with 10% skim milk power and 1% bovine serum albumin in PBS for 1h and hybridized with primary antibodies such as c-Myc (CST), p-416 Src, p-527 Src (Santa Cruz). All primary antibodies were diluted with 1% NaN_3 contained PBS at 1:500 ratio. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature, and the immunoreactive proteins were detected using a Westernbright™ ECL kit (advanstar) according to the manufacturer's instructions. Western

blot bands were visualized using a Davinch-Chmi^{TM} Chemiluminescence Imaging System (Davinch-K).

2.8. Cell migration assay

NIH-3T3 cells were plated at 70~80% confluence on a 24 well plate and incubated at 37°C in a humidified incubator with 5% CO_2 for 12 h. Cells were starved with OPTI-MEM for 30 min and plasmid DNAs were transfected into the cells by Lipofectamine 3000 (Invitrogen). Transfected cells were starved with serum and antibiotic deficient medium for 12 h and stimulated with or without 50 ng/ml Recombinant Human PDGF-BB (Gibco) for 30 min. Stimulated with or without cells were washed twice and scratched with yellow tip. After scratching, cells were washed with medium and examined the photo every 2 h by AE31 microscope with MoticamPre 205A (Motic).

2.9. Immunofluorescence assay

NIH-3T3 cells were plated at 50~60% confluence on a EtOH sterilized cover slip (24 well plate) and incubated with 12 h. Also, cells were starved with OPTI-MEM and plasmid DNAs were transfected with Lipofectamine 3000 (Invitrogen). Transfected cells were starved for 12 h and stimulated with or without 50 ng/ml Recombinant Human PDGF-BB (Gibco) for 30 min. Cells which positioned on the cover slip were fixed in 3.7% formaldehyde for 10 min and permeabilized in 0.1% Triton X-100. Cells were incubated with cortactin antibody for 1 h at room temperature and washed with PBS. Rhodamine-phalloidine, DAPI and Alexa fluor 488 mixture was used for another antibody and incubated for 30 min at room temperature. The fluorescence was examined by AE31 microscope with MoticamPre 205A (Motic).

3. Results

3.1 Manufacture of c-Src WT/YF K321A mutants

First, we manufactured the mutant DNA (c-Src WT/YF K321A) by a polymerase chain reaction. Mutant (WTKA, YFKA), WT, YF c-Src were ligated with pCMV vector, amplified and extracted. Ligation products and pCMV vector were identified by agarose gel electrophoresis. The pCMV vector comprised 4.3 kb and the ligation product of WT, WTKA, YF, YFKA c-Src about 6 kb. Lambda/HindIII was used as a DNA ladder.

3.2 KA mutants induce alteration of c-Src activation in the normal state

Each DNA was transfected into NIH-3T3 cells to compare c-Src activation at the cellular level. Transfection efficiency was determined by c-Myc epitope which exists on the pCMV vector. Phosphorylation of Tyr416 and 527 indicates the active and inactive state of c-Src respectively. Expression of c-Myc was almost the same, but YFKA c-Src was slightly low compared to

other DNAs (Fig. 6a). Phosphorylation of Tyr416 (Fig. 6b) and Tyr527 (Fig. 6c) were divided by c-Myc to express comparative values. YF and YFKA c-Src showed phosphorylation of Tyr416 1.5 to 2 times higher than WT and WTKA c-Src (Fig. 6d). In the case of Tyr527, WTKA showed almost the same level as WT c-Src (Fig. 6e). The KA mutant of WT decreases phosphorylation of Tyr416, which indicates suppression of c-Src activation. However, the KA mutant of YF increases phosphorylation of Tyr416, with an increase in activation. As a result, we found that the KA mutant affects the Tyr416 residue more than it does the Tyr527 in the normal state.

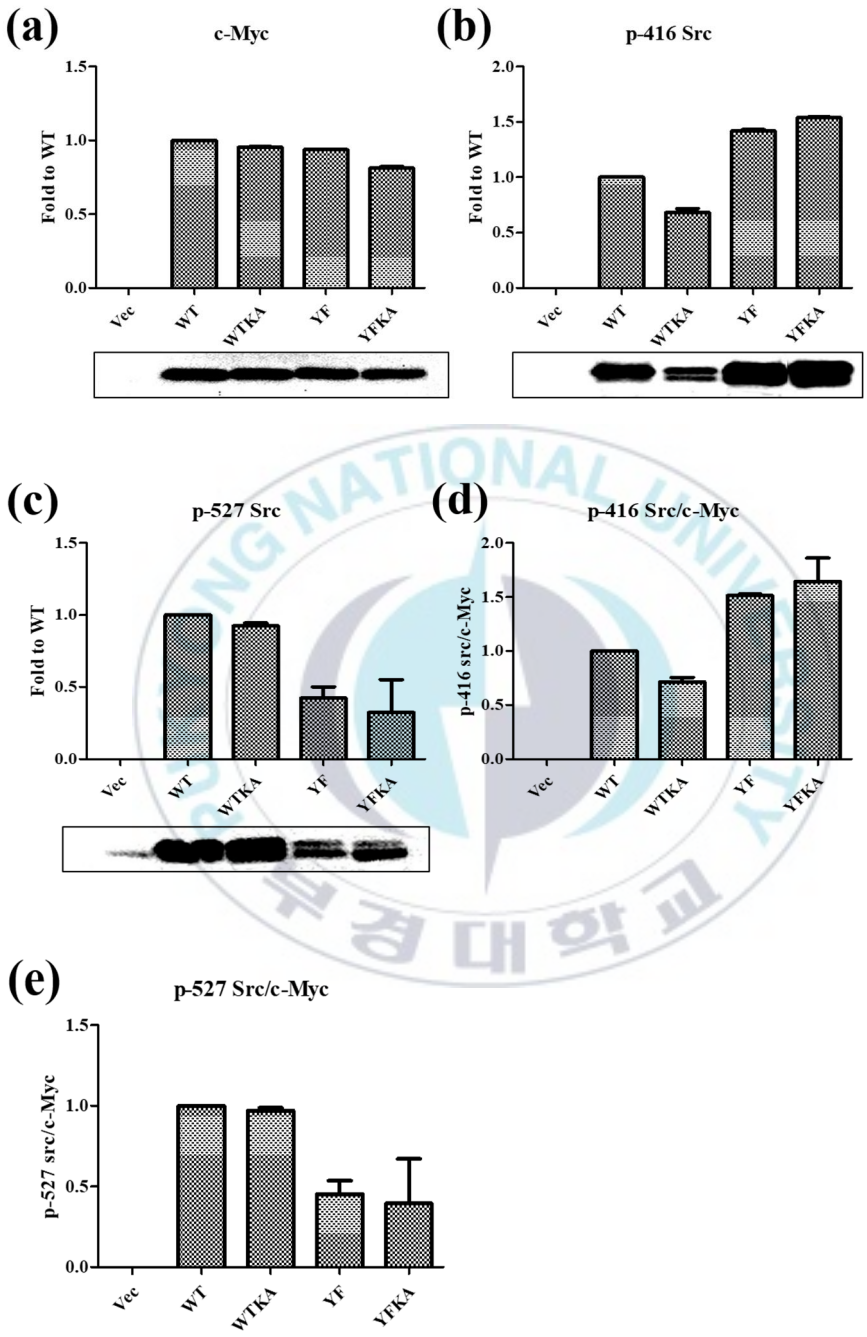


Fig. 6. Western Blot analysis of c-Src activation in the normal state

3.3 KA mutants induce alteration of c-Src activation in a PDGF-BB stimulated state

Conformational change of c-Src is important for its activation. A recent study reveals that KA mutants induce incomplete conformational change of c-Src in computational simulations (Yoon et al., 2018). In order to confirm the change in c-Src activity when going from an inactive to an active state, we introduced the c-Src activator PDGF-BB. PDGF is a growth factor and consists of two polypeptide chains; PDGF-BB is a homodimeric isoform of two b-chains and can bind with PDGF receptor β. As an activator, PDGF-BB directly stimulates the SH2 domain of the Src and causes dephosphorylation of Tyr527 (Mori et al., 1993).

Under the of PDGF-BB stimulated condition, all except the YFKA group show elevation of Tyr416 phosphorylation compared to the normal state. Among them, both normal and stimulated WTKA show a low level of Tyr416 phosphorylation compared to normal WT (Fig. 7b, 7d). In the case of WT, phosphorylation of Tyr527 decreases as it changes from the inactive to active state. However, phosphorylation of Tyr527 in WTKA shows almost the same level in both the normal and stimulated states (Fig. 7e). These results indicate

that KA mutants not only have an effect in the normal state but also in the PDGF-BB stimulated state of c-Src activation.

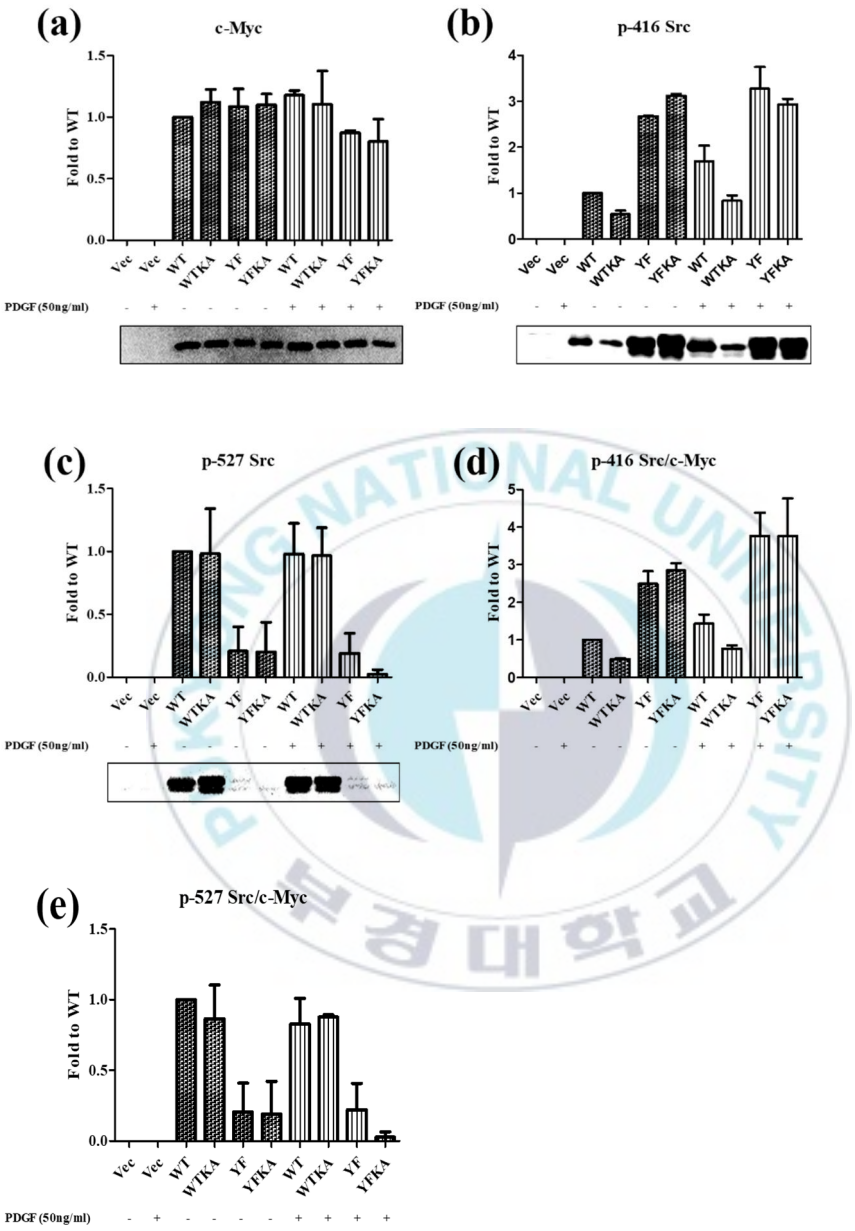
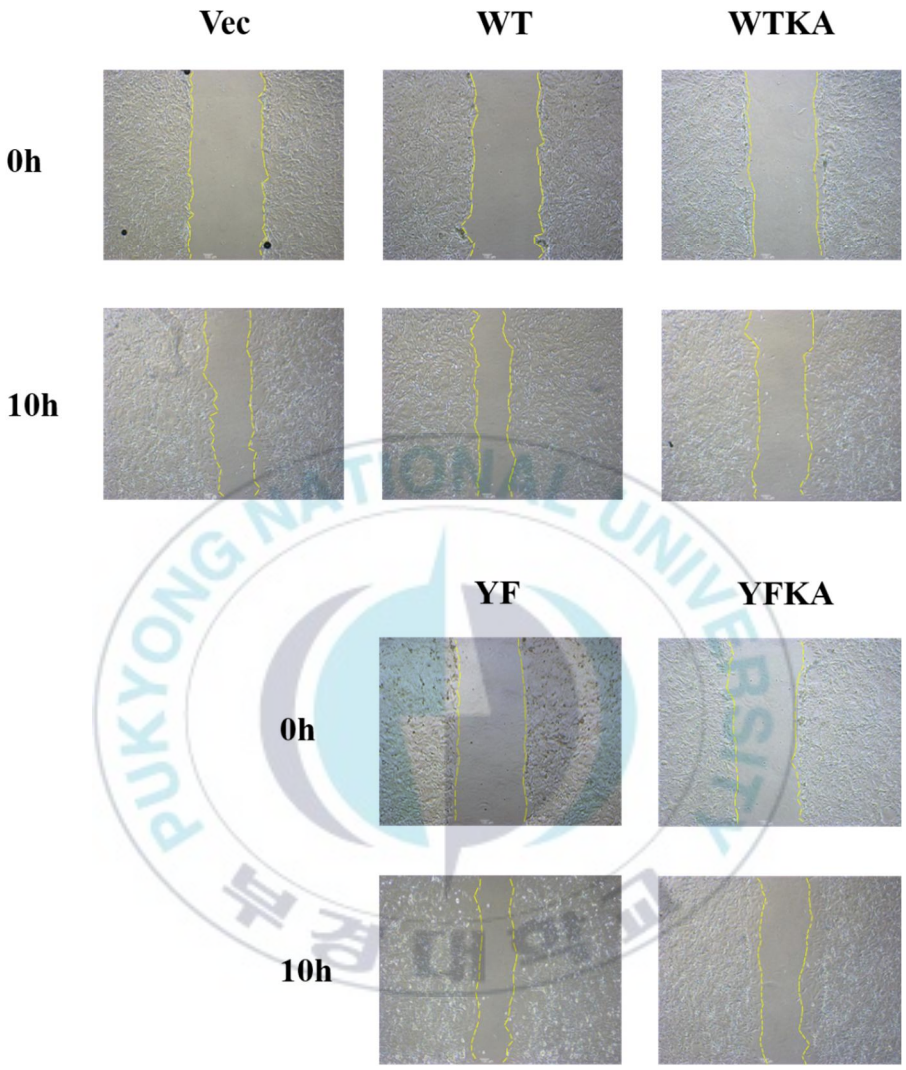


Fig. 7. Western Blot analysis of c-Src activation in the normal state and the PDGF-BB stimulated state

3.4 KA mutants inhibit cell migration activity

Migration is one of the important characteristics of c-Src. To identify the effect of KA mutations on cell migration, we introduced the wound-healing assay. Each DNA was transfected to NIH-3T3 cells, and the scratch gap was measured after 24 hours. To proceed with the same conditions as in the Western blot experiment, PDGF-BB was treated for only 30 minutes and washed out. The measurement scale was a relative scratched gap with 100% at 0 h. As shown in (Fig. 8), the KA mutants decreased migration of NIH-3T3 cells by 20% compared with the WT and YF c-Src; the greatest difference was at 10 h. Therefore, these results suggest that KA mutants inhibit the cell migration of NIH-3T3 cells.


Vec
WT
WTKA
0h
10h
YF
YFKA
0h
10h

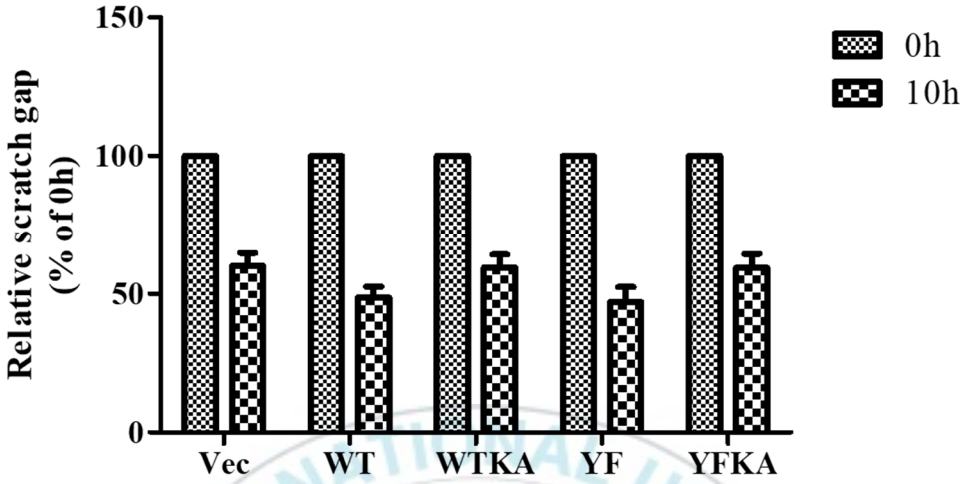
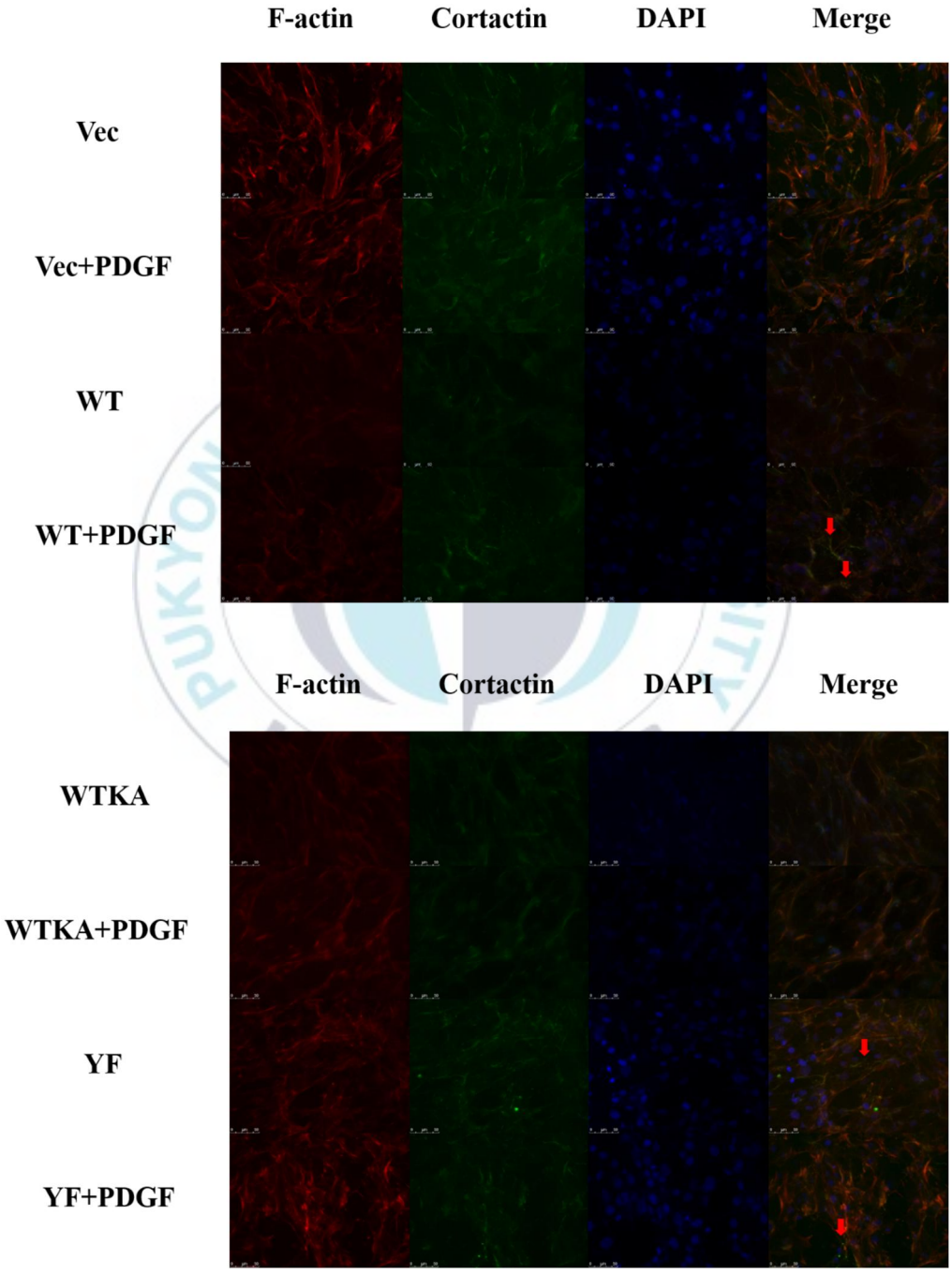


Fig. 8. Relative scratch gap between each transfected NIH-3T3 cell at 10 h

3.5 KA mutants decrease invadopodia formation

Invadopodia are actin-rich protrusions that can help cancer cells to invade effectively. It is already known through a variety of studies that activated c-Src induces invadopodia formation. To identify the effect of KA mutants on invadopodia formation, we used an immunofluorescence assay and checked the invadopodia formation of each group of transfected NIH-3T3 cells (also, PDGF-BB was treated 30 minutes). We used three kinds of dye: rhodamine-phalloidin, F488 (mouse) with cortactin antibody, and DAPI. F-actin identifies the cytoskeleton of the cell and DAPI identifies the nucleus of the cell. Invadopodia consist mainly of cortactin and appear at the ventral surface of the cell. Invadopodia are known to occur in activated c-Src and do not appear if it is not activated or is activated imperfectively. In the fluorescence image, invadopodia appeared as a dot, and we can find invadopodia in the YF, YFKA, PDGF-BB stimulated WT, and YF+YFKA. However, we did not find any invadopodia formation in the other groups (Fig. 9). The results indicate that KA mutation only affects invadopodia formation in the inactive condition.


F-actin
Cortactin
DAPI
Merge
Vec
Vec+PDGF
WT
WT+PDGF
F-actin
Cortactin
DAPI
Merge
WTKA
WTKA+PDGF
YF
YF+PDGF

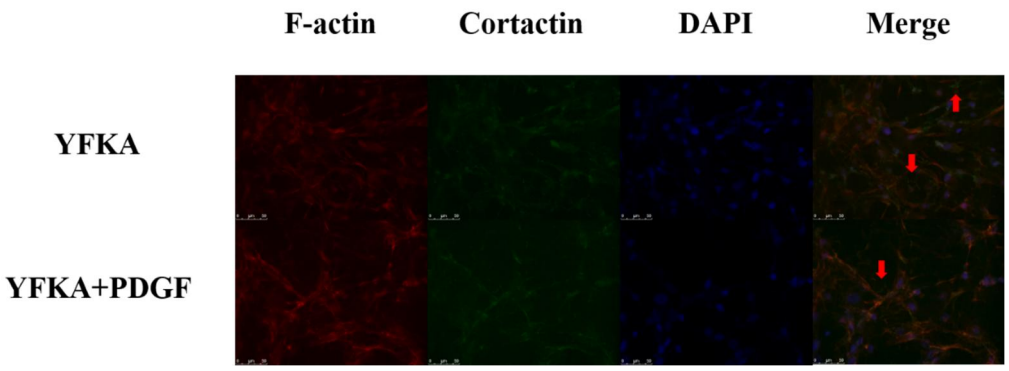


Fig. 9. Invadopodia formation of transfected NIH-3T3 cell with or without PDGF-BB stimulation

4. Discussion

c-Src is a proto-oncogene, and its activated form is present in 40% of all cancers. In this study, we investigated the effects of the K321A (KA) mutation on NIH-3T3 cell migration and invadopodia formation. KA mutants showed different forms of Tyr416 phosphorylation when compared to the normal state of WT and YF. Changes in Tyr416 phosphorylation are related to the structure and activation state of c-Src. To determine the effect of KA on the series of change in the structure of c-Src, we needed to select one of the c-Src activators. Thus, we introduced the activator PDGF-BB. In the PDGF-BB stimulated condition, K321A mutants showed the same result as in the normal state, but Tyr527 phosphorylation of WTKA does not decrease despite PDGF-BB stimulation.

Activated c-Src affects various signals, especially cell motility and invasion (Dehm & Bonham, 2004). So, we checked the migration activity of KA mutants by using a wound-healing assay. We found that the KA mutants decreased cell migration compared to normal WT and YF.

One of the most noticeable characteristics of c-Src activation is that Src-activated cells show invasive structures called invadopodia (Gimona,

Buccione, Courtneidge, & Linder, 2008). Invadopodia are actin-rich structures and are important in cancer cell invasion and metastasis (Caldieri & Buccione, 2010). The shape of invadopodia is like a drill or needle so it can enable the cell to enter other organs. Through the immunofluorescence assay, we checked that WTKA induces elimination of invadopodia formation compared with normal WT (but, there is no effect on the YFKA group). As a result, we found that KA mutants decrease cell migration and invadopodia formation (especially WTKA) through changes in Tyr416 and 527 phosphorylation.

The interaction between c-Src and FAK (focal adhesion kinase) is important for cell migration and invasion (Hauck, Hsia, & Schlaepfer, 2002). A variety of malignant tumors have both high levels of c-Src and FAK activation and high invasive ability (Owens et al., 1995). The FAK and c-Src complex causes alteration of the actin cytoskeleton, including E-cadherin inhibition (Pei, Lan, Lu, Ji, & Hua, 2018), focal adhesion disruption (Nader, Ezratty, & Gundersen, 2016) and expression of MMPs (matrix metalloproteinases) (Hsia et al., 2003).

MMPs are enzymes which are secreted from invadopodia and can dissolve the matrix (Sato et al., 1997). Thus, these enzymes are very important in cell invasion. Existing c-Src-related cancer drugs have many side effects and

limitations. Therefore, we expect that KA mutants may affect these proteins (FAK, E-cadherin, MMPs) and that determining the exact mechanism of KA will be used in the development of novel c-Src anti-cancer therapies.

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